Supplementary Figure 5. Treatment with liposomal clodronate reversed BDL- and CCl_4-induced increase of TNFα and interleukin (IL)-1β mRNA expression in liver. WT C57BL/6 mice were injected with liposomal chlodronate (200 μL/mouse, intravenously) or vehicle. Thereafter, animals were subjected to (A) BDL or (B) CCl_4 intoxication to induce liver fibrosis. Hepatic expression of TNFα and IL-1β mRNA was shown. **$P < .01$ compared with the (A) control diet–sham-operated group or the (B) control diet–corn oil group.
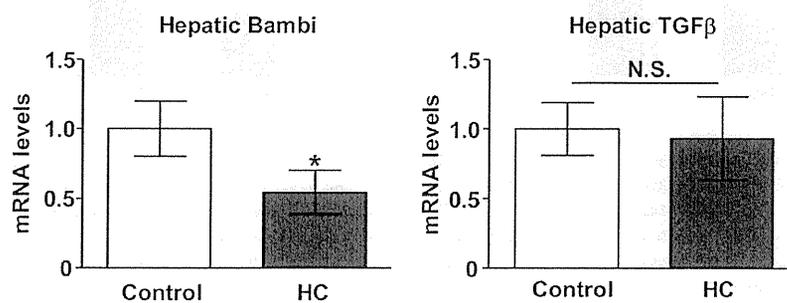
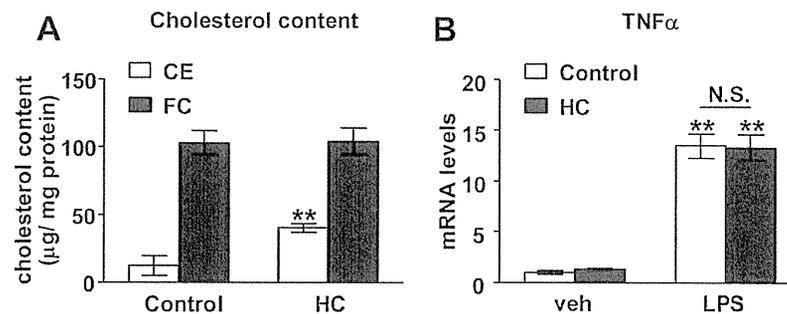**Supplementary Figure 6.** Effects of the HC diet on hepatic expression of Bambi and TGFβ mRNA. Hepatic expression of Bambi and TGFβ mRNA (N = 3/group) after being fed a control or an HC diet for 4 weeks. *$P < .05$ vs control diet group.
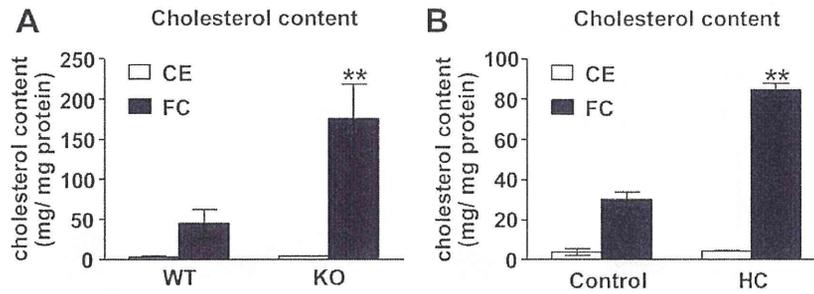
Supplementary Figure 7. Quantification of cellular FC and CE in late endosomes/lysosomes in HSCs. With late endosomes/lysosomes in WT HSCs, the mean ($\pm$SD) TC content was 48.46 $\pm$ 33.57 mg/mg cell protein. In late endosomes/lysosomes in NPC1 KO HSCs, the mean ($\pm$SD) TC content was increased significantly to 178.68 $\pm$ 81.39 mg/mg cell protein. Similarly, with late endosomes/lysosomes in HSCs from the control diet group, the mean ($\pm$SD) TC content was 33.82 $\pm$ 7.34 mg/mg cell protein. In late endosomes/lysosomes in HSCs from the HC diet group, the mean ($\pm$SD) TC content was increased significantly to 89.08 $\pm$ 6.57 mg/mg cell protein. (*A*) Quantification of FC and CE in late endosomes/lysosomes in HSCs from WT mice or NPC1-deficient mice. **$P <$ .01 vs WT mouse group. (*B*) The FC and CE levels in late endosomes/lysosomes in HSCs from control diet–fed mice or HC diet–fed mice. **$P <$ .01 vs control-diet group.
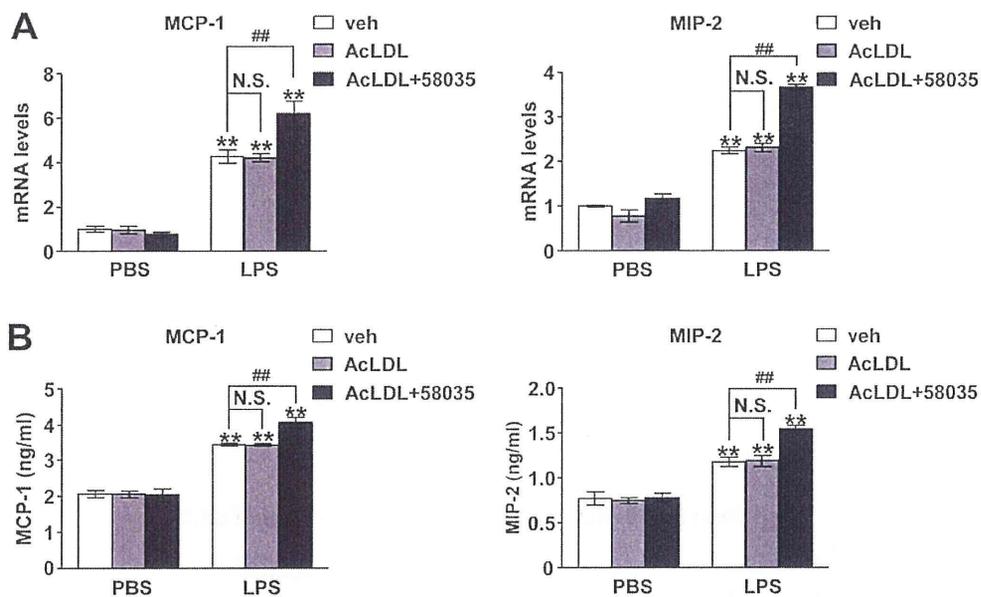
Supplementary Figure 8. FC, but not CE, promotes LPS-induced TLR4 signal transduction in HSCs. Paik et al[3] showed that LPS acts directly through TLR4 and then activates nuclear factor-κB to induce the production of inflammatory cytokines, including interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1), in human activated HSCs. Seki et al[4] also showed a strong up-regulation of MCP-1 and macrophage inflammatory protein-2 (MIP-2: mouse homologue of human IL-8) mRNA in mouse HSCs after LPS stimulation. Based on these reports, we evaluated the LPS responsiveness of HSCs loaded with FC using 2 highly quantitative methods: real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) of TLR4-induced inflammatory cytokines such as MCP-1 and MIP-2. (*A*) Expression of MCP-1 and MIP-2 mRNA in primary HSC cultures. HSCs were incubated with vehicle, AcLDL (50 μg/mL), or AcLDL plus compound 58035 (10 μg/mL) for 16 hours, and then treated with LPS (100 ng/mL) or not for 6 hours (N = 5–7/group). (*B*) Secreted MCP-1 and MIP-2 were quantified by ELISA. HSCs were incubated with vehicle, AcLDL, or AcLDL plus compound 58035 for 16 hours, and then treated with LPS or not for 24 hours (N = 5/group). **$P <$ .01 vs the corresponding culture without LPS treatment in each group. ##$P <$ .01 vs the LPS-treated control culture.

Supplementary Figure 9. Effects of the HC diet on cholesterol contents in Kupffer cells. We determined the level of cholesterol inside Kupffer cells. With Kupffer cells from the control diet group, the mean ($\pm$SD) TC content was 115.03 $\pm$ 22.61 μg/mg cell protein. In Kupffer cells from the HC diet group, the mean ($\pm$SD) TC content was increased significantly to 144.11 $\pm$ 38.83 μg/mg cell protein. (*A*) Quantification of cellular FC and CE in Kupffer cells from control diet–fed or HC diet–fed mice. Cholesterol concentrations were expressed as micrograms per milligrams of cellular proteins (N = 4/group). **P < .01 vs the control diet group. (*B*) Expression of TNFα mRNA in primary Kupffer cell cultures (N = 5/group). Kupffer cells isolated from control diet–fed or HC diet–fed mice were treated with LPS (100 ng/mL) for 6 hours. **P < .01 vs the control diet–control culture.

