between the two markers in 2009 (rho = 0.47, $P = 0.013$, Fig. 1a). This tendency was also seen in the previous 3 years (rho $\geq$0.52).

Next, we obtained DAS28 scores (the standard measurement for evaluating clinical activity of RA) of 40 RA patients in 2009 and found a moderate correlation between ΔIgG and change in DAS28 (rho = 0.39, $P = 0.012$, Fig. 1b). This correlation was observed in the 3 previous years (rho $\geq$0.28).

Discussion

It is widely known that many autoimmune diseases are associated with hypergammaglobulinemia, mainly consisting of IgG. In some diseases, hypergammaglobulinemia has been suggested to be associated with disease activity, such as lymphoid-cell infiltration, treatment responsiveness, and pulmonary arterial hypertension in SS [5, 12]. Another previous report stated that hypergammaglobulinemia in children presenting with SLE-like symptoms is a predictive factor for developing MCTD [13]. However, no previous reports have evaluated the seasonal change in IgG on a large scale. In this study, we show that more than half of the patients with connective tissue diseases demonstrated higher IgG levels in summer than in winter and that some of the seasonal variations may correlate with disease activity to some extent.

When we focussed on patients with connective tissue diseases whose IgG data were frequently measured, IgG titer movement throughout the year suggested that levels recorded in spring and autumn are between the levels in summer and winter (data not shown). This indicates that our comparison between the two seasons is enough to assess seasonal variation of serum IgG. Moreover, comparing two specific seasons avoids multiple testing that increases type 1 statistical error.

We analyzed two independent sets of patients with connective tissue diseases and found the seasonal variation in IgG levels for all 4 years. We observed this tendency in patients with each type of connective tissue disease and in patients with SLE, in particular. As we could not obtain IgG data for healthy people, we used data from 488 patients without connective tissue diseases as reference. These patients had variable diseases from variable departments such as eczema, IgA nephropathy, human T-lymphotropic virus type 1 (HTLV-1) infection, and malignant lymphoma. We found they showed no regular tendency to changes in IgG levels. Although it is more desirable to compare data from age- and sex-matched healthy people, results obtained from the reference group suggest that the seasonal change in serum IgG levels is not seen in general. Moreover, logistic regression analysis did not significantly alter the association between seasonal variation of IgG and connective tissue diseases, even with adjustment by age, sex, treatment, and serum IgG level at baseline. When these variations in IgG were mainly comprised by very small variations (<10 mg/dl), it might be argued that they are not fully convincing and be caused by equipment errors. However, when we set stringent cutoffs for seasonal variation, we still observed changes of serum IgG in patients with connective tissue diseases and its subgroups. This denies the possibility that our results were affected by some tiny changes due to equipment errors.

To analyze the biological meaning of these seasonal changes, we compared ΔIgG with changes in levels of anti-DNA antibody, complement, and urine protein in patients with SLE and those of DAS28 in patients with RA. We found that ΔIgG weakly correlated with changes of serum anti-DNA antibody in SLE patients and with those of DAS28 in RA patients. Because anti-DNA antibody is a small portion of IgG, it is reasonable that they correlate to some extent. However, it is interesting that ΔIgG strongly correlates with stringent change of anti-DNA antibody. These results suggest that ΔIgG reflects changes in disease activity in some patients with connective tissue diseases, although further analysis is necessary.

Seasonal effects on onset, relapse, and disease activity of some connective tissue diseases have been reported. In general, SLE activity is believed to increase in spring and

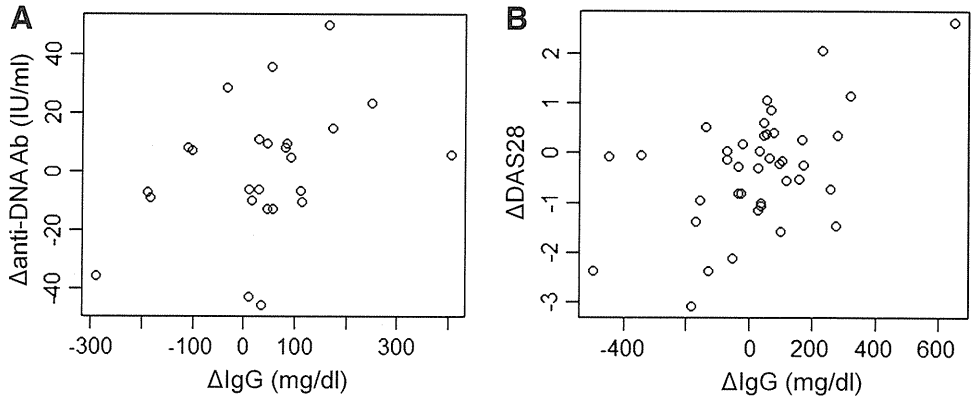

Fig. 1 Correlation between seasonal changes in immunoglobulin G (ΔIgG) and variation of disease activity in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients. Seasonal variation of serum IgG levels was compared with that of anti-DNA antibody in 27 SLE patients (**a**) or of disease activity score of 28 joints (DAS28) in 40 RA patients (**b**); 2009 results are shown as representative

summer due to sunlight exposure [14]. However, there are also conflicting reports showing no seasonal change in lupus activity [15, 16]. The influence of season on PM/DM is also disputed [17, 18]. Therefore, it does not seem that the seasonal IgG changes seen in patients with connective tissue diseases are solely due to disease activity. It is possible that the use of additional medications, including immunosuppressants or corticosteroids to suppress disease flare-up in winter, affected our results. However, logistic regression analysis did not alter the association after treatment adjustment. Moreover, patients with primary SS, in whom immunosuppressants or corticosteroids are scarcely used, also showed the same tendency, suggesting that this cannot fully explain the phenomenon.

IgG change in patients with connective tissue disease seemed to be proportional to the temperature throughout the year, but it is unlikely that the change is caused by temperature changes because the reference group showed no similar tendency. When we compared IgG level with mean temperature in Kyoto, Japan, the correlation was highly variable from patient to patient (data not shown).

The difference between patients with and without connective tissue disease might reflect a difference in B-cell function between the two groups. To elucidate whether other immunoglobulins act in the same manner as IgG, we analyzed IgA and IgM in a similar manner. However, we could not find any regular tendency of seasonal change in IgA or IgM in either controls or patients with connective tissue disease (data not shown). We do not know the underlying mechanisms of IgG fluctuation in patients with connective tissue disease. Further analysis is necessary to address this question. To investigate whether this change is related to temperature, it would be interesting and feasible to investigate ΔIgG in patients with connective tissue diseases in the Southern Hemisphere.

Conflict of interest None.

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PAPER

Interferon-gamma release assay for diagnosing *Mycobacterium tuberculosis* infections in patients with systemic lupus erythematosus

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Our aim was to analyze the performance of an interferon-gamma release assay, QuantiFERON-TB Gold (QFT-2G), for diagnosing *Mycobacterium tuberculosis* (MTB) infection in patients with systemic lupus erythematosus (SLE). We performed the QFT-2G and tuberculin skin test (TST) in 71 SLE patients. The QFT-2G results of 279 patients with other connective tissue diseases (CTD) and 35 healthy controls were analyzed. Of the 71 SLE patients, two (2.8%) were positive and 46 (64.8%) were negative by QFT-2G. All SLE patients had no evidence of active MTB infection, apart from one. QFT-2G produced a significantly higher number of indeterminate results in patients with SLE (23/71, 32.4%) compared with those with other CTD (5.7%) or healthy controls (0%) ($p < 0.0001$ and $p < 0.0001$). Decreased lymphocyte counts and high SLEDAI scores in SLE patients were shown to be risk factors for indeterminate results by multivariate analysis ($p = 0.02$ and $p = 0.04$). Among all patients with CTD, SLE itself and lymphocytopenia were found to be independent risks for indeterminate results ($p = 0.00000625$ and $p = 0.000107$). In conclusion, QFT-2G may have more potential to assist in the diagnosis of active and latent MTB infection than TST in SLE patients. However, because of the high frequency of indeterminate results, caution must be used when interpreting the results of QFT-2G among SLE patients, especially those who have parallel or subsequent flares. *Lupus* (2011) **20,** 792–800.

