et al., 2010]. In addition, a recent report showed the effects of genetic polymorphisms near the *IL28B* gene on the dynamics of HCV during PEG-IFN and ribavirin combination therapy in this patient population [Thompson et al., 2010].

Amino acid substitutions at residue 70 in the HCV core region of patients with HCV 1b have been identified as a virus-related factor that affects the virologic response to combination therapy with PEG-IFN and ribavirin [Akuta et al., 2005, 2007a; Donlin et al., 2007]. Additional studies have showed the effects of this factor on the dynamics of HCV during combination therapy [Akuta et al., 2007b; Toyoda et al., 2010a].

Although several studies have shown a strong association between these factors and the final outcome of PEG-IFN and ribavirin combination therapy or the HCV viral dynamics during combination therapy, the mechanisms that contribute to these associations have not been identified. Is the effect of these factors on the antiviral efficacy of the combination therapy with PEG-IFN and ribavirin related mainly to the pure sensitivity/resistance to IFN or ribavirin? Or does these factors affect HCV replication?

The aim of the present study was to investigate the difference in the sensitivity/resistance to IFN based on these factors. Therefore, the changes in HCV RNA levels after administering a single dose of standard IFN were measured and then these results were compared to the genetic polymorphisms near the *IL28B* gene and amino acid substitutions at residue 70 of the HCV core region.

PATIENTS AND METHODS

Patients

In a previous study, a single dose of standard IFN were administered to 208 patients infected with HCV and the changes in HCV RNA levels were measured 24, 48, 72, and 120 hr after administration in order to investigate the pure sensitivity/resistance to IFN [Toyoda et al., 2009, 2010b]. These patients had pretreatment HCV RNA levels of $>100 \times 10^3$ IU/ml as determined by a quantitative polymerase chain reaction (PCR) assay (Amplicor GT-HCV Monitor, Version 2.0; Roche Molecular Systems, Pleasanton, CA), and were not coinfected with hepatitis B virus or human immunodeficiency virus. None of the patients abused alcohol or were intravenous drug users. Among these 208 patients, 156 patients who had provided written informed consent to use their laboratory data and undergo host genetic analyses were enrolled to the present study. The study protocol was in compliance with the Helsinki Declaration and was approved by the hospital ethics committee.

Single Administration of Standard Interferon and Measurement of Changes in Serum HCV RNA Levels to Evaluate the Sensitivity/Resistance to Interferon

All patients received a single dose of standard IFN-alpha 2b at least 2 weeks before starting the combination therapy with PEG-IFN and ribavirin (Fig. 1). The patients received an injection of six mega-units of standard IFN-alpha 2b (Intron A; Schering–Plough). The HCV RNA levels were measured before and 24, 48, 72, and 120 hr after IFN was administered, and the changes in HCV RNA levels were calculated and compared to the HCV RNA levels before administration.

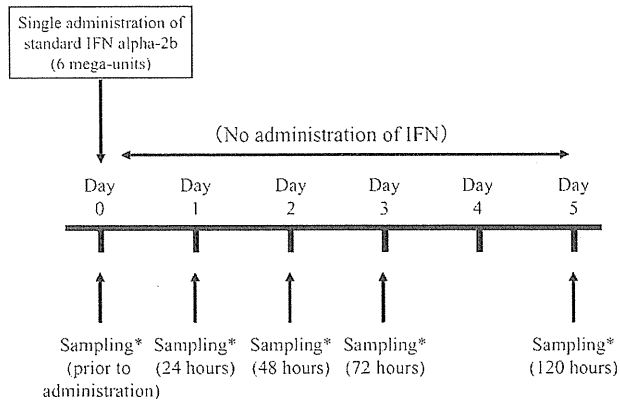

Fig. 1. Schematic representation of administration of standard IFN-alpha and measurements of HCV RNA levels. The serum HCV RNA levels were measured before, and 24, 48, 72, and 120 hr after administration of a singe dose of 6 mega-units of standard IFN-alpha. IFN, interferon; Sampling*, sampling of serum samples to measure HCV RNA levels.

Antiviral Combination Therapy With Peginterferon and Ribavirin

After conducting the single administration examination for standard IFN, all patients started PEG-IFN and ribavirin combination therapy after at least a 2-week interval. The initial doses of PEG-IFN and ribavirin and the dose reductions were according to the manufacturer's recommendations. Patients with HCV genotype 1b were scheduled to receive a 48-week treatment regimen, and those with genotype 2a or 2b were scheduled to receive a 24-week regimen. 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 was detectable after treatment, and no response when the serum HCV RNA remained detectable during and after treatment.

Examination of the Serum HCV RNA Levels, Genetic Polymorphisms Near the *IL28B* Gene, and Amino Acid Substitutions at Residue 70 of the HCV Core

The HCV genotype was determined by PCR amplifying the core gene sequences using genotype-specific primers [Ohno et al., 1997]. The HCV RNA levels in

serum samples were measured using a real-time PCR-based quantitation method for HCV (HCV COBAS AmpliPrep/COBAS TaqMan System; Roche Molecular Systems; lower limit of quantitation: 1.7 $\log_{10}$ IU/ml, lower limit of detection: 1.0 $\log_{10}$ IU/ml). The HCV RNA levels before the administration of a single dose of IFN and before PEG-IFN and ribavirin combination therapy were also examined using the same method on stored serum samples.

Genotyping of rs8099917 polymorphisms near the *IL28B* gene was performed using the TaqMan SNP assays (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).

Amino acid 70 of the HCV core region was analyzed by direct nucleotide sequencing as previously described [Akuta et al., 2007c]. The PCR primer pairs for direct sequencing of the HCV core region were as follows:

5′-GCCATAGTGGTCTGCGGAAC-3′ (outer, sense primer),

5′-GGAGCAGTCCTTCGTGACATG-3′ (outer, antisense primer),

5′-GCTAGCCGAGTAGTGTT-3′ (inner, sense primer), and

5′-GGAGCAGTCCTTCGTGACATG-3′ (inner, antisense primer).

Statistical Analyses

Quantitative values are reported as the means ± SD. Between-group differences were analyzed by a chi-square test. Differences in the quantitative values of two groups were analyzed by the Mann–Whitney *U*-test. Univariate and multivariate analyses using a logistic regression model were performed to identify factors that were associated with a decrease in serum HCV RNA levels at 24 hr after administering standard IFN, 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, genetic polymorphisms near the *IL28B* gene, and amino acid substitutions at residue 70 of the HCV core region (arginine vs. glutamine). All *P*-values were two-tailed, and $P < 0.05$ was considered as significant statistically.

RESULTS

Patient Characteristics and Combination Therapy

The patient characteristics are shown in Table I. The patients included 69 males (44.2%) and 87 females (55.8%) with a mean age of 58.4 ± 9.3 years. The grade of liver fibrosis according to the METAVIR score [The French Cooperative METAVIR Study Group, 1994] was F0 in 8 patients (5.1%), F1 in 97 patients (62.2%), F2 in 35 patients (22.4%), and F3 in 16 patients (10.3%). One hundred one patients (64.8%) were infected with HCV genotype 1b, 42 patients (26.9%) were infected with HCV genotype 2a, and the remaining 13 patients (8.3%) were infected with HCV genotype 2b. An analysis of genetic

TABLE I. Baseline Characteristics of Patients Infected With HCV Genotype 1b and Those With Genotype 2a/2b (n = 156)

	Genotype 1b (n = 101)	Genotype 2a/2b (n = 55)
Age (years)	59.0 ± 8.1	57.1 ± 11.0
Sex (female/male)	51 (50.5)/50 (49.5)	36 (65.5)/19 (34.5)
Body weight (kg)	58.5 ± 9.5	57.8 ± 8.8
Alanine aminotransferase (IU/L)	60.9 ± 63.9	47.6 ± 51.1
Aspartate aminotransferase (IU/L)	50.9 ± 41.2	40.3 ± 36.9
Gamma-glutamyl transpeptidase (IU)	49.0 ± 44.3	41.2 ± 68.6
Alkaline phosphatase (IU/L)	261.3 ± 81.8	281.6 ± 155.8
Albumin (g/dl)	4.18 ± 0.34	4.25 ± 0.35
Total bilirubin (mg/dl)	0.65 ± 0.24	0.62 ± 0.24
White blood cell count (/μl)	5169 ± 1338	5029 ± 1442
Hemoglobin (g/dl)	14.2 ± 1.2	13.8 ± 1.6
Platelet count ($\times 10^3$/μl)	166 ± 49	201 ± 57
Liver histology-activity (A0/A1/A2/A3)	2 (2.0)/64 (63.4)/25 (24.7)/10 (9.9)	1 (1.8)/42 (76.4)/9 (16.4)/3 (5.4)
Liver histology-fibrosis (F0/F1/F2/F3)	4 (4.0)/59 (58.4)/25 (24.7)/13 (12.9)	4 (7.3)/38 (69.1)/10 (18.2)/3 (5.4)
HCV RNA levels ($\log_{10}$ IU/ml)[a]	6.10 ± 0.41	6.04 ± 0.57
Amino acid at HCV core 70 (arginine/glutamine)[b]	71 (70.3)/30 (29.7)	—
Genetic polymorphisms near the *IL28B* gene (TT/TG/GG)[c]	76 (75.2)/24 (23.8)/1 (1.0)	45 (81.8)/10 (18.2)/0
Response (SVR/relapse/NR)[d]	38 (38.8)/38 (38.8)/22 (22.4)	41 (78.9)/10 (19.2)/1 (1.9)

HCV, hepatitis C virus; SVR, sustained virologic response; NR, no response
Percentages are shown in parentheses.
[a]Before the administration of standard interferon.
[b]Analyzed only in HCV genotype 1b-infected patients.
[c]rs8099917 genetic polymorphism.
[d]Six patients (three patients with HCV genotype 1b and three patients with genotype 2a) discontinued treatment.

polymorphisms near the *IL28B* gene indicated that 121 patients had a TT genotype, 1 patient had a GG genotype, and the remaining 34 patients were TG heterozygous. There were no differences in the distribution of the genetic polymorphisms near the *IL28B* gene between patients infected with HCV genotype 1b and those infected with HCV genotype 2a or 2b. An analysis of the amino acid substitutions at residue 70 of the HCV core region in HCV genotype 1b-infected patients showed that 71 and 30 patients arginine and glutamine at this residue, respectively.

Although all patients started PEG-IFN and ribavirin combination therapy after receiving single administration examination of standard IFN, six patients (three patients with genotype 1b and three patients with genotype 2a) discontinued the therapy because of adverse effects (depression in three, severe general fatigue in one, delirium in one, retinopathy in one, and thrombocytopenia in one).

Changes in the Serum HCV RNA Levels After Administering a Single Dose of Standard Interferon-Alpha to Assess the Sensitivity/Resistance to Interferon in Patients Infected With HCV Genotype 1b

Figure 2 shows the changes in the serum HCV RNA levels after a single dose of standard IFN-alpha in patients infected with HCV genotype 1b based on both the genetic polymorphisms near the *IL28B* gene (left panel) and amino acid substitutions at residue 70 of the HCV core region (right panel). Compared to the pretreatment levels, patients with the TT genotype had a more marked reduction in HCV RNA levels than patients with the TG or GG genotype, and this reduction was more marked in patients with arginine than glutamine at residue 70 of the HCV core region. The differences in the reduction in the HCV RNA levels 24 hr after IFN administration were more pronounced based on the genetic polymorphisms near the *IL28B* gene than the amino acid at residue 70 of the HCV core region. These differences were decreased at 48 and 72 hr after IFN administration and disappeared at 120 hr in the case of the TT genotype versus the TG/GG genotype. In contrast, the differences in the reduction in HCV RNA levels based on whether patients had an arginine or glutamine at residue 70 of the HCV core were maintained at 48, 72, and 120 hr after IFN administration.

