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The presence of steatosis and elevation of alanine aminotransferase levels are associated with fibrosis progression in chronic hepatitis C with non-response to interferon therapy☆

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***Background/Aims*: Interferon (IFN) therapy leads to regression of hepatic fibrosis in chronic hepatitis C patients who achieve a sustained virologic response (SVR), while the beneficial effect is limited in those who fail to do so. The aim of the present study was to define factors associated with progression of fibrosis in patients who do not achieve a SVR.**

***Methods*: Fibrosis staging scores were compared between paired liver biopsies before and after IFN in 97 chronic hepatitis C patients who failed therapy. The mean interval between biopsies was 5.9 years. Factors associated with progression of fibrosis were analyzed.**

***Results*: Fibrosis progressed in 23%, remained unchanged in 47% and regressed in 29%. Steatosis and a high average alanine aminotransferase (ALT) between biopsies were independent factors for progression of fibrosis with risk ratios of 5.53 and 4.48, respectively. Incidence and yearly rate of progression of fibrosis was 64% and 0.22 ± 0.29 fibrosis units per year in those with both risk factors compared to 8% and −0.04 ± 0.17 fibrosis units per year in those negative for both factors.**

***Conclusions*: Hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy and therefore may be therapeutic targets to halt the potentially progressive disease.**

Keywords: **Steatosis; ALT; Fibrosis**

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide. Mortality associated with HCV infection results from the development of liver cirrhosis and hepatocellular carcinoma, which now is the leading indication for liver transplantation [1]. Treatment with interferon (IFN), alone or in combination with ribavirin (RBV), can eradicate HCV infection in some patients, leading to sustained nor-

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malization of liver function, improvement of hepatic inflammation and fibrosis and a decreased risk of the development of hepatocellular carcinoma [2,3]. The problem is that only 50% of patients achieve a sustained virological response (SVR) to therapy even with the most highly developed regimens of IFN [4,5]. The remaining patients who fail to clear the virus are left with the risk of progressive disease. In order to halt this potentially progressive disease, there is a need to establish an effective target of therapeutic intervention independent of antiviral therapy. Therefore, it is important to define risk factors for the progression of fibrosis among chronic hepatitis C patients who do not achieve a SVR to IFN therapy.

Several factors that may affect the rate of progression of fibrosis have been investigated extensively, including older age at infection, male gender, obesity, heavy alcohol consumption, and a high grade of necroinflammation [6–8]. Several cross-sectional and longitudinal studies suggest that hepatic steatosis, which is a common histological feature of chronic hepatitis C [9], influences the progression of hepatic fibrosis [10–14], while other studies did not find such an association [15–18]. Besides these conflicting results, no study to date has reported the effect of steatosis on longitudinal progression of fibrosis among patients who fail to respond to IFN therapy. Therefore, we studied factors associated with progression of fibrosis in those who failed IFN therapy by comparing paired pre-treatment and post-treatment liver biopsies.

2. Methods

2.1. Patients

The aim of the study was to identify risk factors associated with progression of fibrosis in chronic hepatitis C patients who failed to achieve a SVR to IFN therapy. To be included in this retrospective study, patients had to have undergone liver biopsy before and after therapy, been treated with IFN and not achieved a SVR. Patients with alcohol consumption of more than 20 g per day, co-infected with HBV or HIV, and those with another known aetiology of liver disease, such as autoimmune hepatitis or metabolic disorders, were excluded. A database of patients who had undergone liver biopsy at Musashino Red Cross Hospital between 1990 and 2004 was reviewed retrospectively and a total of 1241 chronic hepatitis C patients treated with IFN were identified; of these, 407 had a SVR and 834 had not achieved a SVR. Among those with treatment failure, 104 fulfilled the above criteria but seven patients with cirrhosis before treatment were excluded because the endpoint of the study was progression of fibrosis. Therefore, this study cohort consisted of 97 patients. In these patients, second liver biopsies were performed before the second course of IFN therapy. Otherwise, there were no standardized indications for the second liver biopsy which may be the limitation of our study. Demographic characteristics of patients at the time of initial biopsy are shown in Table 1. The time between the paired biopsies was 5.9 years on average, with a range of 1.2–11.6 years. The median interval between first biopsy and IFN therapy was 3 days (range 2–93 days), and that between completion of IFN therapy and second biopsy was 5.4 years (range 0.8–11.2 years). Laboratory tests were performed monthly or bimonthly in all patients and all measurements were taken at our single hospital.

Table 1
Demographic characteristics of patients

Number of patients	97
Age (years)	52 ± 9
Gender: male/female	50/47
BMI (kg/m^2)	23.9 ± 3.2 (median 24.0, range19–33)
BMI <25/25–30/30 ⩽ (kg/m^2)	55/37/5
Route of transmission	
Blood transfusion/unknown	38/59
Duration of infection (years)	30.4 ± 9.2 (median 33.5, range 3–48)
Genotype 1b/2a/2b	85/4/8
Serum HCV-RNA (Meq/ml)	7.7 ± 9.7
Pretreatment AST (IU/l)	73 ± 40
Pretreatment ALT (IU/l)	104 ± 69
Pretreatment GGT (IU/l)	51 ± 44
Histological variables at first biopsy	
Stage of fibrosis 1/2/3	33/38/26
Grade of activity 0/1/2/3	15/36/41/5
Grade of steatosis 0/1/2/3	21/37/25/14
Size of steatosis macro/micro/mixed	16/17/64
Localization of steatosis centrilobular/diffuse	3/94

BMI, body mass index; AST, aspartate aminotransferase, normal range is 7–38 IU; ALT, alanine aminotransferase, normal range is 4–43 IU/l; GGT, gamma-glutamyltransferase, normal range is 0–73 IU/l; macro, macro-vesicular steatosis; micro, micro-vesicular steatosis.

2.2. Histological evaluation

Median length of biopsy specimen and number of portal tracts were 13.0 mm (range 10–40 mm) and 12 (range 6–34). All liver biopsy specimens were evaluated separately by three independent pathologists who were blinded to the clinical data. If there was discordance, the scores assigned by two pathologists were used for the analysis. Fibrosis and activity were scored according to the METAVIR scoring system [19]. Fibrosis was staged on a scale of 0–4: F0 (no fibrosis), F1 (mild fibrosis: portal fibrosis without septa), F2 (moderate fibrosis: few septa), F3 (severe fibrosis: numerous septa without cirrhosis) and F4 (cirrhosis). Activity of necroinflammation was graded on a scale of 0–3: A0 (no activity), A1 (mild activity), A2 (moderate activity) and A3 (severe activity). Percentage of steatosis was quantified by determining the average proportion of hepatocytes affected by steatosis and was graded on a scale of 0–3: grade 0 (no steatosis), grade 1 (0–9%), grade 2 (10–29%), and grade 3 (over 30%). Size of steatosis was categorized into micro-vesicular, macro-vesicular and mixed types. Localization of steatosis was categorized into either centrilobular or diffuse pattern. Definition of changes in the grade of steatosis was as follows: worsening as 1 point or more increase, improvement as 1 point or more decrease, and stability as no change.

2.3. Changes in fibrosis-staging score overtime

Changes in progression of fibrosis were defined as follows: progression of fibrosis was defined as a 1 point or more increase, regression as a 1 point or more decrease and stability as no change in the METAVIR fibrosis-staging score. In addition, because the time between paired biopsies was variable, the yearly rate of progression of fibrosis was calculated as the change in fibrosis-staging score divided by the time between paired biopsies, as originally described by Poynard et al. [6].

2.4. Statistical analysis

The STAT View software package was used for statistical analysis. Categorical data were analyzed using the Fisher's exact test. Continuous variables were compared with the Student's t test. Variables that were statistically significant in univariate analysis were included in multivariate analysis using logistic regression analysis. The Kaplan–Meier method and log-rank test were used to analyze the time to occurrence of fibrosis progression. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Factors associated with the initial stage of fibrosis (cross-sectional study)

All three pathologists assigned the same score in 85% of patients for fibrosis staging and 95% of patients for steatosis-grading. In cases with discordance, at least two pathologists assigned the same scale. The stage of fibrosis in the initial liver biopsy was F1 in 33, F2 in 38 and F3 in 26 patients. Various clinical factors were analyzed in association with the advanced stage of fibrosis. As a result, the presence of F3 fibrosis was associated with older age, (51 ± 9 in F1–2 vs. 55 ± 9 in F3, $p = 0.03$), higher grade of histological activity (A2–3 was 35% in F1–2 vs. 84% in F3, $p = 0.0001$) and higher grade of steatosis (steatosis grade 2–3 was 34% in F1–2 vs. 58% in F3, $p = 0.04$).

The grade of steatosis was 0 in 21, 1 in 37, 2 in 25 and 3 in 14 patients. A higher grade of steatosis was associated with female gender (the male/female ratio was 35/23 in grade 0–1 vs. 15/24 in grade 2–3, $p = 0.04$), increased BMI (BMI over 25 kg/m^2 was 31% in grade 0–1 vs. 62% in grade 2–3, $p = 0.006$), and higher grade of histological activity (A2–3 was 38% in grade 0–1 vs. 62% in grade 2–3, $p = 0.03$). Multivariate logistic regression analysis revealed that increased BMI and female gender were independent factors associated with a high grade of steatosis (Table 2).

