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Prevalence of Hepatitis B Virus Infection in Patients with Rheumatic Diseases in Tohoku Area: A Retrospective Multicenter Survey

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Hepatitis B virus (HBV) reactivation has been increasingly recognized in patients receiving chemotherapy and immunosuppressive therapy; however, the prevalence of HBV infection and rate of HBV screening in patients with rheumatic diseases remains unclear. In this study, we aimed to assess the prevalence of HBV infection and fulminant HBV hepatitis in patients with rheumatic diseases. We also investigated the rate of HBV screening before immunosuppressive therapy in patients with rheumatic diseases. A retrospective questionnaire survey was conducted in the North-east area (Tohoku) of Japan. Questionnaires, comprising 6 questions, were sent to 318 rheumatologists in May 2010, and responses were gathered until June 2011. In total, 71 rheumatologists (22.3%) responded to the survey. We enrolled 7,650 patients with rheumatoid arthritis (RA) and 1,031 patients with systemic lupus erythematosus (SLE). When limited to institutes at which almost all (≥ 90%) patients were tested for HBV serology, 1.1% (40/3,580) patients with RA and 0.3% (3/1,128) patients with SLE were positive for hepatitis B surface antigen (HBsAg), and 25.2% (177/703) patients with RA and 13.7% (34/248) patients with SLE were positive for hepatitis B core antibody (HBcAb).

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About one-third of rheumatologists did not check HBsAg and more than half did not check hepatitis B surface antibody (HBsAb) or HBcAb at all before therapy. Fulminant HBV hepatitis was observed in 1 RA patient who was current HBV carrier. In conclusion, the prevalence of HBV infection is high in patients with RA and SLE. HBV screening before immunosuppressive therapy should be strictly performed.

Keywords: hepatitis B virus; immunosuppressive therapy; rheumatoid arthritis; screening; systemic lupus erythematosus
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Introduction

After the widespread use of rituximab, an anti-CD20 monoclonal antibody, hepatitis B virus (HBV) reactivation has been increasingly recognized in patients receiving chemotherapy and immunosuppressive therapy (Oketani et al. 2012). Some studies have shown that HBV reactivation occurs not only in 'current HBV carriers', who are positive for hepatitis B surface antigen (HBsAg), but also in 'resolved HBV carriers', who are negative for HBsAg but positive for hepatitis B surface antibody (HBsAb) and/or hepatitis B core antibody (HBcAb) (Oketani et al. 2012). Particularly, HBV reactivation in resolved carriers may often cause a type of fulminant hepatitis, termed as *de novo* HBV hepatitis, with an extremely high mortality rate (Umemura et al. 2008).

Based on these results, Centers for Disease Control (CDC) recommended screening for HBV serology before chemotherapy and immunosuppressive therapy (Weinbaum et al. 2008). The American College of Rheumatology (ACR) also recommends HBV screening before immunosuppressive therapy (Singh et al. 2012), and recently, the Japanese College of Rheumatology (JCR) proposed an algorithm for HBV screening (Harigai et al. 2014). According to this algorithm, all patients should be screened for HBsAg before immunosuppressive therapy. In addition, those who are negative for HBsAg should be tested for HBsAb and HBcAb. HBV DNA quantification by real-time polymerase chain reaction (RT-PCR) should be performed in resolved HBV carriers. When HBV DNA becomes positive during and after therapy, prophylactic nucleoside analogs such as entecavir should be administered (Harigai et al. 2014). However, evidence to support validity of this algorithm to prevent severe hepatitis is not sufficient and especially needs to clarify cost-benefit relations.

HBV is endemic in Japan, and approximately 20% Japanese individuals are infected with HBV (Kiyosawa et al. 1994). Therefore, HBV screening before treatment should be more strictly performed in Japan than in other non-endemic countries. However, only few studies have reported the prevalence of HBV infection and rate of HBV screening in Japanese patients with rheumatic diseases (Urata et al. 2011; Mori 2011; Watanabe et al. 2013)

In this study, we assessed the prevalence of HBV infection and fulminant HBV hepatitis in patients with rheumatic diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In addition, we investigated the rate of HBV screening before immunosuppressive therapy in patients with rheumatic diseases.

Methods

A retrospective questionnaire survey was conducted in the North-east area (Tohoku) of Japan. Questionnaires were sent to 318 rheumatologists in May 2010, and we waited for the response until June 2011. Following are the 6 questions listed in the questionnaire: (1) How many patients with RA have you treated? (2) How prevalent is HBV infection in patients with RA? (3) How many patients with SLE have you treated? (4) How prevalent is HBV infection in patients with SLE? (5) Do you examine serological HBV markers before treatment in patients with rheumatic diseases? (6) Have you ever experienced patients with fulminant HBV hepatitis? In this simplified questionnaire survey, we did not check a detail on sex, age, and hepatitis enzymes. We checked each HBV serological marker independently. Therefore, we did not check HBsAg positivity in HBcAb-positive patients.

Diagnoses of RA and SLE were based on RA classification criteria and SLE classification criteria (Arnett et al. 1988; Hochberg 1997; Aletaha et al. 2010). HBV serological tests were performed before starting immunosuppressive therapy at each institute. Immunosuppressive therapy was defined as the use of biologics, immunosuppressive disease-modifying antirheumatic drugs (DMARDs) including methotrexate (MTX), tacrolimus, leflunomide, mizoribine, corticosteroids, and other immunosuppressive agents (Harigai et al. 2014). The study protocol was approved by the ethics committees of Tohoku University Graduate School of Medicine.

Results

Overall response rate

Of the 318 rheumatologists, 71 (22.3%) responded to the questionnaire. Although we waited for the response until June 2011, all the answers obtained were before the Great East-Japan Earthquake (March 11, 2011).

Prevalence of HBV infection in patients with RA

In total, 7,650 patients with RA were enrolled. 0.7% (50/7,650) patients with RA were considered to be current HBV carriers, and 25.6% (214/837) were positive for HBcAb (Table 1). When the patient cohort was limited to institutes at which HBV serology was examined for almost all patients (≥ 90%), 1.1% (40/3,580) patients with RA were current HBV carriers, and 25.2% (177/703) were positive for HBcAb. Among patients receiving biologics, 0.3% (3/1,128) patients were positive for HBsAg, indicating that

Table 1. Positivity rate for each HBV serological marker in patients with RA.

		HBsAg	HBsAb	HBcAb
All patients	Total	50/7,650 (0.7%)	245/1,295 (18.9%)	214/837 (25.6%)
	at institutes ≥ 90% patients were examined	40/3,580 (1.1%)	169/1,011 (16.7%)	177/703 (25.2%)
Patients with biologics	Total	3/1,634 (0.2%)	68/512 (13.3%)	64/274 (23.4%)
	at institutes ≥ 90% patients were examined	3/1,128 (0.3%)	49/391 (12.5%)	57/199 (28.6%)

Table 2. Positivity rate for each HBV serological marker in patients with SLE.

	HBsAg	HBsAb	HBcAb
Total	3/1,031 (0.3%)	26/284 (9.2%)	38/267 (14.2%)
at institutes ≥ 90% patients were examined	3/704 (0.4%)	25/248 (10.1%)	34/248 (13.7%)

Table 3. Screening rate for each HBV marker before starting immunosuppressive therapy in 71 rheumatologists.

	HBsAg	HBsAb	HBcAb	HBV DNA
All patients	18 (25%)	2 (3%)	4 (6%)	0 (0%)
Not all patients	30 (42%)	24 (34%)	29 (41%)	21 (30%)
None	23 (32%)	45 (63%)	38 (54%)	50 (70%)
Total	71	71	71	71

biologics tended to be avoided in current HBV carriers. In contrast, biologics were prescribed for resolved HBV carriers at a similar rate to patients without HBV infection.

Prevalence of HBV infection in patients with SLE

Among 1,031 patients with SLE, 3 patients (0.3%) were positive for HBsAg (Table 2). When limited to institutes at which HBV serology was examined for almost all patients (≥ 90%), 0.4% (3/704) patients were positive for HBsAg and 13.7% (34/248) showed positive results for HBcAb, indicating that the prevalence of HBV infection in patients with SLE was lower than that in patients with RA ($p = 0.0002$, Chi-square test).

Rate of HBV screening before immunosuppressive therapy

The rate of screening for HBV serological markers before initiating treatment is summarized in Table 3. 71 rheumatologists answered it with respect to each HBV serological marker. HBsAg was examined for all patients by a relatively high number of clinicians (18/71, 25%); however, approximately one-third clinicians (23/71, 32%) did not check HBsAg at all and more than half of the clinicians did not check HBsAb or HBcAb at all.

Fulminant HBV hepatitis

Among all answers, fulminant HBV hepatitis was reported in 1 patient with RA. This patient was 70's female and current HBV carrier (HBsAg-positive, and hepatitis Be antigen-negative), but was treated by MTX 8 mg/week and prednisolone (PSL) 5 mg/day without nucleoside analogs in general physician's clinic. HBV DNA quantification was not performed in this clinic. The patient was admitted to a nearest university hospital and treated with plasma exchange and entecavir, but died of fulminant HBV hepatitis confirmed by HBV DNA quantification and autopsy.

