switched to the combination of PEG-IFN and RBV in recent years, it is important to know if a larger dose of IFN is beneficial to patients with chronic hepatitis C.

Many molecular mechanisms through which HCV evades host innate immunity have been reported to date. HCV core, E2 and NS5A proteins have been reported to inhibit the IFN signaling system [Gale et al., 1997; Taylor et al., 1999; Blindenbacher et al., 2003; Bode et al., 2003; Foy et al., 2003; Lin et al., 2006; Ciccaglione et al., 2007]. Variations of amino acid (aa) sequences in the E2 and the NS5A region have been reported to correlate with the effect of IFN therapy [Enomoto et al., 1996; Chayama et al., 1997, 2000; Polyak et al., 1998, 2000; Hashimoto et al., 1999; Puig-Basagoiti et al., 2001; Pascu et al., 2004; Gaudy et al., 2005; Brillet et al., 2007; Torres-Puente et al., 2008]. Recently, Akuta et al. [2005, 2006, 2007a, b] reported that substitution of aa 70 and/or 91 in the core region is an independent and significant predictor of non-virological response.

The aim of the present study was to evaluate the therapeutic efficacy and safety of a large dose of IFN-α-2b combined with RBV. For this purpose, a randomized trial was conducted to compare the therapeutic effects of high-dose (10 MU) versus standard dose (6 MU) of IFN-α-2b combined with RBV in patients with high HCV viral titers. The second endpoint of this study was to analyze the predictive factors associated with virological response including aa substitutions in the core region and the NS5A region.

PATIENTS AND METHODS

Patient Selection

Two hundred adult patients enrolled into the study. The inclusion criteria were positivity for antibody to HCV, HCV RNA levels higher than 100 KIU/ml, and the diagnosis of chronic hepatitis C was confirmed by liver biopsy. The liver biopsy specimens were evaluated as described by Desmet et al. [1994], and classified into F0 to F3. None of the patients included in this study had liver cirrhosis (F4). Other exclusion criteria included leukocytopenia (leukocyte <4,000/mm^3) and anemia (hemoglobin concentration <10 g/dl). Patients with human immunodeficiency or hepatitis B super infection, previous organ transplantation, other causes of liver disease, poorly controlled diabetes, de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia or auto-immune type diseases were also excluded.

Study Design

The double-blind, multi-center randomized clinical trial was conducted in 23 centers in Hiroshima city (The Hiroshima Liver Study Group). The study was approved by the Ethics Committee of Hiroshima University. Written informed consent was obtained form all participants. Eligible patients were assigned randomly into either of the two groups without further stratification using sequentially numbered cards in sealed envelopes. Patients were randomized to treatment with combination of IFN-α-2b (Intron A, Shering Plough, Kenilworth, NJ) at a dose of 6 MU (Group A) or 10 MU (Group B) plus RBV (Rebetol, Shering Plough). IFN-α-2b was administered intramuscularly daily over the initial 2 weeks and three times weekly in the remaining 22 weeks. The dose of RBV was adjusted according to body weight (600 mg/day for ≤60 kg, 800 mg/day for >60 kg). Adverse events were monitored clinically by careful interview and hematological examination throughout the study. The dosage of RBV was reduced in patients who experienced a decrease in hemoglobin concentration to <10 g/dl.

Blood samples were taken 2 and 4 weeks after the beginning of therapy and every 4 weeks thereafter. Biochemical and hematological tests were performed in each center, including alanine amino transferase (ALT). Part of the serum samples were kept frozen at −80°C until further analysis. Viral genotypes were determined by phylogenetic analysis after reverse transcription (RT)-polymerase chain reaction (PCR) and direct sequencing.

Assessment of Efficacy

Serum HCV RNA was detected by nested PCR assay (Cobas Amplicor HCV test v 2.0, Roche Diagnostics, Tokyo, Japan; limit of detection, 50 IU/ml) at weeks 2, 4 and every 4 weeks during treatment and 24 weeks after the cessation of therapy. Positive samples were analyzed further by quantitative assay (Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics; limit of detection, 500 IU/ml).

The primary endpoint of this study was sustained virological response, defined as undetectable serum HCV RNA by qualitative PCR test and normalization of ALT 24 weeks after the treatment. Non-virological response was applied to those patients with positive qualitative HCV RNA PCR tests in all examinations. Virological response was used to define the remaining patients who became PCR negative at least once during the treatment.

Nucleotide Sequencing of the Core and NS5A Gene

The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR] [Enomoto et al., 1996]) sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse) as described by Akuta et al. [2005, 2006, 2007a, b]. After denaturation at 95°C for 5 min, 35

cycles of amplification were set as follows; denaturation for 30 sec at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. (b) Nucleotide sequences of ISDR in NS5A: PCR was performed with IM11 (forward, 5′-TTC CAC TAC GTG ACG GGC AT-3′) and 5OA2KI (reverse, 5′-CCC GTC CAT GTG TAG GAC AT-3′). After denaturation at 98°C for 30 sec, 35 cycles of amplification were set as follows; denaturation for 10 sec at 98°C, annealing of primers for 30 sec at 66°C, and extension for 15 sec at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated in a 2% agarose gel and purified by GENE-CLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [Kato et al., 1990].

Quantitation of HCV Core Antigen

HCV core antigen levels were measured using stored serum samples just before and 4 weeks after the start of the therapy as described previously [Aoyagi et al., 1999].

Statistical Analysis

The baseline characteristics of the patients in the two groups were compared and the differences were assessed by Chi-square test with Yate's correction and Mann–Whitney *U*-test. To assess the sustained virological response rates, an intention-to-treat (ITT) analysis and a per-protocol (PP) analysis were conducted. The response rates and substitutions in the core region and the ISDR were compared by Fisher's exact test. All *P* values reported are two-sided and those less than 0.05 were considered significant. To determine the predictors of sustained virological and non-virological responses, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors included the following variables: age, sex, alcohol consumption, past history of IFN monotherapy, body mass index, ALT, hemoglobin, platelets, HCV RNA level, genotype, liver histology, total RBV dose (adjusted for body weight [mg/kg]) and total dose of IFN-α-2b. The odds ratio and 95% confidence intervals (95% CI) were also calculated. Variables with statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

Patient Demographics

Patient enrollment started in January 2002, and the trial ended in March 2005. The disposition of patients throughout the trial is shown in Figure 1. A total of 200 patients were randomized to treatment, and 198 patients met the eligibility criteria and underwent

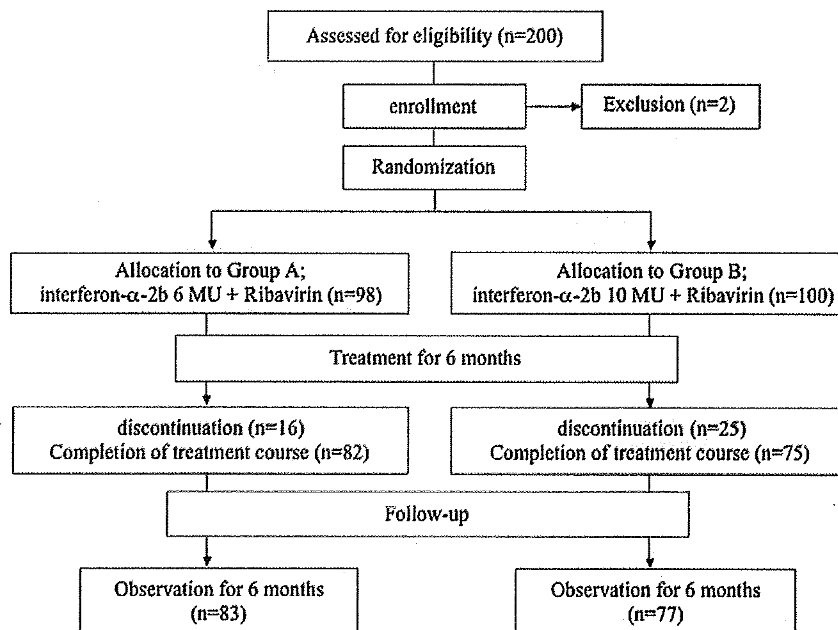

Fig. 1. Flow chart of number of patients throughout the trial. A total of 200 patients were included in this study. One hundred ninety-eight patients met the eligibility criteria and they underwent randomization, 98 patients in Group A and 100 patients in Group B.

TABLE I. Baseline Characteristics of the Patients

Characteristic	Group A (n = 98)	Group B (n = 100)	P
Age (years)[a]	55 ± 10.3	55 ± 11.0	0.43
Male sex (%)	63	75	0.07
Alcohol consumption (%)[b]	23	20	0.61
Past history of IFN monotherapy (%)	33	35	0.72
Body-mass index (kg/m^2)[a]	23.3 ± 2.9	24.2 ± 3.6	0.05
ALT (IU/L)[a]	79.2 ± 45.3	109.4 ± 111.2	0.31
Hemoglobin (g/dl)[a]	14.2 ± 1.4	14.5 ± 1.2	0.02
Platelets (×10^4/mm^3)[a]	14.8 ± 4.8	16.5 ± 5.0	<0.05
HCV RNA (KIU/ml) (%)			
100–850	49	47	
≥850	51	53	0.80
Genotype (%)			
1b	82	72	
2a/2b	17	28	0.32
3a/3b	1	0	
Liver histology[a,c]	2.0 ± 0.84	1.8 ± 0.82	0.05

ALT, alanine aminotransferase.
[a]Values are mean ± SD.
[b]Percentage of patients who consumed alcohol at >30 g/day.
[c]Liver fibrosis was scored 0 (F0), no fibrosis; 1 (F1), periportal expansion; 2 (F2), portoportal septa; 3 (F3), portocentral linkage or bridging fibrosis.

randomization. Ninety-eight patients were assigned to Group A and 100 patients to Group B. Patients were observed for 24 weeks after the treatment. Sixteen patients of Group A and 25 patients of Group B discontinued the treatment because of adverse events. Table I lists the baseline characteristics of the patients. Hemoglobin concentrations and platelet counts were higher in group B patients. The other parameters were similar between the two groups.

Overall Sustained Virological Response

The effect of therapy in the two groups is summarized in Table II. The sustained virological response rate was lower significantly in patients of group B with genotype 2a/b relative to those of group A (ITT analysis). This reflects the fact that a larger number of patients dropped out form the protocol because of the adverse effects (1 [6%] of 16 in group A and 10 [43%] of 23 in group B, $P = 0.02$). All patients who stopped treatment did not achieve sustained virological response. Patients with genotype 1b had a lower sustained virological response rate than those with genotype 2a/b (33/124 [27%] vs. 26/39 [67%], $P < 0.01$).

Dose Reduction or Discontinuation and Adverse Events

Table III summarizes the laboratory abnormalities and the dose reduction and discontinuation of IFN-α-2b and RBV due to adverse events. The overall discontinuation rate was 16% for group A and 25% for group B (not significant). The most frequent adverse event associated with dose reduction was anemia. A larger number of patients of group B developed depression ($P = 0.02$).

Predictive Factors Associated With Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response: age (<60 years, $P = 0.007$); genotype (2a/b, $P < 0.001$); and platelet count ($>15 \times 10^4$/mm^3, $P = 0.01$). Multivariate analysis including the above variables identified two parameters that independently predicted sustained virological response: age ($P = 0.02$) and genotype ($P < 0.001$) (Table IV).