Key words: interferon-γ release assay; *Mycobacterium tuberculosis* infection; systemic lupus erythematosus

Introduction

Patients with systemic lupus erythematosus (SLE) are susceptible to infections because of disease-related disturbances in immune function as well as treatment with immunosuppressive agents.[1–3] An increased incidence of active *Mycobacterium tuberculosis* (MTB) infection has been reported in patients with SLE. In addition, extrapulmonary tuberculosis (TB) and serious infection occur more often in SLE patients.[4–7] However, early diagnosis of active MTB infection is often difficult, because SLE and TB have overlapping chest and central nervous system features, as well as symptoms of fever, malaise and weight loss.[8] Because extrapulmonary TB is more common in SLE patients, it often requires tissue and body fluid analysis for diagnosing TB and thus may take a longer period to establish a definitive diagnosis.[9] The standard diagnostic tool for identifying active MTB infection and latent MTB infection (LTBI) is the tuberculin skin test (TST), which lacks specificity in populations with high bacille Calmette-Guérin (BCG) coverage and non-tuberculosis mycobacteria exposure.[10] Moreover, the TST may have a higher probability of giving false negative results in SLE patients than in the general

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population, because of the immune dysregulation linked to the disease itself or due to immunosuppressive drug use.

Recent studies indicate that interferon-gamma (IFN-γ) release assays (IGRAs) using early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) as MTB-specific stimulants have improved specificity over TST for the diagnosis of active MTB infection and LTBI.[11,12] In the QuantiFERON-TB Gold assay (also known as QFT-2G; Cellestis, Carnegie, Australia), antigens are incubated with whole blood, after which the release of IFN-γ is quantified by enzyme-linked immunosorbent assay (ELISA). According to a recent meta-analysis, the sensitivity was 78% (95% confidence interval [CI], 73% to 82%) for the QFT-2G test and 77% (CI, 71% to 82%) for the TST, and the specificity in BCG-vaccinated populations was 96% (CI, 94% to 98%) for the QFT-2G test and 59% (CI, 46% to 73%) for the TST.[11]

Japan has a moderate TB incidence (about 20 cases per 10^5 inhabitants per year). However, because of the wide usage of BCG (often on multiple occasions for the same individual), we cannot estimate the prevalence of LTBI using TST. Several studies have determined the prevalence of LTBI among patients with rheumatoid arthritis of immunosuppressive status using IGRA. However, little has been reported on the performance of IGRA in SLE patients, whose immunologic impairment may affect the performance of these lymphocyte-based assays. The aim of the present study was to elucidate whether the QFT-2G test is useful in SLE patients.

Patients and methods

Patients

Seventy-one SLE patients were prospectively enrolled in this study at the Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, between July 2006 and September 2008. All patients fulfilled the American College of Rheumatology 1982 revised criteria for SLE.[13] SLE disease activity was calculated according to the SLE Disease Activity Index (SLEDAI).[14] This study was approved by the Ethical Committee of Kyoto University. We obtained written informed consent from all patients. Demographic, clinical and LTBI risk factor information were collected for all patients. The following characteristics were considered risk factors for LTBI in order to assess the usefulness of QFT-2G with that of TST in detecting previous TB infection: a history of household TB contact; chest X-ray findings suggestive of a history of TB (e.g., nodules, fibrotic scars, calcified granulomas, or basal pleural thickenings); or a medically confirmed history of active TB.[15] A diagnosis of active MTB infection was made on the basis of a positive culture for MTB or a positive result on a polymerase chain reaction test for MTB DNA in any clinical specimen associated with compatible TB symptoms and radiographic findings.

We also retrospectively enrolled 279 patients with other connective tissue diseases (non-SLE patients: rheumatoid arthritis (RA) 148, myositis 45, vasculitides 39, systemic scleroderma 19, Sjögren's syndrome 11, Behcet's disease 9, adult-onset Still's disease 8), in whom the QFT-2G test was performed in routine clinical practice because they were suspected of active MTB infection based on radiographic findings and/or clinical symptoms or had clinical indications requiring testing for LTBI at the Department of Rheumatology and Clinical Immunology, Kyoto University Hospital, between July 2006 and September 2008.

As healthy controls, 35 healthcare workers were enrolled, assessed for LTBI by the Department of Infection Control and Prevention, Kyoto University Hospital.

Tuberculin skin test

TST was performed with an intradermal injection of 0.1 ml of tuberculin purified protein derivative (PPD) (approximately equivalent to 3 tuberculin units of PPD-S; Nippon BCG Manufacturing, Tokyo, Japan) into the ventral surface of the forearm of each patient according to the Mantoux method. The size of erythema was measured 48 hours later. If the maximum diameter of erythema was ≥10 mm, the result was defined as positive according to the usual criterion of the TST in Japan.

QuantiFERON TB-2G test

The QFT-2G test was performed according to the manufacturer's instructions (Cellestis). Briefly, four aliquots of heparinized whole blood were incubated with ESAT-6, CFP-10, mitogen (phytohemagglutinin as a positive control) and nil antigen (as a negative control). After 16 to 24 hours of incubation at 37°C in a humidified atmosphere, plasma was aspirated from each well and the amount of IFN-γ was measured by ELISA.

The results of QFT-2G were considered positive if the IFN-γ level in the antigen-stimulated wells (ESAT-6 or CFP-10) was ≥0.35 IU/ml, and negative if the IFN-γ level in the antigen-stimulated wells was <0.35 IU/ml and in the mitogen wells was ≥0.5 IU/ml, after subtracting the negative control value. The results were considered indeterminate if the IFN-γ level in the antigen-stimulated wells was <0.35 IU/ml and in the mitogen wells was <0.5 IU/ml, or if the IFN-γ level in the antigen-stimulated wells was below half of the level of the negative control and in the negative control was >0.7 IU/ml. This judgment was made according to the guidelines proposed by the Centers for Disease Control and Prevention (CDC) for using the QFT-2G test.[16]

Statistical analysis

We performed the chi-squared test or Fisher's exact probability test to compare categorical variables and the *t*-test or Mann–Whitney *U*-test to compare continuous variables as appropriate. The variables that were significant by univariate analysis were used as potential risk factors for the multivariate logistic model. Receiver-operator curve (ROC) and area under the curve (AUC) analyses were used to evaluate the best lymphocyte count cut-off point for discriminating the indeterminate patient group from the determinate patient group in SLE and non-SLE patients respectively. An optimal cut-off point was defined as a point on a ROC curve nearest to the point where both sensitivity and specificity were 1. The discriminatory ability of a test depends on the AUC and this is classified as: 0.9–1.00 = excellent, 0.8–0.9 = good, 0.7–0.8 = fair, 0.6–0.7 = poor, 0.5–0.6 = failed. Statistical analyses were performed using Statview 5.0 software (SAS Institute, Cary, NC, USA) and the open-source R software, version 2.1.0 (http://www.r-project.org). A *p*-value of 0.05 or less was considered statistically significant.