Supplementary Table 1. Effects of the HC Diet on Body Weight, Liver Weight, and Serum Lipid Levels

	Sham				BDL			
	Control diet		HC diet		Control diet		HC diet	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Body weight, g	28.2	0.3	27.2	0.6	27.5	2.3	21.8	0.3
Liver weight, g	1.4	0.1	1.2	0.1	1.7	0.2	2.1	0.2
Serum TG level, mg/dL	130.2	4.5	125.4	6.9	72.1	8.6	75.5	15.6
Serum TC level, mg/dL	103.2	9.8	135.0[a]	3.0	150.7	22.6	407.0[b]	17.7
Serum glucose level, mg/dL	116.6	5.4	120.8	17.6	85.3	26.7	103.0	23.0
	Corn oil				CCl$_4$			
Body weight, g	23.8	0.5	24.3	0.9	23.2	0.2	24.3	0.6
Liver weight, g	1.1	0.0	1.2	0.1	1.1	0.1	1.2	0.0
Serum TG level, mg/dL	229.4	19.6	173.8	20.6	170.8	16.9	193.5	12.0
Serum TC level, mg/dL	120.0	4.5	138.5[c]	9.0	118.0	4.1	143.8[d]	8.2
Serum glucose level, mg/dL	102.2	9.3	88.3	5.9	86.3	6.9	103.5	12.1

SEM, standard error of the mean.
[a]P < .05 vs the control diet-sham-operated group.
[b]P < .01 vs the control diet-BDL-operated group.
[c]P < .05 vs the control diet-corn oil group.
[d]P < .05 vs the control diet-CCl$_4$ group.

Antiviral Combination Therapy With Peginterferon and Ribavirin Does not Induce a Therapeutically Resistant Mutation in the HCV Core Region Regardless of Genetic Polymorphism Near the *IL28B* Gene

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An association has been reported between genetic polymorphism near *IL28B* gene and the prevalence of mutation of hepatitis C virus (HCV) core region residue 70, both of which have been associated with a lack of virologic response to antiviral combination therapy with peginterferon (PEG-IFN) and ribavirin. This study investigated whether PEG-IFN/ribavirin combination therapy induces amino acid (AA) mutation at residue 70 of HCV and whether genetic polymorphism near *IL28B* gene affects it. AA substitutions at residue 70 of the HCV core region were measured and compared before and after combination therapy in 65 non-responders and 88 relapsers to the combination therapy. In three patients in whom both wild-type AA (arginine) and mutant-type AA (glutamine or histidine) were detected at residue 70 before treatment, only mutant-type AA was identified after treatment. In two patients who had wild-type AA solely before treatment, both wild-type and mutant-type AAs were identified at residue 70 after treatment. In five patients, in whom the AA had changed at residue 70 between before and after treatment, four patients carried the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene and one carried the TG/GG genotype. No difference was found in the prevalence of this change of AA at residue 70 between the TT and the TG/GG genotype. Antiviral combination therapy with PEG-IFN and ribavirin does not appear to induce mutation of HCV core region residue 70 regardless of genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. *J. Med. Virol.* **83:1559–1564, 2011.** © 2011 Wiley-Liss, Inc.

INTRODUCTION

Hepatitis C virus (HCV) causes chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998; Kenny-Walsh, 1999]. The current standard therapy for patients with chronic HCV infection is the combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients with sustained virologic response, which indicates the eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieve a sustained virologic response.

Many studies have investigated the potential baseline host- or virus-related factors that are associated

with the lack of virologic response to IFN-based anti-viral therapy. As a host-related factor, recent studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 are strongly associated with a resistance to the combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Patients having the TT genotype at a polymorphic locus (rs8099917) near the *IL28B* gene show a favorable response to the combination therapy with PEG-IFN and ribavirin, whereas patients having the GG genotype or those who are TG heterozygote show a resistance to the therapy. As for virus-related factors, amino acid (AA) mutations at residue 70 in the HCV core region have been reported to be associated strongly with a resistance to PEG-IFN/ribavirin combination therapy in patients infected with HCV genotype 1b [Akuta et al., 2005, 2007a; Donlin et al., 2007]. Patients with the mutant-type AA (glutamine or histidine) at residue 70 in the HCV core region show a resistance to the combination therapy in comparison to those with the wild-type AA (arginine) at this residue. These host- and virus-related factors are both associated with the outcome of the combination therapy with PEG-IFN and ribavirin independently in a previous report [Hayes et al., 2011].

A previous study reported that the percentage of patients with the mutant-type AA at residue 70 of the HCV core region increases with the progression of chronic hepatitis, suggesting that the mutation of AA at residue 70 (from arginine to glutamine or histidine) occurs in the natural course of chronic HCV infection [Kobayashi et al., 2010a]. Several recent studies have reported a higher prevalence of the mutant-type AA at residue 70 in patients who have the TG/GG genotype of genetic polymorphism of rs8099917 near the *IL28B* gene, which is associated with an unfavorable response to the combination therapy with PEG-IFN and ribavirin, than in patients who have the TT genotype [Abe et al., 2010; Kobayashi et al., 2010b]. These reports suggest that the mutation of AA residue 70 of the HCV core region may occur more frequently in patients with the TG/GG genotype. Especially, the induction of this mutation may occur easily in patients who underwent PEG-IFN/ribavirin combination therapy and failed to clear HCV (non-sustained virologic response), wherein HCV obtained a resistance to combination therapy.

Mutation at HCV core region residue 70 has reportedly been associated with a hepatocarcinogenesis and an insulin resistance [Akuta et al., 2007b, 2009; Nakamoto et al., 2010]. In addition, a recent study reported that patients who have both the TG/GG genotype of rs8099917 near the *IL28B* gene and the mutant-type AA at residue 70 of the HCV core region have shown further resistance even to the triple therapy with telaprevir, PEG-IFN, and ribavirin [Akuta et al., 2010]. It is, therefore, important to clarify whether PEG-IFN/ribavirin combination

therapy induces the mutation of the HCV core region residue 70 in patients who failed to eradicate HCV, and whether genetic polymorphism near the *IL28B* gene are correlated with this mutation. If so, some patients should not undergo the current standard combination therapy in order to prevent the acquisition of the resistance (i.e., mutation at residue 70).

The present study investigated the effects of the combination therapy with PEG-IFN and ribavirin and genetic polymorphisms near the *IL28B* gene on the mutation of HCV core region residue 70 in patients who failed to achieve a sustained virologic reponse.

PATIENTS AND METHODS

Patients and Treatment

Three hundred and forty six patients with chronic hepatitis C who had been infected with HCV genotype 1b (as assessed by amplification of core-gene sequences with polymerase chain reaction (PCR) using genotype-specific primers [Ohno et al., 1997]) and pretreatment HCV-RNA level of $>100 \times 10^3$ IU/ml [as assessed by a quantitative PCR assay (Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA)] underwent antiviral combination therapy with PEG-IFN and ribavirin between January, 2007 and December, 2009 at the Ogaki Municipal Hospital or the Nagoya University Hospital. Of these patients, 19 patients dropped out and their outcome could not be defined. Among the remaining 327 patients, 274 patients who gave written informed consent for genetic analyses were enrolled to the study (Fig. 1). No patients were coinfected with hepatitis B virus or human immunodeficiency virus.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough) daily. The initial doses of PEG-IFN and ribavirin and the dose reductions were according to the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of the treatment. Some patients had an extended treatment duration of up to 72 weeks. In some patients, the treatment was discontinued before 48 weeks because they had a low likelihood of achieving a sustained virologic response, when serum HCV-RNA was positive 24 weeks after starting the therapy. The outcomes of the combination therapy were classified as a sustained virologic response when serum HCV-RNA became undetectable during the treatment and remained undetectable for 6 months after the treatment ended (i.e., eradication of HCV), a relapse when the serum HCV-RNA became undetectable during the treatment period but returned detectable after the treatment, and no-response when the serum HCV-RNA remained detectable during and after the treatment period.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Written
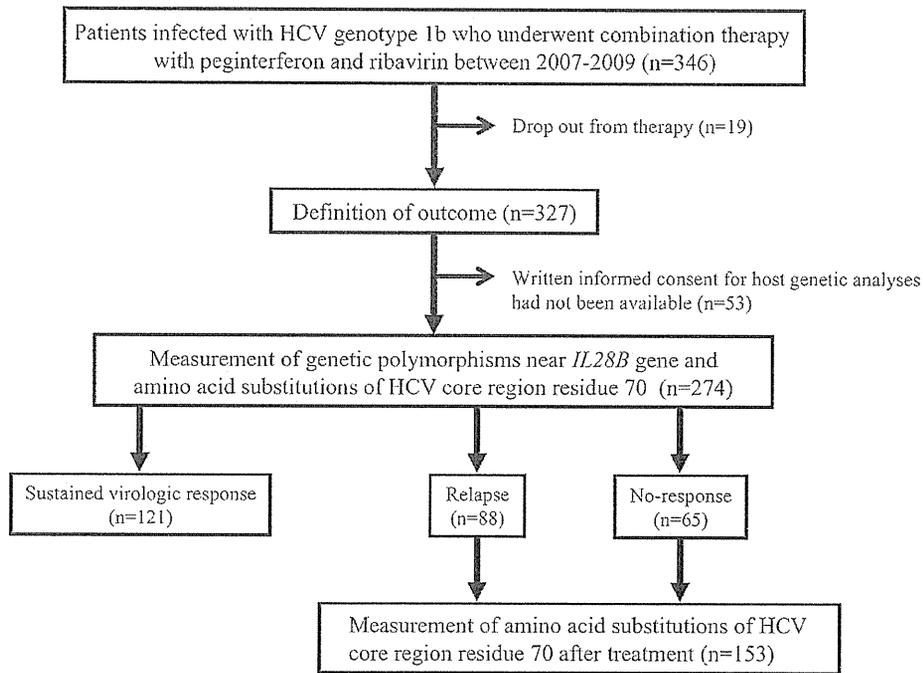
Fig. 1. Schematic representation of the study design.