Discussion

This retrospective multicenter questionnaire survey conducted in the North-east area of Japan demonstrated that approximately 1% patients with RA were current HBV carriers and more than 25% were considered to be resolved carriers. Previous reports in Japan showed similar results estimating that 25% (60/239) patients in Kumamoto and 31.5% (135/428) in Aomori were infected with HBV (Urata et al. 2011; Mori 2011), indicating that more than one-fourth patients with RA may be infected with HBV in Japan. However, the number of patients enrolled in this study is much larger than previous reports. To our knowledge, this is the largest study regarding prevalence of HBV infection in RA patients in Japan. Our data suggest that HBV screening and appropriate management of HBV should be strictly performed when initiating immunosuppressive therapy. Prevalence of HBV infection was significantly lower (16.5%; 41/248) in patients with SLE than that in patients with RA (Watanabe et al. 2013). It has been reported that older adults had a higher frequency of HBV

infection that younger adults in Japan (Tanaka et al. 2011). Therefore, one reason to explain this may be the different ages of onset of RA and SLE. However, because high-dose corticosteroids and intensive immunosuppressive therapy, such as cyclophosphamide, are often required to treat patients with SLE, we should not overlook the risk of HBV reactivation, even in patients with SLE.

This retrospective survey is the first report about the rate of HBV screening before immunosuppressive therapy in Japan and also showed that the rate was low in routine clinical practice. However, previous reports from oncologists showed similar results (Tran et al. 2010; Day et al. 2011a, b; Hwang et al. 2012; Zurawska et al. 2012; Lee et al. 2012). For example, Hwang et al. (2012) reported that among 10,729 patients who received chemotherapy, only 1,787 (16.7%) underwent screening for HBsAg or HBcAb. As for rheumatologists, Stine et al. (2010) conducted a nationwide questionnaire survey about HBV screening in 1,000 ACR members. Responses obtained (153/1,000, 15.3%) were highly variable and more than half of the members did not check HBsAb or HBcAb prior to initiating therapy. The authors concluded that it is necessary to improve education among rheumatologists regarding the risks of HBV reactivation in patients in whom immunosuppressive therapy needs to be started.

Recent advances in treatment have changed the therapeutic goal of patients with RA and other rheumatic diseases (Harigai et al. 2014). Biologics, high-dose MTX, and corticosteroids are being increasingly used to induce remission or low disease activity. Among them, the use of biologics, such as tumor necrosis factor-α inhibitors, may cause HBV reactivation (Urata et al. 2011). However, MTX and corticosteroids also have the potential to induce HBV reactivation and *de novo* hepatitis (Urata et al. 2011; Harigai et al. 2014). The use of biologics is now contraindicated in current HBV carriers (Harigai et al. 2014); however, some rheumatologists prescribe them for current HBV carriers in this study (Table 1). Recently, with an increasing interest in HBV reactivation in Japan, the rate of HBV screening may continue to increase. Nevertheless, a new framework to improve the assessment of the risk of HBV reactivation and fulminant HBV hepatitis by Japanese rheumatologists needs to be developed.

Although fulminant HBV hepatitis was observed in 1 patient who was current carrier, fulminant hepatitis due to HBV reactivation in resolved carriers was not reported in this retrospective study. However, this does not mean that fulminant HBV hepatitis does not occur in resolved carriers because the rate of HBV screening was low. Therefore, we are now conducting a multicenter prospective study to investigate the rate of HBV reactivation in patients with rheumatic diseases under immunosuppressive therapy.

In conclusion, this retrospective questionnaire study demonstrated that approximately 20% or more patients with rheumatic diseases were infected with HBV and the rate of HBV screening before immunosuppressive therapy among rheumatologists was low in routine clinical practice. Although the incidence of fulminant HBV hepatitis was low, we rheumatologists should improve our consciousness regarding the risk of HBV reactivation and management of HBV infection.

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Conflict of Interest

The authors declare no conflict of interest.

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RHEUMATOLOGY

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Original article

An association analysis of *HLA-DRB1* with systemic lupus erythematosus and rheumatoid arthritis in a Japanese population: effects of *09:01 allele on disease phenotypes

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Abstract

Objective. To re-evaluate the roles of *HLA-DRB1* alleles in susceptibility to SLE and RA and their effects on autoantibody status in large-scale Japanese cohorts.

Methods. A total of 656 SLE, 2410 RA and 911 control subjects, who were all Japanese, were genotyped for *HLA-DRB1* alleles using sequence-specific oligonucleotide probes. The association of alleles with disease susceptibility was tested by logistic regression analysis and by the relative predispositional effect method. The association with autoantibody status was examined by the standard χ^2 test.

Results. *HLA-DRB1**15:01, *09:01, *08:02 and *04:01 were significantly associated with SLE susceptibility, while shared epitope (SE) alleles and *DRB1**09:01 were associated with RA susceptibility. The compound heterozygote of *DRB1**09:01/*15:01 conferred an increased risk for SLE compared with the homozygotes for *DRB1**09:01 and *15:01 and was associated with earlier onset of disease, whereas the compound effect of *DRB1*-SE/*09:01 was not clear in RA. *DRB1**09:01 was significantly associated with the appearance of anti-Sm antibody in SLE as well as ACPA in RA, while protectively associated with anti-dsDNA antibody in SLE. No significant interaction was observed between *DRB1**09:01 and smoking status for the appearance of ACPA, unlike that observed in SE alleles in RA.

Conclusion. We identified *HLA-DRB1* alleles associated with SLE and RA in a Japanese population and demonstrated a shared susceptibility of *DRB1**09:01 between the diseases as well as its effect on autoantibody production.

Key words: systemic lupus erythematosus, rheumatoid arthritis, human leukocyte antigen DRB1, autoantibody, genetics, association study, Asian population.

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Introduction

Genome-wide association studies for SLE and RA have discovered many disease susceptibility genes, where the strongest genetic link is seen in the HLA class II region for both diseases [1–3]. In European/Caucasian SLE patients, the HLA-DR3 serotype (or *HLA-DRB1**03:01 for genotype) and DR2 (*DRB1**15:01) have been associated with disease risk [1, 2, 4, 5]. In Asian populations, the association of DR2 was also replicated in many studies, while the exact role of each individual allele comprising the DR2 serotype (*DRB1**15:01 and *15:02 are common in Asians) has not been established [6–10]. In addition, an association of DR9 (*DRB1**09:01), which is rare in European populations, has been implicated in several Asian populations [6–8], although it was not replicated in others [9–11]. This inconsistency may be due to the relatively small sample sizes examined in these individual studies, and it needs to be further tested.

In RA, the disease-associated *DRB1* alleles (DR1/DR4/DR10) are different among European populations but share a conserved amino acid motif (QKRAA/QRRAA/RRRAA) at 70–74, which are now referred to as the shared epitope (SE) [12, 13]. Since ACPA has been recognized in RA, a strong association between SE alleles and the appearance of ACPA has been described [14–16]. Moreover, a gene–environmental interaction between *HLA-DRB1* and cigarette smoking was reported, showing that smokers who carry *DRB1*-SE alleles have a greater risk for the appearance of ACPA [17–19]. In Asian populations, the association of SE alleles has also been repeatedly reported [18, 20]. In addition, *DRB1**09:01, which is not classified as an SE allele, is another risk allele for RA in Asian populations, although the risk is moderate compared with that of SE alleles [18, 20]. The association between the appearance of ACPA and SE alleles has been replicated in Asian populations, but the effect of *DRB1**09:01 on ACPA status is controversial [18, 21–23].

Another issue concerning the association of *HLA-DRB1* alleles with diseases is the genotypic effects, including the gene dosage effect of each allele and the combinational effect of two different alleles. In SLE, compound heterozygotes of susceptible alleles (i.e. DR2/DR3) confer a remarkably increased risk [1]. In European RA patients, compound heterozygotes of SE alleles (i.e. *DRB1**04:01/*04:04) are significantly associated with severe disease in comparison with the homozygotes [24, 25]. In Asia, a study on Koreans demonstrated that the *DRB1**04:05/*09:01 heterozygote conferred a stronger risk for RA than the homozygote for *DRB1**04:05 [26].

Here, to examine and refine the previous findings described so far, we investigated the associations of *HLA-DRB1* alleles with a predisposition to SLE and RA in a Japanese population using a large cohort composed of 656 SLE, 2410 RA and 911 control subjects. We also explored the effect of *DRB1* alleles on disease sub-phenotypes by examining the association between *DRB1* genotypes and autoantibody production in the patients.

