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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 the 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

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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.

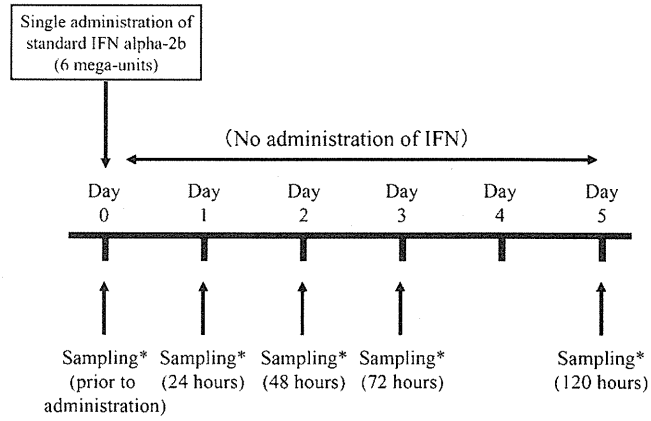

Fig. 1. Schematic representation of administration of standard IFN-alpha and measurements of HCV RNA levels. The serum HCV RNA levels were measured before, and 24, 48, 72, and 120 hr after administration of a singe dose of 6 mega-units of standard IFN-alpha. IFN, interferon; Sampling*, sampling of serum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

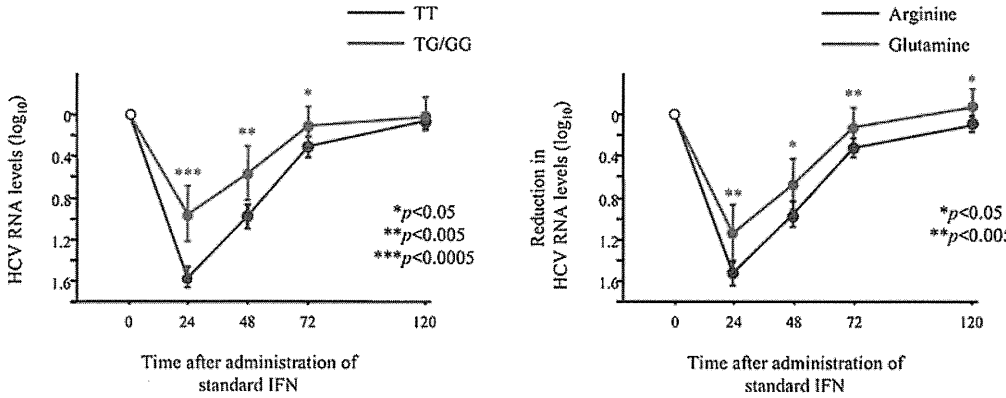

Fig. 2. Changes in HCV RNA levels after administering standard IFN to patients with the TT genotype compared to the TG/GG genotype near the *IL28B* gene (**left panel**), and in patients with an arginine compared to a glutamine at residue 70 of the HCV core region (**right panel**). The decrease in HCV 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.

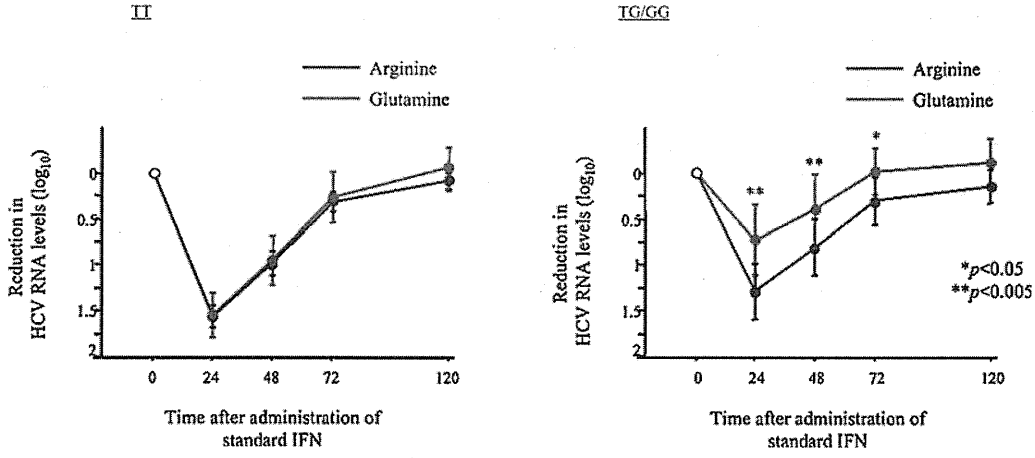

Fig. 3. Changes in HCV RNA levels after administering standard IFN in patients with an arginine compared to a glutamine at residue 70 of the HCV core region in patients with the TT genotype (**left panel**) and in those with the TG/GG genotype (**right panel**) near the *IL28B* gene. The decrease in 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.

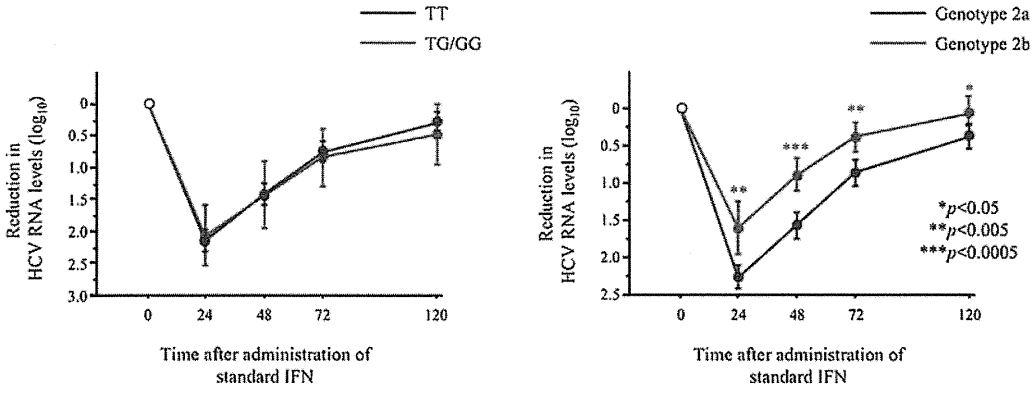

Fig. 4. Changes in HCV RNA levels after administering standard IFN to patients with the TT genotype compared to the TG/GG genotype near the *IL28B* gene (**left panel**) and in patients infected with HCV genotype 2a compared to HCV genotype 2b (**right panel**). The decrease in HCV RNA levels for the 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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A large-scale association study identified multiple HLA-DRB1 alleles associated with ACPA-negative rheumatoid arthritis in Japanese subjects

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ABSTRACT

Background HLA-DRB1 is associated with rheumatoid arthritis (RA). However, it has recently been suggested that HLA-DRB1 is only associated with patients with RA who have anticitrullinated peptide/protein antibodies (ACPA), which are specific to RA.