informed consent was obtained from all patients prior to the study for the measurement of genetic polymorphism of rs8099917 near *IL28B* gene and AA substitution of HCV core region residue 70, and for the use of the laboratory data.

Measurements of Genetic Polymorphism Near the *IL28B* Gene and Amino Acid Substitution of the HCV Core Region Residue 70

Genotyping of polymorphisms of the rs8099917 locus near the *IL28B* gene was carried out in all 274 patients using a Taqman SNP assay (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C__11710096_10, Applied Biosystems).

The AA at residue 70 of the core region of HCV was measured before the treatment in all patients. In patients who failed to achieve a sustained virologic response, that is, patients who showed a relapse or no-response, the AA identity was measured at residue 70 after the treatment and compared pre- to post-treatment AA identity at this residue (Fig. 1). The AA at residue 70 after the treatment was measured in serum samples obtained at the end of treatment in patients who showed no-response. In patients with a relapse, it was measured in serum samples obtained upon the reappearance of HCV-RNA after the completion of the therapy. The AA identity was analyzed by direct nucleotide sequencing according to

a previous report [Akuta et al., 2007c]. The primer pairs used for PCR for direct sequencing the HCV core region were 5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer), 5′-GGAGCAGTCCTTCGTGA-CATG-3′ (outer, antisense primer), 5′-GCTAGCCG-AGTAGTGTT-3′ (inner, sense primer), and 5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer).

Statistical Analysis

The chi-square test was used to analyze the differences in percentages between groups.

RESULTS

Patient Characteristics and the Outcome of the Combination Therapy

The characteristics of study patients are shown in Table I. The study patients comprised 139 males (50.7%) and 135 females (49.3%), with a mean age of 58.0 ± 10.4 years. The grade of liver fibrosis according to the METAVIR score [The French METAVIR Cooperative Study Group, 1994] was F0 in 31 patients (11.6%), F1 in 122 patients (45.9%), F2 in 75 patients (28.2%), and F3 in 38 patients (14.3%). Analysis of the genetic polymorphism of the rs8099917 near the *IL28B* gene indicated 202 patients (73.7%) had the TT genotype, three patients (1.1%) had the GG genotype, and the remaining 69 patients (25.2%) were TG heterozygous. Before the treatment, 204 patients (74.4%)

TABLE I. Baseline Characteristics of the Study Patients (n = 274)

Age (years)	55.9 ± 11.2
Sex (female/male)	135 (49.3)/139 (50.7)
Body weight (kg)	58.0 ± 10.4
Alanine aminotransferase (IU/L)	64.5 ± 56.3
Aspartate aminotransferase (IU/L)	53.7 ± 42.2
Gamma-glutamyl transpeptidase (IU)	49.7 ± 48.5
Alkaline phosphatase (IU/L)	267.9 ± 100.6
Albumin (g/dl)	4.07 ± 0.38
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4933 ± 1331
Hemoglobin (g/dl)	14.0 ± 1.4
Platelet count ($\times 10^3$/μl)	164 ± 50
Liver histology-activity (A0/A1/A2/A3)[a]	2 (0.7)/147 (55.3)/99 (37.2)/18 (6.8)
Liver histology-fibrosis (F0/F1/F2/F3)[a]	31 (11.6)/122 (45.9)/75 (28.2)/38 (14.3)
HCV-RNA concentration ($\log_{10}$ IU/ml)[b]	6.34 ± 0.54
Genetic polymorphisms near the *IL28B* gene (TT/TG/GG)[b]	202 (73.7)/69 (25.2)/6 (2.2)
Amino acid at HCV core 70 (wild type/mutant type/both)[c]	204 (74.4)/64 (23.4)/6 (2.2)
Response (SVR/relapse/NR)	121 (44.2)/88 (32.1)/65 (23.7)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no-response.
Percentages are shown in parentheses.
[a]Liver biopsy was not performed in eight patients.
[b]rs8099917 genetic polymorphism
[c]Before the treatment.

carried HCV with the wild-type AA at residue 70 of the HCV core region, 64 patients (23.4%) carried the mutant-type AA at residue 70, and both the wild-type AA and the mutant-type AA were identified at residue 70 in the remaining six patients (3.5%).

As a final outcome, 121 patients (44.2%) achieved a sustained virologic response, 88 patients (32.1%) relapsed, and the remaining 65 patients (23.7%) showed no-response (Fig. 1). Treatment was discontinued before 48 weeks in 11 of 65 patients who showed no-response because HCV-RNA remained detectable in serum 24 weeks after starting the therapy. The identity of the AA 70 of the core region of HCV was determined after the treatment in serum obtained at the discontinuation of the therapy in these 11 patients. Table II shows the association between the genetic polymorphisms of the rs8099917 near the *IL28B* gene, the AA substitutions of the HCV core region residue 70, and the outcome of the combination therapy. The wild-type AA was more frequently identified at residue 70 in patients with the TT genotype in comparison to those with the TG/GG genotype (82.2% vs.

52.8%, $P < 0.0001$). The rate of a sustained virologic response was significantly higher in patients with the TT genotype than those with the TG/GG genotype (107 of 202 patients, 53.0% vs. 14 of 72 patients, 19.4%, $P < 0.0001$), as well as being higher in patients carrying HCV with the wild-type AA at residue 70 of the core region than those with the mutant-type AA at this residue (101 of 204 patients, 49.5% vs. 19 of 64 patients, 29.7%, $P = 0.0083$, one patient had both the wild-type and the mutant-type AAs).

Comparison of the Amino Acid at Residue 70 of the HCV Core Region Before and After the Combination Therapy in Patients Who Showed a Relapse or No-Response

Table III shows the comparison of the AA at residue 70 of the HCV core region before and after the combination therapy in patients who showed a relapse or no-response, according to the genetic polymorphisms of the rs8099917 near the *IL28B* gene. In three of five

TABLE II. Association Between the Genetic Polymorphisms Near the *IL28B* Gene, the Amino Acid at the HCV Core Region Residue 70, and the Final Outcome of Peginterferon/Ribavirin Combination Therapy

Genetic polymorphism of rs8099917 near *IL28B* gene	Amino acid at residue 70 of the HCV core region		
	Wild type (n = 204)	Mutant type (n = 64)	Wild type + mutant type (n = 6)
TT (n = 202)	166 (92/60/14)	31 (14/9/8)	5 (1/2/2)
TG/GG (n = 72)	38 (9/9/20)	33 (5/7/21)	1 (0/1/0)

Outcomes of the combination therapy with peginterferon and ribavirin are shown in parentheses as sustained virologic response/relapse/no-response.