Results

Characteristics of the study population

The characteristics of the SLE and non-SLE patients and healthy controls are summarized in Table 1. The mean age of the non-SLE patients was significantly higher than the SLE patients and healthy controls ($p < 0.0001$ and $p < 0.0001$, respectively). The number of patients with risk factors for LTBI was significantly higher among the non-SLE patients than the SLE patients and healthy controls ($p = 0.01$ and $p < 0.0001$, respectively). The frequency of suspected TB patients and active TB patients did not differ between the SLE and non-SLE groups (3/71 (4.2%) *vs.* 19/279 (6.8%) and 1/71 (1.4%) *vs.* 5/279 (1.8%), respectively).

Table 1 Characteristics of the study population

Characteristics	*SLE (n = 71)*	*Non-SLE (n = 279)*	*Healthy controls (n = 35)*
Age, mean ± SD; years	38.3 ± 15.2	56.8 ± 14.9[c]	32.3 ± 8.4
Females, *n* (%)	58 (81.7)	207 (74.2)	26 (74.3)
Risk factors for LTBI, *n* (%)[a]	26 (36.6)[d]	149 (53.4)[e]	6 (17.1)
Suspected TB, *n* (%)	3 (4.2)	19 (6.8)	0 (0)
Active TB, *n* (%)	1 (1.4)	5 (1.8)	0 (0)
Treatment regimen, *n* (%)			
Methylprednisolone pulse therapy	2 (2.8)	1 (0.4)	0 (0)
High doses of oral corticosteroid	7 (9.9)	15 (5.4)	0 (0)
Moderate doses of oral corticosteroid	17 (23.9)[f]	21 (7.5)	0 (0)
Low doses of oral corticosteroid	13 (18.3)	46 (16.5)	0 (0)
Immunosuppressive drugs	19 (26.8)	145 (52.0)[g]	0 (0)
NSAIDs or no therapy	13 (18.3)	51 (18.3)	35 (100)
Lymphocyte counts, mean ± SD;/μl[b]	1159 ± 772[h]	1528 ± 1004	N.A.

N.A.: not available.
[a]The number of patients who had a history of anti-TB treatment for active TB was two in SLE, 16 in non-SLE patients and zero in healthy controls.
[b]Data on peripheral blood lymphocytes were available for 71 SLE and 260 non-SLE patients.
[c]$p < 0.0001$ *vs.* SLE and healthy controls.
[d]$p = 0.04$ *vs.* healthy controls.
[e]$p = 0.01$ *vs.* SLE and $p < 0.0001$ *vs.* healthy controls.
[f]$p < 0.0001$ *vs.* non-SLE.
[g]$p = 0.0001$ *vs.* SLE.
[h]$p = 0.002$ *vs.* non-SLE.

QFT-2G and TST results

We show QFT-2G and TST results for each group in Table 2. Among 71 SLE patients, two (2.8%) were positive, 46 (64.8%) were negative and 23 (32.4%) were indeterminate according to the QFT-2G test. One of the two SLE patients with a positive QFT-2G result had active MTB infection but not a positive TST result and the other had a history of insufficient anti-TB treatment in her childhood and was diagnosed as LTBI. Among five non-SLE patients with active MTB infection, three were positive and two were negative in the QFT-2G test. The QFT-2G test produced a significantly higher number of indeterminate results in patients with SLE (32.4%) than in those with other connective tissue diseases (5.7%) or healthy controls (0%) ($p < 0.0001$ and $p < 0.0001$, respectively, Table 2).

Relationship of QFT-2G and TST results to risk factors for LTBI

QFT-2G and TST results according to the presence or absence of risk factors for LTBI in SLE and non-SLE patients and healthy controls are shown in Table 3, Supplementary Table 1 and Supplementary Table 2, respectively. QFT-2G was positive in 7.7% of SLE patients with risk factors for LTBI whereas there were no positives in those without risk factors. In comparison, TST was positive in 7.1% of SLE patients with risk factors and also in 6.9% of those without risk. Univariate analysis shows no significant difference between SLE patients with risk factors for LTBI and those without risk in the QFT-2G and TST results ($p = 0.13$ and $p > 0.99$, respectively, Table 3). In contrast, non-SLE patients showed a higher frequency of positive QFT-2G and TST results

Table 2 QFT-2G and TST results

	SLE No. (%)	*Non-SLE No. (%)*	*Healthy controls No. (%)*
QFT-2G			
Positive	2 (2.8)	22 (7.9)	1 (2.9)
Negative	46 (64.8)	241 (86.4)	34 (97.1)
Indeterminate	23 (32.4)[a]	16 (5.7)	0 (0)
Total	71	279	35
TST[b]			
Positive	3 (7.0)	54 (33.1)[c]	27 (87.1)[d]
Negative	40 (93.0)	109 (66.9)	4 (12.9)
Total	43	163	31

[a] $p < 0.0001$ *vs.* non-SLE and healthy controls.
[b] TST was performed for 43 SLE patients, 163 non-SLE patients and 31 healthy controls.
[c] $p = 0.0004$ *vs.* SLE.
[d] $p < 0.0001$ *vs.* SLE and non-SLE.

Table 3 QFT-2G and TST results according to the presence or absence of risk factors for LTBI in SLE patients

	SLE patients with risk factors for LTBI	*Without risk*
QFT-2G	n (%)	n (%)
Positive	2 (7.7)	0 (0)
Negative	16 (61.5)	30 (66.7)
Indeterminate	8 (30.8)	15 (33.3)
Total	26	45
TST[a]		
Positive	1 (7.1)	2 (6.9)
Negative	13 (92.9)	27 (93.1)
Total	14	29

QFT-2G: $p = 0.13$ (Fisher's exact probability test). The p-value was calculated by comparison of patients with positive results and those with non-positive results (negative and indeterminate results). Among 26 patients with risk factors, two patients had a medically confirmed history of anti-TB treatment for active TB. One was negative and the other was indeterminate according to the QFT-2G test.
TST: $p > 0.99$ (Fisher's exact probability test).
[a] TST was performed for 43 SLE patients. Among 14 patients with risk factors, one patient had a medically confirmed history of anti-TB treatment for active TB. She was negative according to the TST.

Table 4 The clinical characteristics and the IFN-γ levels of 23 SLE patients with indeterminate results in the QFT-2G test