Objective To elucidate whether specific HLA-DR alleles are associated with ACPA-negative RA development.

Methods HLA-DRB1 typing was carried out in 368 Japanese ACPA-negative patients with RA and 1508 healthy volunteers as the first set, followed by HLA-DRB1 typing of 501 cases and 500 controls as the second set. The HLA-DRB1 allele frequency and diplotype frequency were compared in each group, and the results of the two studies were combined to detect HLA-DRB1 alleles or diplotypes associated with ACPA-negative RA.

Results HLA-DRB1*12:01 was identified as a novel susceptibility allele for ACPA-negative RA ($p=0.000088$, OR=1.72, 95% CI 1.31 to 2.26). HLA-DRB1*04:05 and *14:03 showed moderate associations with ACPA-negative RA ($p=0.0063$, OR=1.26, 95% CI 1.07 to 1.49 and $p=0.0043$, OR=1.81, 95% CI 1.20 to 2.73, respectively). The shared epitope was weakly associated with ACPA-negative RA, but no dosage effect was detected ($p=0.016$, OR=1.17, 95% CI 1.03 to 1.34). A combination of HLA-DRB1*12:01 and DRB1*09:01 showed a strong association with susceptibility to ACPA-negative RA ($p=0.00013$, OR=3.62, 95% CI 1.79 to 7.30). Homozygosity for HLA-DR8 was significantly associated with ACPA-negative RA ($p=0.0070$, OR=2.16, 95% CI 1.22 to 3.82). It was also found that HLA-DRB1*15:02 and *13:02 were protective against ACPA-negative RA ($p=0.00010$, OR=0.68, 95% CI 0.56 to 0.83 and $p=0.00059$, OR=0.66, 95% CI 0.52 to 0.84, respectively).

Conclusions In this large-scale association study multiple alleles and diplotypes were found to be associated with susceptibility to, or protection against, ACPA-negative RA.

INTRODUCTION

Rheumatoid arthritis (RA) is one of the most common causes of chronic arthritis and results in severe joint damage and a shorter life span.[1] Genetic factors have been shown to contribute to the onset of RA.[2] Among the genetic susceptibility loci detected to date, HLA-DRB1 has a strong impact on the predisposition to RA and has been repeatedly shown to be associated with RA in an ethnicity-independent manner.[3] It is widely accepted that the shared epitope (SE), a common amino acid sequence located from the 70th to the 74th amino acids of the HLA-DR β chain, explains the associations of specific HLA-DRB1 alleles with RA.[4] Anticitrullinated protein antibodies (ACPA) are a highly specific marker of RA.[5 6] Recent data have shown that the SE is associated with ACPA-positive RA but not associated or only weakly associated with ACPA-negative RA.[7–9] Many of the non-HLA susceptibility genes for RA detected to date, such as *PTPN22*[10] and *CTLA4*[11] have been shown to be associated with ACPA-positive RA alone, and no association between these genes and ACPA-negative RA has been detected. These findings suggest that ACPA-negative RA is genetically distinct from ACPA-positive RA.

Among HLA-DRB1 molecules, HLA-DR3[12] and HLA-DR13[13] were reported to be associated with ACPA-negative RA in populations of European descent, but the same results were not obtained in a meta-analysis of a large Caucasian cohort.[14] In Asian populations, there has only been a small study which showed that HLA-DRB1*09:01 might be associated with ACPA-negative RA,[15] while SEs, especially DRB1*04:05, *04:01 and *01:01, were associated with RA and ACPA-positive RA.[15 16] Thus, no specific alleles that convey susceptibility to, or are protective against, ACPA-negative RA have been identified in populations of European or Asian descent. In this large-scale Japanese case–control association study, we show that HLA-DRB1*12:01, *14:03 and *04:05 are susceptibility alleles for ACPA-negative RA and that HLA-DRB1*13:02 and *15:02 are protective against ACPA-negative RA. We also identified multiple diplotypes that convey susceptibility to, or are protective against, ACPA-negative RA.

MATERIALS AND METHODS

Study subjects

DNA samples were collected at Kyoto University Hospital from 184 patients with RA who were negative for ACPA, as reported previously,[7] and another 184 patients with RA without ACPA were recruited at Tokyo Women's Medical University. These two sample groups were used as the first

set. Independent DNA samples were collected from 501 ACPA-negative patients with RA at RIKEN under the support of BioBank Japan and were used as the second set. The 501 cases in the second set are a fraction of 2410 RA cases included in another manuscript (K Shimane *et al*, unpublished data). All patients were Japanese and diagnosed by rheumatologists to fulfil the 1987 American College of Rheumatology revised criteria for RA.[17] A first set of control DNA samples were collected from 1508 healthy control subjects at Aichi Cancer Center Hospital and from the DNA banks of the Pharma SNP Consortium, which contains DNA samples from healthy Japanese volunteers.[18] The second set of control DNA samples were collected from 500 healthy volunteers at the HLA laboratory. This study was approved by the local ethical committees at each institution, and written informed consent was obtained from all patients. Basic information about cases and controls is shown in table 1.

ACPA detection

ACPA were detected with the MESACUP CCP ELISA kit (Medical and Biological Laboratories Co, Ltd, Nagoya, Japan) according to the manufacturer's instructions at each institution. A cut-off value of 4.5 U/ml was used to assess ACPA positivity.