TABLE III. Amino Acid Substitutions of HCV Core Region Residue 70 Before and After
the Combination Therapy With Peginterferon and Ribavirin in No-Responders or
Relapsers

Amino acid at HCV core region residue 70	After treatment		
Before treatment	Wild type	Wild + Mutant	Mutant type
(A) Genetic polymorphisms near the *IL28B* gene (rs8099917): TT (n = 91)			
No-responders (n = 24)			
Wild type (n = 14)	13	1	0
Wild + mutant (n = 2)	0	0	2
Mutant type (n = 8)	0	0	8
Relapsers (n = 71)			
Wild type (n = 60)	60	0	0
Wild + mutant (n = 2)	0	1	1
Mutant type (n = 9)	0	0	9
(B) Genetic polymorphisms near the *IL28B* gene (rs8099917): TG/GG (n = 57)			
No-responders (n = 41)			
Wild type (n = 20)	19	1	0
Wild + mutant (n = 0)	0	0	0
Mutant type (n = 21)	0	0	21
Relapsers (n = 17)			
Wild type (n = 9)	9	0	0
Wild + mutant (n = 1)	0	1	0
Mutant type (n = 7)	0	0	7

HCV, hepatitis C virus.

patients in whom both the wild-type and mutant-type AAs had been identified at residue 70 of the HCV core region before treatment, only the mutant-type AA was identified at this residue after the treatment. All three of these patients (two no-responders and one relapser) had the TT genotype of the rs8099917. Both the wild-type and mutant-type AAs were identified at residue 70 after the treatment in two no-responders in whom only the wild-type AA had been identified before the treatment. One of them had the TT genotype at the rs 8099917 and the other patient was TG heterozygous. No change in the HCV core region residue 70 was found after the treatment in patients with the mutant-type AA at this residue before the treatment.

DISCUSSION

The present study investigated whether the combination therapy with PEG-IFN and ribavirin causes the mutation of residue 70 of the HCV core region, and whether the genetic polymorphisms of the rs8099917 locus near the *IL28B* gene influence this mutation. It is thought to be important to verify this issue, because it may be advisable to avoid the treatment of patients who have the TG/GG genotypes by the combination therapy with PEG-IFN and ribavirin so as to avoid an acquisition of the further resistance to emerging new therapies against HCV, as well as to avoid a potential enhancement of hepatocarcinogenesis.

The mutation of the AA at residue 70 was not observed before and after the treatment in all patients who had failed to achieve a sustained virologic response. The mutant-type AA was identified solely at residue 70 after the treatment in three patients who had both the wild-type and the mutant-type AAs at residue 70 before the treatment. This could be due to the selection of HCV strains with the mutant-type AA at residue 70 by the combination therapy with PEG-IFN and ribavirin, as reported previously [Kurbanov et al., 2010]. In two patients who carried only the wild-type AA before the treatment, the HCV with the mutant-type AA at residue 70 was also detected with the persistence of the wild-type AA at this residue after the treatment. The very minor HCV strain with the mutant-type AA at residue 70 which were not detected before the treatment may have been detected after the treatment due to the reduction of HCV with the wild-type AA at residue 70 by the combination therapy. Indeed, HCV with the mutant-type AA at core region residue 70 was not detectable in serum 6 months after the end of the combination therapy, suggesting that it returned to being a very minor population (data not shown). These two phenomena were observed in patients with both the TT genotype of the rs8099917, that is associated with a favorable response to the combination therapy and those with the TG/GG genotypes that is associated with an unfavorable response, without difference in the prevalence according to the genetic polymorphisms at the rs8099917 near the *IL28B* gene.

In conclusion, PEG-IFN/ribavirin combination therapy does not appear to induce the mutation of the AA at the HCV core region residue 70 regardless of the genetic polymorphism near the *IL28B* gene in Japanese patients infected with HCV genotype 1b. The combination therapy can be attempted regardless of the genetic polymorphisms near the *IL28B* gene in

treatment-naïve patients without the anxiety for the acquisition of the further resistance to the antiviral therapy. However, future studies should be undertaken to confirm the absence of the mutation at residue 70 of the HCV core region induced by the combination therapy with PEG-IFN and ribavirin. In addition, the effect of the genetic polymorphisms near the *IL28B* gene on the mutation of the AA at the HCV core region residue 70 should be investigated in the long-term observation of the natural course of chronic hepatitis C.

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Predictive Value of Early Viral Dynamics During Peginterferon and Ribavirin Combination Therapy Based on Genetic Polymorphisms Near the *IL28B* Gene in Patients Infected With HCV Genotype 1b

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A study was carried out to determine whether early viral dynamics retain prediction of the outcome of peginterferon (PEG-IFN) and ribavirin combination therapy based on different genetic polymorphisms near the *IL28B* gene, the strongest baseline predictor of response to this therapy. A total of 272 patients infected with hepatitis C virus (HCV) genotype 1b were grouped according to genetic polymorphisms near the *IL28B* gene (rs8099917). The ability of reduced HCV RNA levels at 4 and 12 weeks after starting therapy to predict a sustained virologic response was evaluated based on these genotypes. Among patients with the TT genotype for rs8099917 (associated with a favorable response), the rates of sustained virologic response were higher in patients with a ≥ 3 $\log_{10}$ reduction in serum HCV RNA levels at 4 weeks after starting therapy ($P < 0.0001$). In contrast, among patients with the TG/GG genotype (associated with an unfavorable response), there were no differences in this rate based on the reduction in HCV RNA levels at 4 weeks. Early viral dynamics at 4 weeks after starting therapy retains its predictive value for sustained virologic response in patients with the TT genotype for rs8099917, but not in patients with the TG/GG genotype. Patients who are likely to achieve sustained virologic response despite unfavorable TG/GG genotype cannot be identified based on early viral dynamics during therapy. In contrast, lack of early virologic response at 12 weeks retains a strong predictive value for the failure of sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy. **J. Med. Virol. 84:61–70, 2012.** © 2011 Wiley Periodicals, Inc.

INTRODUCTION

The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although this treatment regimen has increased markedly the number of patients with a sustained virologic response, i.e., the eradication of hepatitis C virus (HCV), only 50% of patients infected with HCV genotype 1 achieved a sustained virologic response approximately.

Many investigators have examined factors that predict the treatment outcome of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1. In addition to the baseline factors, the response of HCV during combination therapy, i.e., the changes in serum HCV RNA levels after starting therapy, has been shown to be an important predictor of the treatment outcome [Zeuzem et al., 2001; Buti

et al., 2002; Berg et al., 2003], with the emphasis on "response-guided therapy" [Lee and Ferenci, 2008; Marcellin and Rizzetto, 2008]. Recent reports have emphasized the importance of evaluating the viral dynamics at 4 weeks after starting therapy to predict a sustained virologic response. A rapid virologic response, in which serum HCV RNA is undetectable at 4 weeks after starting therapy, has been the strongest predictive factor of a sustained virologic response reportedly [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, the predictive value of reduced serum HCV RNA levels at 4 weeks after starting therapy has been clarified further, and a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy has high predictive value that a patient will achieve a sustained virologic response as a final outcome, even in the absence of a rapid virologic response [Toyoda et al., 2011].

In contrast, the lack of an early virologic response, defined as either undetectable serum HCV RNA or HCV RNA levels decreased by >2.0 $\log_{10}$ from the pretreatment level at 12 weeks after starting therapy, has been the most important predictor for the failure of a sustained virologic response in patients infected with HCV genotype 1 reportedly [Fried et al., 2002; Davis et al., 2003]. Therefore, treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment, according to the recommendation in the AASLD guidelines [Ghany et al., 2009].

More recently, several studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19 affect the virologic response to PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch et al., 2010]. Furthermore, genetic polymorphisms near the *IL28B* gene are the strongest baseline predictive factor of the final outcome of combination therapy. An additional report showed the effects of genetic polymorphisms near the *IL28B* gene on HCV viral dynamics during PEG-IFN and ribavirin combination therapy [Thompson et al., 2010].

Although early HCV viral dynamics during therapy was shown originally to have a high predictive value for a sustained virologic response in HCV genotype 1-infected patients before genetic polymorphisms near the *IL28B* gene were linked to a therapeutic response, it is not clear whether early viral dynamics retain their predictive value in light of this additional information. The purpose of the present study was to investigate whether response-guided therapy based on viral dynamics at 4 or 12 weeks after initiating therapy retains its ability to predict the final outcome of PEG-IFN and ribavirin combination therapy after accounting for genetic polymorphisms near the *IL28B* gene.

MATERIALS AND METHODS

Patients and Treatment

Between January 2007 and June 2008, a total of 402 patients with chronic hepatitis C received antiviral combination therapy with PEG-IFN and ribavirin for HCV infection at the Ogaki Municipal Hospital or the Nagoya University Hospital. Among these patients, 272 were infected with HCV genotype 1b and had pretreatment HCV RNA levels >5.0 $\log_{10}$ IU/ml based on a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems, Pleasanton, CA; Lower limit of quantification, 1.7 $\log_{10}$ IU/ml: Lower limit of detection, 1.0 $\log_{10}$ IU/ml) [Colucci et al., 2007; Pittaluga et al., 2008]. This study did not include any patients infected with HCV genotype 1a because this genotype is not found in the general Japanese population.