TABLE II. Rates of Sustained Virological Response According to Adherence

Genotype	Group A	Group B	P
1b	n = 68	n = 56	
ITT	16/68 (24%)	17/56 (30%)	0.39
PP	16/53 (30%)	17/41 (41%)	0.25
2a/b	n = 16	n = 23	
ITT	15/16 (94%)	11/23 (48%)	0.005
PP	15/15 (100%)	11/13 (85%)	0.21

ITT, intention to treatment analysis; PP, per protocol analysis; IFN, interferon; RBV, ribavirin.

TABLE III. Dose Reduction or Discontinuation and Adverse Events

	Group A (n = 98) % (n)	Group B (n = 100) % (n)	P
Discontinuation	16 (16)	25 (25)	0.13
Dose reduction or discontinuation of			
IFN	20 (20)	41 (41)	0.002
RBV	36 (35)	50 (50)	0.04
IFN and/or RBV	37 (36)	55 (55)	0.01
Depression	0 (0)	7 (7)	0.02

IFN, interferon; RBV, ribavirin.

TABLE IV. Factors Associated With Sustained Virological Response to Combination Therapy of Interferon Plus Ribavirin by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Age (years)	0: ≥60	1	
	1: <60	2.420 (1.173–5.002)	0.020
Genotype	0: 1b	1	
	1: 2a/b	5.301 (2.401–11.702)	<0.001

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.

Analysis of aa Sequences in the Core Gene in Genotype 1b Patients

The relationship between aa substitutions in the core region and the viral response to therapy was investigated in patients with genotype 1b using 93 available serum samples. Figure 2 shows the sequences of aa 61–110 of the HCV core region in 93 patients just before commencement of treatment. Table V summarizes the relationship between the response to IFN therapy and

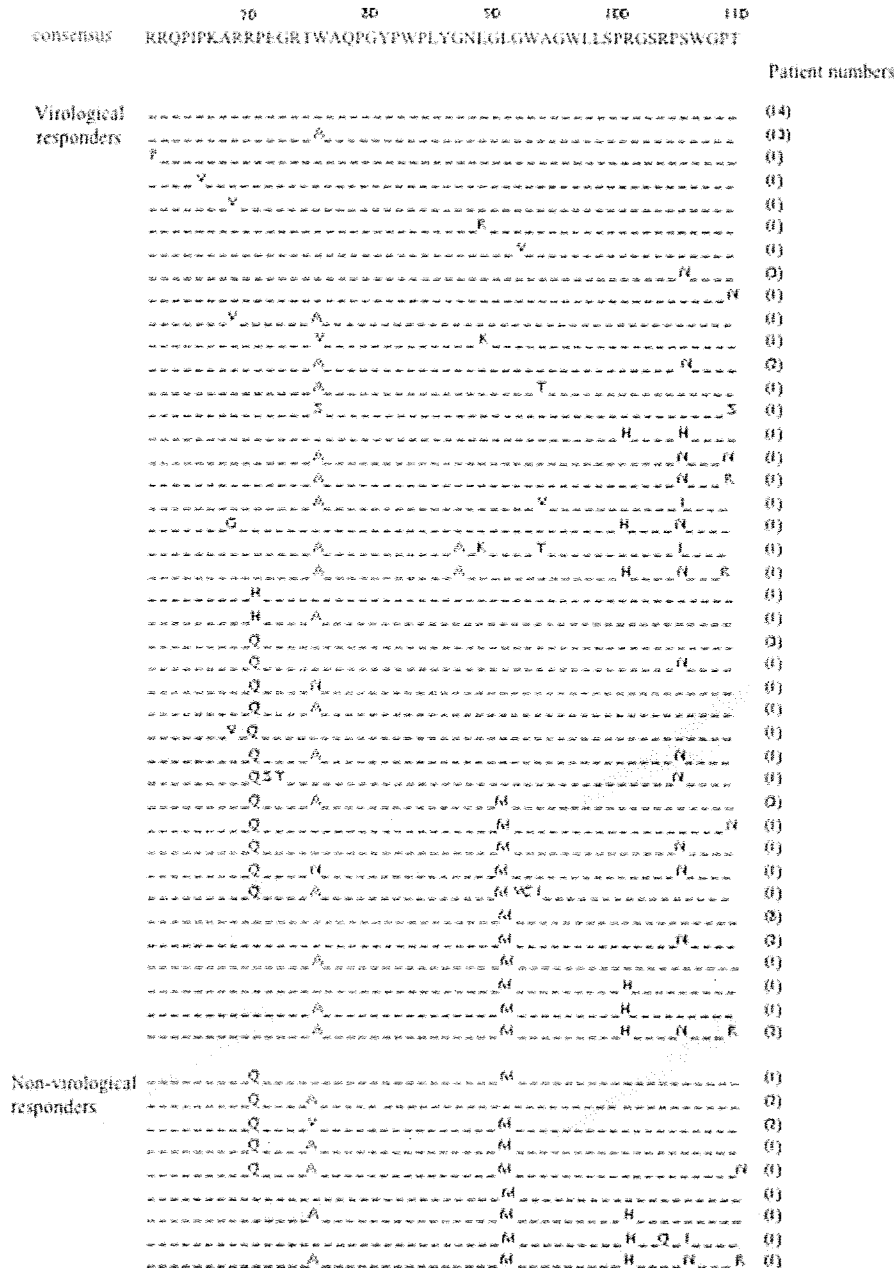

Fig. 2. Sequences of amino acids 61–110 in the core region at commencement of combination therapy in 93 patients infected with hepatitis C virus genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes.

TABLE V. Amino Acid Substitutions in the Core Region in Non-Virologic Responders and Virological Responders in 93 Patients With HCV Genotype 1b

Presence of substitution site	Non-virological response (n = 11) % (n)	Virological response (n = 82) % (n)	P
aa 70	64 (7)	23 (19)	0.01
aa 75	73 (8)	45 (37)	0.11
aa 91	82 (9)	30 (25)	0.001
aa 106	27 (3)	31 (26)	1.0
aa 110	18 (2)	12 (10)	62
aa 70 and 91	45 (5)	10 (8)	0.006
aa 70 and/or 91	100 (11)	44 (36)	<0.001

aa, amino acid.

substitutions of aa. Among aa substitutions, only substitutions of aa 70 and 91 were associated with non-virological response. All non-virological responders had aa substitutions at 70 or 91, or both substitutions. In contrast, only 36 of 82 (44%) virological responders had these substitutions ($P < 0.001$, Table V). In contrast to non-virological response, these substitutions were not predictive for sustained virological response ($P = 0.11$–0.82).

Next, the effect of substitutions of aa 70 and 91 in the core region on early viral kinetics was analyzed by dividing patients into four groups according to the pattern of aa substitutions. As shown in Figure 3, the most rapid decrease in core antigen was noted in patients where both aa 70 and 91 were wild-type (double-wild). In contrast, the poorest reduction was noted in patients with both of aa 70 and 90 substitutions (double-mutant). Patients with either of the two aa substitutions (mutant/wild or wild/mutant) showed decrease in between the above two groups. HCV core antigen decreased below the detectable limit (20 fmol/L) at week 4 in 37 of 40 (93%) patients who had neither aa 70 nor aa 90 substitutions. In contrast, it decreased below the detectable limit in only 5 of 12 patients (42%) who had both aa 70 and 91 substitutions ($P = 0.031$).

Analysis of Nucleotide Sequence of the NS5A Gene

The aa sequences of ISDR in the NS5A gene were determined in 40 patients where PCR for this region was positive. Seventeen of 40 patients had no aa

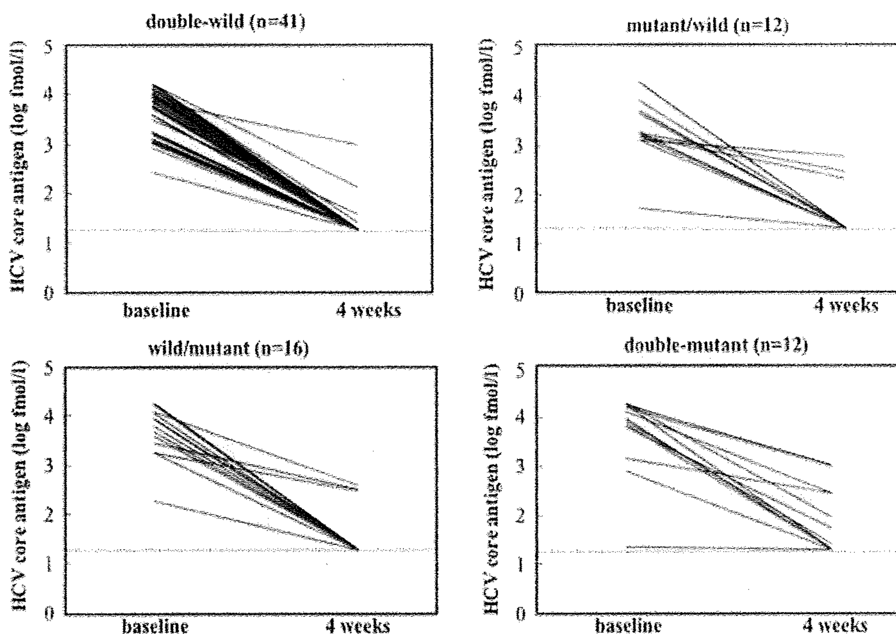

Fig. 3. Reduction of amount of HCV core antigen based on the presence of substitutions at amino acid 70 or 91. Eighty-one patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. The response was divided into four patterns based on the presence of substitution(s) at aa 70 and/or 91. Double-wild; no substitution, neither at aa 70 nor aa 91, mutant/wild; substitution only at aa 70, wild/mutant; substitution only at aa 91, double-mutant; substitutions at both aa 70 and 91. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VI. Amino Acid Substitutions in the IFN-Sensitive Determining Region (ISDR) in Non-Virologic Responders and Virological Responders in 40 Patients With HCV Genotype 1b

ISDR[a]	Non-virological response (n = 8) % (n)	Virological response (n = 32) % (n)	P
Wild-type (n = 17)	36 (6)	64 (11)	
Mutant-type (n = 23)	9 (2)	91 (21)	0.012

aa, amino acid.
[a]Absence of amino acid substitutions was evaluated as wild-type, and presence of one or more amino acid substitutions as mutant-type.

substitutions in ISDR (wild-type), while the remaining 23 patients had one or more substitutions (mutant-type). The relationship between aa substitutions of ISDR and effects of treatment was analyzed. The existence of aa substitution in the ISDR was not predictive for sustained virological response ($P = 0.137$), however, such substitution was observed frequently in virological responders compared to non-virological responders (66% vs. 25%, $P = 0.012$) (Table VI). The use of a different categorization based on the number of substitutions in the ISDR (0/1 vs. ≥ 2) yielded similar results, that is, not predictive for sustained viral response but predictive for virological responders (data not shown).

HCV core antigen decreased more rapidly in patients with ISDR mutant-type compared to those with wild-type (Fig. 4). HCV core antigen decreased below the detectable limit at week 4 in only 6 of 17 (35%) patients with wild-type. In contrast, it decreased below the detectable limit in 19 of 23 (83%) in patients with ISDR mutant-type ($P = 0.006$).

Predictive Factors Associated With Sustained Virological Response and Non-Virological Response in Patients With Genotype 1b

Finally, the predictive factors associated with sustained virological response and non-virological response were analyzed in patients with genotype 1b, including aa substitutions in the core region and ISDR. Univariate analysis showed two parameters correlated with sustained virological response: age (<60 years, $P = 0.004$) and presence of aa substitutions in the core (aa 70 and/or 91, $P = 0.04$). However, multivariate analysis, including the above variables, identified no parameters that influenced sustained virological response independently (age, $P = 0.89$; core, $P = 0.07$). Univariate analysis showed two parameters correlated with non-virological response: age (<65 years, $P = 0.02$) and aa substitutions in the core (double-mutant, $P = 0.01$). Multivariate analysis including the above variables identified aa substitutions in the core as an independent factor that influenced non-virological response (age, $P = 0.40$; core, $P = 0.03$) (Table VII).