Table 2
Multivariate logistic regression analysis of factors associated with hepatic steatosis

	Odds	95% C.I.	p Value
BMI			
$\geqslant$25 kg/m^2	4.23	1.63–10.95	0.003
Gender			
Female	2.75	1.06–7.14	0.04
Activity grade			
2–3	2.30	0.85–6.26	0.10
Fibrosis stage			
3	1.63	0.53–4.97	0.39

3.2. Change in fibrosis-staging scores over time (longitudinal study)

Fibrosis staging progressed in 23% (progression by 2 points in 5% and 1 point in 18%), remained unchanged in 47% and regressed in 29% (regression by 2 points in 2% and 1 point in 27%). At first liver biopsy, laparoscopy was performed in 73 patients and the presence of cirrhosis (F4) was carefully excluded. In another 24 patients, the possibility of mis-diagnosis of F4 as F3 remains. However, the incidence of fibrosis progression did not differ according to the initial stage of fibrosis (21.2% in F1, 26.3% in F2 and 19.2% in F3, $p = 0.78$) which indicates that misdiagnosis of F4 as F3 at initial biopsy is unlikely.

Among various factors, as shown in Table 3, a higher grade of steatosis, higher levels of ALT and AST (average value for the period between the paired liver biopsies) were associated with progression of fibrosis. Since there was significant correlation between ALT and AST levels ($r = 0.684$, $p < 0.0001$), these two variables could not be analyzed together in multivariate analysis.

Table 3
Factors associated with the progression of fibrosis over time

	Progression $n = 22$	Non-progression $n = 75$	p Value
Gender: male/female	9/13	41/34	0.33
Age at biopsy: <60/$\geqslant$60 years	14/8	59/16	0.17
HCV genotype: 1b/non-1b	19/3	66/9	0.99
BMI: <25/$\geqslant$25 kg/m^2	11/11	44/31	0.48
Duration of infection (years)	32.1 ± 5.2	29.9 ± 10.0	0.56
Activity on first biopsy			
Grade: 0–1/2–3	8/14	43/32	0.10
Steatosis on first biopsy			
Grade: 0–1/2–3	6/16	52/23	0.001
Size: macro/micro/mixed	4/4/14	12/13/50	0.96
Location: centrilobular/diffuse	1/21	2/73	0.54
Evolution of steatosis			
Worsening/improvement/stable	2/2/18	9/8/58	0.09
Average ALT: <100/$\geqslant$100 IU/l	13/9	67/8	0.003
Average AST: <75/$\geqslant$75 IU/l	10/12	61/14	0.002
Interval between biopsies (years)	5.1 ± 3.2	6.2 ± 2.4	0.09
Interval between completion of IFN and second biopsy (years)	4.6 ± 3.2	5.7 ± 2.4	0.10
Treatment regimen			
RBV–/RBV+	22/0	71/4	0.27
Response to IFN			
Relapser/non-responder	16/6	53/22	0.99
Evolution of weight			
Gain/loss/stable	5/8/9	29/21/25	0.38

macro, macro-vesicular steatosis; micro, micro-vesicular steatosis; RBV–, interferon monotherapy; RBV+, interferon plus ribavirin combination therapy.
Duration of infection was determined in 38 patients whose source of infection was blood transfusion.

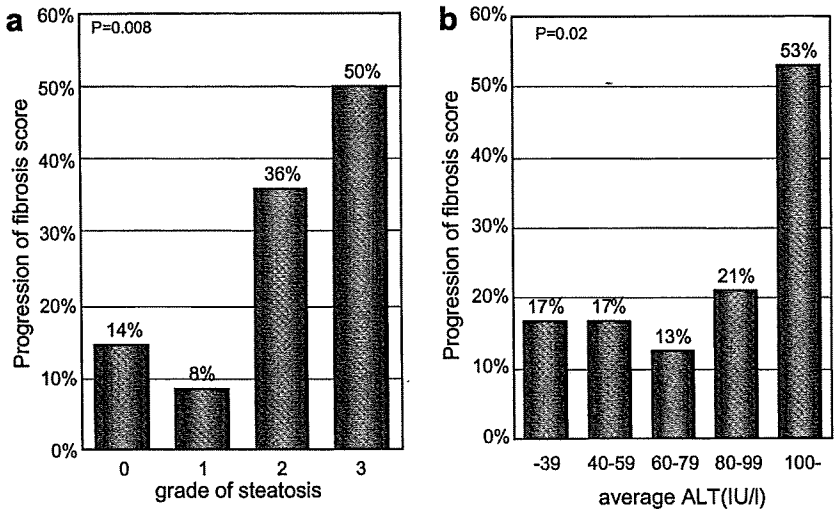

Fig. 1. Progression of fibrosis stage, hepatic steatosis and the average level of ALT. The progression of the fibrosis score over time is illustrated using bar charts. (a) Steatosis grades of 2 or 3 at initial liver biopsy were associated with the increased progression of fibrosis over time. (b) High average ALT levels during the observation period were associated with progression of fibrosis at the threshold of 100 IU/l.

Thus, average level of ALT was used for the following analysis. The probability of progression of fibrosis was 14%, 8%, 36% and 50% in patients with steatosis grades of 0, 1, 2 and 3, respectively ($p = 0.008$), and 17%, 17%, 13%, 21% and 53% in patients with average ALT values of <40, 40–59, 60–79, 80–99 and over 100 IU/l, respectively ($p = 0.02$) (Fig. 1). Multivariate logistic regression analysis revealed that these two were independent risk factors associated with the progression of fibrosis with risk ratios of 5.14 for steatosis ($p = 0.004$) and 5.21 for ALT ($p = 0.01$) (Table 4).

When patients were categorized in terms of these two risk factors, the incidence of progression of fibrosis was as high as 64% in those with both risk factors, compared to 8% in those negative for these factors. Conversely, the incidence of fibrosis regression was only 9% in those with both risk factors, compared to 37% in those negative for these factors ($p = 0.0003$) (Fig. 2).

In order to adjust for the effect of variable intervals between paired biopsies, the yearly rate of progression of fibrosis was calculated as the change in the fibrosis-staging score divided by the time between paired biopsies. The average of all patients was 0.02 ± 0.22 fibrosis units per year. Again, a higher grade of steatosis ($p = 0.004$) and higher average level of ALT ($p = 0.0005$) were associated with a higher rate of progression of fibrosis (Table 5). In addition, the yearly rate of progression of fibrosis was 0.22 ± 0.29 fibrosis units per year in those with both risk factors, 0.12 ± 0.37 in those with elevated ALT alone, 0.05 ± 0.16 in those with steatosis alone and -0.05 ± 0.17 in those negative for these two factors ($p = 0.001$). Time to progression of fibrosis at second biopsy was also analyzed by the Kaplan–Meier method. The cumulative probabilities of progression of fibrosis at five years were 58% in those with both risk factors, 33% in those with elevated ALT alone, 18% in those with steatosis alone and 2% in those negative for these two factors ($p < 0.0001$) (Fig. 3).

Table 4
Multivariate logistic regression analysis of factors associated with progression of fibrosis over time

	Odds	95% C.I.	*p* Value
Steatosis grade ⩾2	5.14	1.67–15.77	0.004
Average ALT ⩾100 IU/l	5.21	1.49–18.20	0.01

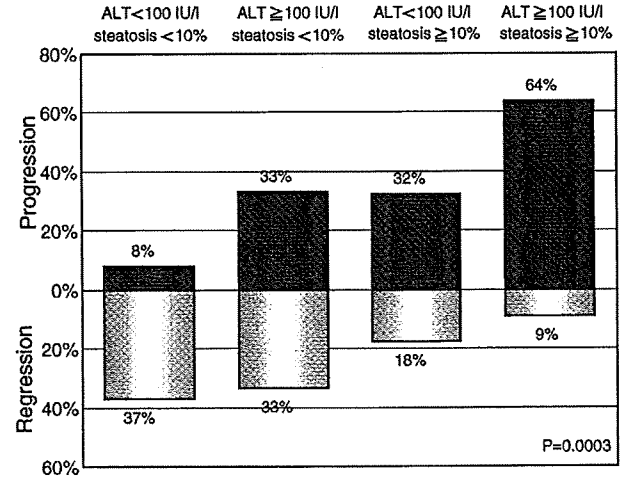

Fig. 2. Evolution of fibrosis stage in terms of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors. The upper bar chart (dark gray) indicates the progression of fibrosis while the lower bar chart (light gray) indicates the regression of fibrosis.