All patients were given PEG-IFN alpha-2b (Pegintron, Schering-Plough, Tokyo, Japan) weekly and ribavirin (Rebetol, Schering-Plough, Kenilworth, NJ) daily. The PEG-IFN and ribavirin doses were adjusted based on the patient's body weight. Patients weighing ≤ 45 kg were given 60 µg of PEG-IFN alpha-2b once a week, those weighing >45 and ≤ 60 kg were given 80 µg, those weighing >60 and ≤ 75 kg were given 100 µg, those weighing >75 and ≤ 90 kg were given 120 µg, and those weighing >90 kg were given 150 µg. Patients weighing ≤ 60 kg were administered 600 mg of ribavirin per day, those weighing >60 and ≤ 80 kg were given 800 mg per day, and those weighing >80 kg were administered 1000 mg per day. The PEG-IFN and ribavirin doses were modified based on the manufacturer's recommendations. All patients were scheduled to undergo 48 weeks of treatment. The treatment duration was extended up to 72 weeks in some patients. In addition, treatment was discontinued before 48 weeks in some patients who had a low likelihood of achieving an eradication of HCV due to the presence of serum HCV RNA at 24 weeks after starting therapy.

A sustained virologic response was defined as undetectable serum HCV RNA at 24 weeks after ending the therapy. A patient was considered to have relapsed when serum HCV RNA was detectable between the end of treatment and 24 weeks after completing treatment, although serum HCV RNA was undetectable during and at the end of therapy. Patients were considered to have non-response if serum HCV RNA was detectable at 24 weeks after initiating therapy (i.e., null response or partial response according to the American guidelines [Ghany et al., 2009]). Patients were considered to have a rapid virologic response if they had undetectable serum HCV RNA at 4 weeks after starting therapy. An early virologic response was defined as the disappearance or decrease in serum HCV RNA levels by at least 2 $\log_{10}$ at 12 weeks after starting therapy. Patients were considered to have a complete early virologic response if serum HCV RNA was undetectable at 12 weeks after starting therapy and a partial early virologic response if the serum

HCV RNA levels had decreased by at least 2 $\log_{10}$ at 12 weeks after initiating therapy. Patients were considered not to have an early virologic response if their HCV RNA levels did not decrease by more than 2 $\log_{10}$ at 12 weeks compared to the pretreatment levels. Patients were considered to have a slow virologic response if the serum HCV RNA became undetectable between 12 and 24 weeks.

The study protocol was in compliance with the Helsinki Declaration and was approved by the ethics committee of the Ogaki Municipal Hospital and the Nagoya University School of Medicine. Prior to initiating the study, each patient provided written informed consent to use the laboratory data, analyze genetic polymorphisms near the *IL28B* gene, and test stored serum samples.

Assessments of Serum HCV RNA Levels and Genetic Polymorphisms Near the *IL28B* Gene

After a patient provided informed consent, serum samples were obtained at the patient's regular hospital visits, just prior to initiating treatment, every 4 weeks during the treatment period, and during the 24-week follow-up period after treatment. Serum samples were stored at −80°C until further use. The HCV RNA levels were measured using a quantitative real-time PCR-based method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System).

Genotyping of rs 8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assay (Applied Biosystems, Foster City, California) according to the manufacturer's guidelines. A pre-designed and functionally tested probe was used for rs8099917 (C__11710096_10, Applied Biosystems).

Statistical analyses. Quantitative values are reported as the mean ± SD. In between-group differences were analyzed by the chi-square test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that predict a sustained virologic response, including age, sex, body weight, serum alanine aminotransferase activity, serum aspartate aminotransferase activity, serum gamma-glutamyl transpeptidase levels, serum alkaline phosphatase values, serum albumin levels, total serum bilirubin values, white blood cell counts, hemoglobin, platelet counts, hepatitis activity grade (A0 and A1 vs. A2 and A3), liver fibrosis grade (F0 and F1 vs. F2 and F3), pretreatment HCV RNA levels (≥6.5 $\log_{10}$ vs. <6.5 $\log_{10}$), reduction in peginterferon dose and ribavirin dose, reduction in HCV RNA levels at 4 weeks after starting therapy (≥3 $\log_{10}$ vs. <3 $\log_{10}$), and the type of an early virologic response. All *P*-values are two-tailed, and *P* < 0.05 was considered significant statistically.

RESULTS

The characteristics of the patients examined in this study are shown in Table I. Liver histology was evaluated according to the METAVIR score [The French

TABLE I. Characteristics of all Study Patients (n = 272)

Age (years)	56.0 ± 10.9
Sex (female/male)	139 (51.1)/133 (48.9)
Body weight (kg)	57.8 ± 10.5
Alanine aminotransferase (IU/L)	64.6 ± 56.4
Aspartate aminotransferase (IU/L)	53.9 ± 42.7
Gamma-glutamyl transpeptidase (IU)	48.5 ± 43.9
Alkaline phosphatase (IU/L)	267.9 ± 101.3
Albumin (g/dl)	4.04 ± 0.37
Total bilirubin (mg/dl)	0.79 ± 0.30
White blood cell count (/μl)	4892 ± 1333
Hemoglobin (g/dl)	14.0 ± 1.3
Platelet count ($\times 10^3$/μl)	163 ± 51
Liver histology-activity (A0/A1/A2/A3)*	3 (1.2)/136 (55.3)/ 92 (37.4)/15 (6.1)
Liver histology-fibrosis (F0/F1/F2/F3)*	27 (11.0)/114 (46.3)/ 70 (28.5)/35 (14.2)
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.35 ± 0.79
Reduction in the peginterferon dose	81 (29.8)
Reduction in the ribavirin dose	130 (47.8)
Final outcomes (sustained virologic response /relapse/ no response)	118 (43.4)/ 84 (30.9)/70 (25.7)

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

METAVIR Cooperative Study Group, 1994]. Although some patients had a reduction in their PEG-IFN and ribavirin doses during therapy, respectively, all patients except for those who discontinued the therapy had more than 80% adhesion to both the PEG-IFN and ribavirin regimens. No patients discontinued the therapy because of adverse effects. The treatment duration was extended up to 72 weeks in 51 of 71 patients (71.8%) who exhibited a slow virologic response. As a final outcome, 118 patients (43.4%) achieved a sustained virologic response, 84 patients (30.9%) relapsed, and the remaining 70 patients (25.7%) had no response.

Reduction in Serum HCV RNA Levels at 4 Weeks after Starting Therapy and Treatment Outcome According to Genetic Polymorphisms Near the *IL28B* Gene

An analysis of genetic polymorphisms at rs8099917 near the *IL28B* gene indicated that 207 patients (76.1%) had a TT genotype, 3 patients had a GG genotype (1.1%), and the remaining 62 patients were TG heterozygote (22.8%). Table II shows the comparison of the background characteristics between patients with the favorable TT genotype and those with the unfavorable TG/GG genotype. As reported previously [Abe et al., 2010], gamma-glutamyl transpeptidase level was higher significantly in patients with the TG/GG genotype. As a final outcome, the rate of a sustained virologic response was higher significantly in patients with the TT genotype. Among 207 patients with the TT genotype, serum HCV RNA became undetectable in 19 patients (9.2%) at 4 weeks after starting therapy (a rapid virologic response). In the remaining 188 patients, the decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.12

TABLE II. Characteristics of Study Patients According to the Genetic Polymorphisms Near the *IL28B* Gene

	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)	*P*-value
Age (years)	56.5 ± 10.4	54.4 ± 12.4	0.4112
Sex (female/male)	107 (51.7)/100 (48.3)	32 (49.2)/33 (50.8)	0.8384
Body weight (kg)	57.8 ± 10.9	57.8 ± 9.4	0.8361
Alanine aminotransferase (IU/L)	65.1 ± 53.3	62.8 ± 65.6	0.2548
Aspartate aminotransferase (IU/L)	53.6 ± 34.8	54.7 ± 62.0	0.3339
Gamma-glutamyl transpeptidase (IU)	44.2 ± 37.1	62.3 ± 59.0	0.0003
Alkaline phosphatase (IU/L)	263.1 ± 90.3	282.8 ± 129.9	0.3875
Albumin (g/dl)	4.04 ± 0.36	4.05 ± 0.43	0.8020
Total bilirubin (mg/dl)	0.79 ± 0.30	0.76 ± 0.32	0.3010
White blood cell count (/μl)	4826 ± 1333	5100 ± 1320	0.1608
Hemoglobin (g/dl)	13.9 ± 1.3	14.1 ± 1.4	0.3339
Platelet count ($\times 10^3$/μl)	161 ± 49	169 ± 57	0.3871
Liver histology-activity (A0/A1/A2/A3)*	2 (1.1)/98 (52.4)/ 74 (39.6)/13 (6.9)	1 (1.7)/38 (64.4)/ 18 (30.5)/2 (3.4)	0.3241
Liver histology-fibrosis (F0/F1/F2/F3)*	21 (11.2)/83 (44.4)/ 57 (30.5)/26 (13.9)	6 (10.2)/31 (52.5)/ 13 (22.0)/9 (15.3)	0.6401
Pretreatment HCV RNA concentration ($\log_{10}$ IU/ml)	6.37 ± 0.85	6.29 ± 0.55	0.0582
Reduction in the peginterferon dose	61 (29.5)	20 (30.8)	0.9644
Reduction in the ribavirin dose	101 (48.8)	29 (44.6)	0.5565
Final outcomes (sustained virologic response /relapse/ no response)	106 (51.2)/ 69 (33.3)/32 (15.5)	12 (18.4)/15 (23.1)/ 38 (58.5)	<0.0001

HCV, hepatitis C virus.
Percentages are shown in parentheses.
*Liver biopsy was not performed in 26 patients.