HLA-DRB1 genotyping

HLA-DRB1 typing was carried out with the WAKFlow system and described in detail elsewhere.[7] In the 184 cases collected at Kyoto University and all the controls in the two sets, genotyping was performed at the HLA laboratory (Kyoto, Japan), whereas it was carried out at RIKEN for all 501 cases in the second set. HLA-DRB1 genotyping of the 184 cases collected at Tokyo Women's Medical University was performed by a sequencing-based typing method using the AlleleSEQR HLA-DRB1 typing kit (Abbott, Tokyo, Japan), and allele assignment was performed using the Assign software.

The following HLA-DRB1 alleles were classified as belonging to the SE: DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *04:10, *04:13, *04:16, *10:01, *13:03, *14:02 and *14:06.

Statistical analysis

The frequency of each genotype or diplotype among the ACPA-negative patients with RA was compared with that in the controls using a χ^2 test or Fisher's exact test. Ninety-five percent CIs, p values and ORs were also calculated. The relative risk (RR) of ACPA-negative susceptibility induced by homozygosity for each allele was calculated to estimate the dosage effect. We performed 1000 permutation tests to confirm the associations found for each allele. Logistic regression analysis was used to evaluate the effects of alleles by adjusting for the influence of other alleles. Statistical analysis was performed using the R statistic system (http://www.R-project.org) or SPSS (version 18). The power calculation was performed using an online power calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/).

RESULTS

Genotyping of the first set

We performed HLA-DRB1 genotyping in the 368 ACPA-negative patients with RA and 1508 healthy controls in the first set to compare the allele frequency of each genotype between the cases and controls (table 1). Tables 2 and 3 show the main results of our association study for single alleles and diplotypes, respectively. More detailed results are given in the online supplementary tables 1 and 2.

The SE showed a weak association with moderate effect (p=0.039), mainly due to HLA-DRB1*04:05. Among the other HLA-DRB1 alleles, HLA-DRB1*14:03, *12:01, and *09:01 resulted in moderate to potential susceptibility to ACPA-negative RA (p=0.022, 0.10, and 0.10, respectively). DRB1*13:02, *04:03, and *15:02 showed moderate to potentially protective effects (p=0.0072, 0.059, and 0.12, respectively).

Replication in the second set and combined analysis

We performed HLA-DRB1 genotyping of samples in the second set to replicate the results found in the first set, using the DNA samples from 501 ACPA-negative patients with RA and 500 sex-matched healthy controls and combined the results of the two association studies.

Among the susceptibility alleles found in the first set, HLA-DRB1*12:01 was confirmed to display a susceptible association (p=0.010 and 0.000088 for the second set and combined study, respectively; table 2). The susceptibility tendencies of *04:05 and *14:03 were replicated in the second set, and these alleles showed moderate associations with susceptibility to ACPA-negative RA in the combined analysis (p=0.0063 and 0.0043, respectively). DRB1*09:01 and *14:05 showed potential susceptibility to ACPA-negative RA in the pooled study (p= 0.062 and 0.080, respectively). The SE showed a weak association with susceptibility to ACPA-negative RA in the combined study (p=0.016), but we could not detect any dosage effect (table 3 and figure 1). Among the protective alleles detected in the first set, the protective effect of DRB1*15:02 was successfully replicated (p=0.002 and 0.00010 in the second set and combined study, respectively; table 2). Although the protective effect of DRB1*13:02 was not replicated in the second set, the combined analysis showed a significant protective effect (p=0.00059). The protective effect of DRB1*04:03 was confirmed in the second set, and the combined study demonstrated a weak protective association (p=0.038). To exclude the possibility that the associations of the susceptibility alleles were induced by the absence of protective alleles or vice versa, we applied logistic regression analysis. The logistic regression analysis suggested that none of the allelic associations—namely, those of HLA-DRB1*12:01, *14:03, *04:05, *13:02, and *15:02, depended on the effects of other alleles (online supplementary table 3). In addition, the permutation tests confirmed the associations of these five alleles (permutation p<0.0070, data not shown).

Next, we analysed the dosage effects of each protective or susceptibility allele. DRB1*12:01 showed a potential dosage effect, but only two patients were homozygous for DRB1*12:01 (figure 1). We could not detect any dosage effects of HLA-DRB1*04:05 or the SE. No patients were homozygous for *14:03

Table 1 Basic information for ACPA-negative patients with RA and controls

Classification	ACPA-negative RA	Control
Set 1		
Number	368	1508
Female (%)	79.7	52.9
Age (mean±SD)	54.7±16.1	46.5±15.3
Set 2		
Number	501	500
Female (%)	80.8	80.0
Age (mean±SD)	62.4±12.2	NA

ACPA, anticitrullinated peptide/protein antibody; NA, not available; RA, rheumatoid arthritis.

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Table 2 Association of the HLA-DRB1 allele with ACPA-negative RA