Table 5
Factors associated with the yearly rate of fibrosis progression

	n	Mean	SD	*p* Value
Gender				
Male	50	−0.01	0.19	0.12
Female	47	0.06	0.23	
Age at biopsy				
<60 years	73	−0.0002	0.21	0.06
⩾60 years	24	0.10	0.23	
HCV genotype				
1b	83	0.02	0.20	0.37
non-1b	14	0.08	0.32	
BMI				
<25 kg/m^2	53	0.004	0.24	0.32
⩾25 kg/m^2	44	0.05	0.19	
Steatosis on first biopsy				
0–1	58	−0.03	0.20	0.004
2–3	39	0.10	0.21	
Activity on first biopsy				
0–1	51	−0.001	0.21	0.24
2–3	46	0.05	0.22	
Fibrosis on first biopsy				
1–2	71	0.03	0.20	0.43
3	26	−0.01	0.25	
Average ALT between paired biopsies				
<100 IU/l	80	−0.01	0.17	0.0005
⩾100 IU/l	17	0.18	0.31	

4. Discussion

In the present study, we found that a higher grade of hepatic steatosis at baseline and a higher average value of ALT are independent risk factors for the progression of fibrosis over time in chronic hepatitis C patients who fail to achieve a SVR to IFN therapy. These two factors may be involved in promoting the progression of fibrosis. The association between steatosis and progression of fibrosis in untreated patients had been suggested by previous studies but this study is the first to demonstrate a similar association for treated patients. These findings are particularly important to establish a rationale for identifying therapeutic targets to halt potentially progressive disease independent of antiviral therapy.

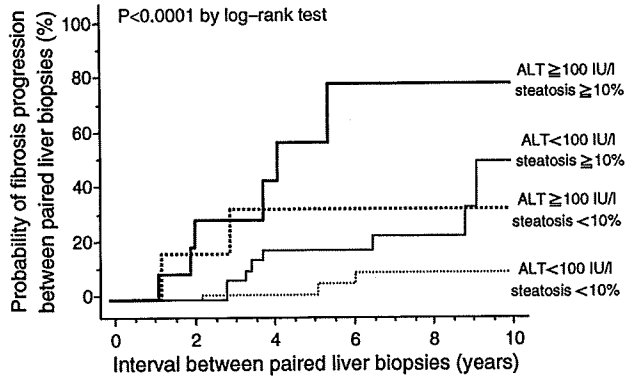

Fig. 3. Probability of fibrosis progression according to the presence of risk factors. Patients were categorized into four groups according to the presence or absence of two risk factors and the time to progression of fibrosis was analyzed.

There have been many studies that analyzed the association between steatosis and progression of liver fibrosis in HCV-infected patients, and the majority have shown a positive association [10–13], including a large scale meta-analysis [14]. However, some studies did not report this association [15–18]. There are two possible reasons for these conflicting results. First, longitudinal studies, rather than cross-sectional studies, are particularly important in the analysis of the role of steatosis in time-dependent progression of hepatic fibrosis, because cross-sectional studies involve patients with an unknown duration of steatosis. Three of four longitudinal studies that analyzed the progression of fibrosis through paired biopsies in untreated patients showed that the presence or worsening of steatosis was associated with the progression of fibrosis [12,13,20], and the probability of progression of fibrosis was significantly related to the grade of steatosis [13]. In one study, however, progression of fibrosis was correlated with older age, periportal necroinflammation and ALT elevations but not with steatosis [17]. Interestingly, steatosis was associated with older age, higher body mass index and ALT elevations in that study, indicating an indirect association of steatosis and fibrosis progression. The authors assumed that steatosis was the result rather than the cause of inflammation. This observation highlights the second reason for the controversies over a correlation between the presence of steatosis and progression of fibrosis, that is, there are so many confounding factors associated with both steatosis and fibrosis progression such as older age, advanced stage of fibrosis, higher degree of inflammation, elevated ALT, increased body mass index and insulin resistance. Because it is very difficult to prove a causal relationship between these confounding factors through clinical observations, steatosis may be a hallmark of the progression of fibrosis but it is unclear whether the effect of steatosis on progression of fibrosis is direct or mediated by other confounding factors.

Hepatic steatosis is a common pathological finding in patients with chronic hepatitis C [9]. Because the proportion of patients with steatosis is higher than would be expected from a chance association, a direct role of HCV in the pathogenesis of steatosis is suggested, at least in some patients with genotype 3 infection [21]. Furthermore, other observations suggest that steatosis may be metabolic; it is correlated with a high body mass index, visceral adiposity and insulin resistance, especially in non-3a genotypes and metabolic steatosis also is correlated with progression of fibrosis [11,22]. The

most reliable evidence that metabolic steatosis is associated with progression of fibrosis is shown by a study indicating that weight reduction in patients with chronic hepatitis C leads to a reduction in steatosis and an improvement in fibrosis, despite the persistence of HCV infection. A reduction in steatosis was significantly associated with a decrease in stellate cell activation and regression of hepatic fibrosis in 56% of patients. Thus, weight reduction may provide an important new adjunct treatment strategy for patients with chronic hepatitis C [23]. A recent study showed that the administration of pioglitazone led to metabolic and histological improvement in subjects with non-alcoholic steatohepatitis [24]. Whether amelioration of insulin resistance could improve steatosis and fibrosis in chronic hepatitis C awaits future investigation.

The mechanism by which steatosis could aggravate hepatic fibrosis in chronic hepatitis C patients remains largely hypothetical. Steatosis related insulin resistance may contribute to hyperinsulinemia and increased hepatic expression of connective tissue growth factor leading to progression of fibrosis [25]. Alternatively, a steatohepatitis-like pathway may be involved where steatosis requires a second hit for progression to fibrosis [26]. The most likely candidate is an oxidative stress with subsequent lipid peroxidation which is reported to correlate with the stage of fibrosis [27]. Another important candidate is an antiviral inflammatory response. It is reported that steatotic liver has increased susceptibility to inflammatory response [28] and that a higher grade of steatosis is correlated with a higher degree of inflammation or elevated ALT [14,15,17]. Higher degree of inflammation or elevated ALTs are associated with the progression of fibrosis [29,30], but hepatic steatosis may be responsible for the amplification of hepatic inflammation and vice versa, and the coexistence of these two factors may lead to further progression of fibrosis, as in patients with non-alcoholic steatohepatitis. In our study, average value of ALT between two biopsies was associated with fibrosis progression, whereas histological inflammation at first liver biopsy was not. The reason for this discordance may be explained by the dynamic process of hepatic necroinflammation. Severity of histological inflammation at the time of biopsy may not reflect subsequent inflammation process, whereas average value of regularly determined ALT may reflect entire fluctuation of hepatic inflammation. If so, our finding may support the hypothesis that co-operation of steatosis as the first hit and dynamic process of hepatic inflammation as the second hit promotes fibrosis progression. On the other hand, elevation of ALT may not be a mere reflection of hepatic inflammation so much as hepatocellular death such as apoptosis. Since it is reported that apoptotic caspase activation is elevated in HCV-associated steatosis [31] and that steatotic liver has increased susceptibility to apoptosis [28], elevation of ALT may also reflect an apoptosis amplified by steatosis which may lead to fibrosis progression.

Regardless of the precise mechanism, the results of the present study suggest that lowering of ALT levels may be beneficial in preventing progression of fibrosis in patients who failed to achieve a SVR. In our population, all patients received 24 weeks of IFN therapy and none received long-term maintenance therapy aiming to ameliorate hepatic inflammation. However, we speculate that amelioration of hepatic inflammation and lowering ALT levels by long-term IFN may prevent fibrosis progression in patients who remain viremic since it has been reported that IFN slowed the natural progression of fibrosis in patients who failed IFN therapy when the rate of progression of fibrosis after IFN therapy was compared to the estimated rate of progression before therapy [2,32], and that treatment duration was associated with the reduction of fibrosis independent of virological response [2]. Another possible approach to lower ALT levels may be the use of ursodeoxycholic acid, which has been reported to induce an almost 30% decrease in serum ALT levels [33,34]. The long-term efficacy of therapies targeted to the reduction of hepatic fibrosis needs future verification.

Some factors related to fibrosis progression in previous studies such as obesity [35] and worsening of steatosis [20] were not significant in our study. In our study where the majority of the population had normal body weight and very few had obesity (BMI $\geqslant 30\ kg/m^2$), impact of increased BMI on fibrosis progression may not be evaluated. Also, a smaller number of patients with worsening of steatosis (11.3% in present study and 34% in previous study [20]) may be the reason for the discrepancy. This may be due to difference in patient selection since no patients in that study had antiviral treatment between two biopsies.

In conclusion, the presence of hepatic steatosis and elevated ALT levels are risk factors for progression of fibrosis in chronic hepatitis C patients who failed to achieve a SVR to IFN therapy. These two factors may be a therapeutic target to halt the potentially progressive disease independent of antiviral therapy.

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Pretreatment Prediction of Virological Response to Peginterferon Plus Ribavirin Therapy in Chronic Hepatitis C Patients Using Viral and Host Factors

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The interferon sensitivity determining region (ISDR) of the hepatitis C virus (HCV) and T-helper type 1 and type 2 (Th1/Th2) ratio were analyzed along with other host and viral factors for their ability to predict the response of patients with chronic hepatitis C to pegylated interferon alpha-2b (Peg-IFN) and ribavirin (RBV) combination therapy. A total of 120 chronic hepatitis C patients with genotype 1 HCV and high baseline viral loads who were to undergo combination therapy scheduled for 48 weeks were enrolled. Sustained virologic response (SVR) was achieved in 54 (45%) of the 120 patients. The pretreatment factors significantly associated with SVR by logistic regression analysis were ISDR mutant [odds ratio (OR) = 86.0, $P = 0.0008$], Th1/Th2 ratio ≤ 15.5 (OR = 9.6, $P = 0.0021$), body weight 59 kg, and neutrophil count 2,300/μL. A logistic regression model to estimate SVR before combination therapy was constructed using these four factors. Patients fell into three groups when plotted according to estimated and actual SVR rates: actual SVR rate was 91% (32/35) in the high sensitivity group, 41% (15/37) in the intermediate sensitivity group, and 15% (7/48) in the low sensitivity group. Rapid or early virological responses were seen in 80% of patients with high sensitivity and who achieved SVR but were found in only 40% of patients with intermediate or low sensitivity. Null- and very late virological responses were quite rare in the high sensitivity group. In conclusion, a logistic regression model that includes the sequence of ISDR of the HCV, Th1/Th2 ratio, body weight, and neutrophil count can be useful for accurately predicting actual SVR rate before combination therapy. (HEPATOLOGY 2008;48:000-000.)