DISCUSSION

Treatment of patients with chronic HCV infection had improved by the advent of PEG-IFN and RBV combination therapy. However, a substantial number of patients do not respond to the combination therapy [Taliani et al., 2006]. Several studies described attempts to improve the sustained virological response rate in such patients. Recent trials showed that a longer treatment period results in a higher sustained virological response rate [Berg et al., 2006; Sánchez-Tapias et al., 2006]. However, there are no conclusive studies that compared a larger dose of IFN with standard dose. Although the treatment had shifted in recent years to PEG-IFN and

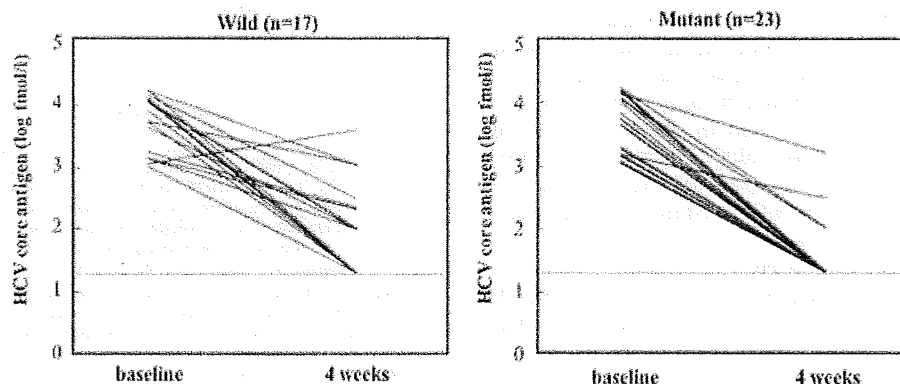

Fig. 4. Reduction of amount of HCV core antigen based on the presence of substitutions in the ISDR. Sixty-five patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. Patients were divided into two groups based on the presence of amino acid substitution(s) in the ISDR. Wild-type; absence of substitutions, mutant-type; presence of one or more substitutions. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VII. Factors Associated With Non-Virological Response to Combination Therapy of Interferon Plus Ribavirin Identified by Multivariate Analysis in Patients With Genotype 1b

Factor	Category	Odds ratio (95% CI)	P
Amino acid substitutions in the core region[a]	0: No double-mutant	1	0.028
	1: Double-mutant	7.000 (1.238–39.566)	

Only the variable that achieved statistical significance ($P < 0.05$) on multivariate logistic regression is shown.
[a]The mutant aa 70 and 91 pattern was evaluated as double-mutant, and other patterns as non-double-mutant.

RBV combination therapy, a different dose of IFN was used in the present study to test whether a larger dosage of IFN improves the outcome of IFN therapy.

In this study, the larger dose did not increase sustained virological response nor decrease non-virological response. Instead, the dose reduction of IFN and/or RBV was significantly higher in the higher dose group (Table III). Furthermore, the incidence of depression was significantly higher in the high-dose group (Table III). These results suggest that a high dose of IFN is not beneficial to patients who receive IFN and RBV combination therapy, and probably who will receive the PEG-IFN and RBV combination therapy.

The predictive factors for sustained virological response and non-virological response to the combination therapy for patients with genotype 1b were analyzed. Logistic regression analyses identified pretreatment substitutions at both aa 70 and 91 in the core region (double-mutant) as a singular predictive factor for non-virological response (Table VII). Furthermore, the existence of aa substitution in the ISDR was significantly more frequent in virological responders compared to non-virological responders (Table VI), in agreement with previous reports [Puig-Basagoiti et al., 2001; Pascu et al., 2004]. It has been reported that the numbers of aa substitutions in the ISDR correlate with serum HCV RNA levels [Enomoto et al., 1996]. However, no apparent correlation was observed in this study. As shown in Figures 3 and 4, patients who had substitutions of aa 70 and/or 91 in the core region or no aa substitutions in ISDR had poor initial reduction in the HCV core antigen. These results are consistent with recent studies that have shown the importance of a rapid initial decline of the viral load in obtaining a better response rate [Fried et al., 2002; Davis et al., 2003]. These results suggest that aa substitution analysis should provide important information on treatment of patients with genotype 1b.

The core protein of the HCV has been reported to disturb the IFN signaling by interacting with STAT1 SH2 domain [Lin et al., 2006] or repressing IRF1 [Ciccaglione et al., 2007]. These studies did not analyze the effect of aa substitutions in the core region. Further study is necessary to clarify the effect of aa substitutions in the core region and to identify a molecular target to improve the therapy.

Although aa substitution in the core region was identified as an important predictor in patients with genotype 1b in this study, aa substitutions of the core region and ISDR in patients with genotype 2a/b infection were not analyzed. Although the sustained virological response rate in patients who completed the therapy was high (26/28 [93%], per protocol analysis), few patients were unable to achieve sustained virological response. Furthermore, a significant number of patients could not complete the treatment course because of adverse effects. A more effective and easy to complete therapy should be developed to treat such patients. The predictive factors in such patients should also be clarified.

The recent development of a new type of drug targeting NS3/4 protease may improve the outcome of treatment in patients with chronic hepatitis C [Reesink et al., 2006; Forestier et al., 2007; Kieffer et al., 2007; Sarrazin et al., 2007a,b]. However, drug resistant mutants might emerge against such a small molecule therapy targeting viral enzyme(s). The functions of virus proteins that resist IFN including core, ISDR and PePHD should be clarified further to develop a better therapy that can achieve a higher sustained virological response rate with fewer and milder side-effects.

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REFERENCES

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2005. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 48:372–380.

Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2006. Predictive factors of virological non-response to interferon-ribavirin combination therapy for patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 78:83–90.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007a. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 46:403–410.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H. 2007b. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype. 1b. J Med Virol 79:1686–1695.

Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S. 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J Clin Microbiol 37:1802–1808.

Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, Buggisch P, Goeser T, Rasenack J, Pape GR, Schmidt WE, Kallinowski B, Klinker H, Spengler U, Martus P, Alshuth U, Zeuzem S. 2006. Extended treatment duration for hepatitis C virus type 1: Comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. Gastroenterology 130:1086–1097.

Blindenbacher A, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, Moradpour D, Blum HE, Alonzi T, Tripodi M, La Monica N, Heim MH. 2003. Expression of hepatitis C virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. Gastroenterology 124:1465–1475.

Bode JLS, Ehrhardt C, Albrecht U, Erhardt A, Schaper F, Heinrich P, Haussinger D. 2003. IFN-α antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling. FASEB J 17:488–490.

Brillet R, Penin F, Hezode C, Chouteau P, Dhumeaux D, Pawlotsky JM. 2007. The nonstructural 5A protein of hepatitis C virus genotype 1b does not contain an interferon sensitivity-determining region. J Infect Dis 195:432–441.

Brouwer JT, Nevens F, Bekkering FC, Bourgeois N, Van Vlierberghe H, Weegink CJ, Lefebvre V, Van Hattum J, Henrion J, Delwaide J, Hansen BE, Schalm SW, for the Benelux Study Group on Treatment of Chronic Hepatitis C. 2004. Reduction of relapse rates by 18-month treatment in chronic hepatitis C. A Benelux randomized trial in 300 patients. J Hepatology 40:689–695.

Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, Koike H, Kobayashi M, Koida I, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Kumada H. 1997. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology 25:745–749.

Chayama K, Suzuki F, Tsubota A, Kobayashi M, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Takahashi N, Kinoshita M, Kumada H. 2000. Association of aa sequence in the PKR-eIF2 phosphorylation homology domain and response to interferon therapy. Hepatology 32:1138–1144.

Ciccaglione AR, Stellacci E, Marcantonio C, Muto V, Equestre M, Marsili G, Rapicetta M, Battistini A. 2007. Repression of interferon regulatory factor 1 by hepatitis C virus core protein results in inhibition of antiviral and immunomodulatory genes. J Virol 81: 202–214.

Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. 2003. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 38:645–652.

Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. 1994. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 19:1513–1520.

Di Marco V, Ferraro D, Almasio P, Vaccaro A, Parisi P, Cappello M, Cino N, Di Stefano R, Craxi A. 2002. Early viral clearance and sustained response in chronic hepatitis C: A controlled trial of interferon and ribavirin after high-dose interferon induction. J Viral Hepat 9:354–359.

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. 1996. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 334:77–81.

Ferenci P, Brunner H, Nachbaur K, Datz C, Gschwantler M, Hofer H, Stauber R, Hackl F, Jessner W, Rosenbeiger M, Munda-Steindl P, Hegenbarth K, Gangl A, Vogel W, Australian Hepatitis Study Group. 2001. Combination of interferon induction therapy and ribavirin in chronic hepatitis C. Hepatology 34:1006–1011.

Forestier N, Reesink HW, Weegink CJ, McNair L, Kieffer TL, Chu HM, Purdy S, Jansen PL, Zeuzem S. 2007. Antiviral activity of telaprevir (VX-950) and peginterferon alfa-2a in patients with hepatitis C. Hepatology 46:640–648.

Foy E, Li K, Wang C, Sumpter R, Jr., Ikeda M, Lemon SM, Gale M, Jr. 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 300:1145–1148.

Fried MW, Shiffman M, Sterling RK, Weinstein J, Crippin J, Garcia G, Wright TL, Conjeevaram H, Reddy KR, Peter J, Cotsonis GA, Nolte FS. 2000. A multicenter, randomized trial of daily high-dose interferon-alfa 2b for the treatment of chronic hepatitis C: Pretreatment stratification by viral burden and genotype. Am J Gastroenterol 95:3225–3229.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL, Jr., Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. 2002. Peginterferon alfa-2a plus ribavirin for patients with chronic hepatitis C virus infection. N Engl J Med 347:975–982.

Gale MJ, Jr., Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. 1997. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology 230:217–227.

Gaudy C, Lambelé M, Moreau A, Veillon P, Lunel F, Goudeau A. 2005. Mutations within the hepatitis C virus genotype 1b E2-PePHD domain do not correlate with treatment outcome. J Clin Microbiol 43:750–754.

Hadziyannis AS, Papaioannou C, Spanou F, Manesis EK, Hadziyannis SJ. 2001. Induction interferon therapy in naive patients with chronic hepatitis C: Increased end-of-treatment virological responses but absence of long-term benefit. Aliment Pharmacol Ther 15:551–557.

Hashimoto M, Chayama K, Kobayashi M, Tsubota A, Arase Y, Saitoh S, Suzuki Y, Ikeda K, Matsuda M, Koike H, Kobayashi M, Handa H, Kumada H. 1999. Fluctuations of hepatitis C virus load are not related to amino acid substitutions in hypervariable region 1 and interferon sensitivity determining region. J Med Virol 58:247–255.

Hoofnagle JH, Ghany MG, Kleiner DE, Doo E, Heller T, Promrat K, Ong J, Khokhar F, Soza A, Herion D, Park Y, Everhart JE, Liang TJ. 2003. Maintenance therapy with ribavirin in patients with chronic hepatitis C who fail to respond to combination therapy with interferon alfa and ribavirin. Hepatology 38:66–74.

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, Shimotohno K. 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA 87:9524–9528.

Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, Kwong AD, Zeuzem S. 2007. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. Hepatology 46:631–639.

Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH, Alter MJ. 1990. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: Analysis by detection of antibody to hepatitis C virus. Hepatology 12:671–675.