			IFN-gamma level (IU/ml)							
Case	*Age*	*Sex*	*Nil*	*Mitogen*	*ESAT-6*	*CFP-10*	*TST*	*Treatment regimen*	*Lymphocyte counts (/μl)*	*SLEDAI*
1	25	F	10.00	0.46	<0.05	0.51	–	PSL 10 mg	144	23
2	27	F	5.50	0.00	0.47	0.26	–	PSL 30 mg	1269	8
3	23	F	4.10	0.42	0.27	0.41	–	PSL 18 mg + MZB	297	9
4	18	F	2.40	1.19	<0.05	0.21	N.A.	None	752	5
5	19	F	1.28	5.96	<0.05	<0.05	–	None	792	14
6	34	F	1.20	1.21	<0.05	<0.05	N.A.	PSL 17 mg	1443	3
7	24	F	0.44	0.04	<0.05	<0.05	–	PSL 45 mg + AZA	292	10
8	58	F	0.27	0.34	<0.05	<0.05	–	PSL 15 mg	360	2
9	47	M	0.18	0.07	<0.05	<0.05	–	None	676	37
10	23	F	0.17	0.21	<0.05	<0.05	–	PSL 25 mg	1908	8
11	17	F	0.15	0.16	<0.05	<0.05	–	None	594	23
12	18	F	0.09	0.00	<0.05	<0.05	N.A.	PSL25mg + AZA	165	14
13	35	F	0.09	0.31	<0.05	<0.05	–	None	1512	22
14	34	M	0.06	0.21	0.09	0.16	–	PSL 30 mg + MZB	440	29
15	41	F	0.04	0.28	<0.05	<0.05	–	PSL 11 mg	496	25
16	36	F	0.04	0.04	<0.05	<0.05	–	PSL 30 mg	1740	24
17	71	M	0.03	0.32	<0.05	<0.05	–	mPSL pulse	288	11
18	39	F	0.02	0.36	<0.05	<0.05	N.A.	PSL 5 mg	536	0
19	28	F	0.01	0.25	<0.05	<0.05	–	PSL 50 mg	2392	16
20	64	F	0.13	0.04	<0.05	<0.05	–	PSL 30 mg + MZB	522	8
21	35	F	0.08	0.07	<0.05	<0.05	N.A.	PSL 7 mg + CyA	1296	8
22	43	F	0.24	0.20	<0.05	<0.05	N.A.	PSL 11 mg + TAC	371	7
23	23	F	0.51	0.45	<0.05	<0.05	N.A.	PSL 25 mg	960	12

N.A., not available; PSL, prednisolone; mPSL, methylprednisolone; MZB, mizoribine; AZA, azathioprine; CyA, cyclosporin A; TAC, tacrolimus.

in patients with risk factors than without them ($p < 0.0001$ and $p = 0.002$, respectively, Supplementary Table 1).

SLE patients with indeterminate QFT-2G results

The clinical characteristics and the IFN-γ levels of 23 SLE patients with indeterminate results following the QFT-2G test are shown in Table 4. The patients had a female to male ratio of 20 : 3 and an average age of 34.0 ± 14.8 years. Among these patients, 17 showed low levels of IFN-γ in the mitogen wells (<0.5 IU/ml: positive control failure) (cases 7–23), three had increased levels in the negative control (>0.7 IU/ml: negative control failure) (cases 4–6) and three displayed both of the above failures (double control failure) (cases 1–3). In RA patients, the positive control failures (five patients) were observed more often than negative control failures (one patient) and double control failures (one patient) (Supplementary Figure 1). Cases 1, 3 and 6 suffered from acute lupus hemophagocytic syndrome. All patients except for cases 8 and 18 had parallel or subsequent flares. Cases 8 and 20 suffered from *Pneumocystis jiroveci* pneumonia. In case 18, SLE activity was well controlled with low dose prednisolone (5 mg/day), but lymphocytopenia (536/μl) was observed, probably due to long-term treatment with corticosteroids.

Analysis of factors affected on indeterminate QFT-2G results

Table 5 shows a comparison of the clinical and laboratory findings of the SLE patients in the indeterminate ($n = 23$) and determinate ($n = 48$) groups. Univariate analysis showed that decreased lymphocyte counts and the increased SLEDAI scores were found significantly more often in patients with indeterminate results than with determinate results ($p = 0.01$ and $p = 0.04$, respectively). Both factors were found to be independent indicators of indeterminate results by multivariate analysis (lymphocyte counts, odds ratio (OR) 0.9990, 95% CI 0.9981–0.9999, $p = 0.02$; SLEDAI, OR 1.0706, 95% CI 1.0005–1.1457, $p = 0.04$). In non-SLE patients, decreased lymphocyte counts were also found significantly more often in patients with indeterminate results than with determinate results ($p = 0.0002$). In all patients with connective tissue diseases (SLE and non-SLE patients), the disease of SLE itself and lymphocytopenia were found to be

Table 5 Comparison of clinical and laboratory findings of the SLE patients between indeterminate patient group and determinate patient group

	QFT-2G test		
Clinical characteristics	*Indeterminate (n = 23)*	*Determinate (n = 48)*	*p-value*
Age, mean ± SD; years	34.0 ± 14.8	40.4 ± 15.2	N.S.
Females, *n* (%)	20 (87.0)	38 (79.2)	N.S.
BCG vaccinated, *n* (%)	19 (82.6)	32 (66.7)	N.S.
Positive results on TST, *n* (%)[a]	0/16 (0)	3/27 (11.1)	N.S.
Risk factors for LTBI, *n* (%)	8 (34.8)	18 (37.5)	N.S.
Active TB, *n* (%)	0 (0)	1 (2.1)	N.S.
Treatment regimen, *n* (%)			
Methylprednisolone pulse therapy	1 (4.3)	1 (2.1)	N.S.
High doses of oral corticosteroid	2 (8.7)	5 (10.4)	N.S.
Moderate doses of oral corticosteroid	5 (21.7)	12 (25.0)	N.S.
Low doses of oral corticosteroid	4 (17.4)	9 (18.8)	N.S.
Immunosuppressive drugs	6 (26.1)	13 (27.1)	N.S.
NSAIDs or no therapy	5 (21.7)	8 (16.7)	N.S.
Total protein, mean ± SD; g/dl	6.5 ± 0.9	6.5 ± 1.0	N.S.
Albumin, mean ± SD; g/dl	3.3 ± 0.5	3.4 ± 0.7	N.S.
Lymphocyte counts, mean ± SD;/μl	837 ± 619	1314 ± 796	0.01
SLEDAI, mean ± SD	13.8 ± 9.5	9.1 ± 7.3	0.04
Lupus nephritis, *n* (%)	11 (47.8)	17 (35.4)	N.S.
CNS lupus, *n* (%)	1 (4.3)	1 (2.1)	N.S.

N.S., not significant.
[a]TST was performed for 43 patients (indeterminate 16, determinate 27).

independent indicators of indeterminate results by multivariate analysis (the disease of SLE, OR 5.6149, 95% CI 2.6159–12.0525, $p = 0.00000625$; lymphocyte counts, OR 0.9986, 95% CI 0.9979–0.9993, $p = 0.000107$). We performed ROC analysis to find the best lymphocyte count cut-off point for discriminating the indeterminate from the determinate group in SLE and non-SLE patients and calculated the AUC. The best cut-off points were estimated at 792/μl in SLE and 980/μl in non-SLE patients. If the best cut-off score is set at 792/μl and 980/μl in these diseases, the sensitivity and specificity were calculated as 0.65 and 0.73 in SLE patients and as 0.75 and 0.75 in non-SLE patients, respectively. The AUC was 0.687 (95% CI 0.566–0.792) in SLE patients (poor discriminatory ability) and 0.781 (95% CI 0.726–0.830) in non-SLE patients (fair discriminatory ability). The AUC was significantly larger than 0.5 in SLE and non-SLE patients ($p = 0.0069$ and $p = 0.0001$, respectively)

Discussion

In the present study, we first compared the performance of the QFT-2G test with the TST test in detecting active MTB infection and LTBI among SLE patients enrolled prospectively.