Chronic infection with hepatitis C virus (HCV) can lead to chronic hepatitis and eventually liver cirrhosis and hepatocellular carcinoma.[1] Administration of antiviral agents such as interferon (IFN) can eradicate HCV in some patients with chronic hepatitis C, and the risk of complicating hepatocellular carcinoma has been reported to decrease remarkably once this is achieved.[2-6]

HCV genotype and viral load are two major factors used to predict the response of patients with chronic hepatitis C to IFN. Patients who have genotype 1 HCV and high viral loads are relatively resistant to IFN therapy.[7] Peg-IFN and RBV combination therapy is currently the first line of therapy for these cases.[8] However, although the sustained virologic response (SVR) rate has been improved with the advent of combination therapy, it remains approximately 50%. The velocity of decrease in viral load during combination therapy is also a good indicator for predicting SVR; high SVR rates are predicted in rapid and early virological responders, whereas low SVR rates are predicted in late and nonvirological responders.[9-12]

It is considered beneficial to predict the response of patients with genotype 1 HCV and high viral load to Peg-IFN and RBV combination therapy before starting treatment because therapy can be long, costly, and have many side effects. However, prediction is often difficult in

Abbreviations: BMI, body mass index; EVR, early virologic response; HCV, hepatitis C virus; IFN, interferon; ISDR, interferon sensitivity determining region; Null-R, null-response; Peg-IFN, peginterferon-alpha-2b; RBV, ribavirin; RVR, rapid virological response; SVR, sustained virological response; Th1/Th2 ratio, T-helper type 1 and type 2 ratio; VLVR, very late virological response.

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these patients because they have already been selected as being poor responders to IFN therapy by the major prediction factors of HCV genotype and viral load.

Amino acid substitutions in the interferon sensitivity determining region (ISDR), located in HCV nonstructural region 5A, have also been reported as useful for predicting the response of patients with genotype 1 to IFN therapy.[13] Several host factors, such as immunological responses, are suggested to be associated with viral response as well.[14-22] Among them, we chose the T-helper type 1 and type 2 (Th1/Th2) ratio as a representative host factor for analysis.

In the current study, we studied whether a combination of viral and host factors, including the presence of ISDR mutants and Th1/Th2 ratio, could predict response to Peg-IFN and RBV therapy in chronic hepatitis C patients with genotype 1 HCV and high viral load.

Patients and Methods

Patients. A total of 120 patients with chronic hepatitis C were treated with Peg-IFN and RBV combination therapy at Shinshu University Hospital and the 21 member hospitals of the Shinshu Interferon Treatment Research Group. The cohort included 65 men and 55 women, ranging from 17 to 75 years of age, who were registered prospectively from December 2004 to December 2005. All patients had HCV genotype 1b and had shown high viral load for at least 6 months. High viral load was defined as serum HCV RNA equal to or greater than 10^5 international units/mL as measured by quantitative Cobas Amplicor assays (Roche Diagnostics Co. Ltd, Tokyo, Japan). Of the 120 patients, 86 had undergone a liver biopsy before combination therapy, and seven of the 86 patients were diagnosed as having cirrhosis. Exclusion criteria for patients not eligible for Peg-IFN and RBV combination therapy were as follows: (1) pregnant women or women of child-bearing potential, nursing mothers, or male patients whose partner might become pregnant; (2) patients with anemia (hemoglobin concentration of 10 g/dL or less), leukopenia (1,500/μL or less), or thrombocytopenia (80,000/μL or less); (3) patients with depression; (4) patients with serious complications in the heart, kidneys, or lungs; (5) patients with autoimmune diseases, such as autoimmune hepatitis; (6) patients infected with hepatitis B virus or human immunodeficiency virus; and (7) patients with hypersensitivity to Peg-IFN or RBV.

This study was approved by the ethics committee of Shinshu University and performed in accordance with the internationally accepted ethical standards for human experimentation. The purpose and the protocol of this study were explained to all patients, and written informed consent was obtained from each participant.

Peg-IFN and RBV Combination Therapy. Peg-IFN-α-2b (Schering-Plough K.K., Tokyo, Japan) was given in weekly doses adjusted to body weight according to manufacturer's instructions (45 kg or less; 60 μg/dose, 46 to 60 kg; 80 μg/dose, 61 to 75 kg; 100 μg/dose, 76 to 90 kg; 120 μg/dose, 91 kg or more; 150 μg/dose). Similarly, RBV (Schering-Plough K.K.) was given in daily doses adjusted to body weight according to manufacturer's instructions (60 kg or less; 600 mg/day, 61 kg to 80 kg; 800 mg/day, 81 kg or more; 1,000 mg/day). The duration of the combination therapy was set at a standard 48 weeks, but treatment extension was permitted to up to 72 weeks if the patient requested.

A rapid virologic response (RVR) was defined as undetectable serum HCV RNA at 4 weeks as measured by qualitative Cobas Amplicor assays (Roche Diagnostics Co. Ltd., Tokyo, Japan). An early virological response (EVR) was defined as detectable serum HCV RNA at 4 weeks but undetectable by 12 weeks, a late virological response was defined as serum HCV RNA detectable at 12 weeks but undetectable by 24 weeks, a very late virological response (VLVR) was defined as serum HCV RNA detectable at 24 weeks but undetectable by 48 weeks, and a null-response (Null-R) was defined as serum HCV RNA not becoming undetectable during the treatment course. An end of treatment response was defined as negative serum HCV RNA by the end of treatment. An SVR was defined as serum HCV RNA becoming undetectable during therapy and remaining so for at least 24 weeks afterwards. Responses other than SVR were regarded as non-SVR.

Achieved rates of Peg-IFN and RBV administration were calculated as the percentage of actual total dose administered of a standard total dose of 48 weeks calculated according to body weight before therapy.

Serological Tests for HCV, Hepatitis B Virus, and Human Immunodeficiency Virus. Antibodies to HCV, hepatitis B virus surface antigen, and human immunodeficiency virus were measured using commercially available enzyme-linked immunosorbent assays (International Reagents Co., Kobe, Japan). Serum HCV RNA was determined using qualitative and quantitative Cobas Amplicor assays (Roche Diagnostics Co. Ltd., Tokyo, Japan), which amplify HCV RNA using reverse transcription polymerase chain reaction. HCV genotypes were determined according to the method reported by Ohno et al.[23] Serum alanine aminotransferase and other relevant biochemical tests were performed using standard methods.

Serum Level of Ribavirin. Serum level of ribavirin was measured using a validated liquid chromatography/

Table 1. Comparison of Pretreatment Factors Between Patients With and Without SVR

Factors	SVR (n = 54)	Non-SVR (n = 66)	P
Age (years)*	59 (26-75)	63 (17-74)	0.032
Sex (male %)	74.1%	37.9%	<0.001
Body weight (kg)*	64 (45-80)	57 (38-92)	<0.001
Body mass index (kg/m^2)*	24 (18-29)	23 (16-31)	0.092
Blood transfusion history	37.0%	31.8%	0.567
Past Interferon therapy	46.3%	50.0%	0.716
Serum ALT at baseline (IU/L)*	57 (25-389)	55 (21-332)	0.516
Serum creatinine (mg/dL)*	0.71 (0.45-1.06)	0.60 (0.47-1.09)	0.011
Serum iron (mg/dL)*	129 (65-293)	144 (19-222)	0.379
White blood cell count (/μL)*	4,990 (2,800-8,770)	4,100 (1,950-7,100)	<0.001
Neutrophil count (/μL)*	2,310 (1,100-5,800)	1,967 (800-4,500)	0.015
Hemoglobin (g/dL)*	15.4 (12.8-17.6)	14.2 (10.9-17.7)	<0.001
Platelets (10^3/μL)*	183 (83-262)	158 (81-319)	0.031
HCV RNA (10^3 IU/mL)*	1215 (100 to >5000)	1850 (100 to >5000)	0.024
ISDR (W:I:M:UD)	19:11:22:2	46:15:4:1	0.002
Th1/Th2 ratio*	13.7 (3.2-63.7)	20.1 (2.0-103.3)	0.001
Fibrosis stage (F1-2:F3-4:UD)	29:7:18	32:18:16	0.508

*Data are expressed as median (range).
W, wild; I, intermediate; M, mutant; UD, undermined.

tandem mass spectrometric assay with a detection limit of 50 ng/mL.[24] Serum samples were taken at 1 and 8 weeks after starting combination therapy.