Patients and methods

Subjects

The DNA samples of SLE patients [$n = 656$; mean (S.D.) age at the time of enrolment = 43.2 (13.7) years; 89.8% female], RA patients [$n = 2410$; mean (S.D.) age = 60.7 (16.7) years; 81.2% female] and control subjects [$n = 911$; mean (S.D.) age = 52.4 (14.4) years; 25.8% female] were recruited through several medical institutes in Japan under the support of the autoimmune disease study group of Research in Intractable Diseases, Ministry of Health, Labor and Welfare, Japan, for SLE patients, the BioBank Japan Project for RA patients [27] and the Midosuji Rotary Club for the controls. All 656 SLE subjects fulfilled the 1997 revised criteria of the ACR for SLE [28], and the RA subjects met the 1987 revised criteria of ACR for RA [29]. The control subjects were the same as those used in a previous study [30]. Subjects having a history of autoimmune diseases were excluded from control subjects based on a questionnaire. All subjects were Japanese and provided informed consent to participate in the study. The study was approved by the ethical committee of each institute (RIKEN Institute, University of Tokyo, Tohoku University, Hokkaido University, University of Occupational and Environmental Health, Wakayama Medical University, Keio University, University of Tsukuba, Juntendo University and Kyoto University).

Genotyping of *HLA-DRB1*

High-resolution (four-digit) genotyping of *HLA-DRB1* was performed by a sequence-specific PCR method with a WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) and a Luminex multi-analyte profiling system (xMAP; Luminex, Austin, TX), according to the manufacturer's instructions. For the analysis of RA patients, *DRB1* alleles that contained amino acid sequences QKRAA/QRRAA/RRRAA at 70–74 were classified as SE+.

Autoantibody and smoking status

Clinical data for 562 SLE subjects (85.7%) were available. All individual ACR criteria were coded as positive, negative or missing. For the study of SLE patients, we obtained autoantibody status from these clinical data, including anti-dsDNA antibody, anti-Sm antibody, aCL and lupus anticoagulant (LAC). The number of subjects available for autoantibody status and the positivity rate for each autoantibody were as follows: anti-dsDNA, $n = 541$, 91.7%; anti-Sm, $n = 451$, 35.5%; anti-CL, $n = 419$, 33.9%; LAC, $n = 349$, 21.2%. All these autoantibodies except for LAC were examined by ELISA. LAC was detected by activated partial thromboplastin time and dilute Russell viper venom time according to the international guideline [31].

Sera from 2384 RA patients (98.9%) were available for the measurement of ACPA. ACPA was measured using the Mesacup CCP test (Medical and Biological Laboratories, Woburn, MA). The positivity rate of ACPA was 79.0%. Smoking status was available for 2294 patients (95.2%) and smoking rates were a total of 32.7%; male

82.2% and female 21.2%. The data were recorded as ever or never smoker. Ever smokers contained former and current smokers.

Statistical analysis

We performed logistic regression analyses in case–control studies to adjust for sex and age. In case-only analyses, we tested the association by the standard χ^2 test using 2×2 contingency tables. Fisher's exact test was applied when a cell value was <5. Only alleles with frequencies >0.01 in control samples were analysed, and Bonferroni's correction was applied for multiple testing (20 tests, $\alpha = 0.0025$). The contingency tables were used to calculate the odds ratio (OR). Disease association was also assessed by the relative predispositional effect (RPE) [32]. When the disease is associated with two or more alleles of a locus, this method identifies the associations sequentially according to their strength. Thus the problem that a strong association with one allele can create misleading deviations in the frequencies of other alleles is alleviated. In the RPE analysis of RA, all the SE-carrying alleles were treated as a single SE allele. We calculated population-attributable risk (PAR) using the following formula: $PAR = f(OR - 1)/(1 + f(OR - 1))$, where f is the allele frequency. The mean age at disease onset was analysed by the Mann–Whitney U test. To evaluate a multiplicative interaction between alleles, we introduced the interaction term in a logistic regression model [19, 33–35]. We also evaluated the interaction by calculating three measures, including the relative excess risk due to interaction (RERI), the proportion attributable to interaction (AP) and the synergy index (S) [36].

We performed k-means cluster analysis to identify cluster groups of SLE patients with similar autoantibody patterns using the clinical data of anti-dsDNA, anti-Sm and anti-CL autoantibodies and LAC, as previously described [37]. We replaced missing data with the mean positive rate of each autoantibody in order to maximize the statistical power [34]. Then we compared autoantibody status and *DRB1* allele frequency among the three groups by χ^2 test using 2×3 tables. All statistical analyses were performed using STATISTICA (StatSoft, Tulsa, OK).

Results

Distribution of *HLA-DRB1* alleles in SLE and RA

The allele distribution of *HLA-DRB1* in SLE, RA and control subjects is presented in Table 1. In SLE, only *DRB1**15:01 showed a significant association with disease susceptibility ($P = 4.2 \times 10^{-9}$, OR 2.90). In the RPE analysis, *DRB1**04:01, *08:02 and *09:01 were significantly associated with SLE susceptibility ($P < 0.0025$). As *DRB1**15:01, *08:02 and *09:01 were the three prominent alleles in our population showing a strong association with SLE and a substantial extent of PAR (0.117, 0.032 and 0.063, respectively), we focused on the *DRB1**15:01, *08:02 and *09:01 alleles for further analysis of SLE.

In RA, SE alleles, including *DRB1**04:01 and *04:05, displayed significant association with disease ($P < 0.0025$), and *DRB1**04:05 showed the highest risk ($P = 4.9 \times 10^{-22}$, OR 2.59). When we evaluated SE alleles collectively, they contributed to a substantial proportion of the RA risk when evaluated by PAR (= 0.233). After SE alleles were excluded collectively in the RPE analysis, only *DRB1**09:01 was significantly associated with RA susceptibility ($P = 3.8 \times 10^{-8}$). Thereafter we focused on SE alleles and *DRB1**09:01 for further analysis of RA.

Genotypic association analyses of *HLA-DRB1* with disease susceptibility

To analyse the combinational effect of the three major risk alleles of SLE, we grouped the SLE patients by the presence of *DRB1**08:02, *09:01 and *15:01 alleles (Table 2). When compared with the reference genotype (denoted as X/X in SLE), the homozygote *DRB1**09:01/*09:01 showed a greater risk (OR 2.37) than the heterozygote *DRB1**09:01/X (OR 1.48), which implied the presence of a gene dosage effect. In contrast, no obvious gene dosage effect was seen in *DRB1**15:01, although a lack of statistical power was undeniable. Strikingly, the compound heterozygote *DRB1**09:01/*15:01 conferred the highest risk for SLE (OR 7.42). The heterozygote *DRB1**09:01/*15:01 showed a significantly increased risk for SLE in comparison with the homozygote for *DRB1**09:01 ($P = 0.0076$, OR 5.05), while no significant difference was observed between the compound heterozygote and the homozygote for *DRB1**15:01 ($P = 0.52$, OR 2.13), in which there was a lack of statistical power. When the multiplicative interaction between *DRB1**15:01 and *09:01 was evaluated in a logistic regression model, the coefficient for the interaction term was not significant but marginal ($P = 0.078$). However, the three measures for interaction as departures from additivity suggested a possible interaction [RERI 6.17 (95% CI −2.04, 14.38), AP 0.63 (95% CI 0.31, 0.96), S 3.39 (95% CI 1.22, 9.48)].

In the RA cohort, we grouped the patients by the presence of *DRB1*-SE and *09:01 (Table 2). In comparison with heterozygote SE/Y, homozygote SE/SE showed a significantly increased risk ($P = 1.1 \times 10^{-6}$, OR 2.69), which confirmed the gene dosage effect of SE alleles as previously described [24]. The homozygote of *DRB1**09:01 suggested a moderately increased risk for RA compared with the heterozygote ($P = 0.093$, OR 1.69). As the risk of compound heterozygote SE/*09:01 did not significantly exceed the risk of homozygote SE/SE nor *09:01/*09:01, there was no dominant compound effect (data not shown). Likewise, no compound effect was detected in *0405/*0901 (data not shown), unlike the previous report in a Korean population [26].