Lin W, Kim SS, Yeung E, Kamegaya Y, Blackard JT, Kim KA, Holtzman MJ, Chung RT. 2006. Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. J Virol 80:9226–9235.

Lindsay KL, Davis GL, Schiff ER, Bodenheimer HC, Balart LA, Dienstag JL, Perrillo RP, Tamburro CH, Goff JS, Everson GT, Silva M, Katkov WN, Goodman Z, Lau JY, Maertens G, Gogate J, Sanghvi B, Albrecht J. 1996. Response to higher doses of interferon alfa-2b in patients with chronic hepatitis C: A randomized multicenter trial. Hepatology 24:1034–1040.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. 2001. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: A randomised trial. Lancet 358:958–965.

McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. 1998. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med 339:1485–1492.

Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, Nawrocki M, Kruska L, Hensel F, Petry W, Haussinger D. 1998. Prognosis of chronic hepatitis C: Results of a large, prospective cohort study. Hepatology 28:1687–1695.

Pascu M, Martus P, Höhne M, Wiedenmann B, Hopf U, Schreier E, Breg T. 2004. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations

within the NS5A-ISDR: A meta-analysis focused on geographical differences. Gut 53:1345–1351.

Polyak SJ, McArdle S, Liu SL, Sullivan DG, Chung M, Hofgärtner WT, Carithers RL, Jr., McMahon BJ, Mullins JI, Corey L, Gretch DR. 1998. Evolution of hepatitis C virus quasispecies in hypervariable region 1 and the putative interferon sensitivity-determining region during interferon therapy and natural infection. J Virol 72:4288–4296.

Polyak SJ, Nousbaum JB, Larson AM, Cotler S, Carithers RL, Jr., Gretch DR. 2000. The protein kinase-interacting domain in the hepatitis C virus envelope glycoprotein-2 gene is highly conserved in genotype 1-infected patients treated with interferon. J Infect Dis 182:397–404.

Puig-Basagoiti F, Sáiz JC, Forns X, Ampurdanès S, Giménez-Barcons M, Franco S, Sánchez-Fueyo A, Costa J, Sánchez-Tapias JM, Rodés J. 2001. Influence of the genetic heterogeneity of the ISDR and PePHD regions of hepatitis C virus on the response to interferon therapy in chronic hepatitis C. J Med Virol 65: 35–44.

Reesink HW, Zeuzem S, Weegink CJ, Forestier N, van Vliet A, van de Wetering de Rooij J, McNair L, Purdy S, Kauffman R, Alam J, Jansen PL. 2006. Rapid decline of viral RNA in hepatitis C patients treated with VX-950: A phase Ib, placebo-controlled, randomized study. Gastroenterology 131:997–1002.

Sánchez-Tapias JM, Diago M, Escartín P, Enríquez J, Romero-Gómez M, Bárcena R, Crespo J, Andrade R, Martínez-Bauer E, Pérez R, Testillano M, Planas R, Solá R, García-Bengoechea M, Garcia-Samaniego J, Muñoz-Sánchez M, Moreno-Otero R, TeraViC-4 Study Group. 2006. Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. Gastroenterology 131:451–460.

Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Müh U, Welker M, Wincheringer D, Zhou Y, Chu HM, Lin C, Weegink C, Reesink H, Zeuzem S, Kwong AD. 2007a. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 132:1767–1777.

Sarrazin C, Rouzier R, Wagner F, Forestier N, Larrey D, Gupta SK, Hussain M, Shah A, Cutler D, Zhang J, Zeuzem S. 2007b. SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. Gastroenterology 132:1270–1278.

Taliani G, Gemignani G, Ferrari C, Aceti A, Bartolozzi D, Blanc PL, Capanni M, Esperti F, Forte P, Guadagnino V, Mari T, Marino N, Milani S, Pasquazzi C, Rosina F, Tacconi D, Toti M, Zignego AL, Messerini L, Stroffolini T, Nonresponder Retreatment Group. 2006. Pegylated interferon alfa-2b plus ribavirin in the retreatment of interferon-ribavirin nonresponder patients. Gastroenterology 130: 1098–1106.

Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. 1999. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science 285:107–110.

Torres-Puente M, Cuevas JM, Jiménez-Hernández N, Bracho MA, García-Robles I, Carnicer F, del Olmo J, Ortega E, Moya A, González-Candelas F. 2008. Hepatitis C virus and the controversial role of the interferon sensitivity determining region in the response to interferon treatment. J Med Virol 80:247–253.

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A Polymorphism in *MAPKAPK3* Affects Response to Interferon Therapy for Chronic Hepatitis C

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Background & Aims: **This study aimed to identify host single nucleotide polymorphisms (SNPs) that are associated with the efficacy of interferon (IFN) therapy in patients with chronic hepatitis C.** **Methods:** **We examined whether 116 tagging-SNPs from 13 genes that are involved in type I IFN signaling associate with the outcome of IFN therapy in Japanese case-control groups; the study included 468 sustained responders and 587 nonresponders.** **Results:** **We identified 2 SNPs (rs3792323 [A/T] and rs616589 [G/A]), located in intron 2 of mitogen-activated protein kinase–activated protein kinase 3 (MAPKAPK3) that were associated with the outcome of IFN therapy in patients infected with hepatitis C virus (HCV) genotype 1b ($P = 4.6 \times 10^{-5}$ and 4.8×10^{-5}, respectively). The 2 SNPs were in strong linkage disequilibrium and multivariate logistic regression analysis showed that rs3792323 is an independent factor associated with the IFN efficacy (genotype 1b; $P = .0011$). *MAPKAPK3* is a kinase involved in the mitogen and stress responses, but the biological significance of MAPKAPK3 in IFN responses is poorly understood. By using an allele-specific transcript quantification assay in liver biopsy, we showed that allele-specific expression of *MAPKAPK3* messenger RNA, corresponding to the risk allele for nonresponse, was significantly higher than that of the other allele. Luciferase reporter assay data indicated that overexpression of MAPKAPK3 inhibits IFN-alfa–induced gene transcription via IFN-stimulated response element and IFN-γ–activated site.** **Conclusions:** ***MAPKAPK3*** **The SNP rs3792323 in** ***MAPKAPK3*** **associates with the outcome of IFN therapy in patients with HCV genotype 1b. Our functional analyses indicate that MAPKAPK3 inhibits IFN-alfa–induced antiviral activity.**

BASIC–LIVER, PANCREAS, AND BILIARY TRACT

Type I interferon (IFN), including IFN-alfa and IFN-β, has been used widely as an antiviral agent for chronic hepatitis C. However, even after the most effective combination therapy of pegylated-IFN-alfa plus ribavirin, more than 50% of patients infected with hepatitis C virus (HCV) genotype 1b and approximately 20% of patients with HCV genotype non-1b fail to eradicate the virus.[1–3]

The mechanisms of modulating the responsiveness to IFN therapy have been studied extensively. Both viral and host factors have been implicated in the resistance to IFN therapy. Viral factors, such as HCV genotype, serum HCV-RNA level, and the interferon sensitivity determining region, have been reported to be associated with the outcome of IFN therapy.[3–5] On the other hand, host factors including age, sex, race, liver fibrosis, and obesity have been shown to associate with the outcome of IFN therapy.[6,7] Furthermore, it has been reported that genetic polymorphisms of cytokines, chemokines, and IFN-stimulated genes are associated with the difference in response to IFN therapy.[7–12]

Recently, genetic polymorphism of type I IFN receptor-1 (IFNAR1) promoter region was reported to be associated with the outcome of IFN therapy in patients with HCV infection.[13] Although the mechanisms of this polymorphism for the different responsiveness to IFN therapy still are unclear, polymorphism of *IFNAR1* promoter region may influence the efficacy of IFN therapy, possibly through modulation of IFNAR1 expression level. Because type I IFN elicits antiviral activity by activation of signaling molecules downstream of type I IFN receptors, genetic polymorphisms in type I IFN signaling molecules

Abbreviations used in this paper: GAS, interferon-γ–activated site; IFN, interferon; IFNAR1, type I interferon receptor-1; ISRE, interferon-stimulated response element; JAK, Janus-activated kinase; MAP, mitogen-activated protein; MAPKAPK, mitogen-activated protein kinase–activated protein kinase; MAPKK, mitogen-activated protein kinase kinases; NR, nonresponders; SNPs, single nucleotide polymorphisms; SR, sustained responders; STAT, signal transducer and activator of transcription.

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also potentially could alter the responsiveness to IFN therapy. However, so far there has been no evidence of associations between polymorphisms of genes involved in type I IFN signal transduction and the efficacy of IFN therapy in patients with chronic hepatitis C.

In the present study, we examined whether single nucleotide polymorphisms (SNPs) in type I IFN signaling molecules are associated with the difference in response to IFN therapy in patients with chronic hepatitis C, using the tagging-SNP approach in a large case-control study. The tagging-SNP serves as a marker to detect associations between a particular gene region and the outcome of IFN therapy. A small set of tagging-SNPs is sufficient to capture genetic variation because polymorphisms that are physically close to each other have a tendency to be in linkage disequilibrium with each other.[14,15] The HapMap online database (http://www.hapmap.org) allows the tagging-SNP approach to be applied readily to many genes or regions.[16]

As for type I IFN signaling molecules, we focused on 2 signaling cascades downstream of type I IFN receptors. First, we examined the Janus-activated kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, which is essential for type I IFN–induced antiviral activity.[17] We selected tagging-SNPs for 7 key genes in this pathway, including *IFNAR1*, *IFNAR2*, *JAK1*, tyrosine kinase 2, *STAT1*, *STAT2*, and IFN regulatory factor 9. Second, we also examined the p38 mitogen-activated protein (MAP) kinase pathway, which has been reported to cooperate with the JAK-STAT pathway in activation of type I IFN–induced antiviral activity.[18–23] We also selected tagging-SNPs for 6 key genes in this pathway, including ras-related C3 botulinum toxin substrate 1,[18] MAP kinase kinases 3 (MAPKK3),[19] *MAPKK6*,[19] p38 MAP kinase,[20,21] MAP kinase-activated protein kinase 2 (MAPKAPK2),[20–23] and *MAPKAPK3*.[20–22]

Here, we provide genetic evidence suggesting that 2 SNPs in *MAPKAPK3* are associated with the responsiveness to IFN therapy. The 2 SNPs may be useful as markers to predict the outcome of IFN therapy, which is very helpful clinically because IFN therapy is expensive and may cause serious adverse effects.[24] In addition, we provided functional evidence that suggests MAPKAPK3 influences IFN-alfa–induced antiviral activity.