Amino Acid Substitutions in the ISDR. ISDR type was determined by the method reported by Enomoto et al.,[13] in which the HCV-J strain of genotype 1b, as reported by Kato et al.,[25] was used as the wild type. Briefly, the nucleotide sequence of ISDRs in the nonstructural 5A region was determined by direct sequencing of polymerase chain reaction amplified materials to deduce amino acid sequence. Wild-type ISDR was defined as having no amino acid substitutions, intermediate-type ISDR was defined as having one amino acid substitution, and mutant-type ISDR was defined as containing two or more amino acid substitutions.

Th1/Th2 Ratio. The Th1/Th2 ratio in peripheral blood was determined using flow cytometry according to the method reported by Kawakami et al.[26] Briefly, CD4-positive cells were extracted, then Th1 (IFN-γ+/interleukin-4−) and Th2 (IFN-γ−/interleukin-4+) cells were classified using monoclonal antibodies to IFN-γ and interleukin-4. The Th1/Th2 ratio was calculated as number of Th1 cells per number of Th2 cells.

Statistical Analyses. The Mann-Whitney *U*-test was used to analyze continuous variables. Chi-squared and Fisher's exact tests were used for analysis of categorical data. Multivariate analysis was performed using a logistic regression model with stepwise method. Each cutoff point for continuous variables was decided by receiver operating characteristic curve analysis. A *P*-value of less than 0.05 was considered significant. Statistical analyses were performed using SPSS for Windows v16.0J (SPSS Inc, Chicago, IL).

Results

Response Rate and Clinical Characteristics. SVR was achieved in 54 (45%) of the 120 patients enrolled in the current study. In total, 18 (15%) patients elected to extend treatment to up to 72 weeks, although SVR rate was similar between patients with (44%) and without (45%) the extension. Discontinuation of Peg-IFN and RBV combination therapy during treatment course was recorded in four (7%) of the 54 patients with SVR and 21 (32%) of the 66 patients with non-SVR. Of the 21 non-SVR patients who discontinued combination therapy, nine were because of side effects and 12 because of insufficient effects, namely serum HCV RNA remaining detectable at 24 weeks.

Factors Associated with SVR. Pretreatment factors that could be associated with responses to Peg-IFN and RBV combination therapy were compared between patients with and without SVR in Table 1. Patients with SVR tended to be younger than those with non-SVR and who were male. Body weight was higher in SVR patients, but body mass index (BMI) did not differ between the two groups. Median counts of white blood cells, neutrophils, and platelets and median concentrations of creatinine and hemoglobin were significantly higher in patients with SVR than in those without. Mutant ISDR was more prevalent in patients with SVR, but the Th1/Th2 ratio was significantly lower.

Predictive factors measured during treatment were also compared between patients with and without SVR (Table 2). Serum concentrations of RBV at 1 and 8 weeks of therapy did not differ between the two groups. Total ad-

Table 2. Comparison of Treatment Factors and Virological Responses During Peg-IFN and RBV Therapy Between Patients With and Without SVR

Factors	SVR (n = 55)	Non-SVR (n = 66)	P
RBV concentration during therapy			
At 1 week (ng/mL)*	1,256 (482-3455)	1453 (227-3496)	0.138
At 8 weeks (ng/mL)*	2558 (1131-12,260)	2507 (1004-6229)	0.746
Total dose administered			
Peg-IFN (μg)*	4240 (1380-5760)	3540 (200-7200)	0.001
RBV (g)*	268.8 (67.2-403.2)	166.6 (11.2-336.0)	<0.001
Achieved administration rate			
Peg-IFN (%)*	91.5 (28.0-133.0)	87.2 (4.2-147.0)	0.146
RBV (%)*	90.5 (24.0-157.0)	75.8 (4.2-138.0)	<0.001
Virological response			
RVR:EVR:LVR:VLVR:Null-R	22:23:9:0:0	4:11:14:4:33	<0.001

*Data are expressed as median (range).
Achieved administration rate for Peg-IFN and RBV was calculated as the percentage of actual dose administered of the scheduled dose for 48 weeks.
RVR, rapid virological response; EVR, early virological response; LVR, late virological response; VLVR, very late virological response; Null-R, null response.

ministered dose of Peg-IFN and RBV was significantly higher in patients with SVR. RVR and EVR were more prevalent in patients with SVR, whereas VLVR and Null-R were more prevalent in patients without.

Factors that were significantly associated with SVR by univariate analysis were then analyzed by multivariate analysis. Both pretreatment and treatment factors were analyzed together to select the pretreatment prediction factors that were independent from the treatment prediction factors. Cutoff points for continuous data were determined by receiver operating characteristic analysis and were as follows: 57 years old, body weight 59 kg, BMI 23 kg/m^2, creatinine 0.75 mg/dL, white blood cells 4,200 cells/μL, neutrophils 2,300 cells/μL, hemoglobin 15.0 g/dL, platelets 135,000 cells/μL, HCV RNA 6.0 $\times$ 10^5 international units/mL, Th1/Th2 ratio 15.5, total Peg-IFN dose 2900 μg, total RBV dose 182 g, achieved rate of Peg-IFN 73% of target amount, and achieved rate of RBV 79% of target amount. The seven factors shown in Table 3 were then evaluated by logistic regression analysis with stepwise method, indicating that mutant ISDR, Th1/Th2 ratio 15.5 or lesser, body weight 59 kg or greater, and neutrophils 2,300 cells/μL or greater were significantly associated with SVR among pretreatment factors. The odds ratio of mutant ISDR was as high as 86.0, and the odds ratios of the remaining three pretreatment factors all fell between 5.0 and 10.0. The positive predictive values of ISDR mutant, Th1/Th2 ratio 15.5 or lesser, body weight 59 kg or greater, and neutrophil count 2300 cells/μL or greater were 82.8%, 61.8%, 63.1%, and 54.1%, respectively, and negative predictive values were 67.0%, 69.2%, 76.4%, and 64.4%, respectively. As for treatment factors, RVR, EVR, and a higher dose of Peg-IFN were also found to be factors predicting SVR.

Pretreatment Prediction of SVR by Logistic Model. A logistic regression model for predicting SVR was constructed using the four pretreatment factors significantly associated with SVR by multivariate analysis:

$$R = -3.615 + 3.117 \times (\text{ISDR mutant}) + 1.732 \times (\text{Th1/Th2} \leq 15.5) + 2.184 \times (\text{body weight} \geq 59\ \text{kg}) + 1.384 \times (\text{neutrophil count} \geq 2300\ \text{cells}/\mu\text{L})$$ (each variable: yes = 1, no = 0)

$$\text{Predicted SVR rate} = 1 / (1 + \exp[-R])$$

Figure 1 shows the distribution of patients according to predicted SVR rates, which are well correlated with

Table 3. Multivariate Logistic Regression Analysis for Factors Associated with SVR

Factors	n	OR	(95% CI)	P
Pretreatment factors				
Mutant ISDR	29	86.0	(6.4-1162.3)	0.0008
Th1/Th2 ratio $\leq$ 15.5	55	9.6	(2.3-40.7)	0.0021
Body weight $\geq$ 59kg	65	6.4	(1.5-26.9)	0.0106
Neutrophil count $\geq$ 2,300/μL	61	5.5	(1.2-26.2)	0.0031
Virological response during therapy				
Rapid virological response	26	6.8	(1.0-45.8)	0.0491
Early virological response	60	12.0	(2.4-60.0)	0.0024
Treatment factor				
Total dose of Peg-IFN $\geq$ 2,900 μg	84	23.1	(2.7-194.9)	0.0039

Cutoff value for each factor was determined by receiver operating characteristic curve (ROC) analysis.

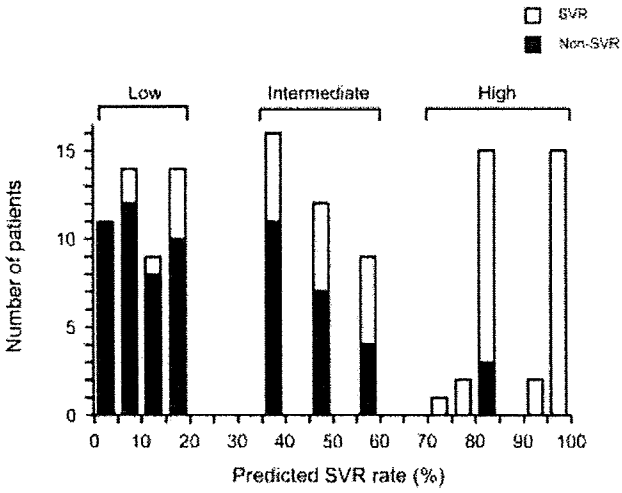

Fig. 1. Distribution of patients with and without SVR according to predicted SVR rate. Patients were classified into three groups according to distribution and named as low, intermediate, and high sensitivity groups to Peg-IFN and RBV combination therapy. Open bars indicate patients with SVR, and closed bars indicate those without SVR.