A recent retrospective study of SLE patients living in a large immigrant population in the United States showed that 7% had active MTB infection and 18% had LTBI using TST.[7] In our study, TST showed a high prevalence of false positive results among healthy controls (Supplementary Table 2) because of the wide usage of BCG in Japan. We also consider that the prevalence of LTBI in SLE and non-SLE patients cannot be estimated using TST. No subject without risk factors for LTBI in the SLE group, as well as non-SLE and healthy control groups, had a positive QFT-2G result. Therefore QFT-2G demonstrated higher specificity compared with TST, as previously reported (Table 3, Supplementary Tables 1 and 2)[11,12] Among our SLE patients, we found only one active TB patient (1.4%) and one LTBI (1.4%) using QFT-2G. In a Japanese report among healthcare workers, age and risk factors for LTBI were independent indicators of positive QFT-2G results by multivariate logistic regression analysis.[17] When we apply the frequency of positive QFT-2G results among these healthcare workers to SLE patients stratified by their age, we can estimate that 3.6 patients (13.8%) are positive for QFT-2G among 26 SLE patients with risk factors. In contrast, the actual frequency of positive QFT-2G results (7.7%, 2/26, Table 3) seems to be lower than expected.

However, among SLE patients with risk factors and only determinate QFT-2G results, the frequency of positive QFT-2G results is 11.1% (2/18), which is considered to be valid.

Because of the high frequency of indeterminate results (32.4%), the application of QFT-2G for detecting LTBI has some limitation in SLE patients. The frequency was not affected by the risk of LTBI (Table 3). The frequency of indeterminate results in patients with connective tissue diseases using QFT-2G and QuantiFERON-TB Gold In-Tube (a recent version also known as QFT-3G) has been reported to be 0–10%.[15,18–20] The frequency of indeterminate results in connective tissue diseases other than SLE in our study was 5.7% (0–18.2%, Table 2, Supplementary Table 3), which was similar to previously reported results. Thus, the level of indeterminate results in our SLE patients was apparently high at 32.4%. Kobashi et al. reported that indeterminate results were significantly associated with elderly patients, patients receiving immunosuppressive treatment, patients with lymphocytopenia and patients with hypoalbuminemia.[21] In contrast, old age and use of immunosuppressive treatment did not correlate with indeterminate results in our SLE patients probably because most of our SLE patients were young and were receiving therapy. Although lymphocytopenia was also associated with indeterminate results both in SLE and non-SLE patients in our study, discriminatory ability between the indeterminate and determinate patient groups using the lymphocyte count cut-off point was poor in SLE compared with non-SLE patients. This suggests that other factors influence the development of indeterminate results in SLE patients. We demonstrated that SLE itself and high SLEDAI scores were the independent risk factors for indeterminate results as well as lymphocytopenia.

IFN-γ is mainly produced by Th1-type T cells and NK cells. In six of 23 SLE patients with indeterminate results, the negative control, which indicates baseline IFN-γ production from unstimulated cells, was abnormally high. Cases 1, 3 and 6 suffered from acute lupus hemophagocytic syndrome, in which increased levels of IFN-γ were reported due to abnormal activation of T cells.[22,23] Cases 2, 4 and 5 had high fever in their flares, which was in agreement with previous reports that serum levels of IFN-γ in the acute inflammatory phase of SLE patients were often increased.[24–27] On the other hand, in 20 of 23 SLE patients, IFN-γ generated by mitogen (phytohemagglutinin) stimulation was abnormally low. In SLE patients, especially during SLE flares, a reduced T-cell count may cause a decrease in the generation of IFN-γ. However, cases 2, 10, 13, 16, 19 and 21 had no severe lymphocytopenia, so the cause of insufficient response to mitogen may be abnormal function of T cells. In SLE, there is an apparent paradox of concomitant T-cell hyperactivity and hypoactivity. SLE T cells show spontaneously increased activation associated with a reduced threshold of activation to self-antigens, yet they are hyporesponsive to further antigenic stimulation.[28] We speculate such immunologic impairment of SLE patients resulted in the indeterminate results in the QFT-2G test.

There are certain limitations to our study. First, our sample size was not sufficiently large to identify active MTB infection, LTBI and various factors associated with indeterminate results. In our study, we detected one patient with active MTB infection, who had a positive QFT-2G result. Tozlu et al. has presented a SLE patient with miliary tuberculosis who had a negative QFT-2G result and severe lymphocytopenia.[29] False negative results may occur in SLE patients for the same reasons as high frequency of indeterminate results. Further studies are required. Second, although SLE patients were prospectively enrolled, non-SLE patients may have some bias due to their retrospective collection. However, since the frequency of indeterminate results was similar to those from previous studies[15,18–20] and lymphocytopenia was also associated with indeterminate results in our population, the selection bias may be small. Third, we chose an erythema of 10 mm as the cut-off for TST positivity. Although the erythema is used to diagnose MTB infection only in Japan, it has been demonstrated that the erythema and induration are highly correlated.[30] Meanwhile, in the TST guidelines of the Centers for Disease Control and Prevention and the American Thoracic Society, an induration of 5 mm or more is interpreted as a positive result for immunosuppressed patients.[31] In 29 of 43 SLE patients, we had data about the diameter of induration in the TST results. Using an induration of 5 mm or more as a threshold, no patient was positive and 29 patients were negative among 29 SLE patients. Univariate analysis showed no significant difference between SLE patients with risk factors for LTBI and those without risk in the TST results regardless of the threshold. The threshold may not influence the sensitivity of the TST in our study. Finally, the QFT-2G test we used in the present study has been shown to

be less sensitive than QFT-3G.[32] The performance of QFT-3G and T-SPOT-TB tests in SLE patients needs to be validated.

In conclusion, the QFT-2G test may have more potential to assist in the diagnosis of active MTB infection and LTBI than TST in SLE patients, but this study was unable to evaluate about one-third of patients due to indeterminate test results. Indeterminate results were associated with lymphocytopenia as well as disease activity and SLE itself. Caution must be taken when interpreting the results of the QFT-2G test among SLE patients, especially those who have parallel or subsequent flares of the disease.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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ORIGINAL ARTICLE

A trans-ethnic genetic study of rheumatoid arthritis identified *FCGR2A* as a candidate common risk factor in Japanese and European populations

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Abstract Rheumatoid arthritis (RA) is a common systemic autoimmune disease and its onset and prognosis are controlled by genetic, immunological, and environmental factors. The *HLA* locus, particularly *HLA-DRB1*, is its strongest genetic risk determinant across ethnicities. Several other genes, including *PTPN22* and *PADI4*, show modest association with RA. However, they cover only a part of its genetic components and their relative contribution is different between populations. To identify novel genetic determinants, we took a candidate gene approach in a trans-ethnic manner. After critical selection of 169 genes based on their immunological function, we performed SNP discovery of these genes by the resequencing of exons and surrounding areas using European and Japanese DNAs. We then generated a panel of 1,509 SNPs for case–control association study in both populations. The DerSimonian–Laird test for meta-analysis, using the combined results of the two populations, identified rs7551957 at the 5′-flanking region of the low-affinity Fc-gamma receptor IIa (*FCGR2A*) gene as the strongest candidate for the association ($p = 8.6 \times 10^{-5}$, odds ratio = 1.58 with 95%CI 1.25–1.99). Suggestive signals were also obtained for three SNPs in the dihydropyrimidine dehydrogenase (*DPYD*) gene (rs6685859; $p = 1.3 \times 10^{-4}$, rs7550959; $p = 1.5 \times 10^{-4}$ and rs7531138; $p = 1.7 \times 10^{-4}$) and an intronic SNP, rs2269310, of the erythrocytic spectrin beta (*SPTB*) gene ($p = 7.9 \times 10^{-4}$).

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Introduction

Rheumatoid arthritis (RA [OMIM: 180300]) is a systemic autoimmune disease [1], and is one of the most common forms of inflammatory arthritis, affecting up to 1% of the adult population [2]. Symptoms are chronic, destructive, and debilitating arthritis with a variation in the number of clinical features, such as the presence of autoantibody and joint erosions [3]. Clinical manifestation of RA is related to the development of a variety of autoantibodies, including antibodies to citrullinated peptide antigens and rheumatoid factor, although their pathological role is still unclear [4, 5].