	Set 1				Set 2				Pooled study			
	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)
Non-SE												
Susceptible												
*12:01	31 (4.2)	91 (3.0)	0.10	1.41 (0.93 to 2.14)	62 (6.2)	37 (3.7)	0.010	1.72 (1.13 to 2.60)	93 (5.4)	128 (3.2)	0.000088	1.72 (1.31 to 2.26)
*14:03	18 (2.4)	39 (1.3)	0.022	1.91 (1.09 to 3.36)	23 (2.3)	14 (1.4)	0.14	1.65 (0.85 to 3.23)	41 (2.4)	53 (1.3)	0.0043	1.81 (1.20 to 2.73)
*09:01	123 (16.7)	432 (14.3)	0.10	1.20 (0.96 to 1.49)	164 (16.4)	154 (15.4)	0.55	1.08 (0.85 to 1.37)	287 (16.5)	586 (14.6)	0.062	1.16 (0.99 to 1.35)
*14:05	19 (2.6)	63 (2.1)	0.41	1.24 (0.74 to 2.09)	29 (2.9)	18 (1.8)	0.11	1.63 (0.9 to 2.95)	48 (2.8)	81 (2.0)	0.080	1.38 (0.96 to 1.98)
Protective												
*15:02	75 (10.2)	369 (12.2)	0.12	0.81 (0.63 to 1.06)	73 (7.3)	113 (11.3)	0.0020	0.62 (0.45 to 0.84)	148 (8.5)	482 (12.0)	0.00010	0.68 (0.56 to 0.83)
*13:02	44 (6.0)	273 (9.1)	0.0072	0.64 (0.46 to 0.89)	52 (5.2)	52 (5.2)	0.99	1.00 (0.67 to 1.48)	96 (5.5)	325 (8.1)	0.00059	0.66 (0.52 to 0.84)
*04:03	14 (1.9)	97 (3.2)	0.059	0.58 (0.33 to 1.03)	23 (2.3)	28 (2.8)	0.47	0.82 (0.47 to 1.43)	37 (2.1)	125 (3.1)	0.038	0.68 (0.47 to 0.98)
SE												
*04:05	103 (14.0)	340 (11.3)	0.040	1.28 (1.01 to 1.62)	145 (14.5)	129 (12.9)	0.31	1.14 (0.89 to 1.47)	248 (14.3)	469 (11.7)	0.0063	1.26 (1.07 to 1.49)
*14:06	16 (2.2)	37 (1.2)	0.051	1.79 (0.99 to 3.23)	14 (1.4)	9 (0.9)	0.30	1.56 (0.67 to 3.62)	30 (1.7)	46 (1.1)	0.076	1.52(0.95 to 2.41)
*10:01	8 (1.9)	13 (0.4)	0.032	2.54 (1.05 to 6.15)	6 (0.6)	5 (0.5)	0.76	1.20 (0.36 to 3.94)	14 (0.8)	18 (0.4)	0.094	1.80 (0.90 to 3.63)
*04:04	4 (0.5)	6 (0.2)	0.10	2.74 (0.77 to 9.74)	3 (0.3)	2 (0.2)	0.66	1.50 (0.25 to 8.99)	7 (0.4)	8 (0.2)	0.16	2.03 (0.73 to 5.60)
*01:01	43 (5.8)	183 (6.1)	0.82	0.96 (0.68 to 1.35)	50 (5.0)	64 (6.4)	0.17	0.77 (0.52 to 1.12)	93 (5.4)	247 (6.2)	0.24	0.86 (0.67 to 1.10)
*04:01	12 (1.6)	35 (1.2)	0.30	1.41 (0.73 to 2.73)	10 (1.0)	10 (1.0)	1.0	1.00 (0.41 to 2.41)	22 (1.3)	45 (1.1)	0.64	1.13 (0.68 to 1.89)
*04:10	6 (0.8)	63 (2.1)	0.021	0.39 (0.17 to 0.89)	25 (2.5)	14 (1.4)	0.076	1.80 (0.93 to 3.49)	31 (1.8)	77 (1.9)	0.73	0.93 (0.61 to 1.41)
All SE	192 (26.1)	677 (22.4)	0.036	1.22 (1.01 to 1.47)	253 (25.3)	233 (23.3)	0.31	1.11 (0.91 to 1.36)	445 (25.6)	910 (22.7)	0.016	1.17 (1.03 to 1.34)

Allele number and the frequency of each HLA-DRB1 allele in ACPA-negative patients with RA (n=368 and allele number=736 in the 1st set and n=501 and allele number=1002 in the 2nd set) and healthy controls (n=1508 and allele number=3016 in the 1st set and n=500 and allele number=1000 in the 2nd set) as well as the p value and OR of each allele for the development of ACPA-negative RA are shown. p Values were calculated using Fisher's exact test or the χ^2 test.
†Number of alleles (allele frequency).
ACPA, anticitrullinated peptide/protein antibody; SE, shared epitope; RA, rheumatoid arthritis.

in the cases or controls. Both DRB1*13:02 and *15:02 showed potential dosage effects.

Diplotype analysis

When we analysed the effects of HLA-DRB1 allele diplotypes on the predisposition to ACPA-negative RA, we found that a combination of DRB1*09:01 and *12:01 demonstrated susceptible effects in both sets (p=0.025, 0.020 and 0.00013 in the first, second and combined study, respectively; table 3). DRB1*08:03 homozygosity showed a weak susceptible association without any dosage effects (table 3,supplementary table 1). Although we found no susceptibility effect of DRB1*08:02 homozygosity, the combination of DRB1*08:02 and *08:03 also resulted in weak susceptibility (supplementary table 2). When we analysed DR8 allele homozygosity, we found that it displayed a moderate susceptibility association in the combined analysis (p=0.0070, table 3). Any combination of two of the three susceptibility alleles—namely, HLA-DRB1*12:01, *14:03, and *04:05, showed a potentially susceptible effect (supplementary table 2).

The HLA-DRB1*08:03 and *15:02 diplotype showed the strongest protective effect (p=0.00011, table 3). We found that the diplotypes with protective effects (*08:03/*15:02, *15:02/*15:02 and *13:02/*15:02) all included HLA-DRB1*15:02 (table 3).

DISCUSSION

Recent studies have suggested that ACPA-negative RA is a genetically different subset of RA.[7 8] While SE is very strongly associated with ACPA-positive RA, it is reported as not associated or only weakly associated with ACPA-negative RA. In populations of European descent, HLA-DR3 and DR13 were reported to be susceptibility alleles,[12 13] but a recent meta-analysis of a large Caucasian cohort did not find any such association.[14] In Japanese subjects, only DRB1*09:01 was reported to be associated with ACPA-negative RA, using small numbers of patients and controls (28 and 265, respectively).[15 16] HLA-DR3 is rare in the Japanese population, and we found only one HLA-DR3 allele in our cohorts.

Although genetic factors contribute to the development of ACPA-negative RA as much as ACPA-positive RA,[19] little is known about the ACPA-negative RA susceptibility alleles of HLA and non-HLA genes.