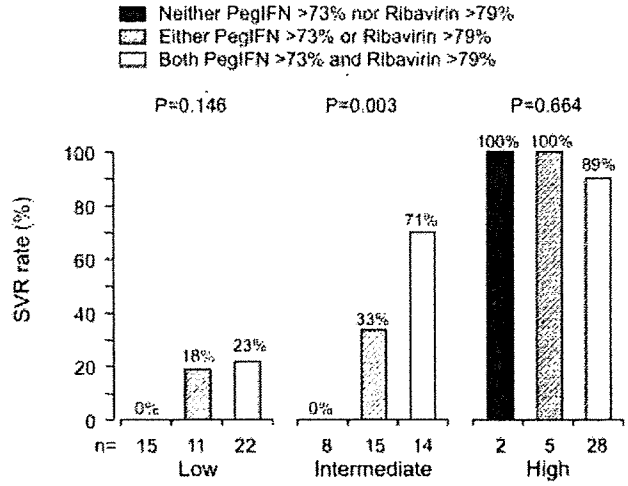

Fig. 2. Actual SVR rates were compared among the three groups of patients classified according to achieved rates of scheduled doses of Peg-IFN and RBV administration. The first group consisted of patients who were given neither a Peg-IFN dose of over 73% nor an RBV dose of over 79% of target amounts. The second group consisted of patients who received either a Peg-IFN dose of over 73% or an RBV dose of over 79% of target amounts. The third group consisted of patients who received both a Peg-IFN dose of over 73% and an RBV dose of over 79% of target amounts.

actual SVR rates. The patients were further divided into three groups: a high-sensitivity group for patients whose predicted SVR rates were in the upper one third (>66%), an intermediate group for SVR rates in the middle one third (33%-66%), and a low-sensitivity group for rates in the lower one third (<33%). The actual SVR rates were 91% (32/35), 41% (15/37), and 15% (7/48) in the high, intermediate, and low sensitivity groups, respectively.

The actual SVR rates were then compared among the three groups classified according to standard doses of Peg-IFN and RBV administration (Fig. 2). The first group consisted of patients who took lesser amounts of treatment (Peg-IFN dose less than 73% and RBV dose less than 79% of target amounts). The second group received Peg-IFN over 73% or RBV over 79% of target amounts. The third group consisted of patients who received both Peg-IFN over 73% and RBV over 79% of target amounts. SVR rates were similarly low among patients with low sensitivity and high among patients with high sensitivity, but rose with increases of Peg-IFN and RBV doses received in patients with intermediate sensitivity.

The SVR rates in patients with RVR, EVR, late virological response, VLVR, and Null-R were 85% (22/26), 68% (23/34), 39% (9/23), 0% (0/5), and 0% (0/32), respectively. Distributions of actual virological responses during therapy are shown according to the estimated sensitivity of low, intermediate, and high in Fig. 3, and are significantly associated with the estimated sensitivities. RVR and EVR were seen in 80% of patients with high sensitivity, but were less than 40% in patients with intermediate or low sensitivity. Null-R and VLVR were quite rare in the high-sensitivity group. All patients with RVR and EVR in the high-sensitivity group achieved SVR.

Discussion

Besides HCV genotype and viral load, several factors, including age, sex, race, BMI, HCV mutations, and host

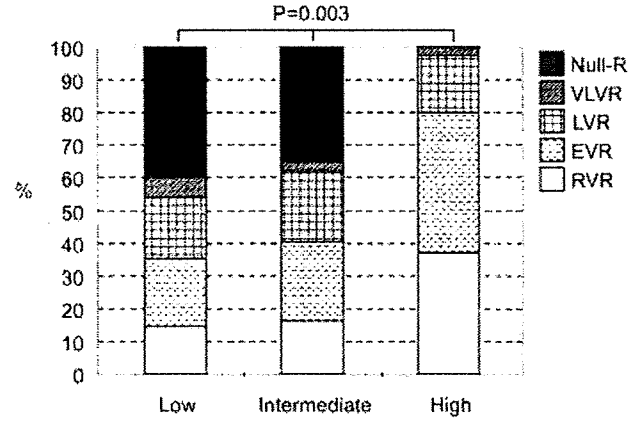

Fig. 3. Distribution of actual virological responses during combination therapy shown according to estimated sensitivities of low, intermediate, and high. Null-R, null-response; VLVR, very late virologic response; LVR, late virological response; EVR: early virological response; RVR, rapid virological response.

immunological parameters, have been reported to be associated with SVR rates in patients with chronic hepatitis C treated with Peg-IFN and RBV combination therapy. However, virological responses during therapy, such as RVR and EVR, are now widely used for predicting final virological response because such treatment factors have even higher predictive value.[9-12] Nonetheless, it is obvious that predictions made before administration of therapy are more desirable than those done during treatment course if accuracy of prediction is comparable. We therefore planned the current study to find such a way to predict SVR before starting Peg-IFN and RBV combination therapy in patients already known to be resistant.

Several regions in the HCV genome have been reported to be associated with sensitivity to interferon therapy. Enomoto et al.[13] reported that a higher number of amino acid substitutions in the ISDR (NS5A, a.a. 2209-2248) were strongly associated with a favorable response to IFN-α monotherapy in patients with genotype 1 HCV.[13] It is postulated that the NS5A protein, in which the ISDR exists, has transcriptional activation functions and represses interferon-induced gene expression.[27] The ISDR overlaps a putative acidic amino acid region that confers transcriptional activity.[28] Akuta et al.[29] reported that amino acid substitutions of R by Q at a.a. 70 or L by M at a.a. 91 in the core region were significantly frequent in patients who showed a null or weak response to combination treatment of 48 weeks. As such, we chose the ISDR among all HCV genetic factors for analysis because it has already been well characterized.[30]

Immunological backgrounds are known to be associated with response to IFN therapy because cellular immune functions are essential to eliminate HCV-infected hepatocytes. Masaki et al.[15] reported that a lower Th1/Th2 ratio before IFN monotherapy was a significant host factor for predicting long-term virological response in Japanese patients with chronic hepatitis C. Lee et al.[31] reported that high baseline sCD30 levels predicted an early and sustained virological response to IFN and RBV therapy, and suggested that therapy might be more effective in patients with a predominant T2 profile. Lagging et al.[32] reported that low levels of a 10-kDa IFN-γ inducible protein predicted rapid and sustained virological response in patients with genotype 1 HCV treated with Peg-IFN and RBV combination therapy. Taken together, these results indicate that an imbalance of Th1 and Th2 subsets before IFN therapy is possibly associated with long-term therapy outcome. In the current study, we chose the Th1/Th2 ratio in peripheral blood as an immunological marker for predicting SVR because identification of helper T cell subpopulations at the cellular level has become practical with the development of intracellular cytokine assays using flow cytometry.

Of the virological and host factors analyzed in the current study, mutant ISDR, Th1/Th2 ratio 15.5 or less, body weight 59 kg, and neutrophil count 2300 cells/μL were selected as significant pretreatment factors predicting a higher rate of SVR. Although our study suggests that higher body weight is a favorable predictor for SVR in the Japanese, several studies on Caucasians have shown that higher body weight or BMI results in a lower SVR rate.[33,34] This difference may be attributed to a difference in average body weight among the studies; whereas median body weight was over 70 kg and patients with body weights of less than 59 kg were quite rare in studies reported from the United States and Europe, the median body weight in our study was 60.7 kg, and patients with body weights of less than 59 kg accounted for 45.8% of our cohort. According to manufacturer's instructions, patients with body weights equal to or less than 60 kg received 80 μg of Peg-IFN-α-2b and 600 mg RBV as initial doses in the current study. It is possible that such doses were insufficient to achieve a high SVR rate despite having been adjusted accordingly. This possibility is further supported by our result that the distribution of BMI did not differ between patients with and without SVR.

A higher neutrophil count was also a significant pretreatment factor predicting a greater likelihood of SVR. Patients with chronic hepatitis C who have advanced fibrosis tend to show lower counts of neutrophils and platelets, which can interfere with administration of Peg-IFN, another significant treatment factor identified in this study. Indeed, pretreatment counts of neutrophils were significantly higher in patients who received sufficient (73% or more) Peg-IFN doses than in those who did not.

Interestingly, a lower Th1/Th2 ratio predicted a higher SVR rate in our study, contrary to the common knowledge that a stronger Th1 response is important to eradicate HCV; Shinohara et al.[35] reported that a higher increase in Th1 response during the early phase of interferon therapy was associated with higher SVR rates. However, these patients also showed a lower Th1/Th2 ratio before starting therapy than those without SVR. Thus, our result that a lower Th1/Th2 ratio predicts a favorable response does not necessarily refute the importance of the Th1 response in eradicating HCV. Further studies are required to clarify the significance of the Th1/Th2 ratio.

The logistic regression model for predicting SVR in this study yielded three patient groups classified according to predicted SVR rate. The actual SVR rates were 91% in the high sensitivity group, 41% in the intermediate sensitivity group, and 15% in the low sensitivity group, and all were well correlated with predicted SVR rates. Earlier

clearance of HCV viremia during combination therapy has been reported to be a good indicator for higher SVR rate. As expected, rapid and early virological responses were seen in 80% of patients with high sensitivity but were found in only 40% of patients with intermediate or low sensitivity. Null or very late virological responders were quite rare in the high-sensitivity group. Because all rapid and early virological responders achieved SVR, patients who are judged to have high sensitivity are strongly recommended to take Peg-IFN and RBV combination therapy.

It is noteworthy that actual SVR rates were similarly low in the low-sensitivity group and high in the high-sensitivity group irrespective of total doses of Peg-IFN and RBV administered, but were significantly associated with total dose in the intermediate-sensitivity group. Thus, efforts to maintain at least standard administration doses of Peg-IFN and RBV are important to achieve higher SVR rates in patients who have intermediate sensitivity to combination therapy.