$\log_{10}$ to 5.71 $\log_{10}$ (mean, 3.12 $\log_{10}$). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in 98 patients (47.3%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 52 patients (25.1%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 23 patients (11.1%), and <1 $\log_{10}$ in 15 patients (7.3%). Figure 1A shows the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TT genotype. The rates were higher significantly in patients who achieved a rapid virologic response or had a ≥3 $\log_{10}$ decrease in
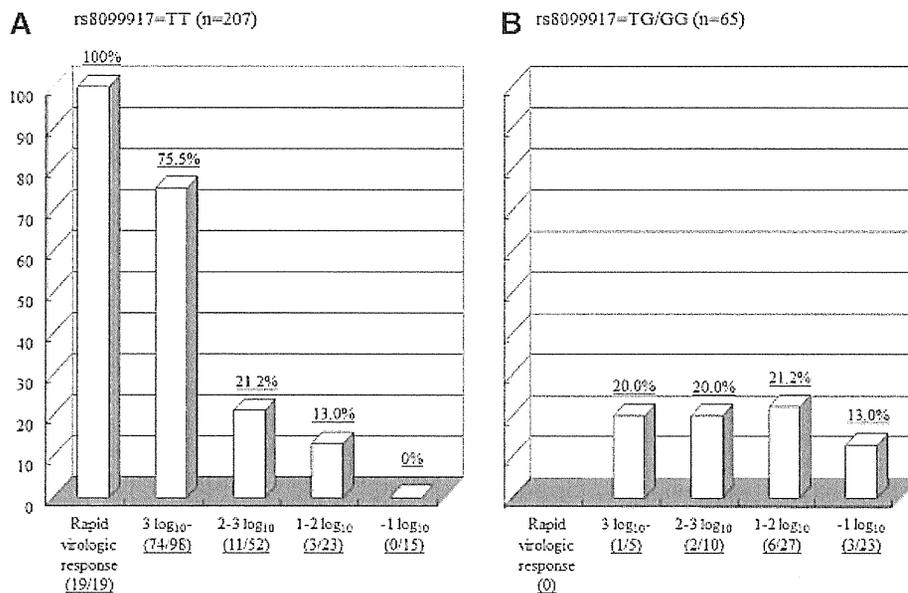

Fig. 1. The rate of sustained virologic responses (%) based on the reduction in serum HCV RNA levels at 4 weeks after starting therapy. A: Patients with the TT genotype for rs8099917, (B) patients with the TG/GG genotype for rs8099917.

serum HCV RNA levels at 4 weeks compared to those with a <3 $\log_{10}$ decrease in serum HCV RNA levels (P < 0.0001). When a 3 $\log_{10}$ decrease in serum HCV RNA levels was defined as the cut-off point, 56.5% of patients were considered to have a ≥3 $\log_{10}$ decrease in serum HCV RNA levels. The sensitivity, specificity, positive predictive value, and negative predictive value for a sustained virologic response were 86.8, 75.2, 78.6, and 84.4%, respectively.

Among the 65 patients who had the TG/GG genotype, no patient achieved a rapid virologic response at 4 weeks after initiating therapy. The decrease in serum HCV RNA levels at 4 weeks after starting therapy ranged from 0.11 $\log_{10}$ to 4.75 $\log_{10}$ (mean, 1.66 $\log_{10}$). The reduction in serum HCV RNA levels at 4 weeks after starting the therapy were smaller in patients with the TG/GG genotype than those with the TT genotype (1.66 ± 1.02 $\log_{10}$ in patients with the TG/GG genotype vs. 3.12 ± 1.37 $\log_{10}$ in patients with TT genotype excluding RVR, P < 0.0001). The reduction in serum HCV RNA levels was ≥3 $\log_{10}$ in five patients (7.7%), <3 $\log_{10}$ and ≥2 $\log_{10}$ in 10 patients (15.4%), <2 $\log_{10}$ and ≥1 $\log_{10}$ in 27 patients (41.5%), and <1 $\log_{10}$ in 23 patients (35.4%). Figure 1B shows the rates of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy in patients with the TG/GG genotype. There were no differences in the rate of a sustained virologic response based on the reduction in HCV RNA levels at 4 weeks after starting therapy; the rate of a sustained virologic response remained at 20% approximately regardless of the reduction in HCV RNA levels in 42 patients with a ≥1 $\log_{10}$ reduction in serum HCV RNA levels.

Association Between an Early Virologic Response at 12 Weeks and Treatment Outcome Based on Genetic Polymorphisms Near the *IL28B* Gene

Figure 2 shows the rate of patients with the TT genotype or TG/GG genotype for rs8099917 who achieved a complete early virologic response, a partial early virologic response, and those who did not achieve early virologic response at 12 weeks after starting therapy based on the reduction in serum HCV RNA level at 4 weeks after initiating therapy. Nearly 75% of patients with the TT genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy achieved a complete early virologic response. In contrast, 80% of patients with the TG/GG genotype whose HCV RNA levels were reduced by ≥3 $\log_{10}$ at 4 weeks after starting the therapy showed a partial early virologic response. The majority of patients with the TT or TG/GG genotypes achieved a partial early virologic response when their reduction in HCV RNA levels was <3 $\log_{10}$ and ≥2 $\log_{10}$ or <2 $\log_{10}$ and ≥1 $\log_{10}$.

Figure 3 shows the rates of a sustained virologic response according to the type of early virologic response in patients with the TT genotype (Fig. 3A) and TG/GG genotype (Fig. 3B). Among patients with the TT genotype, the rate of sustained virologic response was significantly higher in patients with a complete early virologic response than in those with a partial early virologic response (P < 0.0001). In contrast, there was no difference in the rate of a sustained virologic response between patients with a complete early virologic response and those with a partial early virologic response (P = 0.8917) among patients with
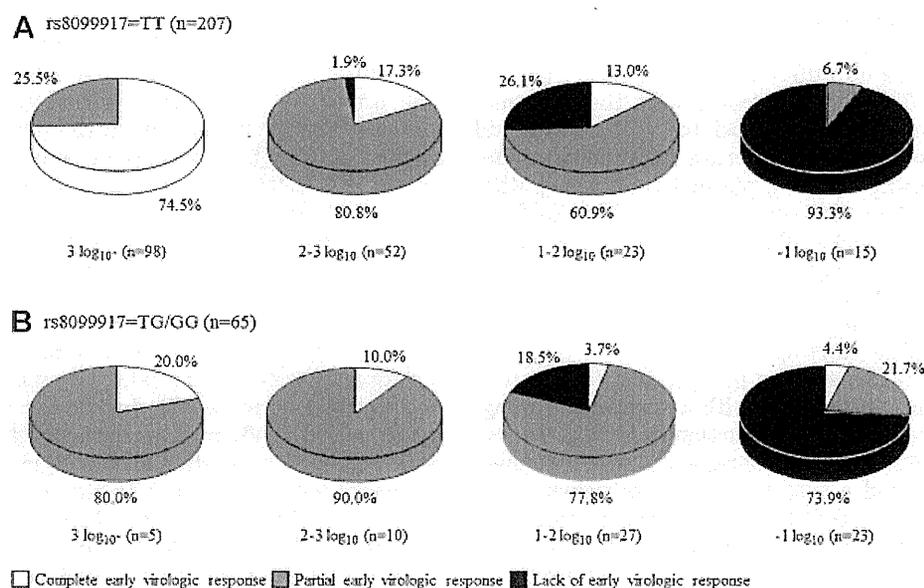