In addition to analysing the genotypic effect of *HLA-DRB1* on disease susceptibility, we also analysed its effect on the age of disease onset. Among the genotypes of SLE patients, only the compound heterozygote *DRB1**09:01/*15:01 conferred a significantly earlier onset of SLE when compared with X/X (24.3 vs 30.9 years, $P = 4.3 \times 10^{-4}$) (Table 3). The same result was obtained when only female patients were analysed. In RA, the homozygote SE/SE and the compound heterozygote SE/*09:01 were significantly associated with an earlier

Table 1 Distribution of *HLA-DRB1* alleles in 656 SLE patients, 2410 RA patients and 911 control subjects

	No. of alleles (%)			SLE				RA				
				Logistic regression		RPE test		Logistic regression		RPE test[b]		
DRB1 allele[a]	SLE cases (n = 1312 alleles)	RA cases (n = 4820 alleles)	Controls (n = 1822 alleles)	OR (95% CI)	P-value	P-value	PAR	OR (95% CI)	P-value	P-value	PAR	SE allele
*01:01	48 (3.7)	321 (6.7)	106 (5.8)	0.58 (0.36, 0.93)	0.024	–		1.14 (0.86, 1.51)	0.35	–		+
*04:01	29 (2.2)	117 (2.4)	18 (1.0)	2.93 (1.29, 6.66)	0.010	3.1×10^{-4}	0.019	2.51 (1.38, 4.55)	0.0025	–		+
*04:03	32 (2.4)	74 (1.5)	62 (3.4)	1.05 (0.57, 1.91)	0.88	–		0.47 (0.30, 0.72)	6.9×10^{-4}	–		
*04:05	126 (9.6)	1208 (25.1)	235 (12.9)	0.62 (0.47, 0.84)	0.0023	–		2.59 (2.14, 6.73)	4.9×10^{-22}	–		+
*04:06	17 (1.3)	116 (2.4)	55 (3.0)	0.50 (0.24, 1.02)	0.058	–		0.80 (0.52, 1.23)	0.30	–		
*04:10	27 (2.1)	123 (2.6)	24 (1.3)	0.98 (0.45, 2.12)	0.95	0.011	<0.001	1.90 (1.14, 3.18)	0.014	–		+
*08:02	81 (6.2)	123 (2.6)	64 (3.5)	1.95 (1.20, 3.15)	0.0066	3.8×10^{-6}	0.032	0.66 (0.44, 0.98)	0.038	–		
*08:03	116 (8.8)	272 (5.6)	164 (9.0)	0.93 (0.67, 1.29)	0.64	–		0.61 (0.48, 0.79)	1.8×10^{-4}	–		
*09:01	273 (20.8)	827 (17.2)	286 (15.7)	1.43 (1.11, 1.85)	0.0062	2.0×10^{-6}	0.063	1.12 (0.94, 1.34)	0.22	3.8×10^{-8}	0.018	
*10:01	4 (0.3)	54 (1.1)	18 (1.0)	0.19 (0.057, 0.65)	0.0082	–		1.30 (0.66, 2.57)	0.45	–		+
*11:01	20 (1.5)	101 (2.1)	43 (2.4)	0.63 (0.31, 1.28)	0.20	–		0.98 (0.60, 1.57)	0.92	–		
*12:01	49 (3.7)	163 (3.4)	72 (4.0)	1.10 (0.66, 1.82)	0.72	–		0.92 (0.64, 1.32)	0.65	–		
*12:02	14 (1.1)	58 (1.2)	27 (1.5)	0.83 (0.33, 2.10)	0.70	–		0.93 (0.53, 1.66)	0.81	–		
*13:02	45 (3.4)	193 (4.0)	97 (5.3)	0.71 (0.42, 1.19)	0.19	–		0.73 (0.53, 1.0)	0.73	–		
*14:03	16 (1.2)	52 (1.1)	20 (1.1)	0.76 (0.33, 1.76)	0.53	–		0.78 (0.42, 1.46)	0.44	–		
*14:05	22 (1.7)	53 (1.1)	30 (1.7)	0.95 (0.46, 1.98)	0.89	–		0.61 (0.34, 1.07)	0.084	–		
*14:06	11 (0.8)	68 (1.4)	21 (1.2)	0.55 (0.22, 1.39)	0.21	–		1.08 (0.60, 1.95)	0.80	–		+
*14:54	44 (3.4)	109 (2.3)	48 (2.6)	0.92 (0.53, 1.59)	0.77	0.012	0.002	0.59 (0.39, 0.91)	0.017	–		
*15:01	181 (13.8)	277 (5.8)	127 (7.0)	2.90 (2.03, 4.13)	4.2×10^{-9}	2.4×10^{-10}	0.117	0.89 (0.68, 1.17)	0.41	–		
*15:02	113 (8.6)	414 (8.6)	246 (13.5)	0.60 (0.43, 0.82)	0.0017	–		0.54 (0.43, 0.67)	3.7×10^{-8}	–		
Other alleles	44 (3.4)	97 (2.0)	59 (3.2)	–	–	–		–	–	–		
Total SE+	–	1907 (39.6)	430 (23.6)	–	–	–		2.29 (1.96, 2.68)	5.1×10^{-25}	5.4×10^{-34}	0.233	
Total SE–	–	2913 (60.4)	1392 (76.4)	–	–	–		–	–	–	–	

[a]Alleles with frequencies of <0.01 are not listed. [b]SE alleles were excluded collectively in the RPE analysis of RA.

TABLE 2 Genotypic association analysis of *HLA-DRB1* with disease susceptibility

DRB1 genotype	No. of subjects (%)		OR (95% CI)	*P*-value
	Cases	Controls		
SLE	$n = 656$	$n = 911$		
*08:02/*15:01	9 (1.4)	7 (0.8)	2.73 (1.00, 7.41)	0.041
*09:01/*15:01	49 (7.5)	14 (1.5)	7.42 (4.02, 13.71)	3.6×10^{-13}
*08:02/*09:01	21 (3.2)	11 (1.2)	4.05 (1.92, 8.53)	8.2×10^{-5}
*15:01/*15:01	4 (0.6)	5 (0.5)	1.69 (0.33, 7.95)	0.48
*15:01/X[a]	115 (17.5)	96 (10.5)	2.54 (1.86, 3.47)	2.8×10^{-9}
*08:02/*08:02	2 (0.3)	0 (0)	-	0.104
*08:02/X	47 (7.2)	46 (5.0)	2.17 (1.40, 3.35)	4.0×10^{-4}
*09:01/*09:01	28 (4.3)	25 (2.7)	2.37 (1.35, 4.16)	0.0020
*09:01/X	147 (22.4)	211 (23.2)	1.48 (1.14, 1.92)	0.0034
X/X	234 (35.7)	496 (54.4)	Reference	
RA	$n = 2410$	$n = 911$		
SE/*09:01	277 (11.5)	70 (7.7)	3.36 (2.49, 4.54)	3.4×10^{-16}
SE/SE	351 (14.6)	45 (4.9)	6.63 (4.71, 9.34)	1.1×10^{-31}
SE/Y[b]	928 (38.5)	270 (29.6)	2.90 (2.38, 3.54)	1.4×10^{-26}
*09:01/*09:01	90 (3.7)	25 (2.7)	3.03 (1.90, 4.83)	1.4×10^{-6}
*09:01/Y	370 (15.4)	166 (18.2)	1.86 (1.47, 2.35)	1.9×10^{-7}
Y/Y	394 (16.3)	335 (36.8)	Reference	

[a]X: alleles other than *DRB1**08:02, *09:01 and *15:01. [b]Y: alleles other than *DRB1*-SE and *09:01.

TABLE 3 *HLA-DRB1* genotype and the age at disease onset in SLE and RA patients

DRB1 genotype	Total patients			Only female patients		
	No. of subjects	Age at onset, mean (S.D.)	*P*-value[a]	No. of subjects	Age at onset, mean (S.D.)	*P*-value[a]
SLE						
*08:02/*15:01	9	26.7 (10.8)	0.45	8	24.6 (9.5)	0.28
*09:01/*15:01	44	24.3 (11.4)	4.3×10^{-4}	37	24.7 (11.4)	0.0037
*08:02/*09:01	15	33.7 (16.0)	0.63	13	36.3 (15.7)	0.15
*15:01/*15:01	3	27.3 (5.5)	0.87	1	22[b]	1.0
*15:01/X[c]	97	29.9 (13.5)	0.49	88	29.5 (12.8)	0.59
*08:02/*08:02	1	20[b]	1.0	1	20[b]	1.0
*08:02/X	39	31.5 (11.8)	0.57	35	31.4 (12.0)	0.48
*09:01/*09:01	20	30.4 (13.6)	0.99	18	28.2 (9.4)	0.83
*09:01/X	122	29.8 (14.4)	0.36	113	29.0 (13.5)	0.40
X/X	191	30.9 (12.9)	Reference	170	30.0 (11.7)	Reference
RA						
SE/*09:01	264	47.1 (14.2)	2.4×10^{-4}	217	45.8 (14.4)	2.3×10^{-4}
SE/SE	327	47.2 (13.9)	1.0×10^{-4}	256	46.4 (13.6)	0.0012
SE/Y[d]	871	50.2 (14.7)	0.19	709	48.9 (14.6)	0.17
*09:01/*09:01	90	48.5 (13.8)	0.086	76	47.2 (13.5)	0.080
*09:01/Y	335	49.0 (14.5)	0.025	273	48.0 (13.9)	0.056
Y/Y	376	51.4 (14.7)	Reference	316	50.3 (14.9)	Reference

[a]Mann–Whitney *U* test was used. [b]Data of a single patient. [c]X: alleles other than *DRB1**08:02, *09:01 and *15:01. [d]Y: alleles other than *DRB1*-SE and *09:01.