Several pretreatment factors other than ours have been reported to be associated with virological response to IFN and RBV combination therapy in patients with genotype 1b HCV infection, including serum levels of low-density lipoprotein cholesterol and insulin resistance. However, the results obtained here are valuable because the estimated sensitivities to combination therapy are very closely associated with actual sensitivities. Our algorithm cannot be applied to other populations directly because clinical backgrounds, such as distribution of body weight, differ globally. However, a number of mutations in the ISDR sequence of the hepatitis C virus have been shown to be associated with response to IFN therapy in a worldwide meta-analysis reported by Pascu et al.[30] Immunological factors, such as Th1/Th2 imbalance, have also been reported to be associated with treatment response in the world.[16,17] As such, the current study is applicable on a global scale because it clearly shows that a combination of viral and host factors, including those of immunological nature, are effective for predicting the response to combination therapy before it is started.

In conclusion, in Japanese patients from Nagano, SVR rates of Peg-IFN and RBV combination therapy can be accurately predicted using the pretreatment factors of ISDR mutations, Th1/Th2 ratios, body weights, and neutrophil counts. This being established, future prospective trials are required to validate our results in other regions of Japan and in other countries.

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Appendix A

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CLINICAL REVIEW

History and prevention of de novo hepatitis B virus-related hepatitis in Japan and the world

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Abstract Hepatitis B virus (HBV) replication has been shown to persist at low levels in the liver for decades, even in patients with resolved HBV infection. In these cases, reactivation of HBV and ensuing hepatitis during or after cytotoxic or immunosuppressive therapy is now recognized as de novo HBV-related hepatitis. The occurrence of de novo HBV-related hepatitis has become more frequent after the introduction of rituximab for the treatment of hematological disorders, such as malignant lymphomas. More alarmingly, reactivation can lead to fatal fulminant hepatic failure, indicating a need to establish guidelines to prevent the occurrence of de novo HBV-related hepatitis. It is possible that lamivudine prophylaxis and close surveillance of serum HBV DNA are effective in this regard. However, such measures are currently not available to hepatitis B surface antigen (HBsAg)-negative patients in Japan. A preliminary guideline for preventing HBV reactivation during and after cytotoxic or immunosuppressive therapies was made in 2008 by two collaborative study groups from the Japanese Ministry of Health, Labour, and Welfare, including measures not only for HBV carriers, but also for patients with resolved HBV infection. Since this recommendation is a tentative one, further testing and improvements are being planned.

Keywords Hepatitis B virus · De novo hepatitis · Reactivation · Immunosuppression · Prevention

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History and prevention

Approximately 3 billion people have been exposed to the hepatitis B virus (HBV), and there are an estimated 350 million chronic carriers worldwide [1–3]. The clearance of circulating hepatitis B surface antigen (HBsAg) and appearance of antibody to HBsAg (anti-HBs) with normalization of liver function were generally considered as evidence of complete clearance of HBV from hosts until the early 1990s. Since then, it has been shown that HBV replication persists at low levels in the liver and peripheral blood mononuclear cells for decades, even in HBsAg-negative patients with resolved HBV infection [4–6]. In such patients, HBV replication is suppressed by immune responses to HBV, including specific cytotoxic T lymphocyte (CTL)-mediated responses [4].

HBV reactivation in patients with resolved infection is being reported in increasing numbers because the number of people undergoing strong immunosuppressive therapy is increasing worldwide, especially patients with malignant neoplasms, autoimmune disorders, and following transplantation for prevention of rejection. In those patients with resolved HBV infection, reactivation of HBV and ensuing hepatitis is recognized as de novo HBV-related hepatitis, which sometimes leads to fulminant hepatic failure (FHF) and is thus becoming an alarming, well-recognized complication of immunosuppressive therapy that needs further attention [7–9].

Wands et al. [10] reported that reseroconversion from anti-HBs to HBsAg was observed in 5 (13%) of 40 patients with myeloproliferative or lymphoproliferative disorders who received cytotoxic chemotherapy in 1975. This might have been the first report suggesting an association between HBV reactivation and chemotherapy in patients who showed the serological markers of resolved HBV infection.

However, the clinical significance of these phenomena was not clear at that time because our knowledge regarding occult HBV infection was limited. In 1991, Lok et al. reported a prospective study on hepatitis associated with HBV reactivation in patients with malignant lymphoma who received cytotoxic chemotherapy [11]. In their study, HBV reactivation and resulting hepatitis was common in patients who were HBV carriers, but rare (2%) in patients with resolved HBV infection. On the other hand, de novo HBV hepatitis was relatively frequent (14–50%) in patients with hematological disorders who received allogenic bone marrow transplantation [12–16]. This difference in frequency could be attributed to the difference in extent of immunosuppression between the treatments; generally, immune responses are suppressed more profoundly in patients with allogenic bone marrow transplantation than in those with ordinary cytotoxic chemotherapy.

The recent occurrence of HBV reactivation hepatitis in the treatment of hematological disorders, such as malignant lymphomas, has drawn the attention of both hepatologists and hematologists [7, 9]. It is considered to be caused mainly by the introduction of rituximab, which is used mainly for the treatment of B-cell-type malignant lymphomas. Accordingly, the US Food and Drug Administration reported a possible relationship between FHF and rituximab use in October 2004. Rituximab is a genetically engineered chimeric murine/human monoclonal antibody against the CD20 antigen found on the surface of normal and malignant B lymphomas and is used alone or in combination with cytotoxic chemotherapeutic drugs [17, 18]. Dervite et al. [19] first reported a possible relationship between HBV reactivation and rituximab use in a patient with resolved HBV infection in 2001. Following that report, several cases of de novo HBV-related hepatitis after treatment with rituximab that proved fatal were reported [20–24]. B cells may act as antigen-presenting cells and prime CTL responses in HBV infection. Rituximab induces profound and durable B cell depletion to an extent of 0% CD20-positive cells, thus possibly enabling reactivation of CTL-suppressed HBV replication to occur.

Hui et al. [25] evaluated the risk of developing de novo HBV-related hepatitis after chemotherapy in Hong Kong. They prospectively followed 244 patients with lymphomas who were negative for HBsAg for a median period of 12.4 months. In their report, eight (3.3%) patients developed de novo HBV-related hepatitis. These eight patients were presumed to have occult HBV infection because they were found to be positive for at least one anti-HBs and for anti-HBc. Of these patients, three developed FHF, one of whom died. Multivariate analysis showed that de novo hepatitis was independently associated with higher risk of FHF, with a relative risk of 29.9. A risk factor for developing de novo hepatitis was the use of a rituximab plus steroid-containing regime. In addition, elevation of HBV DNA, HBsAg, and alanine aminotransaminase (ALT) values were seen after finishing chemotherapy. Since a 100-fold increase in serum HBV DNA preceded the onset of de novo hepatitis by a median period of 18.5 (range 12–28) weeks, Hui recommended close surveillance for such an increase so that antiviral therapy could be initiated as quickly as possible. On the other hand, Liu et al. suggested the possibility that short-term lamivudine prophylaxis during chemotherapy for patients with occult HBV infection is more cost effective than close surveillance of serum HBV DNA [26]. Further studies are required to clarify this matter.

De novo HBV-related hepatitis after orthotopic liver transplantation was first reported by Douglas et al. in 1993 [27]. Uemoto et al. [28] clarified that HBV strains found in a donor liver before transplantation were the same as those found in a corresponding recipient who developed de novo hepatitis by comparing nucleotide sequences in the HBV genome. Rokuhara et al. [29] also showed that those two strains of HBV were identical by determining the full nucleotide sequence of the HBV genome. According to reports so far, the incidence of de novo hepatitis ranges from 33% to 94% when livers are transplanted from donors with anti-HBc, and from 0% to 0.5% when livers are transplanted from donors without [27–32] indicating that de novo HBV-related hepatitis in liver transplantation recipients is closely associated with occult HBV infection in donor livers. Since the occurrence of de novo hepatitis is quite frequent when donors show serological markers of resolved HBV infection, it may be prevented by the administration of hepatitis B immunoglobulins and nucleot(s)ide analogues in combination [33].

The state of de novo HBV-related hepatitis in Japan was surveyed in 2005 by a group directed by Dr. Kumada (Toranomon Hospital) from the Ministry of Health, Labour, and Welfare of Japan (study for standardization of treatment of viral liver diseases including liver cirrhosis) [34]. In this retrospective study, a total of 55 patients with de novo HBV-related hepatitis were seen between January 2000 and December 2004 in 90 hospitals. During the same period, approximately 1,000 patients with typical acute hepatitis B were diagnosed in those hospitals. Among the 55 patients with de novo HBV-related hepatitis, 27% developed FHF, compared with only 7% of patients with acute hepatitis B. It is noteworthy that mortality was as high as 100% in patients with de novo hepatitis who developed FHF [8]. Taken together, it is evident that de novo HBV-related hepatitis with a strong tendency to develop into FHF with high mortality is an important issue that needs to be addressed.