Compelling evidence from genome-wide association (GWA) studies demonstrated that the *HLA* locus is the strongest genetic determinant beyond ethnicity [6]. However, the *HLA* locus contributes to only approximately 37% of the overall genetic susceptibility [7], suggesting the presence of other genes that are genetically associated with pathogenesis, clinical phenotype, and disease heterogeneity. Moreover, the relative contribution of RA-related genes is considered to be different between ethnicities. Indeed, the *PTPN22* gene was shown to be associated with RA in populations of European descent, but not in Asians [8]. Similarly, the *PADI4* gene showed a strong association with RA in Asians [9], but the association was much weaker in Europeans. Intronic SNPs in the *STAT4* gene were identified to be strongly associated with RA in Europeans [10], and their modest statistical association was confirmed in Asians [11], providing the first example of non-*HLA* RA-associated genes in two major ethnicities.

Importantly, however, very few hypothesis-independent GWA studies have succeeded in the identification of non-*HLA* genes associated with RA. A genome scan by the Wellcome Trust study was only able to successfully identify *HLA* and *PTPN22* loci as genome-wide significant [6]. This may be due to the disease heterogeneity with phenocopies, and the insufficient power to detect genes with modest effects [12]. Indeed, SNPs associated with RA in non-*HLA* genes showed only intermediate allelic odds ratios (ORs) of 1.3–2.0. To overcome such shortcomings, studies need to be enlarged, enrolling several thousand patients and a similar number of controls. Alternatively, a candidate gene approach, which focuses on genes critically selected by their biological function, can be an efficient and more cost-effective strategy because SNPs that are associated with RA are mostly within genes that are functionally implicated in the pathogenicity of RA [13]. Above all, comparative genetic analysis across different ethnic groups is ideal.

In this study, we performed a trans-ethnic case–control association study of RA in Japanese and European RA patients by applying an SNP marker panel of 169 genes related to the immune system and drug metabolisms designed for genetic approaches of various immune-related diseases. A total of 1,509 SNPs were exhaustively genotyped in 238 RA cases and 184 controls of Japanese origin, as well as in 182 cases and 273 controls of German and French populations.

Materials and methods

Study populations

Sample collection and genotyping was initiated after approval from the local ethical committees. Written informed consent was obtained from all patients and control individuals after adequate explanation of the study. The disease subjects were recruited in Germany and in Japan. The 420 patients with RA consisted of 182 Germans from the Freiburg area and 238 Japanese from the Kyoto and Kobe areas. All subjects satisfied the American Rheumatism Association's revised criteria (1987) for classification of RA [14]. For population controls, 91 German subjects from the Freiburg area and 184 Japanese subjects were recruited. Additionally, 182 French Caucasian controls from the Epidemiological study on the Genetics and Environment of Asthma (EGEA) were used.

Selection of candidate genes and single nucleotide polymorphisms

A total of 169 genes on autosomes were chosen for case–control association studies of different immune-related diseases, such as autoimmune diseases, immune deficiencies, and allergies, as well as drug response in such diseases. The selection was made according to their biological function in the immune system, such as cytokines and their receptors, cell adhesion molecules, transcriptional factors, and genes involved in signal transduction and cell–cell communication (Table 1). Genes already known for their association with the diseases, in addition to those shown to be related to drug metabolism prior to 2006 when the SNP discovery was performed, were also included in the panel.

The identification of SNPs was performed by an exhaustive resequencing of exons and flanking regions of these genes, using 32 each of French and Japanese control DNAs. Additional SNPs located in the linkage disequilibrium (LD) blocks covering the 169 genes in the International HapMap Project were also included. Among approximately 10,000 SNPs, those having a minor allele frequency (MAF) greater than 0.05 were tested using the

Table 1 Classification of candidate genes by their immunological function

Signal transduction	*ABCB1, ANK1, APOH, CARD4, CARD15, CBLB, CCND2, CCND2P, CCND3, CCNDBP1, CDC37, CDKN1A, CDKN2A2, EIF2AK2, EVI5L, GAB1, GAB2, GRB2, HCK, HSPCA, HSPCB, ITPKB, KIR3DL2, LAT, LCK, LCP2, MBL2, PTPN11, PTPN13, RASGRP1, SOCS3, SOS1, SYK, TANK, TGFB1, VAV1, VAV2, VAV3, XPO1, ZAP70, FTH1, NPM1*
Transcription factors	*AIRE, APOBEC3G, CCNT1, CCNT2, CDK7, CDK9, CD3EAP, NFAT5, NFATC1, NFATC2, NFATC3, NFATC4, STAT1, TBX1, TGFB1I1, TRIM21, TROVE2, TSC22D1, YBX1*
Cell–cell communication	*CD274, CD28, CD36, CD38, CD3Z, CD4, CD58, CD8A, CD8B, COMMD1, CTLA4, FCGR2A, FCGR3A, FER, ICAM2, ICAM3, ITGA1, ITGA2, ITGA4, ITGAL, ITGAV, ITGB1, ITGB4, PECAM1, SELL, TGFB, VCAM1, VIL2, VILL*
Cytokines and receptors	*FAS, FASLG, IFNA2, IFNAR1, IFNG, IFNGR1, IFNGR2, IL10, IL10RA, IL10RB, IL12A, IL12B, IL13, IL15, IL18, IL1A, IL1B, IL1R1, IL1RN, IL2, IL21R, IL2RA, IL2RB, IL4, IL4R, IL6, IL6R, IL6ST, IL7, IL8, L8RA, IL8RB, KIT, TNFRSF11A, TNFRSF13C, TNFRSF18, TNFRSF1A, TNFRSF1B, TNFRSF7, TNFSF15, TNFSF7*
Metabolism	*ALDH2, ANPEP, BCHE, CYP2C19, CYP2D6, CYP2E1, DPP4, DPYD, EPB41, FKBP4, GSTM4, GSTP1, NAT2, NOS2A, NQO1, NQO2, PLCG1, PPIA, SOD1, SOD2, SPTA1, SPTB, TPMT, TRIM5*
Regulation factor of immune response	*GYPA, GYPB, GYPC, OAS1, OAS2, OAS3, THY1*
Genes in the *HLA* locus	*HLA-DOB, PSMB8, PSMB9, TAP2, LTA, CSNK2B*
Other	*C1QBP*: role in complement pathway (binding C1q protein)

GoldenGate technology marker panel selection program by Illumina Inc. (San Diego, CA, USA), and only markers with Illumina Design Scores (IDS) greater than 0.4 were chosen. SNPs in the same LD block with pairwise-r^2 greater than 0.8 were divided into subgroups, and the SNP which showed the highest IDS in each subgroup was selected. When there were multiple SNPs sharing the highest IDS, the one with the highest MAF was chosen. Following these selection steps, a total of 1,509 SNPs were finally chosen as tag SNP markers for the genotyping study.

Genotyping and quality control

Genomic DNAs were extracted from fresh peripheral blood mononuclear cells or from EBV-transformed lymphoblasts in accordance with protocols approved by the appropriate authority.

A panel of 1,509 SNPs was genotyped using a GoldenGate assay on an Illumina BeadArray genotyping platform according to the manufacturer's instructions. DNA samples were tracked using a laboratory information management system (LIMS), and genotypes were called using the Genotyping module of BeadStudio 2 software (Illumina Inc.). The results obtained were filtered on the basis of genotype call rates (success rates of >90% for marker, >95% for DNA sample).