Univariate and multivariate analyses were conducted for factors that are associated with <0.8 $\log_{10}$ decrease in HCV RNA levels 24 hr after administering standard interferon alpha, which was associated strongly with virologic no-response to PEG-IFN and ribavirin combination therapy in our previous study [Toyoda et al., 2010b]. Also in the present study, 38 of 82 patients (46.3%) with reductions in serum HCV RNA levels >0.8 $\log_{10}$ achieved a sustained virologic response. In contrast, of the 16 patients with reduction in serum HCV RNA levels ≤ 0.8 $\log_{10}$, none achieved a sustained virologic response ($P = 0.0014$). A univariate analysis indicated that pretreatment gamma-glutamyl transpeptidase, genetic polymorphisms near the *IL28B* gene, and amino acid substitutions at residue 70 of the HCV core region were associated significantly with a reduction in HCV RNA levels 24 hr after the administration of standard IFN, and pretreatment total bilirubin tended to be

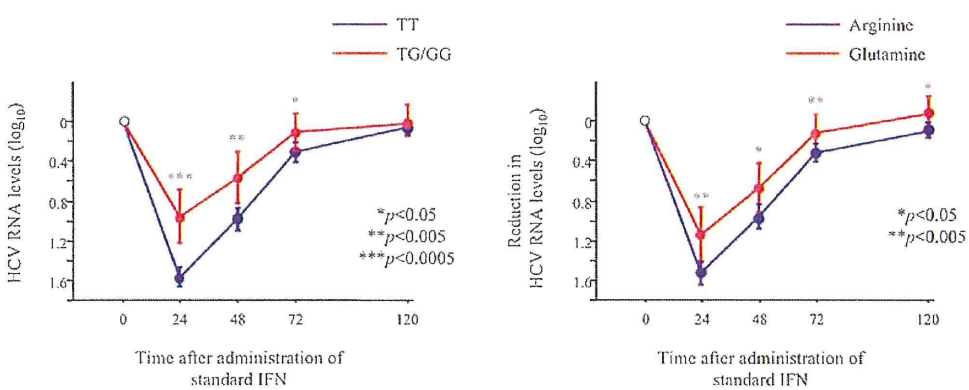

Fig. 2. Changes in HCV RNA levels after administering standard IFN to patients with the TT genotype compared to the TG/GG genotype near the *IL28B* gene (**left panel**), and in patients with an arginine compared to a glutamine at residue 70 of the HCV core region (**right panel**). The decrease in HCV RNA levels for the TT and TG/GG genotypes was 1.56 ± 0.46 $\log_{10}$ IU/ml versus 0.95 ± 0.66 $\log_{10}$ IU/ml ($P < 0.0001$) at 24 hr, 0.98 ± 0.50 $\log_{10}$ IU/ml versus 0.56 ± 0.62 $\log_{10}$ IU/ml ($P = 0.0002$) at 48 hr, 0.31 ± 0.42 $\log_{10}$ IU/ml versus 0.11 ± 0.44 $\log_{10}$ IU/ml ($P = 0.0238$) at 72 hr, and 0.06 ± 0.36 $\log_{10}$ IU/ml versus -0.01 ± 0.41 $\log_{10}$ IU/ml ($P = 0.3856$) at 120 hr after administration of IFN. The decrease in HCV RNA levels for arginine and glutamine was 1.53 ± 0.47 $\log_{10}$ IU/ml versus 1.14 ± 0.71 $\log_{10}$ IU/ml ($P = 0.0013$) at 24 hr, 0.96 ± 0.50 $\log_{10}$ IU/ml versus 0.67 ± 0.65 $\log_{10}$ IU/ml ($P = 0.0058$) at 48 hr, 0.32 ± 0.39 $\log_{10}$ IU/ml versus 0.12 ± 0.49 $\log_{10}$ IU/ml ($P = 0.0043$) at 72 hr, and 0.09 ± 0.32 $\log_{10}$ IU/ml versus -0.08 ± 0.32 $\log_{10}$ IU/ml ($P = 0.0289$) at 120 hr after administration of IFN.

associated with this reduction. A multivariate analysis showed that only genetic polymorphisms near the *IL28B* gene was associated independently with this reduction (Table II).

When patients were stratified according to the TT or TG/GG genotype, we found that there was a significant difference in the reduction in HCV RNA levels 24 hr after IFN administration in patients with TG/GG genotype based on whether the patients had arginine or glutamine at residue 70 of the HCV core (Fig. 3, right panel). However, there were no differences in patients with the TT genotype (Fig. 3, left panel).

Outcome to Combination Therapy With Peginterferon and Ribavirin in Patients Infected With HCV Genotype 1b

As for the final therapeutic outcome, 79 patients (52.7%) achieved a sustained virologic response, 48 patients (32.0%) relapsed, and the remaining 23 patients (15.3%) had no-response. Among 74 patients with the TT genotype of rs8099917 polymorphism near the *IL28B* gene, 36 (48.6%) achieved a sustained virologic response, whereas 2 of 24 patients (8.3%) with TG/GG achieved it. Among 69 patients with arginine at residue 70 of the HCV core region, 32 (46.4%) patients achieved a sustained virologic response, whereas 6 of 29 patients (20.7%) with glutamine at this residue achieved it. The rate of sustained virologic response was significantly higher in patients with the TT genotype ($P = 0.0010$) and in patients with arginine at residue 70 ($P = 0.0312$). When genotype of rs8099917 polymorphism and amino acid at residue 70 of the HCV core region were combined, the rate of sustained virologic response was highest in patients bearing the TT genotype and arginine (50.0%), followed by those with the TT genotype and glutamine (42.8%), those with the TG/GG genotype and arginine (22.2%), and those with the TG/GG genotype and glutamine in this order. None of 15 patients bearing both the TG/GG genotype and glutamine achieved a sustained virologic response.

Univariate and multivariate analyses were conducted for factors that are associated with sustained virologic response to the combination therapy with PEG-IFN and ribavirin. A univariate analysis indicated that pretreatment albumin and platelet counts, genetic polymorphisms near the *IL28B* gene, and amino acid substitutions at residue 70 of the HCV core region were associated significantly with sustained virologic response. A multivariate analysis showed that genetic polymorphisms near the *IL28B* gene and pretreatment platelet counts were associated independently with this reduction (Table III).

Changes in Serum HCV RNA Levels After Administering a Single Dose of Standard Interferon-Alpha to Assess the Sensitivity/Resistance to Interferon in Patients Infected With HCV Genotype 2

Figure 4 shows the changes in the serum HCV RNA levels after a single dose of standard IFN was administered to patients with HCV genotype 2a or 2b based on the genetic polymorphisms near the *IL28B* gene (left panel) and the subtype of HCV genotype 2 (right panel). There was a more marked reduction in HCV RNA levels after IFN administration in patients infected with HCV subtype 2a than in those infected

TABLE II. Univariate and Multivariate Analyses of Factors Associated With <0.8 $\log_{10}$ Decrease in HCV RNA Levels 24 hr After Administering Standard Interferon-Alpha

	Univariate analysis	Multivariate analysis	Odds ratio (95% confidence interval)
Age (years)	0.8801	—	
Sex (female/male)	0.9656	—	
Body weight (kg)	0.7199	—	
Alanine aminotransferase (IU/L)	0.9223	—	
Aspartate aminotransferase (IU/L)	0.7110	—	
Gamma-glutamyl transpeptidase (IU)	0.0290	0.2445	
Alkaline phosphatase (IU/L)	0.3261	—	
Albumin (g/dl)	0.4481	—	
Total bilirubin (mg/dl)	0.0582	0.7530	
White blood cell count (/μl)	0.9814	—	
Hemoglobin (g/dl)	0.6485	—	
Platelet count ($\times 10^3$/μl)	0.3020	—	
Liver histology-activity (A0–1/A2–3)	0.8062	—	
Liver histology-fibrosis (F0–1/F2–3)	0.7220	—	
HCV RNA levels ($\log_{10}$ IU/ml)[a]	0.1954	—	
Genetic polymorphisms near the IL28B gene (TT/TG + GG)[b]	<0.0001	0.0005	15.0446 (3.5533–81.5225)
Amino acid at residue 70 of the HCV core region (arginine/glutamine)	0.0007	0.0983	

HCV, hepatitis C virus.
[a]Before the administration of standard interferon.
[b]rs8099917 genetic polymorphism.

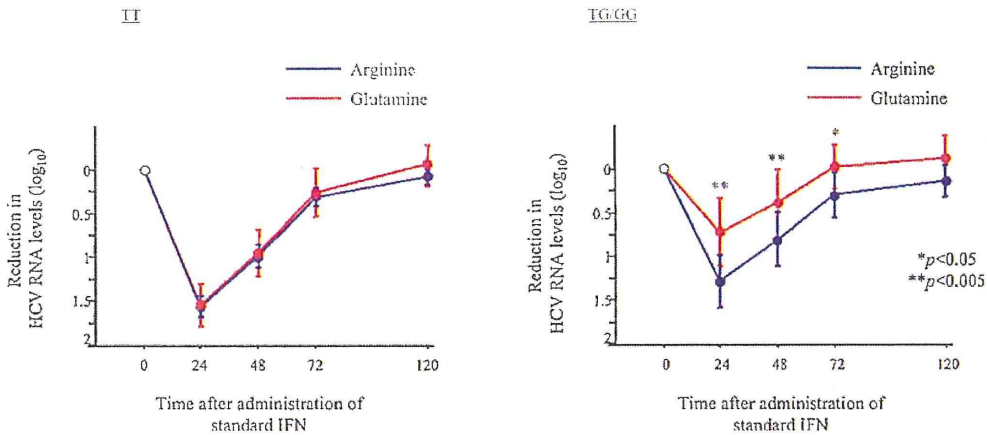

Fig. 3. Changes in HCV RNA levels after administering standard IFN in patients with an arginine compared to a glutamine at residue 70 of the HCV core region in patients with the TT genotype (**left panel**) and in those with the TG/GG genotype (**right panel**) near the *IL28B* gene. The decrease in HCV RNA levels for arginine and glutamine was 1.57 ± 0.47 log_{10} IU/ml versus 1.54 ± 0.44 log_{10} IU/ml ($P = 0.6292$) at 24 hr, 0.98 ± 0.51 log_{10} IU/ml versus 0.95 ± 0.47 log_{10} IU/ml ($P = 0.6810$) at 48 hr, 0.32 ± 0.40 log_{10} IU/ml versus 0.26 ± 0.50 log_{10} IU/ml ($P = 0.2745$) at 72 hr, and 0.08 ± 0.34 log_{10} IU/ml versus -0.05 ± 0.43 log_{10} IU/ml ($P = 0.2230$) at 120 hr after administration of IFN in patients with the TT genotype. The decrease in HCV RNA levels for arginine and glutamine was 1.29 ± 0.42 log_{10} IU/ml versus 0.73 ± 0.70 log_{10} IU/ml ($P = 0.0043$) at 24 hr, 0.81 ± 0.42 log_{10} IU/ml versus 0.39 ± 0.69 log_{10} IU/ml ($P = 0.0047$) at 48 hr, 0.30 ± 0.36 log_{10} IU/ml versus -0.02 ± 0.46 log_{10} IU/ml ($P = 0.0327$) at 72 hr, and 0.14 ± 0.25 log_{10} IU/ml versus -0.11 ± 0.46 log_{10} IU/ml ($P = 0.0672$) at 120 hr after administration of IFN in patients with the TG/GG genotype.

with HCV subtype 2b. In contrast, there were no differences in the reduction in the HCV RNA levels between patients with the TT genotype and the TG/GG genotype. The final outcome of PEG-IFN and ribavirin combination therapy was not different based on either the genetic polymorphisms near the *IL28B* gene or the HCV subtype (data not shown).