TABLE 4 Association of *HLA-DRB1* genotype and autoantibody status in SLE patients

DRB1 genotype	No. of positive/negative patients (positive rate %)	OR (95% CI)	*P*-value
Anti-dsDNA antibody			
*08:02/*15:01	9/0 (100)	-	1.0
*09:01/*15:01	41/1 (97.6)	2.23 (0.30–99.53)	0.69
*08:02/*09:01	16/1 (94.1)	0.87 (0.11–40.29)	1.0
*15:01/*15:01 or X[a]	93/9 (91.2)	0.56 (0.22–1.44)	0.23
*08:02/*08:02 or X	36/2 (94.7)	0.98 (0.20–9.61)	1.0
*09:01/*09:01 or X	119/22 (84.4)	0.30 (0.14–0.65)	0.0014
X/X	183/10 (94.8)	Reference	
Anti-Sm antibody			
*08:02/*15:01	1/6 (14.3)	0.39 (0.008–3.35)	0.67
*09:01/*15:01	11/24 (31.4)	1.07 (0.48–2.36)	0.87
*08:02/*09:01	6/10 (37.5)	1.40 (0.48–4.07)	0.54
*15:01/*15:01 or X	25/55 (31.3)	1.06 (0.59–1.90)	0.85
*08:02/*08:02 or X	13/20 (39.4)	1.51 (0.69–3.30)	0.30
*09:01/*09:01 or X	58/70 (45.3)	1.93 (1.18–3.15)	0.0084
X/X	46/107 (30.1)	Reference	

[a]X: alleles other than *DRB1**08:02, *09:01 and *15:01.

onset of disease as compared with Y/Y ($P = 1.0 \times 10^{-4}$ and 2.4×10^{-4}, respectively) (Table 3).

Association between *DRB1* and autoantibody profile in SLE

Next we examined the difference in autoantibody status between genotypes in SLE patients. Considering that the number of homozygotic patients for *DRB1**08:02, *09:01 and *15:01 was small, a dominant genetic model was used for this analysis. The *DRB1**09:01-bearing genotypes (*09:01/*09:01 or *09:01/X) showed a significant risk for anti-Sm ($P = 0.0084$, OR 1.93), while it showed a protective effect for anti-dsDNA ($P = 0.0014$, OR 0.30) (Table 4). No significant association with autoantibody status was observed for either *DRB1**15:01-bearing genotypes or the *DRB1**09:01/*15:01 heterozygote, although a lack of statistical power was likely.

The relationship observed between *DRB1**09:01 and the status of anti-dsDNA and anti-Sm in the SLE patients prompted us to test whether *HLA-DRB1* affected the autoantibody profiles in SLE. Previously To *et al.* [37] performed a cluster analysis of the autoantibody profiles in European SLE patients and showed that SLE patients could be divided into three distinct clusters: cluster A (anti-Sm and anti-RNP), cluster B (anti-dsDNA, anti-Ro and anti-La) and cluster C (anti-dsDNA, aCL and LAC). Cluster A showed a significantly higher positive rate of anti-Sm antibody and a lower rate of anti-dsDNA antibody than the two other clusters. Therefore we speculated that Japanese SLE patients could also be sub-clustered by autoantibody profiles, where *DRB1* alleles could be involved. We performed k-mean cluster analysis using autoantibody profiles containing anti-dsDNA, anti-Sm antibodies, aCL and LAC. Similar to the results reported, we found three distinct autoantibody clusters (Table 5). The prevalence of each autoantibody was statistically different among clusters, except for the anti-dsDNA antibody for which the difference was marginal. Then we compared each allele frequency of *DRB1**08:02, *09:01 and *15:01 among the three clusters and found that the frequency of *DRB1**09:01 significantly differed among the clusters ($P = 0.010$). In Cluster 1, characterized by a higher positivity rate of anti-Sm and a lower rate of anti-dsDNA, a higher allele frequency of *DRB1**09:01 and lower frequency of *DRB1**15:01 were observed as compared with the other clusters. The allele frequency of *DRB1**09:01 in Cluster 1 was significantly higher than that of Clusters 2 and 3 ($P = 0.0027$ and 0.035 by χ^2 tests, respectively) (Table 5). The approach of replacing missing data with the mean positive rate is one of the established methods [34], but might lead to false-positive findings. Therefore we also performed k-mean cluster analysis based on casewise deletion and identified similar cluster groups and *DRB1**09:01 frequency distributions among the groups. However, the difference of allele frequencies among the clusters was marginal ($P = 0.09$), suggesting that validation studies using larger sample sizes would be required.

Association of *DRB1* and ACPA status in RA and its interaction with smoking

We evaluated the association of *DRB1*-SE alleles as well as *DRB1**09:01 with ACPA status in our population. When genotype frequencies of *DRB1* were compared between ACPA-positive RA patients ($n = 1884$) and negative patients ($n = 501$), the SE-bearing genotypes (SE/SE or SE/Y) as well as *09:01-bearing genotypes (*09:01/*09:01 or *09:01/Y) conferred significantly increased risk of the appearance of ACPA (Table 6). A significant gene dosage effect of SE was observed for the association with ACPA status (SE/SE vs SE/Y, $P = 6.2 \times 10^{-6}$, OR 2.60), while that of *DRB1**09:01 was not significant (*09:01/

Table 5 Cluster analysis of autoantibody profiles in SLE patients

	Cluster 1 Sm ($n=114$)	Cluster 2 dsDNA only ($n=306$)	Cluster 3 dsDNA/CL/LAC ($n=142$)	*P*-value
Autoantibody positivity (%)				
Anti-dsDNA	87.6	91.4	95.4	0.063
Anti-Sm	100	10	38.4	<0.001
aCL	9.2	12.3	100	<0.001
LAC	12.6	13.8	44.1	<0.001
Allele frequency (%)				
*15:01	10.1	14.9	16.2	0.12
*08:02	6.6	5.4	7.4	0.49
*09:01	27.6	18.2	19.7	0.01

CL: cardiolipin.

Table 6 Association of *HLA-DRB1* genotype and ACPA status in RA patients

DRB1 genotype	Smoking status	No. of patients (%) ACPA positive ($n=1884$)	ACPA negative ($n=501$)	OR (95% CI)	*P*-value
SE/*09:01		238 (12.6)	34 (6.8)	4.93 (3.27, 7.44)	1.2×10^{-15}
SE/SE		320 (17.0)	27 (5.4)	8.35 (5.37, 12.98)	1.8×10^{-25}
SE/Y[a]		756 (40.1)	166 (33.1)	3.21 (2.47, 4.17)	4.0×10^{-19}
*09:01/*09:01		71 (3.8)	18 (3.6)	2.78 (1.60, 4.84)	2.0×10^{-4}
*09:01/Y		269 (14.3)	94 (18.8)	2.02 (1.48, 2.75)	7.6×10^{-6}
Y/Y		230 (12.2)	162 (32.3)	Reference	
		$n=1796$	$n=474$		
SE/*0901					
	Ever	67 (3.7)	6 (1.3)	7.60 (3.17, 18.19)	2.7×10^{-7}
	Never	160 (8.9)	26 (5.5)	4.19 (2.57, 6.81)	1.8×10^{-9}
SE/SE					
	Ever	101 (5.6)	5 (1.1)	13.74 (5.40, 34.94)	1.6×10^{-11}
	Never	209 (11.6)	21 (4.4)	6.77 (4.04, 11.34)	3.7×10^{-15}
SE/Y					
	Ever	252 (14.0)	47 (9.9)	3.65 (2.44, 5.45)	8.4×10^{-11}
	Never	470 (26.2)	111 (23.4)	2.88 (2.08, 4.0)	1.1×10^{-10}
*0901/*0901					
	Ever	22 (1.2)	4 (0.8)	3.73 (1.21, 15.32)	0.011
	Never	44 (2.4)	12 (2.5)	2.49 (1.25, 4.96)	0.0076
*0901/Y					
	Ever	88 (4.9)	32 (6.8)	1.87 (1.16, 3.02)	0.0097
	Never	169 (9.4)	59 (12.4)	1.95 (1.32, 2.88)	7.5×10^{-4}
Y/Y					
	Ever	67 (3.7)	51 (10.8)	0.89 (0.57, 1.39)	0.62
	Never	147 (8.2)	100 (21.1)	Reference	

[a]Y: alleles other than *DRB1*-SE and *09:01.

*09:01 vs *09:01/Y, $P=0.27$, OR 1.38). There was no obvious dominant compound effect in neither SE/*09:01 nor *04:05/*09:01 (data not shown).

Next, we assessed gene–environment interactions between smoking and SE or *DRB1**09:01 alleles in developing ACPA. A larger effect on ACPA development was observed in ever smokers than in never smokers, when patients carried double SE alleles (double SE/ever smoker: OR 13.74; double SE/never smoker: OR 6.77) (Table 6). In order to investigate the interactions between smoking and SE or *DRB1**09:01, we examined the interaction terms in a logistic regression model. The logistic coefficient for the interaction term between SE and smoking was significant ($P=0.047$). However, that between *DRB1**09:01 and smoking was not significant ($P=0.34$), although lack of statistical power should be considered.