Fig. 2. The association between the virologic responses at 12 weeks after starting therapy and the reduction in serum HCV RNA levels at 4 weeks after starting therapy. A: Patients with the TT genotype for rs8099917, (B) patients with the TG/GG genotype for rs8099917.
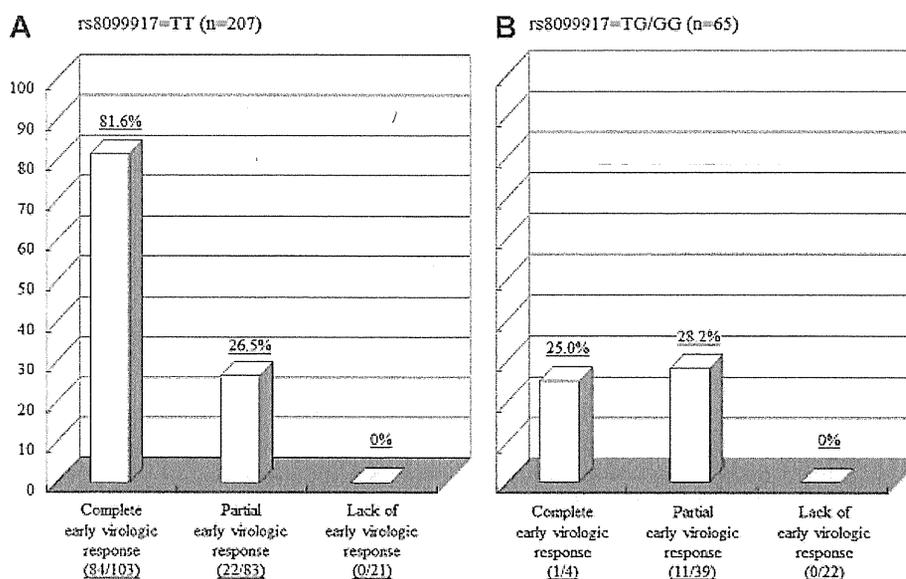
Fig. 3. The rate of sustained virologic responses based on the type of early virologic response. **A**: Patients with the TT genotype for rs8099917, (**B**) patients with the TG/GG genotype for rs8099917.

the TG/GG genotype. None of the patients with the TT genotype or TG/GG genotype who yielded a lack of an early virologic response reached a sustained virologic response.

Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

Univariate and multivariate analyses were conducted for factors associated with a sustained virologic response based on different genetic polymorphisms near the *IL28B* gene. In patients with the TT genotype, the factors that were associated with a sustained virologic response included serum alkaline phosphatase levels, serum albumin, platelet counts, hepatitis activity grade, liver fibrosis grade, reduction in HCV RNA levels at 4 weeks after starting therapy, and a complete early virologic response based on a univariate analysis (Table IIIA). In a multivariate analysis, the serum albumin levels, reduction in HCV RNA levels 4 weeks after starting therapy, and a complete early virologic response were independent factors that were significantly associated with a sustained virologic response (Table IIIB). A reduction in HCV RNA levels 4 weeks after starting therapy was the strongest factor that affected a sustained virologic response. In patients with the TG/GG genotype, the factors that were associated with a sustained virologic response included patient age, platelet counts, and pretreatment HCV RNA levels based on a univariate analysis (Table IIIA). A reduction in the HCV RNA levels at 4 weeks after starting therapy was not associated

with a sustained virologic response. In a multivariate analysis, patient age and pretreatment HCV RNA levels were independent factors that were significantly associated with a sustained virologic response (Table IIIC).

Characteristics of Patients who Achieved a Sustained Virologic Response to the Combination Therapy Despite the Unfavorable TG/GG Genotype Near the *IL28B* Gene

Table IV shows the characteristics of 12 patients who achieved a sustained virologic response despite having the unfavorable TG/GG genotype for rs8099917 near the *IL28B* gene. All but one patient was under 60 years old and had liver fibrosis not more than grade 2 (one patient did not undergo a liver biopsy). Except for one patient, the reduction in the serum HCV RNA levels at 4 weeks after starting therapy was less than 3 $\log_{10}$ and all but one patient showed a partial early virologic response at 12 weeks after starting the therapy. In all 11 patients with a partial early virologic response, the serum HCV RNA was undetectable up to 24 weeks after starting the therapy. All but one patient extended the treatment duration from 48 to 72 weeks (two patients discontinued therapy at 60 weeks during the extended treatment period). When the characteristics of patients who achieved a sustained virologic response were compared between those with the unfavorable TG/GG genotype and those with the favorable TT genotype, patients with the TG/GG genotype were younger (41.8 ± 14.4 years vs. 55.1 ± 10.4 years, $P = 0.0023$) and had lower pretreatment HCV RNA levels (5.91 ± 0.44 $\log_{10}$ IU/ml vs. 6.21 ± 1.05 $\log_{10}$ IU/ml, $P = 0.0199$).

TABLE III. Univariate and Multivariate Analyses for Factors Associated With a Sustained Virologic Response to Peginterferon and Ribavirin Combination Therapy in Patients With the TT and the TG/GG Genotype for the rs8099917

(A) Univariate analyses	P-value	
	Patients with TT genotype of rs8099917 (n = 207)	Patients with TG/GG genotype of rs8099917 (n = 65)
Age (years)	0.0505	0.0007
Sex (female/male)	0.1830	0.2296
Body weight (kg)	0.6891	0.2456
Alanine aminotransferase (IU/L)	0.7988	0.4032
Aspartate aminotransferase (IU/L)	0.5021	0.1705
Gamma-glutamyl transpeptidase (IU)	0.6340	0.6648
Alkaline phosphatase (IU/L)	0.0315	0.0599
Albumin (g/dl)	0.0002	0.6594
Total bilirubin (mg/dl)	0.2929	0.7130
White blood cell count (/μl)	0.2508	0.5549
Hemoglobin (g/dl)	0.0847	0.2289
Platelet count ($\times 10^3$/μl)	0.0454	0.0411
Liver histology-activity (A0–1/A2–3)	0.0445	0.1117
Liver histology-fibrosis (F0–1/F2–3)	0.0002	0.2283
Pretreatment HCV RNA concentration ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.5279	0.0379
Reduction in the peginterferon dose	0.4316	0.5563
Reduction in the ribavirin dose	0.1823	0.4272
Reduction in HCV RNA levels at 4 weeks after starting the therapy ($\geq$3 $\log_{10}$ vs. < 3 $\log_{10}$)	<0.0001	0.9265
Early virologic response (complete vs. partial)	<0.0001	0.9777
Early virologic response (partial vs. non)	0.8632	0.0686

(B) Multivariate analyses: Patients with TT genotype of rs8099917	P-value	Odds ratio (95% confidence interval)
Alkaline phosphatase (IU/L)	0.2617	
Albumin (g/dl)	0.0365	28.287 (1.4107–755.41)
Platelet count ($\times 10^3$/μl)	0.2599	
Liver histology-activity (A0–1/A2–3)	0.6678	
Liver histology-fibrosis (F0–1/F2–3)	0.2307	
Reduction in HCV RNA levels at 4 weeks after starting the therapy ($\geq$3 $\log_{10}$ vs. <3 $\log_{10}$)	<0.0001	16.029 (6.8593–40.406)
Early virologic response (complete vs. partial)	0.0224	0.3685 (0.1557–0.8749)

(C) Multivariate analyses: Patients with TG/GG genotype of rs8099917	P-value	Odds ratio (95% confidence interval)
Age (years)	0.0022	0.0034 (0.0000–0.0840)
Platelet count ($\times 10^3$/μl)	0.3344	
Pretreatment HCV RNA concentration ($\geq$6.5 $\log_{10}$ vs. <6.5 $\log_{10}$)	0.0304	0.0548 (0.0020–0.4950)

HCV, hepatitis C virus.

DISCUSSION

Several previous studies reported that patients who achieved a rapid virologic response, in which serum HCV RNA become undetectable at 4 weeks after starting therapy, had a high likelihood of achieving a sustained virologic response [Martinez-Bauer et al., 2006; Poordad et al., 2008; de Segadas-Soares et al., 2009; Martinot-Peignoux et al., 2009]. In addition, several recent studies reported the predictive value of the degree of reduction in serum HCV RNA levels at 4 weeks after starting therapy [Yu et al., 2007; Huang et al., 2010; Toyoda et al., 2011]. Therefore, the viral dynamics of HCV at 4 as well as 12 weeks after starting therapy is important for response-guided therapy.