DISCUSSION

In the present study, the impact of rs8099917 genetic polymorphisms near the *IL28B* gene on the sensitivity/resistance to IFN was investigated by analyzing the association between genetic polymorphisms and changes in HCV RNA levels after administering a

TABLE III. Univariate and Multivariate Analyses of Factors Associated With Sustained Virologic Response to the Combination Therapy With Peginterferon and Ribavirin

	Univariate analysis	Multivariate analysis	Odds ratio (95% confidence interval)
Age (years)	0.6173	—	
Sex (female/male)	1.0000	—	
Body weight (kg)	0.3904	—	
Alanine aminotransferase (IU/L)	0.3630	—	
Aspartate aminotransferase (IU/L)	0.4537	—	
Gamma-glutamyl transpeptidase (IU)	0.2782	—	
Alkaline phosphatase (IU/L)	0.2500	—	
Albumin (g/dl)	0.0473	0.1203	
Total bilirubin (mg/dl)	0.9748	—	
White blood cell count (/μl)	0.4362	—	
Hemoglobin (g/dl)	0.5580	—	
Platelet count ($\times 10^3$/μl)	0.0445	0.0408	14.9668 (1.2103–230.4323)
Liver histology-activity (A0–1/A2–3)	0.8789	—	
Liver histology-fibrosis (F0–1/F2–3)	0.1119	—	
HCV RNA levels (log_{10} IU/ml)[a]	0.9591	—	
Genetic polymorphisms near the IL28B gene (TT/TG + GG)[b]	0.0025	0.0020	0.06233 (0.00780–0.29468)
Amino acid at residue 70 of the HCV core region (arginine/glutamine)	0.0207	0.5067	

HCV, hepatitis C virus.
[a]Before the administration of standard interferon.
[b]rs8099917 genetic polymorphism.

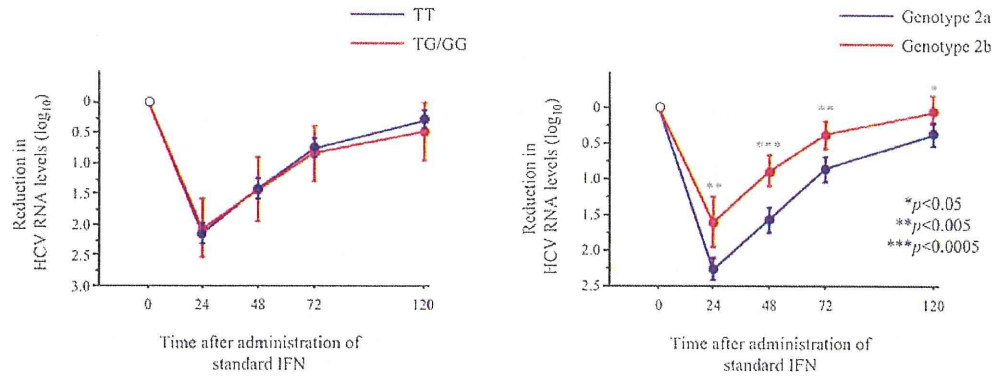

Fig. 4. Changes in HCV RNA levels after administering standard IFN to patients with the TT genotype compared to the TG/GG genotype near the *IL28B* gene (**left panel**) and in patients infected with HCV genotype 2a compared to HCV genotype 2b (**right panel**). The decrease in HCV RNA levels for the TT and TG/GG genotypes was 2.12 ± 0.58 log_{10} IU/ml versus 2.07 ± 0.66 log_{10} IU/ml ($P = 0.9652$) at 24 hr, 1.40 ± 0.56 log_{10} IU/ml versus 1.43 ± 0.74 log_{10} IU/ml ($P = 0.8872$) at 48 hr, 0.73 ± 0.53 log_{10} IU/ml versus 0.85 ± 0.64 log_{10} IU/ml ($P = 0.6005$) at 72 hr, and 0.47 ± 1.53 log_{10} IU/ml versus 0.48 ± 0.67 log_{10} IU/ml ($P = 0.6372$) at 120 hr after administration of IFN. The decrease in HCV RNA levels in patients infected with HCV genotype 2a and 2b was 2.27 ± 0.51 log_{10} IU/ml versus 1.60 ± 0.59 log_{10} IU/ml ($P = 0.0007$) at 24 hr, 1.57 ± 0.55 log_{10} IU/ml versus 0.89 ± 0.36 log_{10} IU/ml ($P = 0.0002$) at 48 hr, 0.86 ± 0.55 log_{10} IU/ml versus 0.38 ± 0.33 log_{10} IU/ml ($P = 0.0012$) at 72 hr, and 0.60 ± 1.58 log_{10} IU/ml versus 0.04 ± 0.33 log_{10} IU/ml ($P = 0.0354$) at 120 hr after administration of IFN.

single dose of standard IFN. A previous study by Thompson et al. [2010] reported that genetic polymorphisms near the *IL28B* gene was associated strongly with early viral kinetics during the combination therapy with PEG-IFN and ribavirin. However, the viral response in their study reflected the response of HCV to both PEG-IFN and ribavirin that were administered in combination, and the response did not represent a pure sensitivity/resistance to IFN in the absence of ribavirin. In a previous study, the decrease in HCV RNA levels 24 hr after administering standard IFN was investigated and the decrease was shown to be associated strongly with the outcome of PEG-IFN and ribavirin combination therapy [Toyoda et al., 2010b]. In the present study, a difference in the decrease in HCV RNA levels 24 hr after administering standard IFN was observed in patients with the TT genotype compared to those with the TG/GG genotype. The rs8099917 genetic polymorphisms near the *IL28B* gene was an only independent factor that was associated with a decrease in HCV RNA levels 24 hr after IFN administration. This finding indicates that genetic polymorphisms near the *IL28B* gene affect the pure sensitivity/resistance to IFN in patients infected with HCV genotype 1b.

In the absence of subsequent IFN administration, HCV RNA levels increased after 24 hr and were restored to the pretreatment levels in both patients with the TT genotype and those with the TG/GG genotype. The differences in HCV RNA levels between patients with the TT and TG/GG genotypes decreased rapidly, and there were no differences in HCV RNA levels 120 hr after administering standard IFN. Based on this finding, rs8099917 genetic polymorphisms do not appear to affect HCV replication.

A difference in the decrease in HCV RNA levels 24 hr after administering a single dose of standard IFN was observed also based on amino acid substitutions at residue 70 of the HCV core region, although this difference was less marked compared to the differences associated with the genetic polymorphisms. In contrast to the differences associated with these genetic polymorphisms, the differences associated with the amino acid substitutions in the HCV core region were maintained until 120 hr after IFN administration. Therefore, the effects on HCV of host genetic polymorphisms and amino acid substitutions in the core protein of infected HCV during administration of IFN or PEG-IFN may be by a different mechanism.

When patients were stratified according to the TT genotype or the TG/GG genotype and the changes in HCV RNA levels 24 hr after IFN administration were compared, there were no differences between patients with arginine and those with glutamine at residue 70 of the HCV core among patients with the TT genotype. However, there was a difference in the changes in HCV RNA levels among patients with the TG/GG genotype. Therefore, genetic polymorphisms near the *IL28B* gene are a strong factor that affects the reduction in HCV RNA levels and amino acid substitutions at residue 70 of the HCV core region have an effect only in patients with the TG/GG genotype. These findings are consistent with the rate at which patients achieved a sustained virologic response as a final outcome and a result of the multivariate analysis for sustained virologic response.

In a previous study by Hayes et al. [2011], genetic polymorphisms near the *IL28B* gene, amino acid substitutions at residue 70 of the HCV core region, and

mutations in the interferon sensitivity-determining region of HCV NS5A region were evaluated as a predictor of response to the combination therapy with PEG-IFN and ribavirin in 817 Japanese patients with chronic HCV genotype 1b infection. They reported that genetic polymorphisms near the *IL28B* gene and amino acid substitutions at residue 70 of the HCV core contributed independently to a sustained virologic response to the combination therapy, indicating the different effects of these two factors on the response to PEG-IFN and ribavirin combination therapy. In contrast, amino acid substitutions at residue 70 of the HCV core region failed to be an independent predictor by multivariate analysis in the present study. This discrepancy may be simply due to a small number of patients in our study population. Indeed, the difference of changes in the serum HCV RNA levels after administering a single dose of standard IFN in the present study also indicated the different mechanism of resistance to IFN between genetic polymorphisms near the *IL28B* gene and amino acid substitutions at HCV residue 70.

In patients infected with HCV genotype 2a or 2b, there were no differences in the changes in HCV RNA levels after a single dose of standard IFN based on genetic polymorphisms near the *IL28B* gene. Rather, there was a significant difference in the reduction in HCV RNA levels in patients infected with HCV genotype 2a compared to those infected with genotype 2b, as our previous report [Toyoda et al., 2009]. The genetic polymorphisms near the *IL28B* gene appeared to have few effects on the reduction in HCV RNA levels after IFN administration in patients infected with HCV genotype 2.

There are several limitations on this study. The data were based on Japanese patients infected with HCV genotype 1b, because there are so few patients infected with HCV genotype 1a in Japan. Therefore, these results should be confirmed in patients of other ethnicities and patients infected with HCV genotype 1a. In addition, the number of patients was small in comparison to previous studies. This was because of the difficulty to conduct the examination of single administration of standard IFN and measurement of changes in serum HCV RNA levels. As a result, only 25 patients with HCV genotype 1b were bearing minor allele of polymorphisms near the *IL28B* gene (GG genotype or TG heterozygote); 10 had arginine and 15 had glutamine at residue 70 of the HCV core region. Finally, only standard IFN-alpha 2b and PEG-IFN-alpha 2b were used in this study. Results may differ with the use of IFN/PEG-IFN alpha-2a, as the pharmacokinetics of PEG-IFN are different between PEG-IFN alpha-2b and PEG-IFN alpha-2a.

In conclusion, rs8099917 genetic polymorphisms near the *IL28B* gene are associated with the sensitivity/resistance to IFN in patients infected with HCV genotype 1b. In addition, amino acid substitutions at residue 70 of the HCV core region are related to the sensitivity/resistance to IFN only in patients with the TG/GG genotype. These associations were not seen in patients infected with HCV genotype 2.