Here, we performed a case–control association study using a large number of ACPA-negative patients with RA and controls and showed that multiple alleles and diplotypes are associated

Table 3 Associations between HLA-DRB1 allele diplotypes and ACPA-negative RA

			Set 1				Set 2				Pooled study			
Effect	Allele 1	Allele 2	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)	†ACPA-negative RA, N (%)	†Control, N (%)	p Value	OR (95% CI)
Non-SE														
Susceptible	*09:01	*12:01	7 (1.9)	10 (0.7)	0.025	2.90 (1.1 to 7.68)	13 (2.6)	3 (0.6)	0.020	4.41 (1.25 to 15.58)	20 (2.3)	13 (0.7)	0.00013	3.62 (1.79 to 7.30)
	*08:03	*08:03	5 (1.4)	7 (0.5)	0.054	2.95 (0.93 to 9.36)	7 (1.4)	4 (0.8)	0.36	1.76 (0.51 to 6.04)	12 (1.4)	11 (0.6)	0.021	2.54 (1.12 to 5.78)
	*04:05	*14:05	5 (1.4)	7 (0.5)	0.054	2.95 (0.93 to 9.36)	5 (1.0)	2 (0.4)	0.26	2.51 (0.48 to 13.00)	10 (1.2)	9 (0.5)	0.033	2.59 (1.05 to 6.39)
Protective	*08:03	*15:02	3 (0.8)	35 (2.3)	0.095	0.35 (0.11 to 1.13)	1 (0.2)	14 (2.8)	0.00047	0.070 (0.010 to 0.53)	4 (0.5)	49 (2.4)	0.00011	0.18 (0.07 to 0.51)
	*15:02	*15:02	2 (0.5)	16 (1.1)	0.36	0.51 (0.12 to 2.23)	1 (0.2)	9 (1.8)	0.011	0.11 (0.010 to 0.86)	3 (0.4)	25 (1.3)	0.024	0.27 (0.08 to 0.91)
	*13:02	*15:02	3 (0.8)	28 (1.9)	0.16	0.43 (0.13 to 1.44)	3 (0.6)	7 (1.4)	0.20	0.42 (0.11 to 1.65)	6 (0.7)	35 (1.7)	0.029	0.39 (0.16 to 0.94)
SE	SE	SE	26 (7.1)	87 (5.8)	0.35	1.24 (0.79 to 1.95)	27 (5.4)	30 (6.0)	0.68	0.89 (0.52 to 1.52)	53 (6.1)	117 (5.8)	0.78	1.05 (0.75 to 1.47)
Serotype	DR8	DR15	8 (2.2)	72 (4.8)	0.027	0.44 (0.21 to 0.93)	10 (2.0)	23 (4.6)	0.021	0.42 (0.20 to 0.90)	18 (2.1)	95 (4.7)	0.00075	0.43 (0.26 to 0.71)
	DR13	DR15	6 (1.6)	55 (3.7)	0.051	0.44 (0.19 to 1.02)	6 (1.2)	11 (2.2)	0.22	0.54 (0.20 to 1.47)	12 (1.4)	66 (3.3)	0.0039	0.41 (0.22 to 0.77)
	DR8	DR8	13 (3.5)	17 (1.1)	0.00097	3.21 (1.55 to 6.67)	10 (2.0)	8 (1.6)	0.64	1.25 (0.49 to 3.20)	23 (2.7)	25 (1.3)	0.0070	2.16 (1.22 to 3.82)

Diplotype number and the frequency of each HLA-DRB1 diplotype in ACPA-negative patients with RA (n=368 and 501 in the 1st and 2nd set, respectively) and healthy controls (n=1508 and 500 in the 1st set and 2nd set, respectively) as well as the p value and OR of each diplotype for the development of ACPA-negative RA are shown. p Values were calculated using Fisher's exact test or the χ^2 test.
†Number of alleles (allele frequency).
ACPA, anticitrullinated peptide/protein antibody; SE, shared epitope; RA, rheumatoid arthritis.

Figure 1 Suggestive dosage effect of associated alleles on anticitrullinated peptide/protein antibody (ACPA)-negative rheumatoid arthritis susceptibility. The OR for each genotype is shown. Different colours indicate the number of copies of each allele. The numbers of homozygotes of *12:01, *14:05, *15:02, and *13:02 in cases are limited (2, 2, 3 and 3, respectively). Since no patients in this study were homozygous for DRB1*14:03, only the result for *14:05 is shown in this figure. SE in the figure includes DRB1*04:05, which is shown separately.

with ACPA-negative RA in Japanese people. Although the controls in the first set had different age and sex ratio values from those of the patients and we could not obtain age data for the 500 controls in the second set, the effects of the above-mentioned difference and lack of data on our results were considered to be limited. The HLA locus is located on chromosome 6 and is not affected by sex or age. Indeed, regression analysis did not significantly alter our association results (data not shown).

Our study showed that HLA-DRB1*12:01 is strongly associated with ACPA-negative RA and that HLA-DRB1*14:03 and HLA-DRB1*04:05 in SE are moderately associated with ACPA-negative RA in Japanese people. All three susceptibility alleles showed susceptibility associations with ACPA-negative RA when found in combination with one of the other two alleles. Our data also suggested a dosage effect of HLA-DRB1*12:01, while no dosage effect of HLA-DRB1*04:05 was detected, with decreased OR of DRB1*04:05 in homozygotes compared with heterozygous patients. In addition, we showed that the HLA-DRB1*09:01 and HLADRB1*12:01 diplotype and HLA-DR8 homozygosity are strong susceptibility combinations for ACPA-negative RA. We also determined HLA-DRB1*13:02 and *15:02 as protective alleles against ACPA-negative RA with a potential dosage effect. The combination of DRB1*08:03 and *15:02 had a strong protective effect in our study. Using logistic regression analysis, we confirmed that the effects of these susceptibility and protective alleles do not depend on each other (supplementary table 3). Although we searched for common amino acid sequences among the susceptibility alleles, we could not detect any meaningful sequences common to HLA-DRB1*12:01, *14:03, and/or *04:05. We also failed to detect a common amino acid sequence among the protective alleles HLA-DRB1*15:02 and *13:02.