There seem to be no official guidelines for preventing de novo HBV-related hepatitis occurring during or after cytotoxic or immunosuppressive therapy in the world. The American Association for the Study of Liver Diseases has

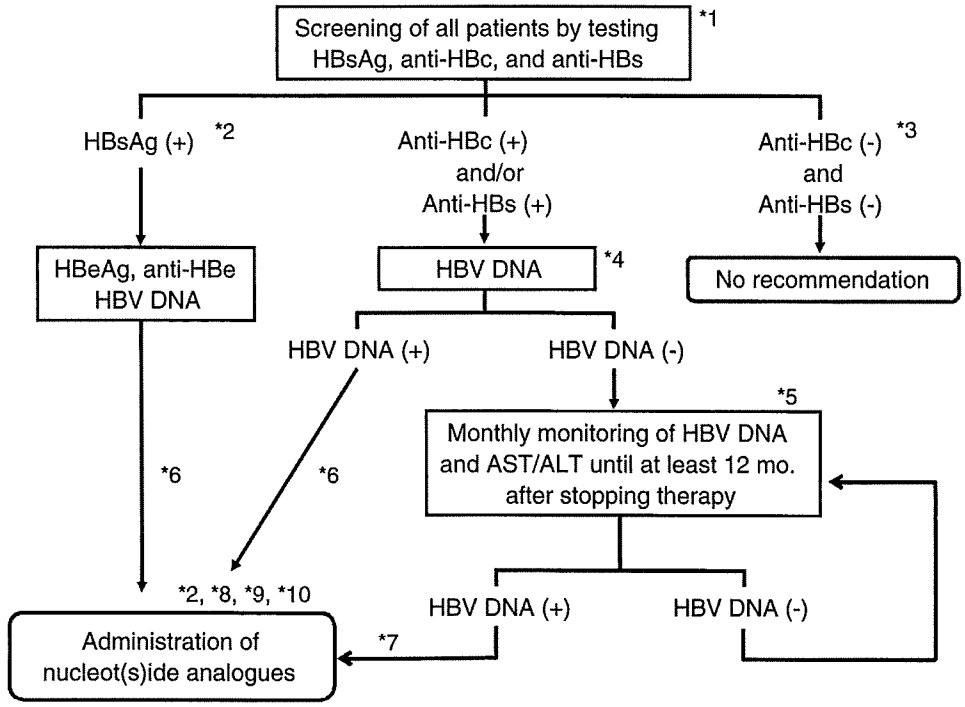

Fig. 1 Recommendation for preventing hepatitis B due to reactivation of HBV during and after immunosuppressive or cytotoxic therapies. The prophylactic administration of nucleot(s)ide analogues recommended here is not completely based on evidence, and thus does not necessarily guarantee the prevention of fulminant hepatic failure. *1 Measurement using chemiluminescence immunosorbent assay is recommended. *2 Consultation with a hepatologist is recommended. *3 It is possible that anti-HBc and anti-HBs becomes undetectable during immunosuppressive or cytotoxic therapies. Additional measurement of HBV DNA is recommended in these cases. *4 HBV DNA is recommended to be tested by methods having the highest sensitivity available. *5 A high risk of developing de novo HBV-related hepatitis should be noted in patients who receive a rituximab plus steroids regime or in those who underwent hematopoietic cell transplantation. Fuldarabine can suppress immune responses profoundly and thus its use requires attention; however, its potential for causing reactivation of HBV is currently unknown. *6 Administration of nucleot(s)ide analogues is recommended as early as possible before starting immunosuppressive or cytotoxic therapies. *7 Administration of nucleot(s)ide analogues is recommended as soon as possible when HBV DNA becomes detectable during immunosuppressive or cytotoxic therapies. *8 Entecavir is recommended among nucleot(s)ide analogues. *9 In HBV-carrier patients, discontinuation of nucleot(s)ide analogues can be considered when patients meet the conditions shown in the guidelines for the treatment of hepatitis B patients prepared by Dr. Kumada and the Ministry of Health, Labour, and Welfare of Japan in 2008. In patients with resolved HBV infection at screening, discontinuation of nucleot(s)ide analogues can be considered when patients meet all of the following conditions: (1) the period after discontinuing immunosuppressive or cytotoxic therapies is at least 12 months, (2) ALT levels are within normal range during this period, and (3) HBV DNA levels are under detection limits during this period. *10 Patients should be followed carefully for 12 months after discontinuing nucleot(s)ide analogues. If serum HBV DNA becomes detectable during the follow-up period, administration of nucleot(s)ide analogues should be recommenced as soon as possible

published the antiviral prophylaxis for HBV carriers, but not for patients with resolved HBV infection [35]. It is possible that nucleot(s)ide analogue prophylaxis and close surveillance of serum HBV DNA are effective to prevent occurrence of de novo HBV-related hepatitis [25, 26]. However, such measures are currently not available to HBsAg-negative patients in Japan. The incidence of de novo HBV-related hepatitis is expected to increase in the future with the advent of stronger immunosuppressive and cytotoxic drugs, such as rituximab. As de novo hepatitis sometimes causes fatal FHF that cannot be controlled with nucleot(s)ide analogues after the onset of hepatitis, guidelines to prevent the occurrence of reactivation are needed.

A preliminary recommendation for preventing HBV reactivation during and after cytotoxic or immunosuppressive therapies was prepared in 2008 by two study groups from the Ministry of Health, Labour, and Welfare of Japan in collaboration: the study group for the standardization of treatment of viral liver diseases including liver cirrhosis directed by Dr. Kumada (Toranomon Hospital), and the study group for intractable liver and biliary tract diseases directed by Dr. Tsubouchi (Kagoshima University). This recommendation includes measures not only for HBV carriers, but also for patients with resolved HBV infection, as shown in Fig. 1. Although the guideline is tentative and may need future amendments, it nonetheless represents a first step in addressing the increasing global problem of de novo HBV-related hepatitis.

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BRIEF REPORT

Mortality Secondary to Fulminant Hepatic Failure in Patients with Prior Resolution of Hepatitis B Virus Infection in Japan

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Hepatitis B virus (HBV) reactivation in patients with resolved HBV infection was found in 23 (4%) of 552 newly hepatitis B surface antigen–positive patients in Japan. Because one-fourth of cases develop into fulminant hepatic failure and mortality is 100%, management of HBV reactivation in patients with resolved HBV infection should be discussed.

Reactivation of hepatitis B virus (HBV) is becoming a well-recognized complication in patients with chronic HBV infection who are undergoing cytotoxic chemotherapy or immunosuppressive therapy [1–5]. HBV reactivation has a variety of manifestations, ranging from subclinical increases in transaminase activity to severe and potentially fatal fulminant hepatic failure (FHF). Because clinical studies have demonstrated that lamivudine therapy reduces the rate of HBV reactivation and mortality [6–9], prophylactic antiviral therapy is advised for HBV carriers at the onset of chemotherapy [10].

The clearance of hepatitis B surface antigen (HBsAg) and the appearance of antibody to HBsAg, with normalization of liver function, is generally accepted as evidence of clinical and serologic recovery from acute hepatitis B. However, HBV replication has been shown to persist at low levels in the liver for decades [11–13], which may explain the recent increase in the rate of HBV reactivation in patients with resolved infection during or after chemotherapy and transplantation [1, 5, 14–16]. Although reactivation led to FHF and even death in some cases [17–22], the incidence of and mortality associated with HBV reactivation have not been fully clarified in this context. Recently, a prospective study [23] from Hong Kong revealed that 3.3% of HBsAg-negative patients developed HBV reactivation after chemotherapy. In Japan, because ~20% of individuals are positive for at least 1 HBV marker [24], HBV reactivation during or after immunosuppressive treatment may become a critical issue in the near future. Thus, we investigated the mortality associated with and prevalence and clinical significance of HBV reactivation in Japanese patients with resolved HBV infection in a multicenter, cross-sectional study.

Methods. In 2005, we sent a questionnaire to 230 hospitals certified by the Japan Society of Hepatology; this included questions about patients who had become newly positive for serum HBsAg from January 2000 through December 2004 [25]. A total of 1239 patients were registered by 93 hospitals (40%). Of those patients, 55 were recorded as having experienced HBV reactivation after having resolved HBV infection, and the remaining 1184 patients were classified as having acute hepatitis B. Sixty-three (68%) of 93 hospitals responded to a second survey and provided information on 552 patients enrolled in this study; 23 of these patients developed HBV reactivation, and 529 had acute hepatitis B.

HBV reactivation was defined (according to a slight modification of the report by Hui et al. [23]) as a decrease in the level of antibody to HBsAg that was associated with the reappearance of HBsAg, a 3-fold elevation of serum alanine aminotransferase (ALT) level above normal, and detection of HBV DNA in serum during or after chemotherapy. The diagnoses of acute hepatitis B and FHF were defined as reported elsewhere [26]. Patients with other liver diseases were excluded. Serum HBV markers were determined as reported elsewhere [26]. Serum levels of HBV DNA were determined with use of Amplicor HBV Monitor kits (Roche Diagnostics) at each hospital when the patients were admitted. HBV genotypes were determined with use of the PCR-invader method, with genotype-specific probes [27]. This study was approved by the ethics committees of appropriate institutional review boards. Informed consent was obtained from each patient in accordance with the Helsinki Declaration.

The Mann-Whitney *U* test was used to analyze continuous variables. The χ^2 test with Yate's correction was used for analysis of categorical data. In cases in which the number of patients was <5, Fisher's exact test was used. $P \leq .05$ was considered to

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