Patients and Methods

Study Subjects and DNA Preparation

We enrolled 1055 patients with chronic HCV infection who were treated with IFN monotherapy before 2001, at the Department of Hepatology, Toranomon Hospital, Hiroshima University Hospital, and Hiroshima University affiliated hospitals. Each patient was treated with 6×10^6 units of IFN intramuscularly every day for 8 weeks, followed by the same dose twice a week for 16 weeks, with a total dose of 528 million units. The characteristics of participating patients are described in Table 1. All patients had abnormal serum alanine transaminase levels for more than 6 months, and were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, and had not received immunosuppressive or antiviral therapy before enrollment in the study. Patients were classified into the following 2 groups: sustained responders (SRs) and nonresponders (NRs). SRs had normal alanine transaminase levels and no evidence of viremia at 6 months after completion of IFN therapy. Relapsed responders were excluded. NRs remained viremic at 6 months after completion of IFN therapy. HCV-RNA levels were determined by Amplicor-monitor assay (Roche Diagnostics, Basel, Switzerland) or branched-chain DNA assay and stratified into 2 categories according to cut-off values that have been reported previously.[25,26] It has been reported that having serum HCV-RNA levels higher than 1.0 mEq/mL by branched-chain DNA assay or 100 KIU/mL by Amplicor-monitor assay is a predictor of poor responsiveness to IFN therapy. Histologic staging was determined according to the previously described criteria using biopsy specimens of liver tissue.[27] All subjects in the present study were ethnically Japanese and gave written informed consent to participate in the study according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama. Genomic DNA samples were obtained from peripheral blood of the participating pa-

Table 1. Characteristics of Patients With Chronic Hepatitis C

	SRs	NRs	*P* value
Patients, n	468	587	—
Mean age ± SD, *y*	54.6 ± 11.8	55.9 ± 10.3	.1[a]
Sex			.002
Male	325	354	
Female	143	233	
HCV genotype			<.0001[b]
1a	3	3	
1b	208	434	
2a	209	101	
2b	48	49	
HCV-RNA level[c]			<.0001[d]
High	177	420	
Low	216	69	
No data	75	98	
Fibrosis stage			.19[e]
F0	5	5	
F1	213	254	
F2	130	173	
F3	24	43	
F4	17	28	
No biopsy	79	84	

[a]Mann–Whitney U test.
[b]Chi-square test between HCV genotype 1b and non-1b.
[c]Low HCV-RNA level: <100 KIU/mL by Amplicor-monitor assay and <1.0 mEq/mL by branched-chain DNA assay.
[d]Chi-square test between HCV-RNA level high and low.
[e]Chi-square test between F0-1 and F2-4.

tients. DNA extraction was performed according to a standard phenol-chloroform protocol.[28]

Selection of Tagging-SNPs and Genotyping

As shown in Supplementary Table 1, we selected 116 tagging-SNPs for a total set of 13 candidate genes related to the type I IFN pathway, using the HapMap database (public release 21a; phase II of the January 2007 National Center for Biotechnology Information build 35 assembly; dbSNP build 125) and the Haploview program (available: http://www.broad.mit.edu/mpg/haploview). With the selection criteria of r^2 greater than 0.8 and minor allele frequency of greater than 0.05 for the Japanese population, tagging-SNPs were selected from all bins that cover the entire gene region from approximately 2000 bp upstream of the transcription start site to 1500 bp of the 3′ untranslated region in each gene. The number of tagging-SNPs in each candidate gene were as follows: *IFNAR1*, 3; *IFNAR2*, 6; *JAK1*, 8; tyrosine kinase 2, 3; *STAT1*, 23; *STAT2*, 1; IFN regulatory factor 9, 5; ras-related C3 botulinum toxin substrate 1, 7; *MAPKK3*, 5; *MAPKK6*, 35; p38 MAP kinase, 10; *MAPKAPK2*, 6; and *MAPKAPK3*, 4 (Supplementary Table 1). SNPs were genotyped by using the Invader assay[29] and the TaqMan assay[30] as described previously. The probe sets for the Invader assay were designed and synthesized by Third Wave Technologies (Madison, WI) and those for the TaqMan assay by Applied Biosystems (Foster City, CA).

SNP Discovery

To identify genetic polymorphisms within the coding region of *MAPKAPK3*, we amplified appropriate fragments of genomic DNA from 48 patients by polymerase chain reaction and sequenced the products to identify SNPs using previously described methods.[28,31]

Cells and Cell Culture

Human hepatoma cell line, Huh7, was purchased from RIKEN Cell Bank (Tsukuba, Japan). Huh7 cells were cultured in Dulbecco's modified minimal essential medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% CO_2.

Allele-Specific Transcript Quantification for MAPKAPK3

Allele-specific transcript quantification was performed as described previously,[32,33] with some modifications. Liver biopsy samples were collected from 5 patients with informed consent before IFN therapy for chronic hepatitis C. Total RNA was isolated using the RNeasy Micro kit (Qiagen, Hilden, Germany) and treated with 5 IU/mL RNase-Free DNase I (Qiagen). First-strand complementary DNA (cDNA) was prepared using the SuperScript III Platinum Two-Step quantitative reverse-transcription polymerase chain reaction kit (Invitrogen, Carlsbad, CA). Genomic DNA from peripheral blood of the 5 patients was prepared as described earlier. Both cDNA and genomic DNA were amplified with specific primers for the 3′ untranslated region of *MAPKAPK3*. The primers were as follows: forward, 5′-CCTGTGAATGCTGAGTGAGCGAGTA-3′; reverse, 5′-AGTCACCCTTTGGGTCGGGAATAGT-3′.

For determination of allele-specific *MAPKAPK3* messenger RNA (mRNA) expression, probes for SNP rs1385025 (A/G) were designed and synthesized by Third Wave Technologies. The invader assays were performed in a 5-μL reaction volume containing 1 × signal buffer, 1 × FRET Mix (FRET22/FTRE7), 30 ng cleavase VIII enzyme, 0.3 μL of probe mixture (all reagents from Third Wave Technologies), and 2 ng polymerase chain reaction product in a 96-well plate format. The thermal profile was 95°C for 5 minutes, followed by 40 cycles at 63°C for 1 minute, and a real-time intensity of fluorescence (FAM for G allele, and VIC for A of rs1385025) was measured by use of the Mx3000P Multiplex Quantitative polymerase chain reaction system (Stratagene, La Jolla, CA). For the construction of standard curves for each allele, sequential dilution of an amplified product from genomic DNA of patients with double heterozygosity for rs1385025 and rs3792323 was used. Each experiment was performed in triplicate assay at least 3 times.

Luciferase Reporter Assay

One day before transfection, 7 × 10^3 of Huh7 cells were seeded in a 96-well culture plate. We used 2 types of firefly luciferase expression vector that contain promoter element IFN-stimulated response element (ISRE) or IFN-γ-activated site (GAS). Huh7 cells were transfected with both 1 ng renilla luciferase expression vector pRL-TK (Promega, Madison, WI) and 10 ng firefly luciferase expression vector pISRE-TA-Luc or pGAS-TA-Luc (BD Biosciences, San Jose, CA), in conjunction with 40 ng expression plasmid pDEST51/mock (empty vector), pDEST51/*MAPKAPK3*, or pDEST51/suppressor of cytokine signaling 1, by use of the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). After 24 hours, cells were stimulated with IFN-alfa (Dainippon Sumitomo Pharma, Osaka, Japan) for 20 hours followed by Dual-Luciferase Assay (Promega). Firefly luciferase activity was normalized by renilla luciferase activity. Each experiment was performed at least 3 times. Data are expressed as mean ± SD in triplicate assay.

Statistical Analysis

We calculated allele frequencies and tested fit to Hardy-Weinberg equilibrium by the chi-square test at each SNP, using Excel software (Microsoft, Redmond, WA).[34] Then, we compared differences in genotype distribution of each SNP between cases and controls with the chi-square test and the Cochran-Armitage trend test (Excel).[35] LD coefficients (r^2) were calculated as described previously (Excel).[36] The age of the SRs and NRs was

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compared by the Mann–Whitney *U* test. Differences in categoric data of patients in the 2 groups were analyzed by the chi-square test. To evaluate internal consistency of the results shown by association study, a 2-stage replication design was simulated by the Monte Carlo method. We assessed population stratification by analyzing the data from 116 tagging-SNPs in patients with HCV genotype 1b, using a genomic control method that has been reported previously.[37] Multivariate logistic regression with stepwise forward selection was performed with a significance level of 0.05 for including variables, by use of the StatFlex 5.0 software package (Artec Inc., Osaka, Japan). For case-control haplotype analysis we estimated haplotype frequencies and tested for association by chi-square analysis, to detect differences in haplotype distribution between groups we used using Haploview 3.2 software. For allele-specific transcript quantification assay, statistical differences between allelic *MAPKAPK3* mRNA expression corresponding to haplotype 1 and haplotype 2 were analyzed by Mann–Whitney *U* test. For luciferase reporter assay, comparisons among the 3 groups were analyzed by the Kruskal–Wallis test, followed by the Scheffe test to evaluate statistical differences between the 2 groups (StatFlex 5.0 software package).

Results

Association Between Tagging-SNPs in MAPKAPK3 *and the Outcome of IFN Therapy*

We searched for the association between 116 tagging-SNPs for 13 candidate genes and the outcome of IFN therapy, using 468 SR and 587 NR subjects. We were successful in genotyping all 116 tagging-SNPs (Supplementary Table 2). The mean call rate was 99.4% across all tagging-SNPs. None of the tagging-SNPs showed a significant deviation from Hardy–Weinberg equilibrium. Because HCV genotype 1b, which is the most common in Japan, is associated with poor response to IFN treatment, we divided the patients into 2 subgroups according to the genotypes of HCV with which they were infected (1b vs non-1b), and performed the comparison separately.

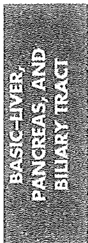
We found that 2 SNPs, rs3792323 (A/T) and rs616589 (G/A), located in intron 2 of MAPKAPK3, are associated with the outcome of IFN therapy in patients with HCV genotype 1b; the T allele for rs3792323 was significantly more frequent in NRs than in SRs (Table 2; 33.4% vs 22.4%; $P = 5.2 \times 10^{-5}$; odds ratio, 0.57; 95% confidence interval, 0.44–0.75). Similarly, the A allele for rs616589 was significantly more frequent in NRs than in SRs (37.8% vs 26.4%; $P = 5.6 \times 10^{-5}$; odds ratio, 0.59; 95% confidence interval, 0.45–0.76).

In Table 2, the Cochran–Armitage trend test (assuming an additive model for minor alleles) revealed an allele dose-dependent association of rs3792323 with the outcome of IFN therapy ($P = 4.6 \times 10^{-5}$), with decreased

Table 2. Associations Between the Two SNPs in *MAPKAPK3* and the Outcome of IFN Therapy

dbSNP ID	Allele 1/2	HCV genotype	Patients, n	Allele frequency, % 1	2	*P* value	OR (95% CI)	Genotype (%) 11	12	22	Cochran–Armitage trend test, *P* value	OR (95% CI) 11 vs 12	11 vs 22	Dominant model for allele 2 (11 vs 12, 22) *P* value	OR (95% CI)	Recessive model for allele 2 (11, 12 vs 22) *P* value	OR (95% CI)
rs3792323	A/T	total	SR (n = 468)	71.8	28.2	.088	0.85 (0.70–1.02)	242 (51.8)	187 (40.0)	38 (8.1)	.089	0.83 (0.65–1.08)	0.74 (0.47–1.15)	.10	0.82 (0.64–1.04)	.32	0.80 (0.52–1.23)
			NR (n = 587)	68.4	31.6			273 (46.7)	253 (43.3)	58 (9.9)							
		1b	SR (n = 208)	77.6	22.4	.000052	0.57 (0.44–0.75)	124 (59.6)	75 (36.1)	9 (4.3)	.000046	0.58 (0.41–0.83)	0.30 (0.14–0.63)	.00019	0.53 (0.38–0.74)	.0074	0.38 (0.18–0.79)
			NR (n = 434)	66.6	33.4			189 (43.9)	196 (45.5)	46 (10.7)							
		non-1b	SR (n = 260)	67.2	32.8	.056	1.36 (0.99–1.86)	118 (45.6)	112 (43.2)	29 (11.2)	.06	1.40 (0.92–2.14)	1.72 (0.83–3.57)	.067	1.45 (0.97–2.17)	.27	1.48 (0.73–2.99)
			NR (n = 153)	73.5	26.5			84 (54.9)	57 (37.3)	12 (7.8)							
rs616589	G/A	total	SR (n = 468)	68.0	32.0	.034	0.82 (0.68–0.99)	215 (46.0)	205 (43.9)	47 (10.1)	.033	0.83 (0.64–1.07)	0.67 (0.44–1.00)	.062	0.79 (0.62–1.01)	.12	0.74 (0.50–1.08)
			NR (n = 587)	63.6	36.4			235 (40.3)	271 (46.5)	77 (13.2)							
		1b	SR (n = 208)	73.6	26.4	.000056	0.59 (0.45–0.76)	111 (53.4)	84 (40.4)	13 (6.3)	.000048	0.59 (0.42–0.84)	0.33 (0.17–0.62)	.00023	0.53 (0.38–0.75)	.0055	0.42 (0.23–0.79)
			NR (n = 434)	62.2	37.8			164 (38.0)	209 (48.4)	59 (13.7)							
		non-1b	SR (n = 260)	63.5	36.5	.24	1.20 (0.89–1.61)	104 (40.2)	121 (46.7)	34 (13.1)	.25	1.33 (0.87–2.05)	1.29 (0.68–2.46)	.18	1.32 (0.88–1.98)	.72	1.12 (0.61–2.06)
			NR (n = 153)	67.5	32.5			71 (47.0)	62 (41.1)	18 (11.9)							