Genetic polymorphisms near the *IL28B* gene have emerged as the strongest predictive factor of a sustained virologic response in patients infected with HCV genotype 1 [Hayes et al., 2011; Kurosaki et al., 2011]. In addition, Thompson et al. [2010] reported that genetic polymorphisms near the *IL28B* gene were associated strongly with early viral dynamics during PEG-IFN and ribavirin combination therapy. These findings raised an important issue of whether response-guided therapy, based on the reduction in serum HCV RNA levels at 4 or 12 weeks after starting

TABLE IV. Patients who Achieved a Sustained Virologic Response Despite the TG/GG Genotype for the rs8099917

	Age (years)	Sex	Liver histology	Pretreatment HCV RNA level ($\log_{10}$ IU/ml)	HCV RNA reduction at 4 weeks	Response at 12 weeks	HCV RNA became undetectable (weeks)	Treatment duration (weeks)
1.	31	Female	A1/F1	6.13	2.19	partial EVR	20	48
2.	55	Male	A1/F1	5.80	1.77	partial EVR	16	72
3.	57	Female	A1/F1	5.58	3.01	partial EVR	16	72
4.	57	Female	A1/F1	6.21	1.81	partial EVR	20	72
5.	62	Male	N.D.	6.23	1.13	partial EVR	24	72
6.	21	Male	A1/F2	6.04	1.83	partial EVR	24	72
7.	42	Male	A1/F1	6.27	0.57	partial EVR	24	72
8.	29	Female	A1/F2	5.83	1.83	partial EVR	20	60
9.	52	Male	A1/F0	5.91	2.12	complete EVR	12	48
10.	40	Male	A2/F1	5.84	1.34	partial EVR	20	72
11.	27	Male	N.D.	5.63	0.42	partial EVR	24	72
12.	28	Male	A1/F0	6.59	0.76	partial EVR	20	60

N.D., not done; HCV, hepatitis C virus; EVR, early virologic response.

therapy, retains a predictive value when considering genetic polymorphisms near the *IL28B* gene.

In the present study, the predictive value of the decrease in serum HCV RNA levels was evaluated at 4 and 12 weeks after starting therapy in Japanese patients infected with HCV genotype 1b based on genetic polymorphisms near the *IL28B* gene. Consistent with previous reports, patients with the TG/GG genotype for rs8099917 had a smaller reduction in serum HCV RNA levels at 4 weeks after starting treatment ($P < 0.0001$), which indicates an unfavorable response to the combination therapy. Patients with the TT genotype for rs8099917, which is associated with a favorable response to the combination therapy, exhibited a significant difference in the rate of a sustained virologic response based on the reduction in serum HCV RNA levels at 4 weeks after initiating the therapy. Patients with a rapid virologic response or with a ≥ 3 $\log_{10}$ reduction in HCV RNA levels had a higher likelihood of achieving a sustained virologic response.

In contrast, these factors did not have any predictive value in patients with the TG/GG genotype. Only 18.5% of patients achieved a sustained virologic response (12 of 65 patients), and it was difficult to identify these patients based on the reduction in HCV RNA levels at 4 weeks or the type of an early virologic response at 12 weeks after starting therapy. Patients who achieved a sustained virologic response, despite the TG/GG genotype for rs8099917, were identified among those with a <2 $\log_{10}$ and ≥ 1 $\log_{10}$ or even <1 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy. Interestingly and paradoxically, the possibility of a sustained virologic response can be expected in patients with a <1 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy only when they have the unfavorable TG/GG genotype.

In the evaluation at 12 weeks after starting therapy, patients with the TT genotype who achieved a complete early virologic response had a higher rate of a sustained virologic response significantly than patients who achieved a partial early virologic

response, whereas this difference was not found in patients with the TG/GG genotype. No patients who failed to achieve an early virologic response achieved a sustained virologic response regardless of the genetic polymorphisms near the *IL28B* gene. Thus, the lack of an early virologic response retained a strong predictive value for the failure of achieving a sustained virologic response. This result supports the recommendation in the AASLD guidelines, in which treatment may be discontinued in patients without an early virologic response at 12 weeks of treatment.

The characteristics of patients who achieved a sustained virologic response despite the unfavorable TG/GG genotype were younger in age and lower pretreatment HCV RNA levels. Most patients with the TG/GG genotype who achieved a sustained virologic response showed a partial early virologic response and extended the treatment duration. It was difficult to identify these patients according to viral dynamics at 4 or 12 weeks after starting therapy.

There are several limitations in this study. Some patients with a slow virologic response did not have their treatment period extended from 48 to 72 weeks. This is because the effectiveness of a 72-week combination therapy regimen in patients with HCV genotype 1 with a slow virologic response [Berg et al., 2006; Pearlman et al., 2007] had not been established in Japan in the earlier part of this study. This fact might have influenced the treatment outcome especially in patients with the unfavorable TG/GG genotype. Another limitation is a smaller sample size of patients with the TG/GG genotype in comparison to that of patients with the TT genotype. This sample size could have caused the lack of statistical significance in the rate of a sustained virologic response according to the reduction in HCV RNA levels at 4 weeks after starting therapy or according to the type of an early virologic response in patients with the TG/GG genotype. In addition, the data were based on Japanese patients infected with HCV genotype 1b. Therefore, these results should be confirmed in other ethnicities and patients infected with HCV genotype 1a.

In conclusion, among patients infected with HCV genotype 1b with the TT genotype for rs8099917, a rapid virologic response or a ≥ 3 $\log_{10}$ reduction in HCV RNA levels at 4 weeks after starting therapy, or a complete early virologic response indicate strongly that these patients will achieve a sustained virologic response as a final outcome for PEG-IFN and ribavirin combination therapy. Early viral dynamics retain the predictive value in this patient subpopulation. A reduction in HCV RNA levels at 4 weeks after starting therapy or the type of an early virologic response does not predict the likelihood that patients with the TG/GG genotype will achieve a sustained virologic response. In contrast, the lack of an early virologic response retains a strong predictive value for the failure to achieve a sustained virologic response regardless of *IL28B* polymorphisms, which remains useful as a factor to stop therapy.

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Impact of Genetic Polymorphisms Near the *IL28B* Gene and Amino Acid Substitutions in the Hepatitis C Virus Core Region on Interferon Sensitivity/Resistance in Patients With Chronic Hepatitis C

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It has been reported that genetic polymorphisms near the *IL28B* gene or amino acid substitutions in hepatitis C virus (HCV) core protein are associated with the clinical outcome of peginterferon (PEG-IFN) and ribavirin combination therapy. The impact of these factors on the pure sensitivity/resistance to interferon was evaluated. Changes in the HCV RNA levels 24, 48, 72, and 120 hr after administering a single dose of standard interferon (IFN) were measured in 156 HCV-infected patients. The changes were compared based on the genetic polymorphisms near the *IL28B* gene or amino acid substitutions in the HCV core region. Among patients with HCV genotype 1b, there were differences in the reduction and subsequent increase in HCV RNA levels after administering IFN based on rs8099917 genetic polymorphisms. Amino acid substitutions at residue 70 were associated with differences in the changes in HCV RNA levels only in patients with TG/GG genotype. Multivariate analyses showed that genetic polymorphisms near the *IL28B* gene was the sole independent factor that was associated with the reduction in HCV RNA levels after administering IFN and the final response to the combination therapy. Among patients infected with HCV genotype 2a or 2b, there were no differences in the changes in HCV RNA levels based on genetic polymorphisms near the *IL28B* gene. In HCV genotype 1b, genetic variations near the *IL28B* gene affected the sensitivity/resistance to IFN strongly. Genetic polymorphisms near the *IL28B* gene did not affect the sensitivity/resistance to IFN in HCV genotype 2. *J. Med. Virol.* **83:1203–1211, 2011.** © 2011 Wiley-Liss, Inc.

KEY WORDS: chronic hepatitis C; genetic polymorphism near the *IL28B* gene; amino acid substitution at residue 70 of the HCV core region; resistance to interferon

INTRODUCTION

Hepatitis C virus (HCV) causes a chronic infection that can result in chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma [Niederau et al., 1998]. The current standard antiviral therapy for patients with chronic hepatitis C is combination therapy with peginterferon (PEG-IFN) and ribavirin [Ghany et al., 2009]. Although the current treatment regimen has markedly increased the rate of patients who achieve a sustained virologic response, which is an eradication of HCV, only approximately 50% of patients infected with HCV genotype 1 achieved sustained virologic response.

Many studies have examined baseline host- or virus-related factors that affect potentially the outcome of IFN-based antiviral therapy. Recently, several studies reported that genetic polymorphisms near the *IL28B* gene (rs8099917, rs12979860) on chromosome 19, which encodes IFN-λ-3, affect the virologic response to a 48-week regimen of PEG-IFN and ribavirin combination therapy in patients infected with HCV genotype 1 [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009; McCarthy et al., 2010; Rauch