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Effect of maintenance therapy with low-dose peginterferon for recurrent hepatitis C after living donor liver transplantation

Y. Ueda,[1] H. Marusawa,[1] T. Kaido,[2] Y. Ogura,[2] F. Oike,[2] A. Mori,[2] K. Ogawa,[2] A. Yoshizawa,[2] E. Hatano,[2] A. Miyagawa-Hayashino,[3] H. Haga,[3] H. Egawa,[2] Y. Takada,[2] S. Uemoto[2] and T. Chiba[1]

[1]*Department of Gastroenterology and Hepatology,* [2]*Department of Surgery and* [3]*Department of Diagnostic Pathology, Graduate School of Medicine, Kyoto University, Shogoin, Sakyo-ku, Kyoto, Japan*

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SUMMARY. Approximately 30% of patients who have recurrent hepatitis C after liver transplantation achieve sustained virological response (SVR) by taking a combination therapy of pegylated interferon and ribavirin. For the remaining non-SVR patients, an effective management treatment has not yet been established. In this study, efficacy of long-term peginterferon maintenance therapy for non-SVR patients was evaluated. Forty patients who had previously received the combination therapy for hepatitis C after living donor liver transplantation were classified into one of the following three groups: the SVR group (n = 11); the non-SVR-IFN group (n = 17), which received low-dose peginterferon maintenance therapy for non-SVR patients; and the non-SVR-Withdrawal group (n = 12), which discontinued the interferon treatment. We then compared histological changes among these three groups after 2 or more years follow-up. Activity grade of liver histology improved or remained stable in patients in the SVR and non-SVR-IFN groups, but deteriorated in half of the patients in the non-SVR-Withdrawal group. Fibrosis improved or remained stable in 10 of 11 SVR patients and in 13 of 17 non-SVR-IFN patients, but deteriorated in all non-SVR-Withdrawal patients. Mean changes in fibrosis stage between pretreatment and final liver biopsy were −0.18, +0.06 and +2.2 in the SVR, non-SVR-IFN and non-SVR-Withdrawal groups, respectively. Fibrosis stage deteriorated to F3 or F4 significantly more rapidly in the non-SVR-Withdrawal group than in the other two groups. In conclusion, continuing long-term maintenance therapy with peginterferon prevented histological progression of hepatitis C in patients who had undergone living donor liver transplantation.

Keywords: fibrosis, hepatitis C, liver transplantation, maintenance therapy, peginterferon, ribavirin.

INTRODUCTION

Cirrhosis and hepatocellular carcinoma caused by hepatitis C virus (HCV) infection is the leading indication for liver transplantation in Japan, the United States and western Europe. However, liver allograft infection with HCV following liver transplantation is universal, and almost all patients develop recurrent liver injury [1–6]. The progression of recurrent hepatitis C is often accelerated and, without appropriate antiviral therapy, 10–25% of patients develop cirrhosis within 5 years after transplantation, resulting in poorer prognosis for HCV-positive recipients than HCV-negative recipients [7].

To prevent the progression of hepatitis C after liver transplantation, a combined therapy of pegylated interferon plus ribavirin is commonly administered [8,9]. However, the efficacy of this combination therapy is limited: The mean sustained virological response (SVR) rate among patients with recurrent hepatitis C after liver transplantation was only 30% (range, 8–50%) [10]. Effective management of the remaining 70% of the patients who are unable to achieve SVR has not been established [11].

We recently reported the change in liver histology after combination therapy with interferon plus ribavirin in patients who have recurrent hepatitis C after living donor liver transplantations (LDLT). Among patients who did not achieve SVR, activity grade was not improved and fibrosis stage deteriorated. On the other hand, SVR was associated with reduced hepatic inflammation and suppression of liver fibrosis progression [12]. Because the histological progression of non-SVR patients occurred mainly after interferon

Abbreviations: AIH, autoimmune hepatitis; ALT, alanine aminotransferase; HCV, hepatitis C virus; LDLT, living donor liver transplantations; MMF, mycophenolate mofetil; PCR, polymerase chain reaction; SVR, sustained virological response.

Correspondence: Yoshihide Ueda, MD, PhD, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: yueda@kuhp.kyoto-u.ac.jp

therapy was discontinued, we hypothesized that long-term, continuous interferon administration might be effective in slowing the progression of liver damage in these patients. Therefore, after our previous study, we prescribed a low-dose peginterferon maintenance therapy for non-SVR patients. Here, we evaluated the efficacy of this treatment by investigating long-term histological changes in these patients, as well as comparing them to the changes observed in SVR patients and non-SVR patients who did not receive maintenance treatment.

METHODS

Eighty patients who had previously received the combination therapy with interferon and ribavirin ($n = 40$) or peginterferon and ribavirin ($n = 40$) for recurrent hepatitis C after LDLT at Kyoto University between January 2001 and April 2007 were retrospectively analysed.

Patients

Between March 1999 and December 2006, 141 patients with HCV-related liver diseases underwent LDLT at Kyoto University. Of these, 100 patients had been followed up for more than 6 months after LDLT in our hospital. Antiviral therapy was given to 80 patients with recurrent hepatitis C between January 2001 and April 2007. The remaining 20 patients did not receive the antiviral therapy because of no histological recurrence of hepatitis C in the follow-up period. To evaluate the histological progression caused by hepatitis C, patients who were diagnosed as having other causes of liver injury, such as biliary complications, chronic rejection, and *de novo* autoimmune hepatitis (AIH), were excluded. Patients who discontinued the treatment within 3 months because of worsening of liver function caused by hepatitis C were also excluded, because the rapid progression of these patients is not comparable to the long-term progression and inclusion of these patients would have led to overestimation of the progression in the patients who discontinued treatment. Patients were also excluded if they did not have a liver biopsy more than 2 years after the initiation of treatment, because this prevented an analysis of long-term histological changes.

Treatment protocol and definition of responses to treatment

After liver transplantation, patients with recurrent HCV liver disease underwent treatment with interferon-α-2b (3 or 6 mega units thrice weekly) plus ribavirin (400–800 mg/day orally) for the first 6 months. This was followed by interferon monotherapy for 6 months [12]. This treatment protocol was employed between January 2001 through April 2004 inclusive. From May 2004 to April 2007, patients underwent combination antiviral therapy comprising peginterferon-α-2b (1.5 μg/kg body weight, weekly) and ribavirin (400–800 mg/day orally) [13]. Patients who became negative for serum HCV RNA within 12 months after initiating the treatment continued to receive the full (initial) dose for 8–22 months to achieve SVR; then, the treatment ended. Patients who were negative for serum HCV RNA for more than 6 months after completion of interferon therapy were defined as achieving SVR.

Patients who did not become negative for serum HCV RNA within 12 months of initiating the combination therapy, as well as patients who experienced a relapse after transient discontinuation of the treatment, continued to receive a low-dose peginterferon maintenance therapy (0.5–0.75 μg/kg of peginterferon-α-2b with or without ribavirin at 200 mg/day). Treatments occurred during the study period, May 2005–December 2009. During this time, the therapy was discontinued in patients with severe adverse events. Additionally, peginterferon treatments were discontinued when neutrophil and platelet counts fell below 500 and 30 000/μL, respectively, and ribavirin was discontinued when haemoglobin levels fell below 8 g/dL.

Histological assessment

Liver biopsies were performed when patients' alanine aminotransferase (ALT) levels were more than twice the upper limit of normal, or at yearly intervals, with informed consent. Biopsy specimens were evaluated by two pathologists (H.H. and A.M.) with extensive experience in the pathology of liver transplantation. Necroinflammatory activity (A0–A3) and fibrosis stage (F0–F4) were assessed using METAVIR scores [14,15]. Grading was defined as A0 (no activity), A1 (mild activity), A2 (moderate activity) or A3 (severe activity); staging was defined as F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) or F4 (cirrhosis) [14,15].

The following equations were used to analyse the histological changes:

(1) Changes in activity grade = grade at final biopsy − grade at pretreatment biopsy, and
(2) Changes in fibrosis stage = stage at final biopsy − stage at pretreatment biopsy.

Immunosuppression

Tacrolimus and low-dose steroid therapies were administered to induce immunosuppression [12,13,16]. The lower limit of the target for whole blood tacrolimus level was 10–15 ng/mL during the first 2 weeks, 10 ng/mL during weeks 2–8 and 5–8 ng/mL thereafter. Four patients received cyclosporine microemulsions, rather than tacrolimus, to induce immunosuppression (Table 1). Steroid therapy was initiated at a dose of 10 mg/kg before graft reperfusion and then tapered from 1 mg/kg per day on the first day to 0.3 mg/kg per day until the end of the first month, followed by 0.1 mg/kg per day until the end of the third month. After

Table 1 Baseline characteristics of 40 enrolled patients with recurrent hepatitis C after LDLT before interferon therapy

		Non-SVR		
	SVR (n = 11)	IFN (n = 17)	Withdrawal (n = 12)	P
Age (years)	55 (17–68)	57 (39–66)	58 (15–70)	0.724*
Males/Females	7/4	12/5	5/7	0.281†
Time since LDLT (months)	11.5 (4.2–39.1)	10.6 (1.1–51.2)	5.9 (1.8–85.3)	0.316*
HCV genotype 1/non-1	8/3	15/2	12/0	0.141†
HCV RNA (kIU/mL)	1120 (289–5000)	2810 (74–5000)	2320 (498–5000)	0.850*
White cell count (/μL)	4000 (2200–9000)	4600 (1300–6900)	4400 (1700–6900)	0.991*
Neutrophil count (/μL)	2220 (1235–4140)	2040 (793–4816)	2642 (836–4623)	0.884*
Haemoglobin (g/dL)	12.4 (11.6–17)	11.6 (9.2–15.5)	11.65 (8.9–15.2)	0.096*
Platelet count (10^4/μL)	11.7 (5.9–58.1)	11.3 (4.8–32.4)	14.9 (7.6–40)	0.529*
PT (INR)	1.00 (0.92–1.19)	1.04 (0.93–1.67)	1.07 (0.87–1.34)	0.561*
AST (IU/L)	106 (27–352)	78 (30–258)	107 (44–464)	0.539*
ALT (IU/L)	106 (38–395)	82 (37–275)	157.5 (40–354)	0.619*
ALP (IU/L)	492 (233–1954)	479 (234–828)	636 (306–2977)	0.221*
γ-GTP (IU/L)	293 (41–1447)	107 (29–457)	122.5 (23–1417)	0.147*
Bilirubin (mg/dL)	0.9 (0.4–1.8)	0.9 (0.4–2.6)	1.25 (0.3–10.4)	0.530*
Albumin(g/dL)	3.7 (3.3–4.7)	3.8 (2.7–4.5)	3.5 (2.9–4.4)	0.329*
METAVIR score				
A 0/1/2/3	0/8/3/0	0/8/8/1	0/7/5/0	0.594†
F 0/1/2/3/4	1/8/2/0/0	1/9/7/0/0	5/5/2/0/0	0.066†
Immunosuppression				
Tacrolimus	8	16	7	0.257†
Tacrolimus + MMF	2	0	3	
Tacrolimus + prednisolone	1	0	2	
Cyclosporine	0	1	0	
Cyclosporine + MMF	0	1	0	
Trough level for tacrolimus (ng/mL)	5.9 (3.4–8.7)	5.95 (3.3–10.9)	6.4 (3.8–9.1)	0.752*

PT, prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; MMF, mycophenolate mofetil; LDLT, living donor liver transplantations; SVR, sustained virological response. Qualitative variables are shown in number; and quantitative variables expressed as median (range). *Kruskal–Wallis test, †chi-square test.

that, steroid administration was terminated. Mycophenolate mofetil (MMF) was administered to patients who experienced refractory rejection or required reduction in tacrolimus or cyclosporine doses because of adverse events.