Statistical analysis

Genotype distribution was evaluated by the Hardy–Weinberg equilibrium in the control group (χ^2 test), and the markers with p values less than 0.05 were excluded from the tests [15]. Allele frequency of each SNP was compared for association with RA between cases and controls in each population using the trend χ^2 test and a non-biased exact trend test [16], as well as the DerSimonian–Laird test for meta-analysis, using the combined results of two populations [17].

Expression analyses of the FCGR gene family

A gene-expression dataset in lymphoblastoid cell lines derived from 210 unrelated HapMap populations (GSE6536) was obtained from the Gene Expression Omnibus (GEO) database [18]. The correlation between the expression data of *FCGR1A*, *2A*, *2B*, *2C*, *3A*, and *3B*, and rs7551957 genotypes of 268 individuals (89 European, 44 Japanese, 45 Chinese, and 90 West African Yoruba) available from the HapMap phase 2 data, was examined using the calculation program recommended by the GEO. The association p values were obtained by the Joncheere–Terepstra method using R software or SPSS (version 18).

Results

Association analysis in Japanese and European populations

In order to identify genetic loci associated with susceptibility to RA, 1,509 SNP markers representing 169 candidate genes with an average number of 8.9 SNPs (ranging from 1 to 75) per gene, were genotyped in DNA samples of Japanese and European origins (summarized in Table 1). After quality control of the results, 1,375 and 1,330 SNPs in the Japanese and Europeans, respectively, were tested for association.

In the Japanese case–control analysis, a total of 41 SNPs tagging 26 genes showed a nominal p value <0.005, of which 13 SNPs belonging to ten genes were less than 0.001 (Supplementary Table 1). Of those 41 SNPs, rs17587 in exon4 of the *PSMB9* gene was non-synonymous (arginine to histidine), while the others were either in the gene-flanking regions (11 SNPs), 3′-untranslated region (one SNP) or in introns (28 SNPs). The association analysis using the European sample set identified a putative difference in allele frequency of 20 SNPs in 16 genes with a nominal p value <0.005, of which five SNPs corresponding to three genes showed a p value <0.001 (Supplementary Table 2). One synonymous SNP, rs2302872, was located in exon14 of the *DPP4* gene, while the others were either in the gene-flanking regions (11 SNPs) or in introns (nine SNPs). Markers that are located in the *HLA* locus showed association with RA in both populations. The strongest p value was observed for rs1894408, located adjacent to the *HLA-DOB* gene ($p = 7.5 \times 10^{-5}$) in the Japanese, and for rs1383266 near the *PSMB9* gene ($p = 0.0011$) in the Europeans (Supplementary Tables 1 and 2).

Meta-analysis using combined results of Japanese and European studies

To identify the genes/variants that are associated with RA in both Japanese and Europeans, we performed a meta-analysis combining the genotyping results of the two populations by using the DerSimonian–Laird test. In total, 22 SNPs corresponding to 15 genes showed a p value (*DLp*) less than 0.01 (Table 2). Among them, five SNPs, namely, rs6685859, rs7551957, rs2269304, rs4819522, and rs5746834, showed relatively modest association with RA in both populations ($p = <0.05$).

The strongest association was obtained for rs7551957 of the *FCGR2A* (low affinity Fc-gamma receptor IIa) gene, with $DLp = 8.6 \times 10^{-5}$ and OR = 1.58 with 95% confidence interval (CI) 1.25–1.99. This SNP showed a strong association with RA in both populations (trend χ^2 $p =$ 0.0035 in the Japanese and $p = 0.0062$ in the Europeans). Three suggestive signals were obtained in the *DPYD* (dihydropyrimidine dehydrogenase) gene by meta-analysis: rs6685859 ($DLp = 1.3 \times 10^{-4}$ and OR = 1.64 with CI 1.27–2.12), rs7550959 ($DLp = 1.5 \times 10^{-4}$ and OR = 1.52 with CI 1.52–1.89), rs7531138 ($DLp = 1.7 \times 10^{-4}$ and OR = 1.51 with CI 1.22–1.88; Table 2). One SNP, rs2269310, in intron1 of the *SPTB* (erythrocytic spectrin beta) gene also showed a modest association ($DLp = 7.9 \times 10^{-4}$ and OR = 1.51 with CI 1.18–1.92) with RA.

Discussion

To our knowledge, this is the first report of a candidate gene-based trans-ethnic association analysis of RA. A panel of 169 genes that play important roles in immune response was extensively studied by SNP genotyping in Japanese and European populations. The *HLA* gene cluster is the major gene locus that contributes to RA susceptibility, and the *HLA-DRB1* gene is reported to be the strongest candidate [6]. In our study, the association between SNPs in the *HLA* region and RA was confirmed in both populations when the results were analyzed separately. However, these SNPs were not found to be significant in the meta-analysis. It is well known that the LD structure in this region is markedly different between ethnic groups. Hence, a larger sample size is required for sufficient statistical power to detect the same SNP as significant in both populations. Moreover, the panel used in this study only contains a single *HLA* gene, *HLA-DOB*, and not the others that are proven to be associated with RA.

The strongest association was obtained in the *FCGR2A* gene, which is located within the cluster of low affinity Fc-gamma receptor genes (*FCGR2A*, *FCGR3A*, *FCGR2C*, *FCGR3B*, and *FCGR2B*) on chromosome 1q22-23 [19]. Rs7551957 showed a strong association with RA in the two ethnicities ($p = 0.0035$ in the Japanese and $p = 0.0062$ in the Europeans), which was further confirmed by meta-analysis ($DLp = 8.6 \times 10^{-5}$;Table 2). In both populations, the variant allele frequency is higher in the RA cases (0.170 in the Japanese and 0.466 in the Europeans) compared with the controls (0.098 in the Japanese and 0.372 in the Europeans). Another SNP in the *FCGR2A* gene, rs1801274, showed a modest association in the meta-analysis ($DLp = 0.0021$). The difference in trend of p value between the two populations ($p = 0.098$ in the Japanese and $p = 0.011$ in the Europeans) may well be explained by the difference in the allele frequencies (0.225 in the Japanese and 0.500 in the Europeans). In line with our results, a recent meta-analysis of European genome-wide association studies confirmed the association of *FCGR2A* with RA risk ($p = 0.0004$) [20]. FCGRs are expressed on the surface of cells involved in the immune