We also evaluated the interaction between smoking and *DRB1* alleles by calculating the three measures (RERI, AP and S). We found interactions only in SE alleles [double SE: RERI 7.34 (95% CI −6.55, 21.24), AP 0.51 (95% CI 0.01, 1.0), S 2.23 (95% CI 0.72, 6.94); single SE: RERI 1.32 (95% CI −0.35, 2.99), AP 0.31 (95% CI 0.01, 0.61), S 1.68 (95% CI 0.92, 3.06)], but not in *DRB1**09:01 (data not shown).

Discussion

In the present analysis of *HLA-DRB1* locus in Japanese SLE and RA patients using one of the largest cohorts ever examined in Asian populations, we demonstrated that *DRB1* alleles were associated with susceptibility as well as disease subphenotype, where the *DRB1**09:01 allele, prominent in Asian populations, has a significant impact in both diseases.

In SLE, *HLA-DRB1**15:01, *09:01, *08:02 and *04:01 were significantly associated with disease susceptibility in our population, where the association of *08:02 and *04:01 has not been previously described in Asian populations. In contrast to *15:01, we did not observe a significant association in *DRB1**15:02. As the *15:02 allele is rarely observed in European populations, and the majority of previous studies in Asian populations examined these alleles together as DR2 (or DR15) serotypes, the differential role of these two alleles had not been clearly defined. As for the amino acid sequence, these alleles only differ at position 86 (a valine for *DRB1**15:01 and a glycine for *DRB1**15:02). This amino acid constitutes the P1 pocket of the DRβ molecule and is considered to be critical for antigen presentation [38]. The influence of other HLA genes on the haplotype of *DRB1*, such as *DQB1*, should also be considered. In this context, the positive association of *DRB1**15:02 observed in Taiwanese and Thais [39, 40], unlike in our population, could be explained by the primary association of *DQB1**05:01, considering that the *DRB1**15:02 is in strong linkage disequilibrium with *DQB1**06:01, not with *DQB1**05:01, in a Japanese population.

In the genotypic comparison, we found that the compound heterozygote *DRB1**09:01/*15:01 showed a remarkable contribution to increased risk of SLE as well as earlier disease onset. A compound heterozygote of *DRB1* haplotypes may have an increased probability of presenting self-antigens, leading to increased predisposition and earlier onset of disease. Alternatively, the *trans*-complementing heterodimers formed by *DQA* and *DQB* genes on the haplotype of *DRB1**09:01 and *15:01 could also explain the increased effect of heterozygote, as was suggested in a study of Type 1 diabetes (T1D) [41]. In contrast to SLE, no obvious compound heterozygote effect of *DRB1*-SE/*09:01 (or *04:05/*09:01) was observed in RA, possibly because the stronger effect of SE alleles may dominate that of *DRB1**09:01 in RA.

We demonstrated a positive association of *DRB1**09:01 with anti-Sm antibody formation, but a negative association with anti-dsDNA antibody in SLE patients, implying that differential mechanisms involving different DR molecules underlie the appearance of these autoantibodies. A recent study in European patients reported that an SNP in the HLA class II region was associated with anti-dsDNA antibody-negative patients, which may support our findings [42]. To *et al.* [37] found that SLE patients could be grouped into several autoantibody patterns, and one of the groups was characterized by a higher positivity rate of anti-RNP and anti-Sm and a lower rate of anti-dsDNA antibodies. This grouping by autoantibody patterns was also applicable to the population we examined. In addition, as differential distribution of *DRB1* alleles was also observed among the groups in our population, combinations of the class II haplotypes should affect autoantibody production in SLE and may influence clinical subphenotypes of SLE patients.

The association of SE alleles in ACPA-positive RA has been well established in both European and Asian populations [14–16, 18, 19]. This suggests an essential role of the SE alleles in presenting citrullinated self-antigens. In addition, a gene–environment interaction between SE alleles and smoking has been demonstrated for the onset of RA [18, 19]. These suggest a pathological series of smoking, citrullination of self-proteins and antigen presentation by SE alleles [43]. However, whether this is also the case for *DRB1**09:01 is not clear. Two previous studies in Japanese populations suggested that *DRB1**09:01 but not SE alleles is associated with ACPA-negative RA [21, 22]. The study by Furuya *et al.* [21] indicated a significant association of *DRB1**09:01 with ACPA-negative RA ($P = 0.016$, OR 2.2). Another study by Terao *et al.* [22], in which the ACPA-negative patients (502 subjects) used in the present study were also included, showed a marginal association with ACPA-negative RA ($P = 0.062$, OR 1.16). However, the effect of *DRB1**09:01, independent of SE alleles, on ACPA-positive RA was not evaluated in these studies. Another study in a Korean population showed that *DRB1**09:01 made a significant contribution to the appearance of ACPA independently of SE ($P = 1.10 \times 10^{-3}$, OR 2.49) [18], which corresponds to our observation (Table 6). Taken together, this evidence suggests that *DRB1**09:01 confers risk for both ACPA-positive and ACPA-negative RA. Moreover, as no significant gene–environmental interaction was observed between smoking and *DRB1**09:01 for the appearance of ACPA, *DRB1**09:01 may not function in the same pathological series of SE alleles, as mentioned.

The amino acid sequence of *DRB1**09:01 at 70–74 (RRRAE) is different from that of the SE alleles. As both *DRB1**14:05 and *14:54, the only alleles that share the RRRAE epitope in the Japanese population, displayed a negative trend for the risk of RA, it may be unlikely that this epitope alone can explain the susceptibility of *DRB1**09:01 to RA. A recent study examining all possible epitopes in the DR molecule showed that two new epitopes ($VH^{11,13}$ and $LA^{67,74}$) in addition to the classical SE exhibited a predisposition to RA in European populations [44]. Haplotypes involving *DRB1**09:01 have been reported to be associated with autoimmune diseases other than RA and SLE, including T1D, Graves' disease,

mixed CTD and microscopic polyangiitis in Asian populations [45–48], suggesting that these autoimmune diseases share the same pathological pathway. Although the DR9 molecule itself may play an important role in disease pathogenesis, it is also known that *DRB1**09:01 is in linkage with *DRB4*, a paralogue of *DRB1* that also encodes DRβ chains. *DRB4* is only present in haplotypes of *DRB1**04, *07 and *09. Its presence has been found to be associated with the progression of RA [49] and the presence of ACPA [50]. Therefore the association of *DRB1**09:01 could be secondary to the effect of *DRB4* in RA. In addition, the exclusive presence of *DRB4* in *DRB1**04 among SE alleles might explain the difference in the effect size between *DRB1**04 alleles and other SE alleles (i.e. *01:01).

In conclusion, we identified *HLA-DRB1* alleles associated with SLE and RA in a Japanese population and demonstrated a shared susceptibility of *DRB1**09:01 between the diseases as well as its effect on autoantibody production. Further studies, such as structural analysis of the *DRB1**09:01 product itself and analysis of other candidate causal variants of the *DRB1**09:01 haplotype, are needed to clarify the exact role of the *DRB1**09:01 haplotype in disease pathogenesis.

Rheumatology key messages

- A large-scale association study identified *HLA-DRB1* alleles associated with SLE and RA in the Japanese population.
- *HLA-DRB1**09:01 allele is associated with susceptibility to both SLE and RA in the Japanese population.
- *HLA-DRB1**09:01 allele affects autoantibody profiles of SLE and RA in Japanese patients.

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Clinical vignette

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Critical reversible bilateral internal carotid artery stenosis associated with SLE

A 44-year-old teacher presented with intense headache. She had mouth ulcers and dry mouth. Investigations revealed positive ANA of 1:160, DNA antibodies of 343 units (positive > 60) and aCL IgG of 26.4 units (<17). Magnetic resonance angiography (MRA) revealed a long irregular stenosis of the left internal carotid artery and a 20% stenosis of the right internal carotid artery (Fig. 1A). Five days later an MRA with different sequences revealed an irregular 80% stenosis of the left internal carotid artery and an irregular stenosis of the right internal carotid artery, a new finding, indicating rapid progression of the disease (Fig. 1B).

The oral ulcers, positive ANA, DNA and aCL IgG antibodies were consistent with SLE [1]. We started prednisolone 60 mg/day and aspirin 75 mg/day and the headache resolved. HCQ and MTX were added. Prednisolone was gradually tapered to 5 mg/day and the MTX was gradually increased to 20 mg weekly. The most recent MRA showed mild irregularity of the left internal carotid artery and a normal right internal carotid artery.

Our patient had headache and critical rapidly progressive reversible bilateral internal carotid artery stenosis associated with SLE. With prompt immunosuppression her symptoms resolved and the radiographic appearance of the carotid arteries improved.