Virological assays

Hepatitis C virus genotype was determined using a genotyping system based on polymerase chain reaction (PCR) of the core region using genotype-specific PCR primers [17]. Serum HCV RNA load was evaluated once a month during treatment and 24 weeks after treatment, using PCR and an Amplicor HCV assay (Cobas Amplicor HCV Monitor; Roche Molecular Systems, Pleasanton, CA, USA).

Statistical analysis

Wilcoxon and Kruskal–Wallis tests, chi-square tests and t-tests were used to analyse the continuous variables, categorical variables and histological changes, respectively. The Kaplan–Meier method was used to estimate the rates of patients who showed a progression of fibrosis to stage F3 or F4 after the initiation of the interferon therapy; log-rank tests were used to compare these rates among groups. Significance was defined as $P < 0.05$.

RESULTS

Characteristics of patients

Hepatitis C virus RNA concentrations and histological evidence were used to diagnose 80 patients with recurrent hepatitis C after LDLT. These patients were given one of two combination therapies: interferon and ribavirin (n = 40) or peginterferon and ribavirin (n = 40) at Kyoto University between January 2001 and April 2007. Thirty-one of the 80 patients who received the combination therapy achieved SVR (Fig. 1). Among the remaining 49 non-SVR patients,

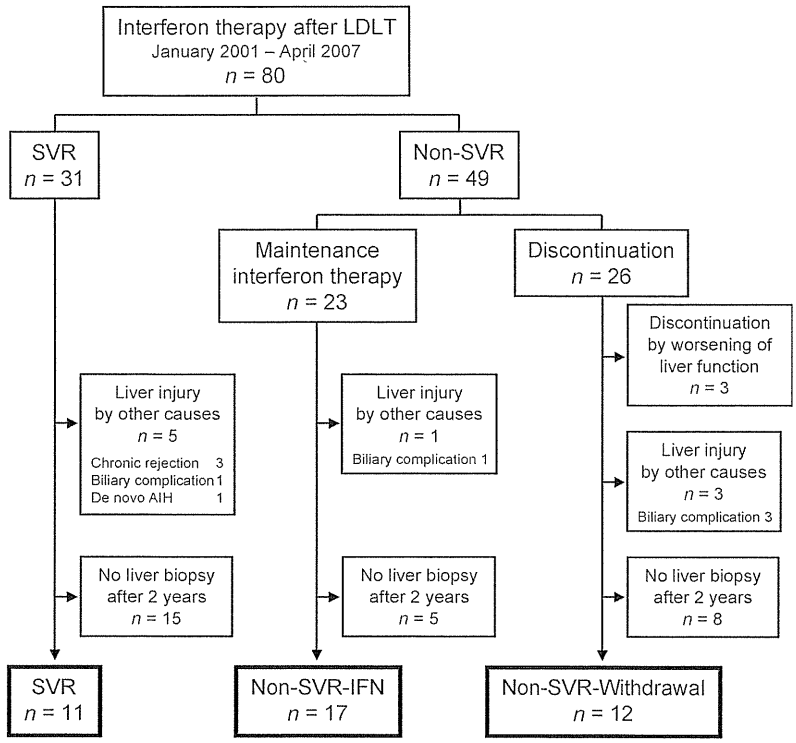

Fig. 1 Flow diagram showing the outcome of interferon therapy for patients with recurrent hepatitis C after living donor liver transplantation and indicating the classification of patients in this study.

23 (47%) received the low-dose peginterferon maintenance therapy, while 26 (53%) discontinued treatment within 12 months and did not receive low-dose peginterferon maintenance therapy as this was the patients' wish (n = 4), because of general fatigue (n = 4), recurrent hepatocellular carcinoma (n = 4), worsening of liver function (n = 3), biliary complications (n = 3), heart failure (n = 2), brain haemorrhage (n = 1), dementia (n = 1), sinusitis (n = 1), anaemia (n = 1), neutropenia (n = 1), and haemosputum (n = 1).

Of the 31 SVR patients, five were excluded because of chronic rejection (n = 3), biliary complications (n = 1) and *de novo* AIH (n = 1). Fifteen patients did not have liver biopsies more than 2 years after the initiation of the interferon therapy, mainly because liver function tests were normal. The remaining 11 patients were classified as the SVR group for analysis in this study. Among the 23 patients who received maintenance therapy, one patient with biliary complications and five patients who did not have liver biopsy more than 2 years after the initiation of therapy were excluded from the study. The remaining 17 patients were classified into the non-SVR-IFN group. Among the 26 patients who discontinued treatment within 12 months, three patients who initially experienced worsening of liver function were excluded because of the rapid progression of HCV; an additional three patients were excluded because of biliary complications. Eight patients were excluded because they had no liver biopsies taken more than 2 years after the initiation of the treatment. The remaining 12 patients were classified into the non-SVR-Withdrawal group. Cumulatively, we analysed the long-term histological changes of 40 patients: 11 in the SVR group (27.5% of the total), 17 in the non-SVR-IFN group (42.5% of the total) and 12 in the non-SVR-Withdrawal group (30% of the total).

There were no significant differences in the baseline characteristics among patients in the SVR, non-SVR-IFN, and non-SVR-Withdrawal groups (Table 1). The median age of patients at the beginning of therapy was 56.5 years (range, 15–70 years). The treatment started at a median of 9.5 months (range, 1.1–85.3 months) after LDLT. Thirty-five patients (88%) were infected with HCV genotype 1b. HCV genotypes of the remaining patients were 2a (n = 3), 2b (n = 1) and undetermined (n = 1). Median serum HCV RNA load was 2290 kIU/mL (range, 73.7–5000 kIU/mL); i.e. most patients had an extremely high viral load. Before the treatment, the necroinflammatory activity of all patients was A1 or greater, and 33 patients (83%) had a fibrosis score of F1 or greater. Among patients receiving tacrolimus for immunosuppression, the median serum trough level was 5.95 ng/mL (range, 3.3–10.9).

Effect of maintenance interferon therapy on liver histology

To evaluate the efficacy of long-term peginterferon therapy on histological changes, we compared scores between final biopsy samples (median, 44.0 months; range, 24.0–81.3 months) and those taken prior to treatment. Five patients in the non-SVR-IFN group discontinued maintenance

therapy between 26.5 and 53.1 months after the initiation of the treatment because of the adverse events. For these patients, the biopsies taken just before or within 3 months after discontinuation of the treatment were analysed as final biopsies. Despite the variation in time between pretreatment and final biopsy sample collection, there were no significant differences in the duration among the three groups ($P = 0.547$). Median duration from initiation of interferon therapy to final liver biopsy was 41.9 months (range, 24.0–81.3 months) in the SVR group, 41.7 months (range, 26.5–68.4 months) in the non-SVR-IFN group and 46.5 months (range, 30.4–79.6 months) in the non-SVR-Withdrawal group.

There were no significant differences in baseline activity grades or fibrosis stages of patients in the three treatment groups when they were first diagnosed with recurrent hepatitis C (Table 1). However, there were noticeable differences among the three groups by the end of treatment (Fig. 2a). The activity grade of all patients in the SVR and non-SVR-IFN groups improved or remained stable, whereas it deteriorated in 6 (50%) of 12 patients in the non-SVR-Withdrawal group. The fibrosis stage deteriorated in all patients in the non-SVR-Withdrawal group; nine of these patients (75%) deteriorated by more than one stage. In contrast, only four patients (24%) in the non-SVR-IFN group deteriorated, all by only a single stage. Furthermore, three patients actually improved. In the SVR group, fibrosis stage decreased or remained stable in 10 of 11 patients (91%).

In patients in the SVR and non-SVR-IFN groups, the mean activity grade was markedly reduced in the final biopsy, compared to the pretreatment biopsy (Fig. 2b). In contrast, patients in the non-SVR-Withdrawal group experienced an increase in activity grade. The differences between the non-SVR-Withdrawal group and both the SVR and the non-SVR-IFN groups were statistically significant ($P < 0.001$). The mean changes in fibrosis stage in the SVR and non-SVR-IFN groups were −0.18 and +0.06, respectively, suggesting that fibrosis did not change during the follow-up period. However, there was an obvious increase (+2.2) among patients in the non-SVR-Withdrawal group, indicating marked progression of fibrosis.

The Kaplan–Meier analysis allowed us to investigate whether patients in the three treatment groups experienced different progression rates to late-stage fibrosis (Fig. 2c). No patient in the SVR group and only 1 patient (6%) in the non-SVR-IFN group developed fibrosis stage F3 or F4, whereas nine patients (75%) in the non-SVR-Withdrawal group progressed to these stages. The rates of fibrosis progression were significantly higher in the non-SVR-Withdrawal group than in the non-SVR-IFN and SVR groups ($P = 0.0049$ and $P = 0.0086$, respectively). There was no significant difference between the SVR group and the non-SVR-IFN group ($P = 0.3980$). Five-year progression rates to F3 or F4 were 0% in the SVR group, 14% in the non-SVR-IFN group and 54% in the non-SVR-Withdrawal group.

Safety and tolerability of maintenance interferon therapy

Five of 17 patients (29%) who received low-dose maintenance peginterferon treatment discontinued interferon therapy because of biliary complications ($n = 2$), neutropenia ($n = 1$), anaemia ($n = 1$) and *de novo* AIH ($n = 1$), between 26.5 and 53.1 months after its initiation. The biliary complications were not related to interferon therapy. Patients with neutropenia and anaemia recovered after discontinuing interferon therapy and were able to resume therapy within months (3 and 10, respectively). Steroid therapy alleviated the *de novo* AIH, but the patients did not resume interferon therapy.

DISCUSSION

Studies have repeatedly shown the benefits of achieving SVR via interferon therapy after liver transplantation. For instance, the durability of the SVR is associated with improvements in hepatic inflammation and histological regression of fibrosis over the long-term [18–23]. In contrast, efficacy of interferon therapy for non-SVR patients after liver transplantation had not previously been investigated. Here, we have demonstrated that long-term peginterferon maintenance therapy suppresses histological progression of recurrent hepatitis C after LDLT.

Maintenance interferon therapy was recently shown to have no influence on either histological or clinical outcomes in patients with nontransplant hepatitis C [24]. This conclusion was drawn after observing that the rate of fibrosis progression was similar between treatment and control groups following a 3.5-year randomized controlled trial of low-dose peginterferon. As a large number of patients with advanced fibrosis were enrolled in the randomized controlled trial, it is difficult to compare with our study in which the number of patients studied is much smaller and patients with advanced fibrosis were not enrolled. In the current study after liver transplantation, however, we demonstrated that low-dose maintenance interferon therapy reduced necroinflammatory activity and fibrosis scores in non-SVR patients to levels similar to those in SVR patients. Furthermore, we found that non-SVR patients who discontinued treatment had significantly worse scores once no longer receiving therapy.