Table 2 Single nucleotide polymorphisms associated with rheumatoid arthritis in meta-analysis using the combined results of Japanese and European populations

dbSNP ID	Chr.	Gene	Location	Nucleotide		Amino acid		Japanese freq. A2		Nominal trend *p*	European freq. A2		Nominal trend *p*	Meta-analysis	
				Ref. (A1)	Var. (A2)	Ref.	Var.	Case	Cont		Case	Cont		*DLp*	OR (95%CI)
rs6685859	1p21.3	*DPYD*	intron16	G	C			0.023	0.050	0.042	0.506	0.619	5.1×10^{-4}	1.3×10^{-4}	1.64 (1.27–2.12)
rs7550959			intron13	G	A			0.166	0.215	0.079	0.387	0.504	6.4×10^{-4}	1.5×10^{-4}	1.52 (1.22–1.89)
rs7531138			intron13	T	A			0.168	0.215	0.090	0.392	0.509	5.9×10^{-4}	1.7×10^{-4}	1.51 (1.22–1.88)
rs7551957	1q23.3	*FCGR2A*	5′-flanking	T	C			0.098	0.170	0.0035	0.372	0.466	0.0062	8.6×10^{-5}	1.58 (1.25–1.99)
rs1801274			exon4	A	G	His	Arg	0.176	0.225	0.098	0.412	0.500	0.011	0.0021	1.40 (1.13–1.74)
rs697846	1q42.12	*ITPKB*	3′-flanking	A	G			0.202	0.283	0.010	0.140	0.181	0.14	0.0042	1.44 (1.12–1.85)
rs1050567	2p15	*XPO1*	3′UTR	C	T			0.236	0.331	0.0053	0.102	0.133	0.17	0.0021	1.50 (1.15–1.94)
rs3770768	2p22.2	*EIF2AK2*	intron13	G	A			0.004	0.014	0.15	0.099	0.155	0.015	0.0082	1.72 (1.15–2.58)
rs926169	2q33.2	*CTLA4*	5′-flanking	G*	T			0.660	0.597	0.091	0.423	0.357	0.047	0.0087	1.31 (1.07–1.61)
rs2686399	3q.26.1	*BCHE*	3′-flanking	C*	G			0.844	0.819	0.36	0.732	0.648	0.0064	0.0077	1.37 (1.08–1.73)
rs6946119	7q21.12	*ABCB1*	3′-flanking	T	C			0.121	0.159	0.12	0.238	0.304	0.028	0.0076	1.39 (1.09–1.78)
rs2269310	14q23.3	*SPTB*	intron26	G	A			0.368	0.475	0.0054	0.108	0.147	0.073	7.9×10^{-4}	1.51 (1.18–1.92)
rs229670			intron1	A*	C			0.413	0.349	0.061	0.268	0.177	0.0011	0.0024	1.48 (1.14–1.91)
rs2269304			intron14	C*	A			0.118	0.075	0.049	0.213	0.153	0.018	0.0025	1.54 (1.16–2.05)
rs4787426	16p12.1	*IL4R*	3′-flanking	T	G			0.104	0.170	0.012	0.122	0.153	0.21	0.0076	1.49 (1.11–2.01)
rs4968681	17q23.3	*ICAM2*	5′-flanking	G	A			0.323	0.409	0.016	0.326	0.371	0.17	0.0082	1.32 (1.07–1.62)
rs7503550	17q23.3	*PECAM1*	intron4	G*	A			0.529	0.467	0.096	0.566	0.474	0.0072	0.0018	1.37 (1.12–1.67)
rs537188	19p13.2	*EVI5L*	intron11	G	A			0.085	0.105	0.34	0.140	0.212	0.0032	0.0045	1.52 (1.13–2.04)
rs347033	19p13.3	*VAV1*	intron4	T	C			0.144	0.227	0.0045	0.163	0.205	0.11	0.0028	1.50 (1.15–1.97)
rs2866370	20q12	*PLCG1*	intron1	G	A			0.039	0.077	0.022	0.050	0.078	0.080	0.0054	1.81 (1.19–2.75)
rs4819522	22q11.21	*TBX1*	exon9	C	T	Thr	Met	0.058	0.097	0.042	0.182	0.254	0.013	0.0014	1.58 (1.19–2.09)
rs5746834			3′-flanking	G	T			0.037	0.069	0.039	0.190	0.248	0.039	0.0049	1.52 (1.13–2.03)

SNPs with a *p* value (*DLp*) less than 0.01 according to the DerSimonian–Laird test are listed with odds ratio (OR) and 95% confidence interval (95%CI).

The risk allele is indicated by an asterisk if it is the reference allele.

Arg arginine, *His* histidine, *Met* methionine, *Thr* threonine, *Ref.* reference allele, *Var.* variant allele

system, and participate in diverse functions such as phagocytosis of immune complexes and modulation of antibody production by B cells. Various genetic polymorphisms of these receptors were reported to be associated with several autoimmune diseases [21, 22]. In particular, *FCGR2A* was shown to be associated to systemic lupus erythematosus [23, 24]. In the mouse model, *Fcgr3*-deficient hosts exhibit resistance to arthritis induced by collagen type II or anti-glucose-6-phosphate isomerase antibody [25]. In contrast, mice deficient for *Fcgr2b* lead to increased susceptibility to collagen-induced arthritis [26]. Theses findings suggest that expression of FCGRs on synovial cells may contribute to the antibody-triggered inflammation in joints [27]. In addition, we examined the association of rs7551957 with the expression level of the other *FCGR* family members according to the four population groups (European, Japanese, Chinese, and West African Yoruba), but linear regression analyses failed to reveal any significant associations (results not shown).

Variation in gene copy number is postulated to influence clinical phenotype. There have been conflicting reports regarding the association of copy number variations (CNV) in the *FCGR* locus with RA in Caucasian studies [28, 29]. In the vicinity of the *FCGR* locus there are at least three reported regions showing CNV, but rs7551957 is located more than 19-kb away from the nearest CNV region, which extends from the 3′-UTR of *FCGR2A* to the 3′-UTR of *FCGR2C* [30]. In addition, a careful examination of the rs7551957 genotype results did not reveal any indications of CNV in the observed cluster signals. Hence the association observed for rs7551957 with *FCGR2A* is unlikely to be caused by CNV of the *FCGR* locus.

Rs6685859 in intron16 of the *DPYD* gene showed $DLp = 1.3 \times 10^{-4}$, although trend p value was at a marginal level ($p = 0.042$) in the Japanese compared with the Europeans ($p = 5.1 \times 10^{-4}$). Again this may be due to the difference in allele frequencies (case versus control; 0.023 versus 0.050 in the Japanese and 0.506 versus 0.619 in the Europeans), because the risk allele is the same in both populations. The two other markers, rs7550959 and rs7531138, showed similar trends as rs6685859. DPYD is a pyrimidine catabolic enzyme, mutation of which leads to dihydropyrimidine dehydrogenase deficiency, putting cancer patients receiving 5-fluorouracil chemotherapy at an increased risk of toxicity [31]. To our knowledge, there have been no published reports on its association with RA or other autoimmune-related diseases to date.

Among the SNPs in the *SPTB* gene examined, three (rs2269310, rs229670, and rs2269304) showed significant *DLp* values. Rs2269310, which showed $DLp = 7.9 \times 10^{-4}$ and a significant trend p value of 0.0054 in the Japanese, did not show a similar level of association in the Europeans ($p = 0.073$). Again this may be due to a lower frequency of the variant allele in Europeans. SPTB acts to stabilize erythrocyte membranes, and mutations in the *SPTB* gene have been associated with spherocytosis type 2 [32], and neonatal hemolytic anemia [33]. Again, its association with RA is not known, although it has been previously reported that there was no significant immunoreactivity observed against spectrin in the sera of 50 RA patients [34].

In the current study, none of the SNPs remained significant after nominal trend p values were corrected for multiple testing using the Bonferroni method (data not shown). Therefore, detection of significant association requires replication analyses using independent sample sets. Nonetheless, we succeeded in the identification of several genetic variants as being associated with RA susceptibility across ethnicities, indicating the usefulness of a trans-ethnic comparison. Additional research is necessary to further confirm their association with the disease, and to elucidate their biological role in the pathophysiology of RA.

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Conflict of interest None.

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

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

KEY WORDS: chronic hepatitis C; peginterferon and ribavirin; amino acid substitution of HCV core region residue 70; genetic polymorphisms near the *IL28B* gene; mutation; non-sustained virologic responder

INTRODUCTION

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

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

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