Although the association of SE with ACPA-negative RA cannot be concluded, our large-scale study showed that it is weakly associated with ACPA-negative RA. As we observed a lower OR of the SE in homozygotes than in heterozygous patients, confirmation of this association in other studies are needed. We consider that the SE is associated with ACPA-negative RA but has a much weaker effect than in ACPA-positive RA. Both the

relatively small effect of SE on ACPA-negative RA and the small number of cases in previous reports might have resulted in non-significant p values for such tendencies.

HLA-DRB1*12:01, which was found to be associated with ACPA-negative RA susceptibility in our study, was reported to be associated with type 1 diabetes mellitus (T1D) in Latin America, but no similar association has been reported in Japan.[20 21] While a Japanese study showed RA with the anti-glucose-6-phosphate isomerase antibody is associated with HLA-DRB1*12:01,[22] no large-scale studies have reported an association between HLA-DRB1*12:01 and RA. As RA shares susceptibility genes with T1D such as *PTPN22*,[23] the determination of HLA-DRB1*12:01 as a potential common risk allele for both T1D and ACPA-negative RA is interesting. Although HLA-DRB1*12:01 showed a possible dosage effect, further confirmation is necessary as only two homozygous patients were among the cases. The allele frequency of HLA-DRB1*12:01 in a European population is 1–4%,[24] and so far there are no reports showing an association with ACPA-negative RA.[14] HLA-DRB1*12:02, the other allele of HLA-DR12, showed no association with ACPA-negative RA.

HLA-DRB1*14:03 was reported to be associated with Grave's disease in Japanese patients,[25] but its role in RA is unknown. Although our samples did not contain any patients who were homozygous for the allele owing to its low allele frequency, it showed a moderate association with ACPA-negative RA susceptibility. Among the other non-SE DR14 alleles, DRB1*14:05 displayed a tendency towards ACPA-negative RA susceptibility, while *14:01 and *14:07 did not. In total, DR14 alleles, including *14:06 in SE, showed moderate susceptibility effects on ACPA-negative RA (supplementary table 1).

Although one European study suggested that HLA-DR15 has a protective effect against ACPA-negative RA, its effect on ACPA-negative RA has not been fully examined.[13] We showed that HLA-DR15 has strong protective effect against ACPA-negative RA and a possible dosage effect. HLA-DRB1*15:02 is reported to be associated with Japanese T1D in a protective manner.[21]

Among HLA-DR13 alleles, HLADRB1*13:02 was reported to be protective against ACPA-positive RA.[26 27] Its protective effect was also reported in Japanese patients with RA.[16] Its effect on ACPA-negative RA has not been established.[13 14] Our study suggested that HLA-DRB1*13:02 has a protective effect against ACPA-negative RA. As the second set in our study did not show any differences in allele frequency between the patients and controls, further validation of our findings is necessary. HLA-DRB1*13:01, a major component of DR13 in populations of European descent, had no effect in our study, where we included DRB1*13:01 in eight alleles in cases and 23 alleles in controls (p=0.59).

HLA-DR8 has also been reported to be associated with some arthropathic autoimmune diseases, such as juvenile idiopathic arthritis[28] and psoriatic arthritis[29] in European subjects. The associations indicate that these arthropathies share common pathological mechanisms. Interestingly, the combination of DR8 and DR15 had a strong protective effect against ACPA-negative RA. Considering that DR8 did not show susceptibility association as a single allele, it seems to induce ACPA-negative RA susceptibility in a recessive manner. Among the DR8 alleles, DRB1*08:03 appeared to have a strong effect on ACPA-negative RA susceptibility.

Although we did not detect a dosage effect of HLA-DRB1*04:03, it showed a potentially protective effect against ACPA-negative RA in the combined study. Further studies are necessary to confirm the association.

As DRB1*09:01 has been shown to be associated with a decreased ACPA titre in ACPA-positive RA,[30] it is likely to be associated with ACPA-negative RA. While DRB1*09:01 showed a potential susceptibility association (p=0.062), the combination of DRB1*09:01 and *12:01 showed strong susceptibility association (p=0.00013). DRB1*09:01 also showed a possible dosage effect. From this viewpoint, we consider that DRB1*09:01 has a potential susceptibility effect on ACPA-negative RA. Owing to the relatively high allele frequency of DRB1*09:01, another independent association study or appropriate classification of ACPA-negative RA could produce significant results.

In addition to the different associations of the SE with ACPA-negative RA and ACPA-positive RA, we found multiple alleles associated with ACPA-negative RA that are not shared by ACPA-positive RA. These showed that ACPA-negative RA is a distinct subset of RA. Moreover, when we focused on ACPA-negative erosive RA to exclude the possibility of our results being affected by non-RA arthritic diseases, the effects of all the following alleles were maintained: *12:01, *14:03, *04:05, *13:02 and *15:02 (data not shown).

This is the first large-scale association study involving Japanese ACPA-negative patients with RA and the detection of multiple alleles and diplotypes associated with susceptibility to, or protection against, ACPA-negative RA. To evaluate whether our cohort had sufficient power to detect HLA-DRB1 genotype associations, we applied a risk allele with 5% frequency in the general population (see 'Materials and methods'). Our power calculation showed that this study had power values of 81% for finding genotype associations with an OR of 1.4 at the 0.05 significance level. When we set the OR to 1.2, our study had power values of 31%. These results suggest that our study has sufficient power to detect associated alleles that are present in relatively high frequencies (such as 5%) and a moderate OR of 1.4. On the contrary, our study has insufficient power to detect associations involving a weak OR such as 1.2. There is a possibility that ACPA-negative RA is associated with more HLA-DRB1 alleles or diplotypes that display a low allele frequency and/or a low OR. Further studies using ACPA-negative RA samples in Japan are necessary to find such associations.

While association studies using ACPA-negative patients with RA of European descent only found a few weak associations and none of them were subsequently replicated, our study successfully determined multiple alleles with relatively strong effects on ACPA-negative RA. From this viewpoint, we suppose that Japanese ACPA-negative patients with RA have a relatively similar genetic background compared to European patients. Population stratification within European population may also be assumed. Nevertheless, the validation of our results in Asian countries is necessary, and large-scale genome-wide association studies of ACPA-negative RA are also required to elucidate the pathogenesis of ACPA-negative RA.