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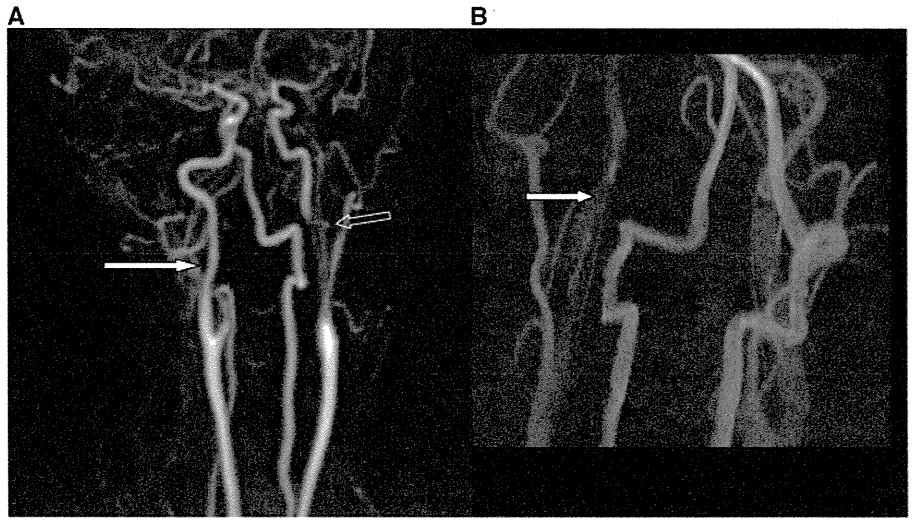
FIG. 1 MRA scans of the carotids demonstrating critical rapidly progressive bilateral internal carotid artery stenosis.



(**A**) MRI/MRA brain and carotids (19 September 2010) showing irregular stenosis of the left internal carotid artery (ICA, open arrow) with an almost normal right internal carotid artery (white arrow). (**B**) Non-contrast MRA 3DI of the carotids with different sequences (24 September 2010) demonstrating irregular stenosis of the right ICA (white arrow), which is a new finding in comparison with the scan in (A).

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Review Article

An Innovative Method to Identify Autoantigens Expressed on the Endothelial Cell Surface: Serological Identification System for Autoantigens Using a Retroviral Vector and Flow Cytometry (SARF)

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Autoantibodies against integral membrane proteins are usually pathogenic. Although anti-endothelial cell antibodies (AECAs) are considered to be critical, especially for vascular lesions in collagen diseases, most molecules identified as autoantigens for AECAs are localized within the cell and not expressed on the cell surface. For identification of autoantigens, proteomics and expression library analyses have been performed for many years with some success. To specifically target cell-surface molecules in identification of autoantigens, we constructed a serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF). Here, we present an overview of recent research in AECAs and their target molecules and discuss the principle and the application of SARF. Using SARF, we successfully identified three different membrane proteins: fibronectin leucine-rich transmembrane protein 2 (FLRT2) from patients with systemic lupus erythematosus (SLE), intercellular adhesion molecule 1 (ICAM-1) from a patient with rheumatoid arthritis, and Pk (Gb3/CD77) from an SLE patient with hemolytic anemia, as targets for AECAs. SARF is useful for specific identification of autoantigens expressed on the cell surface, and identification of such interactions of the cell-surface autoantigens and pathogenic autoantibodies may enable the development of more specific intervention strategies in autoimmune diseases.

1. Introduction

Inappropriate humoral and cellular immune responses mediate the tissue damage in autoimmune diseases, and the outcome of an autoimmune disease is influenced mainly by the tissue distribution of target self antigens [1]. The pathogenesis of most autoimmune diseases is highly complex and involves multiple cellular and humoral pathways. One part of the humoral arm of the immune assault is caused by autoantibodies, and the mechanisms of autoimmune damage mediated by many autoantibodies have been studied [2]. Clinically, specific autoantibodies are critical for the diagnosis, classification, and monitoring of autoimmune diseases [2].

Autoantibodies cause damage through a number of mechanisms, including the formation of immune complexes, cytolysis or phagocytosis of target cells, and interference with cellular physiology [3]. The cellular localization of the target antigen is believed to play a critical role in the pathogenetic potential of autoantibodies [4]. Intracellular proteins are preferential targets of autoantibodies in autoimmune diseases, but many questions remain unanswered regarding how autoantibodies against intracellular proteins play pathogenic roles. In contrast, it is generally accepted that autoantibodies against integral membrane proteins are usually pathogenic [1]. Some autoantibodies have been clearly confirmed to be pathogenic in several autoimmune diseases, and a model

Table 1: Prevalence of anti-endothelial cell antibodies.

Disease	% of positive sera
Systemic lupus erythematosus	15–85
Rheumatoid arthritis	0–87
Mixed connective tissue disease	33–45
Systemic sclerosis	15–84
Polymyositis/dermatomyositis	44–64
Antiphospholipid syndrome	0–64
Sjögren's syndrome	24-25
Polyarteritis nodosa	50–56
Microscopic polyangiitis	2–60
Granulomatosis with polyangiitis	19–80
Eosinophilic granulomatosis with polyangiitis	50–69
Takayasu arteritis	54–95
Giant-cell arteritis	33–50
Behçet's disease	14–80
Kawasaki disease	65

for customized and specific therapeutic approaches against a highly pathogenic subset of autoantibodies using small molecules have been reported [5].

In 1971, Lindqvist and Osterland first described autoantibodies to vascular endothelium based on indirect immunofluorescence (IIF) experiments [6]. These autoantibodies were called anti-endothelial cell antibodies (AECAs) and were defined as autoantibodies targeting antigens present on the endothelial cell (EC) membrane [7]. As target antigens of AECAs are present on the ECs, which are always in contact with these circulating antibodies, AECAs have the potential to induce vascular lesions directly. Here, we present a review of AECAs and a novel method for identification of cell-surface autoantigens.

2. AECAs

2.1. AECAs and Disease. The presence of AECAs has been reported in patients with a wide variety of diseases, including collagen diseases (Table 1), inflammatory bowel disease, diabetes, thyroid diseases, thrombotic thrombocytopenic purpura, primary sclerosing cholangitis, interstitial lung disease, chronic obstructive lung disease, uveoretinitis, renal transplantation, Susac syndrome, masked hypertension, and atherosclerosis [8–23]. AECAs are correlated to disease activity in some collagen diseases, and are thought to be critical especially for vascular lesions in collagen diseases [23]. In addition, AECAs have been shown to be clinical signs of vasculitis in patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [24]. AECAs were also reported to play critical roles in several pathophysiological conditions, including pulmonary hypertension, digital ulcers, and gangrene [21, 22].

AECAs are detected even in healthy subjects [25, 26]. These natural autoantibodies interact with living ECs with lower affinity as compared to pathologic AECAs, and their antigens are highly conserved protein families. They contribute to modulate endothelial function with protective anti-inflammatory and anti-thrombotic functions [26].

2.2. Detection and Identification of AECAs. Methods for detection of AECAs have not been standardized, and a number of methods have been reported, including IIF, cell-based-enzyme linked immunosorbent assay (ELISA), flow cytometry, radioimmunoassay, western blotting (WB), and immunoprecipitation [22, 23]. As these each of methods have advantages and disadvantages, use of different technical approaches to obtain more robust data is recommended [7].

Human umbilical vein endothelial cells (HUVECs) are commonly used as a substrate, but antigen patterns of ECs differ among other ECs, passage numbers, and culture conditions [27]. It is also important whether ECs are fixed or not because fixation induces permeabilization of the EC membrane, and intracellular antigens become accessible to antibodies [22]. The results of AECA positivity were therefore not considered in the same light, and the prevalence of AECAs differed among studies (Table 1). Miura et al. recently reported a novel solubilized cell-surface protein capture ELISA for detection of AECAs [28], and further evaluation and standardization are needed.

2.3. Pathogenicity of AECAs. An experimental animal model for pathogenicity of AECAs was reported by Damianovich et al. [29]. In their experiment, BALB/c mice were actively immunized with the purified AECAs from a patient with granulomatosis with polyangiitis. Three months after a booster injection with human AECAs, mice developed endogenous AECAs, and histological examination of lungs and kidneys revealed both lymphoid cell infiltration surrounding arterioles and venules.

AECAs have been shown to be correlated with disease activities, and have the potential to induce vascular lesions because their targets are expressed on ECs that are readily accessible to these circulating antibodies. AECAs are also considered to play roles in the development of pathological lesions by a number of methods as described below [22, 23, 30–32].

The first is the cytotoxicity of ECs through complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). CDC activity of AECAs was reported in patients with SLE, Takayasu arteritis, hemolytic-uremic syndrome, and Kawasaki disease [7, 24, 33–35]. Recently, we confirmed that fibronectin leucine-rich transmembrane protein 2 (FLRT2) is a novel target antigen of AECAs in SLE, which exerts direct cytotoxic effects through CDC [9].