Although these results clearly suggest that low-dose peginterferon maintenance therapy is beneficial for non-SVR patients with recurrent hepatitis C after liver transplantation, the mechanism behind this positive response is unknown. Progression of hepatitis C and development of fibrosis after discontinuation of interferon treatment has been shown to proceed more rapidly in patients who have undergone liver transplantation [20,21]. Our results, indicating that activity grade and fibrosis stage markedly deteriorated in non-SVR patients who discontinued maintenance treatment, support these previous findings. Thus, such a

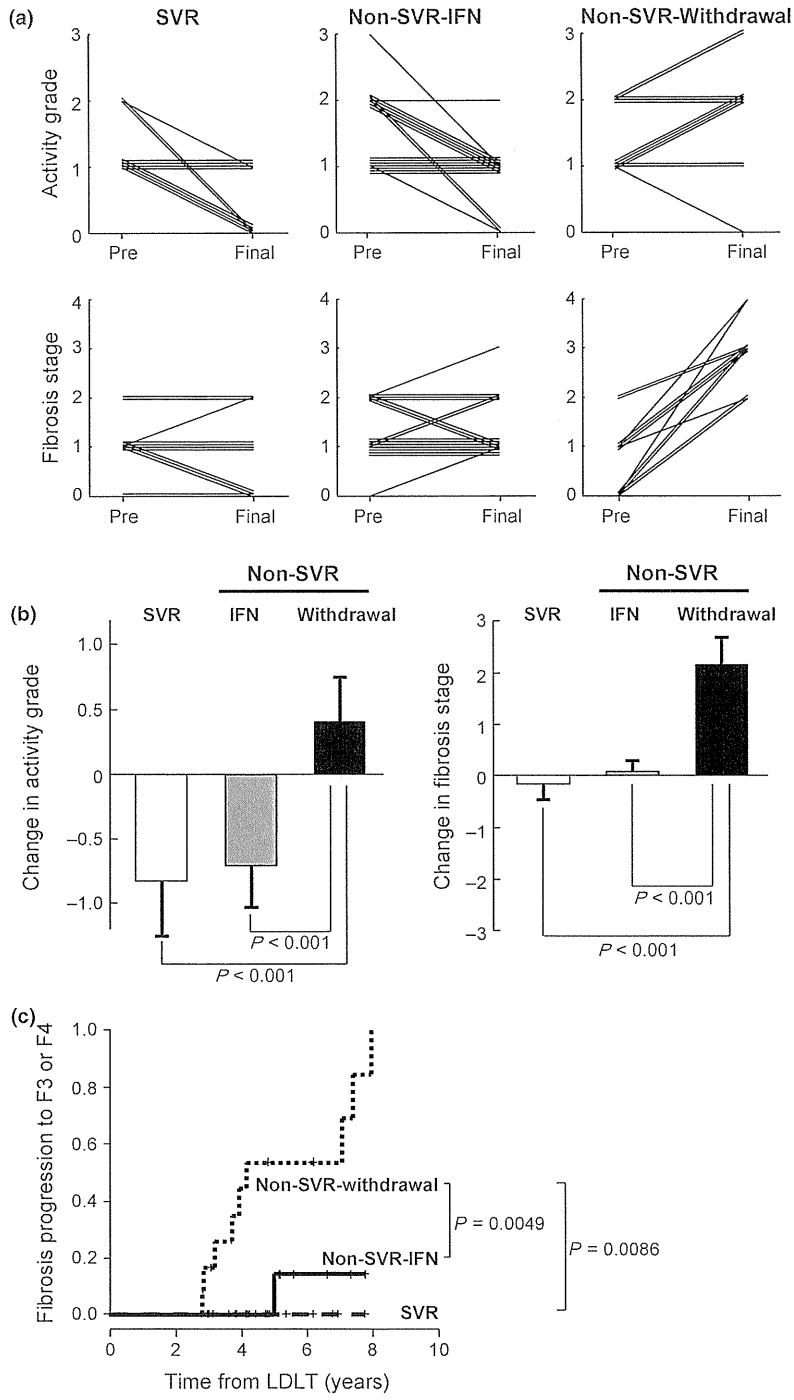

Fig. 2 Effect of maintenance interferon therapy on liver histology: (a) Changes in activity grade (upper) and fibrosis score (lower) of individual patients before interferon therapy (Pre) and at final biopsy (final). (b) Mean changes of liver activity grade (left) and fibrosis stage (right) between pretreatment liver biopsy and the final liver biopsy in each of the three treatment groups. The error bars represent 2 SEs. (c) Kaplan–Meier estimates of the progression rates among patients whose fibrosis advanced to F3 or F4. The dashed line indicates the sustained virological response (SVR) group, the solid line indicates the non-SVR-IFN group and the dotted line indicates the non-SVR-Withdrawal group.

rapid progression of recurrent hepatitis C in patients who discontinued interferon therapy may have highlighted the beneficial effect of the low-dose peginterferon maintenance therapy.

Another issue is the tolerability and safety of long-term peginterferon maintenance treatment. In this study, five patients (29%) discontinued the treatment during the peginterferon maintenance treatment, but only three did so for reasons directly related to the treatment. While two of these patients recovered simply by discontinuing the treatment, the third did require steroid pulse therapy to treat *de novo* AIH. Overall, however, the maintenance therapy did not result in the incidence of major adverse events, suggesting that it is both a tolerable and a safe treatment method.

Our work shows that long-term, low-dose peginterferon administration is an effective method for inhibiting the

progression of liver damage for recurrent hepatitis C after liver transplantation. Unfortunately, this was not a randomized control study, and only a small number of patients were eligible for research. Therefore, we recommend further work to more fully explore the effects of this treatment and to improve the outcomes for patients who do not achieve SVR.

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ORIGINAL ARTICLE

Targeting activation-induced cytidine deaminase prevents colon cancer development despite persistent colonic inflammation

A Takai[1], H Marusawa[1], Y Minaki[1], T Watanabe[1], H Nakase[1], K Kinoshita[2], G Tsujimoto[3] and T Chiba[1]

[1]*Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, Kyoto, Japan;* [2]*Shiga Medical Center Research Institute, Moriyama, Japan and* [3]*Department of Genomic Drug Discovery Science, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan*

Inflammatory bowel disease (IBD) is an important etiologic factor in the development of colorectal cancer. However, the mechanism underlying carcinogenesis through chronic inflammation is still unknown. Activation-induced cytidine deaminase (AID) is induced by the inflammation and involved in various human carcinogenesis via its mutagenic activity. In the current study, we investigated whether the inflammation/AID axis plays an integral role in the development of colitis-associated cancers. Inflammation in the cecum was more severe than that in other colonic regions, and endogenous AID expression was enhanced most prominently in the inflamed cecal mucosa of interleukin (IL)-10$^{-/-}$ mice. Blockade of tumor necrosis factor (TNF)-α and IL-12 significantly suppressed AID expression. Although proinflammatory cytokine expression was comparable between IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice, sequencing analyses revealed a significantly lower incidence of somatic mutations in *Trp53* gene in the colonic mucosa of IL-10$^{-/-}$AID$^{-/-}$ than IL-10$^{-/-}$AID$^{+/+}$ mice. Colon cancers spontaneously developed in the cecum in 6 of 22 (27.2%) IL-10$^{-/-}$AID$^{+/+}$ mice. In contrast, none of the IL-10$^{-/-}$AID$^{-/-}$ mice developed cancers except only one case of neoplasia in the distal colon. These findings suggest that the proinflammatory cytokine-induced aberrant production of AID links colonic inflammation to an enhanced genetic susceptibility to oncogenic mutagenesis. Targeting AID could be a novel strategy to prevent colitis-associated colon carcinogenesis irrespective of ongoing colonic inflammation.
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Keywords: AID; colitis-associated cancer; colonic inflammation; IL-10$^{-/-}$ mouse

Correspondence: Dr H Marusawa, Department of Gastroenterology and Hepatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: maru@kuhp.kyoto-u.ac.jp
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Introduction

Chronic inflammation in epithelial tissues predisposes to the development of cancers (Mantovani *et al.*, 2008). For example, epidemiologic studies demonstrate that patients with chronic hepatitis caused by hepatitis virus infection and chronic gastritis caused by *Helicobacter pylori* infection leads to the development of hepatocellular carcinoma and gastric cancers, respectively (Chiba *et al.*, 2006; Ikeda *et al.*, 2007). Similarly, it is well recognized that the incidence of colorectal cancer is significantly higher in those with inflammatory bowel disease (IBD) than in the general population (Podolsky, 2002). Indeed, the cumulative risk of developing colorectal cancer for any patient with ulcerative colitis is estimated to be 1.6% at 10 years, 8.3% at 20 years and 18.4% at 30 years from disease onset (Eaden *et al.*, 2001). The mechanisms of colon carcinogenesis in chronically inflamed tissue remain unclear, but it is reasonable to assume that multistep gene alterations required for malignant transformation occur in the constitutively inflamed colonic mucosa. Consistent with this idea, genetic alterations such as the *tumor protein p53* (*TP53*) mutation appear to be an early event and are already present in colonic mucosa of patients with ulcerative colitis before cancer onset (Yin *et al.*, 1993; Kern *et al.*, 1994; Hussain *et al.*, 2000; Leedham *et al.*, 2009). Therefore, identifying the molecular pathway that links inflammation and genetic alterations in tumor-related genes is an important step to understand colitis-associated carcinogenesis.

Recently, we demonstrated that one of the human nucleotide-editing enzymes, activation-induced cytidine deaminase (AID), induces somatic mutations in several tumor-related genes, including *TP53*, in gastrointestinal epithelial cells (Endo *et al.*, 2007, 2008; Kou *et al.*, 2007; Matsumoto *et al.*, 2007; Komori *et al.*, 2008). AID was originally identified as an inducer of somatic mutations and class switch recombination of immunoglobulin genes, which diversifies the antibody production in B lymphocytes (Muramatsu *et al.*, 2000). Although AID expression is restricted to activated B cells under physiologic conditions, the inflammatory response can trigger aberrant AID expression in various epithelial organs. Stimulation of proinflammatory cytokines such

as tumor necrosis factor (TNF)-α induces AID expression in hepatocytes, cholangiocytes and gastric epithelial cells (Endo *et al.*, 2007; Matsumoto *et al.*, 2007; Komori *et al.*, 2008). More importantly, aberrant AID expression in these epithelial cells results in the generation of nucleotide alterations in tumor-related genes and possible malignant transformation of the AID-expressing cells. Consistent with this hypothesis, animal models with constitutive and ubiquitous AID expression revealed that AID expression in epithelial tissues results in the accumulation of genetic mutations in various genes, leading to the development of liver, lung and gastric cancers (Morisawa *et al.*, 2008; Takai *et al.*, 2009). These findings strongly suggest that AID nucleotide-editing activity is intimately involved in the pathogenesis of inflammation-associated carcinogenesis (Chiba and Marusawa, 2009).

Chronic production of various proinflammatory cytokines is thought to be responsible for tumor development and progression in colitis-associated cancers (Lin and Karin, 2007). One example of the oncogenic effect of proinflammatory cytokines on colonic cells is provided by the colon carcinogenesis model, in which TNF-α blockade reverses azoxymethane and dextran sodium sulfate-induced colonic mucosal damage and attenuates subsequent colon cancer development (Popivanova *et al.*, 2008). What is noteworthy is that aberrant AID expression is induced in colonic epithelial cells in response to TNF-α via the IκB kinase-dependent nuclear factor (NF)-κB signaling pathways (Endo *et al.*, 2008). Moreover, we showed that enhanced expression of endogenous AID protein is detectable in the inflamed colonic mucosa of patients with ulcerative colitis or Crohn's disease (Endo *et al.*, 2008). These findings suggest that the inflammatory cytokine/AID axis may actually promote colon carcinogenesis by its genotoxic activity in the background of IBD. To clarify whether AID is a crucial mediator of the genetic alterations required for inflammation-mediated carcinogenesis, we investigated the impact of AID deficiency in the pathogenesis of colitis-associated colon cancer.