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A large-scale association study identified multiple HLA-DRB1 alleles associated with ACPA-negative rheumatoid arthritis in Japanese subjects

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ORIGINAL ARTICLE

Serum IgG levels demonstrate seasonal change in connective tissue diseases: a large-scale, 4-year analysis in Japanese

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Abstract Hypergammaglobulinemia is often found in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE), and its level may correlate with disease activity. However, it is unclear whether immunoglobulin G (IgG) displays seasonal changes. We analyzed the seasonal change in serum IgG by assessing 450 patients with connective tissue disease. The serum IgG levels in summer were compared with those in winter from 2006 to 2009. Independent samples from 355 patients were analyzed to confirm results in the first set. The differences in the IgG levels between the two seasons were analyzed in each disease and compared with disease activity. 488 patients without connective tissue disease were analyzed as reference instead of healthy people as control. We found that connective tissue disease patients tended to show higher levels of serum IgG in summer than in winter every year from 2006 to 2009, whereas patients without connective tissue disease did not demonstrate such a tendency. We observed this seasonal tendency in each disease. Seasonal changes weakly correlated with those of anti-DNA antibody in SLE patients and those of disease activity score in rheumatoid arthritis (RA) patients. Serum IgG levels of patients with connective tissue diseases display seasonal variations. Biological and clinical significance of these variations should be elucidated.

Keywords Immunoglobulin · Connective tissue disease · Serologic marker · Systemic lupus erythematosus · Rheumatoid arthritis

Introduction

Immunoglobulin G (IgG) is a major portion of immunoglobulins produced by plasma cells in lymphoid organs and comprises ~20% of serum proteins. IgG binds to antigens to induce an inflammatory response or form immune complexes [1]. It is widely known that patients with connective tissue diseases, especially those with Sjögren's syndrome (SS), systemic lupus erythematosus (SLE), or mixed connective tissue disease (MCTD), display hypergammaglobulinemia or a high IgG titer. IgG is used to evaluate the effects of immunosuppressive therapy, and some autoantibody titers are known to be related to disease activity; for example, anti-double-strand DNA antibody titers are related to lupus nephritis and its activity [2–4], and a previous report demonstrated that IgG level is associated with lymphoid infiltration in SS [5]. However, the effects of seasonal changes in IgG levels of patients with connective tissue diseases have never been considered. During our daily medical practice, we noticed that patients with connective tissue diseases show seasonal changes in IgG levels regardless of their medication; therefore, we conducted a retrospective chart review of a large number of patients with connective tissue diseases at our hospital to verify our hypothesis.

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Materials and methods

Cases and controls

We enrolled 450 patients with connective tissue diseases who were followed up at Kyoto University Hospital and for whom both summer (July and August) and winter (January and February) IgG measurements were available for the period 2006–2009. There were 195 rheumatoid arthritis (RA), 140 SLE, 46 systemic sclerosis (SSc), 19 MCTD, 41 primary SS, and 28 polymyositis/dermatomyositis (PM/DM) patients. We analyzed 355 patients with connective tissue diseases followed up at Kyoto University to replicate results in the first set. This group consisted of 155 RA, 123 SLE, 33 SSc, 13 MCTD, 32 primary SS, and 23 PM/DM patients. A small number of patients had more than two connective tissue diseases, and overlap in more than two disease subgroups was allowed. We extracted 488 patients without connective tissue diseases whose IgG data were available from 39,089 outpatients at Kyoto University Hospital on January and February in 2010 and analyzed them as reference instead of healthy people as control. Basic information for each group is shown in Table 1. Connective tissue diseases patients fulfilled criteria for each disease, namely, American College of Rheumatology (ACR) criteria for RA [6], SLE [7], SSc [8]; Japanese criteria for primary SS [9]; criteria for MCTD [10]; and criteria for PM and DM [11]. This study was designed in accordance with the Declaration of Helsinki and approved by Kyoto University Graduate School and Faculty of Medicine, Ethics Committee. Information regarding the study was disclosed to all patients instead of obtaining written informed consent.

IgG levels and disease markers

A retrospective chart review of the enrolled patients was performed to evaluate their serum IgG levels from 2006 to 2009. We obtained disease activity score for 28 joints (DAS28) in patients with RA and titers of serum C3, C4, CH50, anti-DNA antibody, and urine protein in patients with SLE from 2006 to 2009. These markers were evaluated on the same date as IgG evaluation.

Table 1 Basic patient information

	Connective tissue disease	Nonconnective tissue disease
	1st set + 2nd set	Reference set
Number	805	488
Sex	Female: 714, male: 91	Female: 259, male: 229
Age[a]	54.3 ± 15.3	45.0 ± 24.8
	1st set	
Number	450	
Sex	Female: 402, male: 48	
Age[a]	54.7 ± 15.4	
	2nd set	
Number	355	
Sex	Female: 312, male: 43	
Age[a]	53.8 ± 15.2	

[a] Mean ± standard deviation

Statistical analyses

Serum IgG levels in summer were compared with those in winter for each patient from 2006 to 2009. The difference between the two seasons was defined as ΔIgG (ΔIgG = IgGsummer − IgGwinter). The ratio of positive ΔIgG in each group for each year was compared with the null hypothesis that the ratio is not different from 50% in binomial test. Logistic regression analyses were used to adjust other factors, such as age, sex, and treatment. Statistical analyses were performed using R software (http://www.r-project.org/) or SPSS (version 18).

Results

The 450 patients with connective tissue diseases were selected and IgG levels were evaluated in a retrospective manner from 2006 to 2009. These patients showed higher IgG levels in summer than in winter in 2009 ($P = 0.00070$, Table 2). This tendency held over the previous 3 years (Table 2). To confirm these results, we evaluated another independent set containing 355 patients with connective tissue disease who were followed up around the same time as the first set. The second set also showed that patients with connective tissue disease had higher IgG levels in summer than in winter in all 4 years (Table 2). When we combined the two data sets, the ratio of positive ΔIgG in patients with connective tissue disease reached significant levels in 2006, 2007, and 2009 (Table 2). Although the ratio did not show significant P value in 2008, the tendency remained (Table 2). In particular, patients with SLE demonstrated a high ratio value of positive ΔIgG (Table 2).