NOTE. *P* values were calculated from case-control analysis by the chi-square test and unadjusted for multiple testing. Odds ratios of having a sustained response to IFN therapy were calculated. Allele 1 and allele 2 denote a major and a minor allele, respectively.
OR, odds ratio; CI, confidence interval.



odds ratios of 0.58 and 0.30 for AT and TT genotypes, respectively (95% confidence interval, 0.41–0.83 for AT; 0.14–0.63 for TT). Under a dominant model for the T allele of rs3792323, a significant association also was seen in patients infected with HCV genotype 1b ($P = 1.9 \times 10^{-4}$; odds ratio, 0.53; 95% confidence interval, 0.38–0.74).

Similarly, an allele dose-dependent association of rs616589 with the responsiveness to IFN therapy was revealed in Table 2 ($P = 4.8 \times 10^{-5}$), with decreased odds ratios of 0.59 and 0.33 for GA and AA genotypes, respectively (95% confidence interval, 0.42–0.84 for GA; 0.17–0.62 for AA). Under a dominant model for the A allele of rs616589, a significant association also was seen in HCV genotype 1b-infected patients ($P = 2.3 \times 10^{-4}$; odds ratio, 0.53; 95% confidence interval, 0.38–0.75).

To adjust the P values for multiple testing, we applied a Bonferroni correction with each individual SNP as an independent variable (total, 116 SNPs). Despite this conservative adjustment, our results for rs3792323 and rs616589 about patients with HCV genotype 1b remained highly significant ($P < .05$). On the other hand, the other tagging-SNPs did not show significant associations with the outcome of IFN therapy after Bonferroni corrections (Supplementary Table 2).

Internal Validation of the Observed Associations

To evaluate internal consistency of the results shown by the association study, 2-stage replication design was simulated by the Monte Carlo method. Half of the cases and half of the controls were selected randomly from HCV-1b-infected patients in this study, and used for the first-stage test with a significance level $\alpha 1$ (the probability of making a Type I error) in the allele-frequency model. Only the SNPs that were judged to be associated significantly with the phenotype then underwent the second-stage test. In the second stage, the remaining independent cases and controls were used to test the association between the selected SNPs and the phenotype with a significance level $\alpha 2$. We set $\alpha 1$ at 0.01, 0.02, and 0.05, and calculated $\alpha 2$ as $(0.05/116)/\alpha 1$ because the global significance level after Bonferroni's correction is 0.05/116 (total, 116 SNPs). The number of iterations was 100,000 for each condition. By this method, the results of the test in the first stage are validated by the test in the second stage.

When the level of significance was set at $\alpha 1 = .01, .02$, and .05, the proportions of significant results of SNP rs3792323 were 0.563, 0.595, and 0.557, respectively (Table 3). Similarly, those of SNP rs616589 were 0.554, 0.579, and 0.540, respectively when the same values of $\alpha 1$ were used. These results suggest that the results of the first-stage test could be replicated in many cases if 2 halves of the patients, the first-stage set and the second-stage set, were tested independently for the association.

Table 3. Internal Validation Analysis of the Observed Associations Between the Two SNPs in *MAPKAPK3* and the IFN Efficacy in Patients With HCV Genotype 1b

SNP	$\alpha 1$	Proportion of significant results	95% CI
rs3792323	0.01	0.563	0.560–0.566
	0.02	0.595	0.591–0.598
	0.05	0.557	0.554–0.560
rs616589	0.01	0.554	0.551–0.557
	0.02	0.579	0.576–0.582
	0.05	0.540	0.537–0.543

$\alpha 1$, a significance level for the first-stage test; CI, confidence interval.

Population Stratification Analysis

We assessed population stratification by analyzing the data from 116 tagging-SNPs in patients with HCV genotype 1b by using the genomic control method. We estimated the inflation factor, which effectively can adjust for the confounding effect of population stratification regardless of its extent (inflation factor, 1.18; 95% confidence interval, 0.87–1.65). The corrected P values for rs3792323 and rs616589 in *MAPKAPK3* were .00017 and .00018, respectively. After a Bonferroni correction, the results for the 2 SNPs remained highly significant ($P < .05$). These results suggest that population stratification in our patients was negligible.

Haplotype Analysis

We examined whether *MAPKAPK3* haplotypes show more significant associations with the effect of IFN therapy than single-marker analysis. Because the 2 SNPs rs3792323 and rs616589 were in strong linkage disequilibrium with an r-squared value of 0.82 in our genotype data of 1055 patients, we constructed *MAPKAPK3* haplotypes from 3 tagging-SNPs (rs3792323 A>T, rs3804628 G>A, and rs2040397 C>T), using the Haploview 3.2 software. As a result, 4 haplotypes with frequencies greater than 5% were deduced in patients with HCV genotype 1b: AGC 43.9%, TGC 29.8%, AGT 20.6%, and AAC 5.7%. Although haplotype TGC was associated the most significantly with the outcome of IFN therapy in 4 haplotypes ($P = .000051$), this P value was comparable with that for single-marker analysis ($P = .000046$ for rs3792323 in the Cochran-Armitage trend test).

Results of Multivariate Logistic Regression Analysis

To determine independent factors on the outcome of IFN therapy in patients infected with HCV genotype 1b, we used multivariate logistic regression analysis with stepwise forward selection. We evaluated the following 6 factors: SNP rs3792323 (A allele vs T allele), rs616589 (G allele vs A allele), age (per year increase), sex (male vs female), fibrosis stage (F0–F1 vs F2–F4), and HCV-RNA level before treatment (low vs high).

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Table 4. Predictive Factors Associated Independently With the Response to IFN Therapy in Patients With HCV Genotype 1b by Multivariate Logistic Regression Analysis

	HCV genotype 1b		
Variable	*P* value	OR	95% CI
rs3792323 (T allele/A allele)	.0011	0.29	0.14–0.61
Age (per year increase)	.0096	0.97	0.95–0.99
Fibrosis stage (F0–F1/F2–F4)	.035	1.66	1.04–2.66
HCV-RNA level (low/high)[a]	<.00001	8.25	5.05–13.50

OR, odds ratio; CI, confidence interval.
[a]Low HCV-RNA level: <100 KIU/mL by Amplicor-monitor assay and <1.0 mEq/mL by branched-chain DNA assay. Odds ratios of having a sustained response to IFN therapy were calculated.

We found that SNP rs3792323 is an independent factor associated with IFN efficacy (Table 4; P = .0011; odds ratio, 0.29; 95% confidence interval, 0.14–0.61). On the other hand, SNP rs616589 was removed from this model, suggesting that the 2 SNPs are not associated independently with the outcome of IFN therapy. This is consistent with the result that the 2 SNPs were in strong linkage disequilibrium in our genotype data.

Next, to eliminate the effect of confounding factors, we also tried running models by forcing in the earlier-mentioned 4 factors (age, sex, HCV-RNA level, and fibrosis stage) and SNP rs3792323 into multivariate logistic regression analysis in patients with HCV genotype 1b. We identified that SNP rs3792323 is associated with the outcome of IFN therapy (P = .0014; odds ratio, 0.30; 95% confidence interval, 0.14–0.63).

SNP Discovery Within the Coding Region of MAPKAPK3

To investigate whether there is any genetic polymorphism in *MAPKAPK3* that results in amino acid substitution, we sequenced the coding region of *MAPKAPK3* from genomic DNA isolated from 48 patients. We did not find any nonsynonymous allelic variants in *MAPKAPK3*.

Allele-Specific Transcript Quantification of MAPKAPK3

Next, we examined the possibility that SNP rs3792323 associates with *MAPKAPK3* expression in liver biopsy specimens from patients with chronic hepatitis C. Because rs3792323 in *MAPKAPK3* intron 2 was not present in mRNA, we selected SNP rs1385025 (A/G) in the 3′ untranslated region as a marker SNP. We confirmed that rs1385025 showed complete linkage disequilibrium to rs3792323 (D' values = 1), using HapMap data and the Haploview program. We selected 5 patients who were doubly heterozygous with genotype rs1385025 A/G and rs3792323 A/T for this assay. Haplotype pairs of these patients theoretically were specified to be haplotype 1 (rs1385025A rs3792323A) and haplotype 2 (rs1385025G rs3792323T).

We measured the relative contribution of each haplotype to *MAPKAPK3* transcription in these patients, using probes that detect each allele of rs1385025. As shown in Figure 1, allele-specific *MAPKAPK3* mRNA expression corresponding to haplotype 2 was 1.15- to 1.76-fold higher than that of haplotype 1 (P = .009). This result indicated that SNP rs1385025 and rs3792323 associate with the expression level of *MAPKAPK3*.

Effects of MAPKAPK3 on IFN-Alfa–Induced Gene Transcription Via ISRE and GAS Elements

We tested whether transient overexpression of MAPKAPK3 influences IFN-alfa–induced gene transcription via ISRE and GAS elements, which are essential promoter elements for type I IFN–induced antiviral activity, by use of luciferase reporter assay. When MAPKAPK3 was overexpressed in Huh7 cells, IFN-alfa–induced luciferase activities via ISRE and GAS elements were suppressed significantly by various doses of IFN-alfa, compared with the negative control (Figure 2). Similar results were obtained in suppressor of cytokine signaling 1, which has been reported to suppress IFN-alfa–induced gene expressions. In addition, we also overexpressed β-galactosidase gene as a negative control. In comparison with this control, MAPKAPK3 also significantly inhibited IFN-alfa–induced luciferase activities via ISRE and GAS elements (data not shown). These results suggest that MAPKAPK3 can inhibit IFN-alfa–induced gene transcription via ISRE and GAS elements.

Discussion

We identified that SNP rs3792323 (A/T) and rs616589 (G/A), located in *MAPKAPK3*, are associated

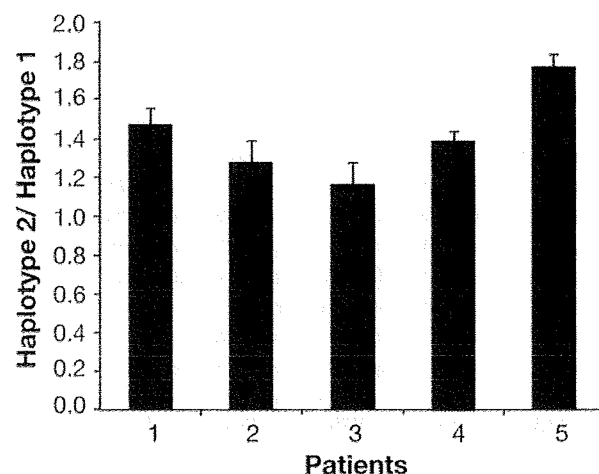

Figure 1. Allele-specific transcript quantification of *MAPKAPK3*. The allele-specific *MAPKAPK3* mRNA expression ratio for haplotype 2 to haplotype 1 is shown. Individual data from liver biopsies from 5 patients are indicated. Each experiment was performed in triplicate assay at least 3 times. Data represent the mean ± SD.