Results

AID gene expression in inflamed colonic mucosa of interleukin (IL)-10$^{-/-}$ mice

We first investigated endogenous AID expression in association with the degree of colonic inflammation in IL-10$^{-/-}$ mice, a representative model of human IBD. IL-10$^{-/-}$ mice were maintained under pathogen-free conditions and histological analysis was performed using paraffin-embedded tissue sections from the cecum, the proximal colon, the distal colon and the small intestine. IL-10$^{-/-}$ mice spontaneously developed intestinal inflammation after 8 weeks of age, whereas no inflammatory change was observed in the colons of the wild-type (WT) mice (Figure 1a). In 52-week-old IL-10$^{-/-}$ mice, the histological findings from the colonic tissue revealed epithelial hyperplasia, inflammatory cell infiltration and goblet cell loss (Figure 1a). Inflammatory lesions occurred at greater severity in the cecum compared with the proximal colon, the distal colon and the small intestine.

Colonic mucosal inflammation in IL-10$^{-/-}$ mice is mediated by proinflammatory cytokines as well as an excessive Th1 T-cell response associated with increased interferon (IFN)-γ and IL-12 secretion (Berg *et al.*, 1996; Davidson *et al.*, 1996). Consistent with previous studies, expression levels of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 were elevated in the inflamed cecal mucosa of the 52-week-old IL-10$^{-/-}$ mice (Figure 1b). Moreover, enhanced expression of Th1 cytokines, including IL-12 and IFN-γ, was observed in the 52-week-old IL-10$^{-/-}$ mice, but little expression of inflammatory cytokines was detected in 8-week-old IL-10$^{-/-}$ mice. In contrast, the expression levels of Th2 cytokines such as IL-4 and IL-13 did not differ between control and the IL-10$^{-/-}$ mice (Figure 1b).

We next examined whether aberrant AID expression appeared in association with colonic inflammation in IL-10$^{-/-}$ mice. Quantitative reverse transcription–PCR (RT–PCR) revealed a marked elevation of endogenous AID expression in the cecal epithelial cells of IL-10$^{-/-}$ mice >20 weeks of age, whereas only trace amounts of AID expression in younger IL-10$^{-/-}$ mice and in WT mice (Figure 1c, Supplementary Figure 1). In the 20- and 52-week-old IL-10$^{-/-}$ mice, AID was strongly expressed in the cecal mucosa compared with the epithelium of the proximal and distal colon (Figure 1d).

To determine whether the increased AID expression derives from epithelial cells or infiltrating B lymphocytes, we examined the expression pattern of endogenous AID in the inflamed cecal mucosa of IL-10$^{-/-}$ mice using *in situ* hybridization. The specificity of the *in situ* hybridization results was confirmed by control staining performed on an intestinal lymphoid follicle containing mostly activated B cells or the tissues derived from the transgenic mice with constitutive AID expression (Figure 1e, Supplementary Figure 2). No AID expression was detected in the normal cecal mucosa, liver and kidney of WT mice, or the murine B lymphoma cells with the small interfering RNA-mediated knockdown of endogenous AID transcripts (Figure 1e, Supplementary Figure 2). In contrast, high AID expression was observed mainly in the cytoplasm of both cecal epithelium and lymphocytes in inflamed intestinal tissues (Figure 1e, Supplementary Figure 3).

These findings suggest that persistent inflammation in the cecum of the older IL-10$^{-/-}$ mice is closely associated with the enhanced production of various inflammatory cytokines, leading to the induction of aberrant AID expression in inflamed colonic mucosa.

Inhibition of TNF-α and IL-12 suppressed AID expression with the decrease of colonic inflammation in IL-10$^{-/-}$ mice

To clarify the role of TNF-α and IL-12 in the pathophysiology of colonic inflammation in association with aberrant AID expression in the IL-10$^{-/-}$ mice, the biologic activity of TNF-α and IL-12 was inhibited using the TNF antagonist etanercept and neutralizing

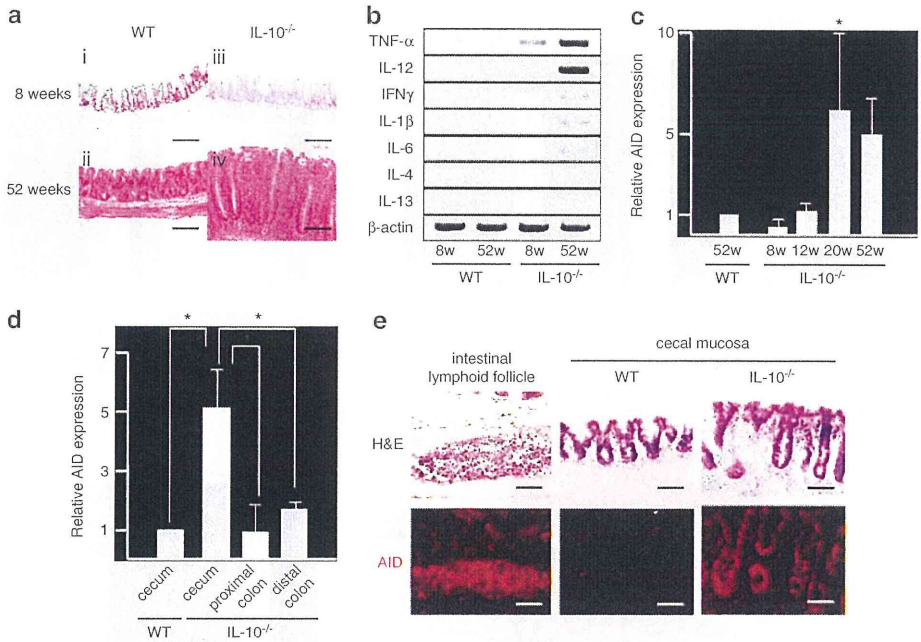

Figure 1 Endogenous AID expression in inflamed colonic mucosa of IL-10$^{-/-}$ mice. (**a**) Microscopic (hematoxylin and eosin (H&E) stain) images of the cecum of IL-10$^{-/-}$ mice and its WT littermate. The images of 8-week-old mice (i, iii) and 52-week-old mice (ii, iv) are shown. Scale bars are 200 μm. (**b**) Representative results of RT–PCR for the expression of various proinflammatory cytokines in the cecal mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from cecal mucosa of 8- and 52-week-old IL-10$^{-/-}$ mice and their WT littermates. RT–PCR was performed using oligonucleotides specific for murine TNF-α, IL-12, IFN-γ, IL-1β, IL-6, IL-4, IL-13, and β-actin. (**c**) Time course changes of AID expression in the cecal mucosa of IL-10$^{-/-}$ mice. Total RNA was isolated from mucosa at the cecum of 8-, 12-, 20- and 52-week-old IL-10$^{-/-}$ mice and WT littermates of 52-week-old IL-10$^{-/-}$ mice. Quantitative RT–PCR was performed using oligonucleotides specific for murine AID. *$P<0.05$ versus WT mice. (**d**) AID expression in various regions of colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, and the proximal and distal colon of 52-week-old IL-10$^{-/-}$ mice and WT littermates. Quantitative RT–PCR was performed using oligonucleotides specific for murine AID. *$P<0.05$. (**e**) Representative images of AID expression in inflamed cecal mucosa determined by *in situ* hybridization assay. The images show the intestinal lymphoid follicle (left panels), the cecal mucosa of WT (middle panels) and IL-10$^{-/-}$ mice (right panels) stained with H&E (upper panels) or hybridized with the probe specific for the murine AID transcript (lower panels). Scale bars are 100 μm.

IL-12p40 monoclonal antibody (mAb), respectively (Liu *et al.*, 2006; Watanabe *et al.*, 2006; Popivanova *et al.*, 2008). First, we confirmed that the expression of both TNF-α and IL-12 was significantly upregulated in the cecum compared with the proximal and distal colon ($P<0.05$, Figure 2a). In agreement with established findings that TNF-α augments the expression of various cytokines and chemokines (Marra *et al.*, 1993; Popivanova *et al.*, 2008), etanercept treatment resulted in the suppression of a variety of proinflammatory cytokines and chemokines such as TNF-α, IL-12, IFN-γ, IL-1β, IL-6, and monocyte chemoattractant protein-1 (Figure 2b, Supplementary Figure 4). In contrast, there was no apparent difference in the expression levels of IL-5, a Th2 cytokine, between the etanercept and control groups (Supplementary Figure 4). Histopathological findings revealed that the cecal inflammation was substantially reduced in mice treated with etanercept compared with the control mice (Figure 2c, left and middle panels). Similar to etanercept, IL-12p40 mAb treatment effectively suppressed cecal inflammation in association with reduced levels of proinflammatory cytokines and chemokines other than IL-5 compared with the control mice (Figure 2b, Supplementary Figure 4).

NF-κB p65 is strongly activated in the inflamed colonic mucosa of IL-10$^{-/-}$ mice (Neurath *et al.*, 1996; Inoue *et al.*, 2009). To determine the effect of inhibition of the TNF-α- or IL-12-mediated signaling pathways on NF-κB activity, immunohistochemical staining of the cecal tissue from IL-10$^{-/-}$ mice was performed using the phospho-NF-κB p65 antibody. In the vehicle-treated IL-10$^{-/-}$ mice, phospho-NF-κB p65 was strongly positive in the nucleus of the epithelial cells, whereas NF-κB activation was markedly suppressed in the cecal mucosa of mice treated with the etanercept or IL-12p40 mAb (Figure 2c, right panels).