As we could not obtain successive serum IgG data from healthy people, we used 488 patients without connective tissue diseases as reference to investigate whether this IgG seasonal change was observed in general and found no regular tendency of ratio value of positive ΔIgG in this group (Table 2). Logistic regression analysis demonstrated that positive ΔIgG was associated with connective tissue disease even after adjustment by serum IgG levels at baseline, age, sex, and treatment (Table 3).

Next, we analyzed data with a stringent cutoff (ΔIgG > 10 mg/dl as positive change and −10 mg/dl > ΔIgG as negative change) to exclude the possibility that subtle seasonal movement of serum IgG levels greatly influenced the overall ratio value of positive ΔIgG. There is a possibility that change of temperature, humidity, and so on between

Table 2 Ratio of patients each year whose serum immunoglobulin G (IgG) levels were higher in summer than in winter

	2006		2007		2008		2009	
	Positive ratio	*P*	Positive ratio	*P*	Positive ratio	*P*	Positive ratio	*P*
1st set connective tissue disease	136/236	0.090	176/298	0.017	186/349	0.41	212/346	0.00070
	57.6 (51.3–63.9)		59.1 (53.5–64.6)		53.3 (48.1–58.5)		61.3 (56.1–66.4)	
2nd set connective tissue disease	97/160	0.046	122/221	0.29	151/277	0.31	166/272	0.0042
	60.6 (53.1–68.2)		55.2 (48.6–61.8)		54.5 (48.6–60.4)		61.0 (55.2–66.8)	
1st set + 2nd set connective tissue disease	233/396	0.0059	298/519	0.0087	337/626	0.18	378/618	2.7×10^{-6}
	58.8 (54.0–63.7)		57.4 (53.2–61.7)		53.8 (49.9–57.7)		61.2 (57.3–65.0)	
RA	91/153		110/224		141/275		159/276	
	59.5 (51.7–67.3)		49.1 (42.6–55.7)		51.3 (45.4–57.2)		57.6 (51.8–63.4)	
SLE	87/145		116/193		117/213		139/213	
	60.0 (52.0–68.0)		60.1 (53.2–67.0)		54.9 (48.2–61.6)		65.3 (58.9–71.7)	
SSc	25/44		20/45		31/54		30/48	
	56.8 (42.2–71.5)		44.4 (29.9–59.0)		57.4 (44.2–70.6)		62.5 (48.8–76.2)	
Primary SS	23/39		25/35		25/49		28/44	
	59.0 (43.5–74.4)		71.4 (56.5–86.4)		51.0 (37.0–65.0)		63.6 (49.4–77.9)	
MCTD	13/18		17/22		11/24		17/27	
	72.2 (51.5–92.9)		77.3 (59.8–94.8)		45.8 (25.9–65.8)		63.0 (44.7–81.2)	
PM/DM	8/20		22/33		26/41		27/44	
	40.0 (18.5–61.5)		66.7 (50.6–82.8)		63.4 (48.7–78.2)		61.4 (47.0–75.8)	
Reference set	76/148		104/199		131/273		149/314	
	51.4 (43.3–59.4)		52.3 (45.3–59.2)		48.0 (42.1–53.9)		47.5 (41.9–53.0)	

Seasonal change of serum IgG in patients with and without connective tissue diseases. When a patient had more than two connective tissue diseases, overlapping was allowed

Positive ratio IgG levels higher in summer than in winter. , RA rheumatoid arthritis, SLE systemic lupus erythematosus, SSc systemic sclerosis, *MCTD* mixed connective tissue disease, *PM/DM* polymyositis/dermatomyositis

P values calculated using the binomial test

seasons may influence laboratory equipment to cause subtle change. We observed the same IgG movement tendency even with stringent cutoff in connective tissue diseases and each subgroup (Supplementary Table 1). The reference group showed no regular tendency. When we set a more stringent cutoff ($\Delta IgG > 100$ mg/dl as positive change and -100 mg/dl $> \Delta IgG$ as negative change), we observed the same results in all groups (Supplementary Table 2). We thus concluded that seasonal variations were not due to equipment errors or sampling bias.

As the SLE subgroup showed clear seasonal variations in serum IgG we chose this subgroup to assess the correlation of ΔIgG with disease activity. We subsequently collected successive data of anti-DNA antibody, C3, C4, CH50, and urine protein and compared changes of these markers for SLE activity with ΔIgG in the patients. Whereas we observed no meaningful association between ΔIgG and seasonal change of C3, C4, CH50 or urine protein, we found that ΔIgG was moderately correlated with the seasonal change of anti-DNA antibody in 2009 [rho (Spearman's rank-sum coefficient) $= 0.35$, $P = 1.6 \times 10^{-5}$]. We found this correlation occurred in the 3 previous years (rho ≥ 0.30). Even when we chose SLE patients demonstrating stringent changes in anti-DNA antibody (Δanti-DNA ≥ 5 IU/ml or -5 IU/ml $\geq \Delta$anti-DNA), we observed a rather strong correlation

Table 3 Association of connective tissue diseases with positive ΔIgG

	P value	Odds ratio (95% CI)
IgG in winter[a]	0.021	0.97 (0.95–1.00)
ICS	0.66	0.93 (0.66–1.30)
Corticosteroid	0.50	0.91 (0.67–1.22)
Connective tissue disease	0.0078	1.58 (1.12–2.23)
Age	0.94	1.00 (0.99–1.01)
Female	0.043	1.42 (1.00–2.00)

Logistic regression analysis using positive ΔIgG as a dependent variable and IgG in winter, immunosuppressant or corticosteroid use, having connective tissue diseases, age, and sex as independent variables. The 2009 data of 618 patients with and 314 patients without connective tissue disease are used as a representative

IgG immunoglobulin G, *CI* confidence interval, *ICS* immunosuppressant

[a] Odds ratio in winter indicates odds ratio of increase of 100 mg/dl serum IgG