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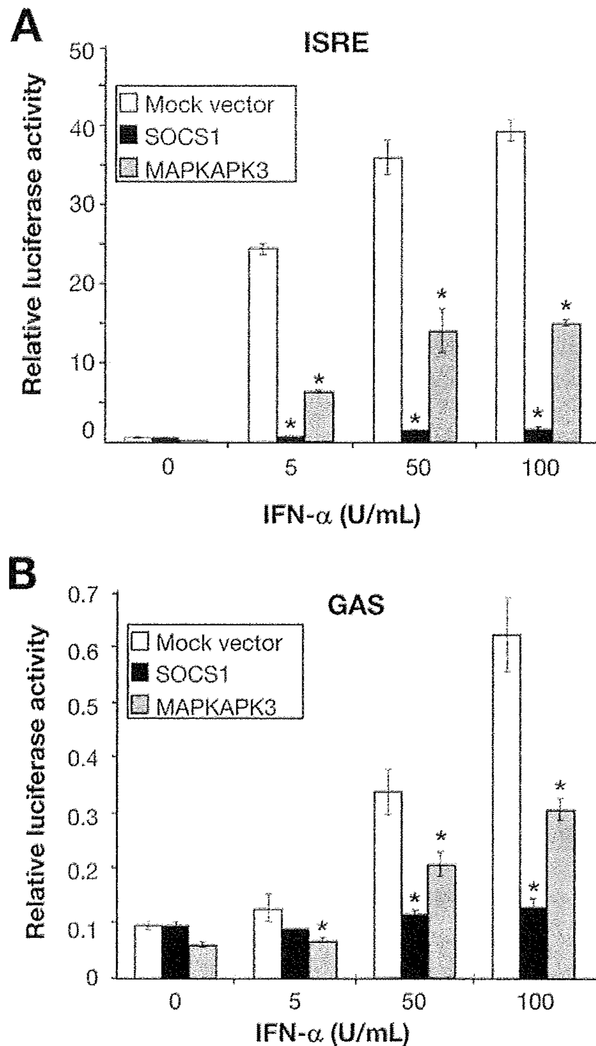

Figure 2. Effects of *MAPKAPK3* on IFN-α–induced gene transcription via ISRE and GAS elements. Huh7 cells were transfected with both 1 ng renilla luciferase expression vector pRL-TK (internal control) and 10 ng firefly luciferase expression vector (*A*) pISRE-TA-Luc or (*B*) pGAS-TA-Luc, in conjunction with 40 ng expression plasmid pDEST51/mock (negative control, *open bar*), pDEST51/suppressor of cytokine signaling 1 (socs1) (positive control, *black bar*), or pDEST51/*MAPKAPK3* (*gray bar*). After 24 hours, cells were stimulated with IFN-alfa for another 20 hours, and luciferase induction was measured. Firefly luciferase activity was normalized by renilla luciferase activity. Data represent the mean ± SD of triplicate assay. *$P < .05$ for comparison with mock.

with the outcome of IFN therapy in patients infected with HCV genotype 1b (Tables 2–4). In our genotype data of 1055 patients, the 2 SNPs were in strong linkage disequilibrium with an r^2 value of 0.82. Multivariate logistic regression analysis showed that rs3792323 is an independent factor associated with IFN efficacy (Table 4).

MAPKAPK3 is expressed in every human tissue.[38] *MAPKAPK3* encodes a serine/threonine-specific protein kinase and functions as a mitogen-activated protein kinase–activated protein kinase in both mitogen and stress responses.[39] MAPKAPK3 shares 72% nucleotide and 75% amino acid identity with MAPKAPK2. MAPKAPK3 and MAPKAPK2 act as downstream kinases of p38 MAP kinase under type I IFN stimulation.[22] It has been shown that disruption of p38α MAP kinase gene results in defective transcription of genes that are regulated by ISRE and GAS elements.[22] It also was reported that pharmacologic inhibition of p38 MAP kinase partially inhibits type I IFN-induced antiviral activity.[21] In mouse embryonic fibroblasts with targeted disruption of MAPKAPK2, it was indicated that type I IFN-induced antiviral activity was decreased.[22] On the other hand, little is known about the role of MAPKAPK3 in the responses to type I IFN.

In the present study, we hypothesized that enhanced expression of MAPKAPK3 is associated with resistance to IFN therapy for the following 3 reasons. First, carriers of the T allele for rs3792323, rather than the A allele, were more likely to show no response to IFN therapy (Tables 2 and 4). Thus, the T allele for rs3792323 was considered as a risk allele for nonresponse. Similarly, the A allele for rs616589 also was considered as a risk allele for nonresponse. Second, allele-specific *MAPKAPK3* mRNA expression corresponding to the T allele for rs3792323 (risk allele for nonresponse) was significantly higher than that of the A allele for rs3792323 in liver biopsy specimens of participating patients (Figure 1). Third, we did not find any nonsynonymous allelic variants in *MAPKAPK3* from the analysis of genomic DNA from 48 patients.

To examine our hypothesis, we examined whether enhanced MAPKAPK3 expression influences IFN-alfa–induced gene transcription. In reporter gene assay, overexpression of MAPKAPK3 inhibited IFN-alfa–induced gene transcription via ISRE and GAS elements (Figure 2), suggesting that MAPKAPK3 plays an important role in inhibition of IFN-α–induced antiviral activity. Several downstream effectors of MAPKAPK3 have been reported, including actin-binding protein, such as heat shock protein 27,[39] and transcription factors such as basic helix-loop-helix transcription factor E47[40] and cyclic AMP responsive element binding protein.[41] However, the mechanisms by which MAPKAPK3 inhibits IFN-α–induced gene transcription still are unclear, and further investigations are required. It also is interesting to examine the allele-specific *MAPKAPK3* mRNA levels during interferon therapy. Although we have no direct information, MAPKAKP3 mRNA expression is not inducible by IFN-alfa stimulation in human hepatoma cell line Huh-7 and HepG2 (data not shown).

In this study, the association between the *MAPKAPK3* polymorphisms and the efficacy of IFN therapy was observed in HCV genotype 1b, but not found in genotype non-1b. The reason for this difference between the 2 groups is yet to be seen. As one possible reason, the high susceptibility of genotype non-1b to IFN treatment may make the fine effect of SNPs obscure. As another possible

explanation, the effect of MAPKAPK3 on IFN efficacy may vary among different HCV genotypes. It was reported that associations between SNPs in the osteopontin gene and the efficacy of IFN therapy were particularly evident in patients with genotype 1b and a high virus titer, rather than in patients with genotype non-1b.[26] Until now, in various HCV genotypes including 1a, 1b, and 2a, HCV subgenomic replicon cell lines, which show autonomous HCV-RNA replication in the human hepatoma cell line, have been established. In the case that MAPKAPK3 is overexpressed in these HCV replicon cell lines, it is interesting to examine whether the effect of MAPKAPK3 on IFN efficacy is different or not among these HCV genotypes. It also is important to test associations between the *MAPKAPK3* SNPs and the IFN efficacy in each subgroup of patients infected with each HCV genotype including 1a, 2a, and 2b.

Recently, it was reported that the combination treatment of IFN-alfa plus ribavirin results in higher rates of sustained response than IFN monotherapy.[2,3] Various mechanisms of ribavirin activity against HCV have been proposed.[42] However, it is notable that treatment with ribavirin alone has no effect on serum HCV-RNA level.[43,44] On the other hand, the addition of ribavirin to IFN-alfa monotherapy leads to marked reduction of the serum HCV-RNA level. These facts suggest that IFN-alfa signaling is important for the induction of antiviral activity not only in IFN-alfa monotherapy but also in IFN-alfa combination therapy with ribavirin. Therefore, it is likely that the inhibitory effect of MAPKAPK3 on IFN signaling influences the efficacy of IFN-alfa combination therapy as well as IFN-alfa monotherapy. At present, about 50% of patients infected with HCV genotype 1b fail to eradicate the virus even after combination therapy of IFN-α plus ribavirin.[1–3] It is important to examine whether the 2 SNPs are associated with the responsiveness to combination therapy of IFN-alfa plus ribavirin.

It remains to be seen whether or not rs3792323 and rs616589 are functional cis-acting polymorphisms affecting *MAPKAPK3* expression. Even if the 2 SNPs do not have functional effects, it is expected that the 2 SNPs can serve as marker SNPs in linkage disequilibrium with functional cis-acting polymorphisms.[45] Furthermore, the 2 SNPs may be useful as genetic markers to predict the efficacy of IFN therapy. In our patients with IFN-alfa monotherapy, patients with risk alleles for nonresponse (T allele for rs3792323, A allele for rs616589) were more likely to be NRs compared with risk allele–negative patients (Tables 2 and 4). Although the results of our internal validation suggest that the observed association between the 2 SNPs in *MAPKAPK3* and the IFN efficacy is internally consistent, further replication with an independent cohort is needed to confirm the association. It also is interesting to test associations between the 2 SNPs and the phenotypes in relapsed patients. At present, we do not enroll enough of these patients to examine the association. In the future, we will enroll enough patients and test the association.

It was reported that polymorphism of GT-repeat length in the IFNAR1 promoter region was associated with the outcome of IFN therapy for chronic HCV infection in a study of 157 Japanese patients (HCV genotype total, P = .008).[13] In our study, we could not find a similar association for analyzed tagging-SNPs in IFNAR1 (Supplementary Table 2). The reason for the discrepancy between the 2 studies is not clear at present. Unfortunately, the GT-repeat polymorphism in IFNAR1 is not included in the HapMap database. Therefore, it is not clear whether tagging-SNPs have strong linkage disequilibrium with the GT-repeat polymorphism in IFNAR1. Possibly, tagging-SNPs may not capture the GT-repeat polymorphism in IFNAR1. To explain the discrepancy between the present study and the previous study, it is desirable to genotype the GT-repeat polymorphism in IFNAR1 by direct sequencing.

In conclusion, we identified that SNP rs3792323 in *MAPKAPK3* is associated strongly with the outcome of IFN therapy in patients infected with HCV genotype 1b. In addition, we showed that SNP rs3792323 associates with the expression level of *MAPKAPK3* and *MAPKAPK3* inhibits IFN-alfa–induced gene transcription via ISRE and GAS elements. Therefore, MAPKAPK3 may play an important role in the inhibition of IFN-alfa–induced antiviral activity.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.01.061.