Endogenous AID expression is induced in response to TNF-α or IL-12 treatment and AID transcription is regulated via IκB kinase-dependent NF-κB signaling pathways in human colonic cells (Endo *et al.*, 2008). Thus, we investigated AID expression levels when TNF-α or IL-12 activity was inhibited in the colonic tissue of IL-10$^{-/-}$ mice. Quantitative RT–PCR analyses showed that endogenous AID expression was markedly decreased in the cecal mucosa in both the etanercept and IL-12p40 mAb groups when compared with the control group (Figure 2d). These findings suggest that TNF-α and IL-12 play a critical role in not only intestinal

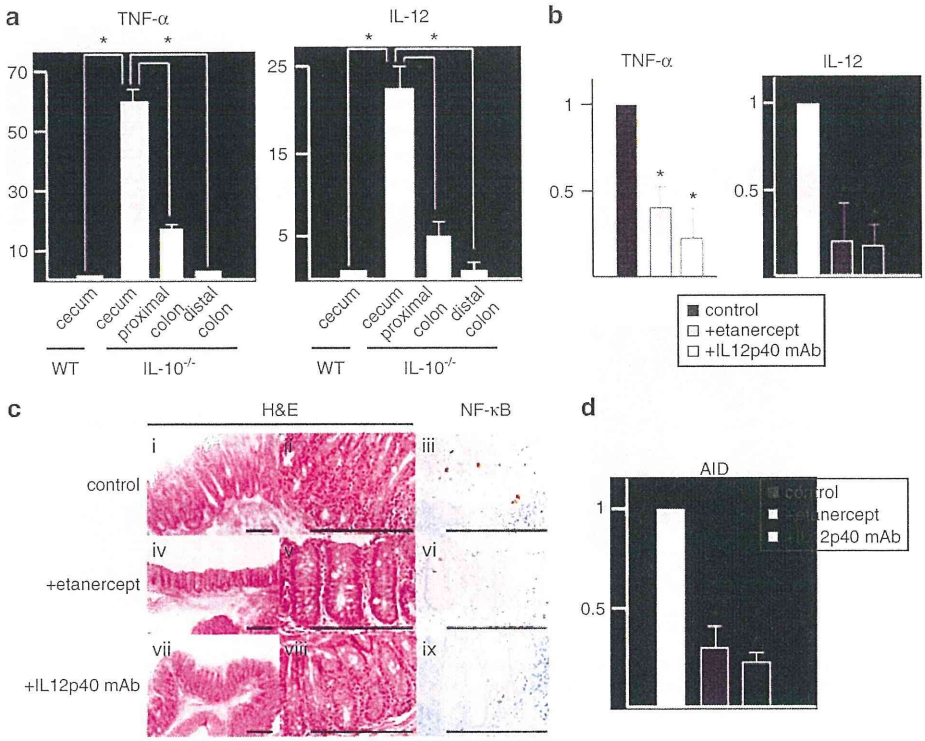

Figure 2 Effects of TNF-α and IL-12 blockade on various cytokine expression, NF-κB activation and AID expression in the colonic mucosa of IL-10$^{-/-}$ mice. (**a**) TNF-α and IL-12 expression in various regions in colonic mucosa of IL-10$^{-/-}$ mice. Total RNA was extracted from mucosa at the cecum, and the proximal and distal colon of 52-week-old IL-10$^{-/-}$ mice and the cecum of the WT littermates. Quantitative RT–PCR was performed using oligonucleotides specific for murine TNF-α and IL-12. *$P<0.05$. (**b**) Changes of various cytokine expression under the inhibition of the biologic activity of TNF-α and IL-12. Total RNA was isolated from mucosa at the cecum of IL-10$^{-/-}$ mice treated with phosphate-buffered saline (PBS), etanercept and IL-12p40 mAb. Quantitative RT–PCR was performed using oligonucleotides specific for murine TNF-α and IL-12. Relative values of these cytokines are shown. *$P<0.05$ versus control mice. (**c**) Changes of histopathological image and NF-κB activation induced by administration of etanercept and IL-12p40 mAb. Microscopic images (hematoxylin and eosin (H&E) stain) of the cecum of IL-10$^{-/-}$ mice treated with PBS (i, ii), etanercept (iv, v) and IL-12p40 mAb (vii, viii). Immunohistochemical staining for phospho-NF-κB p65 was performed using each sample including control group (iii), etanercept-treated group (vi) and IL-12p40 mAb-treated group (ix). Scale bars are 500 μm. (**d**) Results of quantitative RT–PCR for AID expression in the cecum of IL-10$^{-/-}$ mice treated with PBS, etanercept and IL-12p40 mAb. *$P<0.05$ versus control mice.

inflammation but also the aberrant AID expression in the cecal mucosa of the IL-10$^{-/-}$ mice.

AID deficiency did not affect the expression levels of proinflammatory cytokines in colonic mucosa

To gain insight into the role of AID expression in the inflamed colonic mucosa, we evaluated the effect of AID deficiency on the inflammatory response by crossing AID$^{-/-}$ mice with IL-10$^{-/-}$ mice (Figure 3a), and the cecal mucosa isolated from IL-10$^{-/-}$AID$^{-/-}$ mice were subjected to further analysis. Histological findings revealed that mucosal inflammatory changes in IL-10$^{-/-}$AID$^{-/-}$ mice, including pronounced inflammatory infiltration and marked epithelial hyperplasia, were comparable with those of the IL-10$^{-/-}$AID$^{+/+}$ littermates (Figure 3b). In agreement with a previous study (Fagarasan *et al.*, 2002), hyperplasia of isolated lymphoid follicles developed in the cecum of 52-week-old AID$^{-/-}$ mice as well as in IL-10$^{-/-}$AID$^{-/-}$ mice (data not shown). RT–PCR analyses revealed only trace amounts of cytokine expression in WT or AID$^{-/-}$ mice (Figure 3c). In contrast, enhanced expression levels of various cytokines such as TNF-α, IL-12, IFN-γ, IL-1β and IL-6 observed in the IL-10$^{-/-}$AID$^{-/-}$ mice were comparable with those in the IL-10$^{-/-}$AID$^{+/+}$ mice (Figure 3c). Quantitative RT–PCR analyses also showed markedly enhanced expression levels of monocyte chemoattractant protein-1 in the cecum of IL-10$^{-/-}$AID$^{-/-}$ mice to almost the same levels as that in IL-10$^{-/-}$AID$^{+/+}$ mice (Figure 3d). Thus, the production levels of inflammatory cytokines and chemokines in the cecal mucosa did not differ between IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice.

Deficiency of endogenous AID resulted in the reduced frequencies of nucleotide alterations in the Trp53 *gene in IL-10$^{-/-}$ mice*

To clarify whether the AID upregulation induced by chronic colitis is genotoxic in colonic mucosa, we determined the nucleotide sequences of the *Trp53*, *Apc*, *Ctnnb1* and *Kras* genes, all of which are thought to be involved in human colorectal carcinogenesis (Fearon and Vogelstein, 1990). We first confirmed that the incidence of nucleotide alterations was <1.00

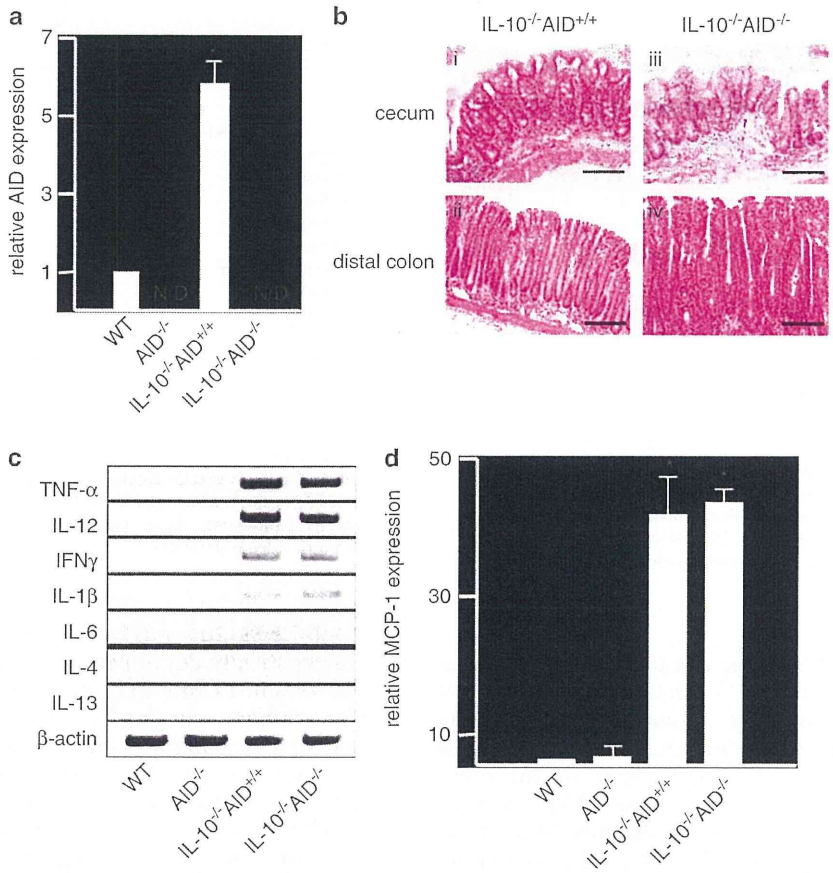

Figure 3 Effects of AID deficiency in the colonic mucosa of IL-10$^{-/-}$ mice. (**a**) Results of quantitative RT–PCR for AID expression. Total RNA was extracted from the mucosa at the cecum of 52-week-old WT, AID$^{-/-}$, IL-10$^{-/-}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. Quantitative RT–PCR was performed using oligonucleotides specific for murine AID. *P<0.05 versus WT mice. N/D, not detected. (**b**) Microscopic images (hematoxylin and eosin (H&E) stain) of the large intestine of 52-week-old IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$AID$^{-/-}$ mice. Upper panels (i, iii) are the images of the cecum and lower panels (ii, iv) are the images of the distal colon. Scale bars are 200 μm. (**c**) Representative results of semiquantitative RT–PCR for the expression of various proinflammatory cytokines in the cecum of 52-week-old WT, AID$^{-/-}$, IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice. RT–PCR analyses were performed using specific primers for murine TNF-α, IL-12, IFN-γ, IL-1β, IL-6, IL-4, IL-13 and β-actin. (**d**) Results of quantitative RT–PCR for monocyte chemoattractant protein-1 (MCP-1) expression in the cecum of each genotype. *P<0.05 versus WT mice.

substitution per 10^4 nucleotides in these four tumor-related genes obtained from over 40 randomly picked clones from the normal cecal epithelial cells of WT mice, determined by conventional sequencing analyses with high-fidelity PCR amplification (data not shown). We then determined the sequences of the *Trp53, Apc, Ctnnb1* and *Kras* genes in 50 randomly selected clones amplified from the cecal epithelial cells of three IL-10$^{-/-}$AID$^{+/+}$ as well as three IL-10$^{-/-}$AID$^{-/-}$ mice (representative data are shown in Table 1). Nucleotide alterations had accumulated in the *Trp53* gene in the inflamed cecal mucosa of the IL-10$^{-/-}$AID$^{+/+}$ mice with a frequency of 2.19 substitutions per 10^4 nucleotides (Table 1). Although all the nucleotide changes determined in the *Trp53* gene were different in each clone, 9 of 12 (75%) alterations were accumulated in the regions corresponding to the DNA-binding motif of the human *TP53* gene (Figure 4). Among the nucleotide changes observed in the cecal epithelial cells of IL-10$^{-/-}$AID$^{+/+}$

Table 1 Gene mutation frequencies in inflamed cecal mucosa of the IL-10$^{-/-}$AID$^{+/+}$ mice and IL-10$^{-/-}$ AID$^{-/-}$ mice

Gene	*Genotype*	*Nucleotide alterations*		P-*value**
		Number[a]	*Frequency (/10^4)*	
Trp53	AID (+/+)	12/54 787	2.19	<0.05
	AID (−/−)	5/70 380	0.71	
Apc	AID (+/+)	2/33 717	0.59	0.43
	AID (−/−)	1/42 762	0.23	
Ctnnb1	AID (+/+)	5/38 988	1.28	0.51
	AID (−/−)	2/36 037	0.55	
Kras	AID (+/+)	4/36 603	1.09	0.84
	AID (−/−)	3/31 955	0.94	

Abbreviations: AID, activation-induced cytidine deaminase; IL-10, interleukin-10.
Representative results of mutation frequencies in *Trp53*, *Apc*, *Ctnnb1* and *Kras* genes in inflamed cecal mucosa of IL-10$^{-/-}$AID$^{+/+}$ and IL-10$^{-/-}$AID$^{-/-}$ mice are shown.
[a]Number of mutated nucleotides/number of total nucleotides examined.
**P*-value is calculated using the χ^2 test.