Appendix

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References

1. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.
2. Management of hepatitis C. NIH Consensus Statement 1997; available at http://consensus.nih.gov/1997/1997HepatitisC105html.htm.
3. Liang TJ, Rehermann B, Seeff LB, et al. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000;132:296–305.
4. Martinot-Peignoux M, Marcellin P, Pouteau M, et al. Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. Hepatology 1995;22:1050–1056.
5. Trepo C. Genotype and viral load as prognostic indicators in the treatment of hepatitis C. J Viral Hepat 2000;7:250–257.
6. Walsh MJ, Jonsson JR, Richardson MM, et al. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. Gut 2006;55: 529–535.
7. Gao B, Hong F, Radaeva S. Host factors and failure of interferon-alpha treatment in hepatitis C virus. Hepatology 2004;39:880–890.
8. Huang Y, Yang H, Borg BB, et al. A functional SNP of interferon-gamma gene is important for interferon-alpha-induced and spontaneous recovery from hepatitis C virus infection. Proc Natl Acad Sci U S A 2007;104:985–990.
9. Yee LJ, Tang J, Gibson AW, et al. Interleukin 10 polymorphisms as predictors of sustained response in antiviral therapy for chronic hepatitis C infection. Hepatology 2001;33:708–712.
10. Naito M, Matsui A, Inao M, et al. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. J Gastroenterol 2005;40:381–388.
11. Suzuki F, Arase Y, Suzuki Y, et al. Single nucleotide polymorphism of the MxA gene promoter influences the response to interferon monotherapy in patients with hepatitis C viral infection. J Viral Hepat 2004;11:271–276.
12. Vidigal PG, Germer JJ, Zein NN. Polymorphisms in the interleukin-10, tumor necrosis factor-alpha, and transforming growth factor-beta1 genes in chronic hepatitis C patients treated with interferon and ribavirin. J Hepatol 2002;36:271–277.
13. Matsuyama N, Mishiro S, Sugimoto M, et al. The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. Hepatol Res 2003;25:221–225.
14. Pharoah PD, Dunning AM, Ponder BA, et al. Association studies for finding cancer-susceptibility genetic variants. Nat Rev Cancer 2004;4:850–860.

15. Johnson GC, Esposito L, Barratt BJ, et al. Haplotype tagging for the identification of common disease genes. Nat Genet 2001; 29:233–237.
16. Gibbs RA, Belmont JW, Hardenbol P, et al. The International HapMap Project. Nature 2003;426:789–796.
17. Darnell JE Jr, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 1994;264:1415–1421.
18. Uddin S, Lekmine F, Sharma N, et al. The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins. J Biol Chem 2000;275:27634–27640.
19. Li Y, Batra S, Sassano A, et al. Activation of mitogen-activated protein kinase kinase (MKK) 3 and MKK6 by type I interferons. J Biol Chem 2005;280:10001–10010.
20. Uddin S, Majchrzak B, Woodson J, et al. Activation of the p38 mitogen-activated protein kinase by type I interferons. J Biol Chem 1999;274:30127–30131.
21. Mayer IA, Verma A, Grumbach IM, et al. The p38 MAPK pathway mediates the growth inhibitory effects of interferon-alpha in BCR-ABL-expressing cells. J Biol Chem 2001;276:28570–28577.
22. Li Y, Sassano A, Majchrzak B, et al. Role of p38alpha Map kinase in type I interferon signaling. J Biol Chem 2004;279:970–979.
23. Ishida H, Ohkawa K, Hosui A, et al. Involvement of p38 signaling pathway in interferon-alpha-mediated antiviral activity toward hepatitis C virus. Biochem Biophys Res Commun 2004;321:722–727.
24. Hoofnagle JH. Therapy of acute and chronic viral hepatitis. Adv Intern Med 1994;39:241–275.
25. Shiratori Y, Kato N, Yokosuka O, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Tokyo-Chiba Hepatitis Research Group. Gastroenterology 1997;113: 558–566.
26. Naito M, Matsui A, Inao M, et al. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. J Gastroenterol 2005;40:381–388.
27. Desmet VJ, Gerber M, Hoofnagle JH, et al. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520.
28. Ozaki K, Ohnishi Y, Iida A, et al. Functional SNPs in the lymphotoxin-alpha gene that are associated with susceptibility to myocardial infarction. Nat Genet 2002;32:650–654.
29. Ohnishi Y, Tanaka T, Ozaki K, et al. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001; 46:471–477.
30. Suzuki A, Yamada R, Chang X, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34: 395–402.
31. Iida A, Saito S, Sekine A, et al. Catalog of 258 single-nucleotide polymorphisms (SNPs) in genes encoding three organic anion transporters, three organic anion-transporting polypeptides, and three NADH:ubiquinone oxidoreductase flavoproteins. J Hum Genet 2001;46:668–683.
32. Osawa N, Koya D, Araki S, et al. Combinational effect of genes for the renin-angiotensin system in conferring susceptibility to diabetic nephropathy. J Hum Genet 2007;52:143–151.
33. Kamiyama M, Kobayashi M, Araki SI, et al. Polymorphisms in the 3′ untranslated region in the neurocalcin delta gene affect mRNA stability, and confer susceptibility to diabetic nephropathy. Hum Genet 2007;122:397–407.
34. Nielsen DM, Ehm MG, Weir BS. Detecting marker-disease association by testing for Hardy-Weinberg disequilibrium at a marker locus. Am J Hum Genet 1998;63:1531–1540.
35. Sladek R, Rocheleau G, Rung J, et al. A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 2007; 445:881–885.
36. Devlin B, Risch N. A comparison of linkage disequilibrium measures for fine-scale mapping. Genomics 1995;29:311–322.
37. Freedman ML, Reich D, Penney KL, et al. Assessing the impact of population stratification on genetic association studies. Nat Genet 2004;36:388–393.
38. Sithanandam G, Latif F, Duh FM, et al. 3pK, a new mitogen-activated protein kinase-activated protein kinase located in the small cell lung cancer tumor suppressor gene region. Mol Cell Biol 1996;16:868–876.
39. Ludwig S, Engel K, Hoffmeyer A, et al. 3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways. Mol Cell Biol 1996;16: 6687–6697.
40. Neufeld B, Grosse-Wilde A, Hoffmeyer A, et al. Serine/threonine kinases 3pK and MAPK-activated protein kinase 2 interact with the basic helix-loop-helix transcription factor E47 and repress its transcriptional activity. J Biol Chem 2000;275:20239–20242.
41. Maizels ET, Mukherjee A, Sithanandam G, et al. Developmental regulation of mitogen-activated protein kinase-activated kinases-2 and -3 (MAPKAPK-2/-3) in vivo during corpus luteum formation in the rat. Mol Endocrinol 2001;15:716–733.
42. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967–972.
43. Lau JY, Tam RC, Liang TJ, et al. Mechanism of action of ribavirin in the combination treatment of chronic HCV infection. Hepatology 2002;35:1002–1009.
44. Hoofnagle JH, Lau D, Conjeevaram H, et al. Prolonged therapy of chronic hepatitis C with ribavirin. J Viral Hepat 1996;3:247–252.
45. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. Science 2002;296: 2225–2229.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Table 1. Candidate Genes Related to Type I IFN Pathway and Selected 116 Tagging-SNPs

dbSNP ID	Gene symbol	Alleles	SNP bin	SNP location
rs2243594	*IFNAR1*	A/G	Bin1	Intron2_108
rs2243600	*IFNAR1*	G/T	Bin2	Intron8_1812
rs2252930	*IFNAR1*	C/G	Bin3	Intron1_6765
rs2252650	*IFNAR2*	A/T	Bin1	Intron4_571
rs6517154	*IFNAR2*	T/C	Bin2	Intron1_6159
rs2073362	*IFNAR2*	A/G	Bin3	Intron5_1606
rs2248202	*IFNAR2*	A/C	Bin4	Intron1_1359
rs10211925	*IFNAR2*	G/A	Bin5	Intron2_1450
rs2248412	*IFNAR2*	A/G	Bin7	Intron1_3010
rs310209	*JAK1*	C/A	Bin1	Intron2_3344
rs3790541	*JAK1*	C/T	Bin2	Intron3_3141
rs310247	*JAK1*	A/G	Bin3	Intron16_2338
rs3790532	*JAK1*	G/A	Bin4	Intron21_225
rs2254002	*JAK1*	A/C	Bin5	Intron22_112
rs3818753	*JAK1*	A/G	Bin6	Intron3_5476
rs17127024	*JAK1*	G/T	Bin7	Intron21_484
rs2274948	*JAK1*	T/C	Bin8	Intron9_1930
rs280523	Tyrosine kinase2	G/A	Bin1	Exon6_51
rs280519	Tyrosine kinase2	A/G	Bin2	Intron11_7
rs280496	Tyrosine kinase2	C/G	Bin3	Intron22_122
rs11885069	*STAT1*	C/T	Bin1	Intron5_1665
rs9789428	*STAT1*	C/A	Bin2	Intron21_656
rs2280233	*STAT1*	T/C	Bin3	Intron14_1014
rs13395505	*STAT1*	A/G	Bin4	3′flank_1750
rs12693589	*STAT1*	C/T	Bin5	3′flank_7602
rs2066805	*STAT1*	T/C	Bin6	Intron8_42
rs1400657	*STAT1*	T/G	Bin7	3′flank_6724
rs3771300	*STAT1*	T/G	Bin8	3′flank_4668
rs11677408	*STAT1*	C/T	Bin9	Intron5_5674
rs11887698	*STAT1*	A/G	Bin10	Intron11_1080
rs2030171	*STAT1*	A/G	Bin11	Intron5_3126
rs1467199	*STAT1*	C/G	Bin12	5′flank_-1566
rs10199181	*STAT1*	T/A	Bin13	Intron4_136
rs2066802	*STAT1*	A/G	Bin15	Exon3_64
rs16833155	*STAT1*	C/T	Bin16	Intron9_1205
rs2066799	*STAT1*	C/T	Bin17	Intron14_95
rs11693463	*STAT1*	A/G	Bin18	Intron5_2378
rs10208033	*STAT1*	C/T	Bin19	5′flank_-481
rs3755312	*STAT1*	C/G	Bin20	Intron18_1118
rs2280232	*STAT1*	A/C	Bin21	Intron14_814
rs13029532	*STAT1*	A/C	Bin22	Intron2_2350
rs7562024	*STAT1*	T/C	Bin23	Intron11_433
rs1914408	*STAT1*	C/T	Bin24	3′flank_288
rs2066807	*STAT2*	C/G	Bin1	Exon20_58
rs12432194	IFN regulatory factor 9	C/T	Bin1	3′flank_251
rs4981494	IFN regulatory factor 9	G/A	Bin2	5′flank_-331
rs12432304	IFN regulatory factor 9	C/T	Bin3	3′flank_1029
rs2277484	IFN regulatory factor 9	G/A	Bin4	5′flank_-678
rs2236350	IFN regulatory factor 9	C/A	Bin5	Intron1_463
rs2303364	Ras-related C3 botulinum toxin substrate 1	C/T	Bin1	Intron6_30
rs836483	Ras-related C3 botulinum toxin substrate 1	G/A	Bin2	Intron3_827
rs6954996	Ras-related C3 botulinum toxin substrate 1	G/A	Bin3	Intron5_1439
rs7456834	Ras-related C3 botulinum toxin substrate 1	G/C	Bin4	Intron2_1041
rs702484	Ras-related C3 botulinum toxin substrate 1	G/C	Bin5	Intron4_261
rs2347339	Ras-related C3 botulinum toxin substrate 1	C/G	Bin6	Intron2_8177
rs768409	Ras-related C3 botulinum toxin substrate 1	A/T	Bin7	Intron1_9471
rs2305871	*MAPKK3*	G/C	Bin1	Intron6_35
rs9901404	*MAPKK3*	G/A	Bin2	3′flank_5917
rs12602109	*MAPKK3*	G/A	Bin3	3′flank_8737
rs3760201	*MAPKK3*	A/G	Bin4	Intron1_5337
rs8074866	*MAPKK3*	C/T	Bin5	Intron1_2246
rs2074028	*MAPKK6*	T/C	Bin1	Intron7_308
rs2034100	*MAPKK6*	G/A	Bin2	Intron1_1022