The second is the induction of coagulation. AECAs may exhibit procoagulant effects by the production of tissue factor in SLE and the release of heparin sulfate in systemic sclerosis (SSc) [36, 37].

The third is the induction of apoptosis. AECAs may induce EC apoptosis through CD95 or cross-reaction with anti-phospholipid antibodies [38–40]. Dieudé et al. reported that heat-shock protein (Hsp60) bound to ECs and induced phosphatidylserine exposure and then apoptosis [41].

Margutti et al. identified antibodies to the C-terminus of Ral-binding protein 1 (RLIP76), and these autoantibodies induced oxidative stress-mediated EC apoptosis [42].

The fourth is the activation of ECs. AECAs were reported to induce the secretion of interleukin (IL)-1β, IL-6, IL-8, and monocyte chemotactic protein-1, (MCP-1), and the expression of adhesion molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) [8, 24, 31], which cause leukocyte recruitment and adhesion.

Alard et al. reported that recognition of cell-surface adenosine triphosphate (ATP) synthase in the low pH microenvironment contributes to intracellular acidification of ECs, which may induce cell death and trigger inflammation [43].

As described above, there is a great deal of evidence that AECAs play pathogenic roles in collagen diseases. Identification of targets of AECAs is required because (a) antigen-specific detection systems are important for establishing diagnostic tools and standardization of AECAs measurement, (b) identification will enable thorough analysis of the pathogenicity of AECAs, and (c) AECA-autoantigen interactions may be good targets for specific therapeutic approaches against highly pathogenic autoantibodies.

3. Technologies for Identification of Autoantigens for AECAs

The prevalence of AECAs varies according to the type of ECs used for detection [44]. It was demonstrated that AECAs cross-react with human fibroblasts [45], and partial inhibition of AECA activity was documented by absorption of the AECA-containing sera with mononuclear cells [8]. It was also reported that a structure shared by platelets and ECs was recognized by a subset of AECAs [46]. These data suggested that the target antigens of AECAs may include not only EC-specific but also non-EC-specific molecules.

Target antigens of AECAs have been investigated intensively, but they are heterogeneous, and the following classification of target antigens was proposed: membrane component, ligand-receptor complex, and molecule adhering to the plasma membrane [8]. The EC autoantigens may be either constitutively expressed or translocated from intracellular compartment to membrane by cytokines, such as IL-1 and tumor necrosis factor α (TNFα), or physical effects [8, 47]. The reported autoantigens and their pathogenicities are summarized in Table 2 [7, 9, 22–24, 42, 43, 47–56].

Several molecules can bind to ECs and are called "planted antigens" for AECA presumably via charge-mediated mechanisms, a DNA-histone bridge, or a specific receptor. Myeloperoxidase, DNA, and β2-glycoprotein I (β2-GPI) are thought to adhere to ECs during incubation of ECs with sera from patients. Extracellular matrix components, such as vimentin, may also be target antigens for AECAs [57]. Proteinase 3 (PR3) could represent another potential cryptic target antigen [58]. PR3 has been maintained to migrate to the plasma membrane of ECs, following stimulation [8].

As methods for identification of target antigens of AECAs, immunoprecipitation and WB of glycoproteins from the EC membrane with AECA-positive sera have been used [8, 23]. Although numerous protein bands were reported as candidates for target antigens by this method, some of the bands were considered to be artifacts [8], and further identification of given bands was also sometimes difficult.

Alternative methods have been developed, such as proteomics analysis using two-dimensional electrophoresis followed by matrix-assisted laser desorption ionization time of flight mass spectrometry [8, 23] and expression libraries [8, 42, 56].

Proteomics analysis identified vimentin, Hsp60, voltage-dependent anion-selective channel 1 (VDAC-1), peroxiredoxin 2, and ATP synthase as targets for AECAs [41, 43, 48–50]. Expression libraries also identified tropomyosin, T-plastin, and RLIP76 [42, 56], and these technologies are therefore promising. The problem is that most of the molecules reported to date as targets for AECAs are intracellular proteins (Table 2) although AECAs must be directed against the cell surface. These two methods are not specific for detecting cell-surface molecules rather than intracellular molecules. In addition, extraction of some membrane proteins has been reported to be difficult in proteomics analysis, and this may make it difficult to identify such proteins as AECA targets [7].

To overcome this problem, we constructed a novel expression cloning system for specific identification of cell-surface antigens [9], which we call serological identification system for autoantigens using a retroviral vector and flow cytometry (SARF) (Figure 1), and we have confirmed that this system is useful to identify autoantigens expressed on the EC surface [9].

4. Strategy for Identification of Cell-Surface Autoantigens: SARF

4.1. Generation of HUVEC cDNA-Expressing Cells (Figure 1(a)). Our strategy to identify AECA target molecules involves use of a retroviral vector system and flow cytometry [9]. As described previously, antigen patterns of ECs differ among other ECs [27]. Because we used HUVECs as a substrate for AECAs measurement, we generated a HUVEC cDNA library using HUVECs grown in the same conditions as for AECAs measurement and ligated it into the retroviral vector, pMX [59]. Then, the HUVEC cDNA library in pMX was retrovirally transfected into the YB2/0 rat myeloma cell line [60]. As the localization of cellular molecules depends on their structures, only cell-surface molecules are expressed on the surface of YB2/0 cells transfected with the HUVEC cDNA library.

4.2. Sorting of Cells Expressing Cell-Surface Autoantigens (Figure 1(b)). AECAs can bind only to cell-surface molecules in flow cytometry. Therefore, sorting of IgG-binding cells can concentrate and isolate cells expressing target molecules for AECAs on the cell surface. After staining of HUVEC cDNA-expressing YB2/0 cells with AECA IgG and secondary antibody, cells with strong fluorescent signals are sorted by flow cytometry. This step of sorting is repeated for several rounds to concentrate AECA IgG-binding cells. After concentration,

Table 2: Reported target antigens of anti-endothelial cell antibodies.

Disease	Target antigen	Pathogenicity
Systemic lupus erythematosus	DNA-DNA-histone	
	Ribosomal P protein PO	
	Ribosomal protein L6	
	Elongation factor 1-alpha	
	Adenylyl cyclase-associated protein	
	Profilin 2	
	Plasminogen activator inhibitor	
	Fibronectin	
	Heparan sulfate	
	β2-glycoprotein I	
	Heat-shock protein 60 (Hsp 60)	Apoptosis
	Heat-shock protein 70 (Hsp 70)	
	Fibronectin leucine-rich transmembrane protein 2 (FLRT2)	Complement-dependent cytotoxicity
Mixed connective tissue disease	Voltage-dependent anion-selective channel 1 (VDAC-1)	
Systemic sclerosis	Topoisomerase I	
	Centromere protein B (CENP-B)	
Vasculitis	Proteinase 3	
	Myeloperoxidase	
	Peroxiredoxin 2	Cytokine secretion
	Adenosine triphosphate (ATP) synthase	Intracellular acidification
Microscopic polyangiitis	Human lysosomal-associated membrane protein 2	
Behçet's disease	Alpha-enolase	
	C-terminus of Ral-binding protein 1 (RLIP76)	Apoptosis
Kawasaki disease	Tropomyosin	
	T-plastin	
Transplantation	Vimentin	
	Keratin-like protein	
Thrombotic thrombocytopenic purpura	Glycoprotein CD36	
Heparin-induced thrombocytopenia	Platelet factor 4 (PF4)	
	Heparin sulfate	

several cell clones can be established from the AECA IgG-binding cell population by the limiting dilution method.

4.3. Identification of Novel Cell-Surface Autoantigens. After polymerase chain reaction (PCR) amplification and cloning of HUVEC cDNA inserted into the genomic DNA of cloned cells, DNA sequencing can be performed followed by BLAST analysis, which enables the identification of the inserted cDNA. In this step, microarray analysis is an alternative method to identify the inserted cDNA. Next, an expression vector of the identified cDNA is generated and transfected into a cell line that does not express the identified protein. Finally, it is necessary to confirm that AECA IgG shows binding activity to 7-amino-actinomycin D-(7-AAD-) negative identified protein-expressing cells. If the binding activity is confirmed, it can be concluded that the identified protein is a novel autoantigen.

5. Novel Autoantigens Identified by SARF

5.1. FLRT2. We reported the membrane protein FLRT2 as a novel autoantigen of AECAs in patients with SLE based on results obtained using SARF [9]. FLRT2 is type I transmembrane protein located on the plasma membrane [61]. FLRT2 was shown to be expressed in the pancreas, skeletal muscle, brain, and heart with Northern blotting [61], and we confirmed the expression of FLRT2 on HUVECs and other ECs by flow cytometry and IIF [9]. Anti-FLRT2 antibody activity accounted for 21.4% of AECAs in SLE, and anti-FLRT2 activity was significantly correlated with low levels of complement C3, C4, and CH50 [9]. Anti-FLRT2 antibody induced CDC against FLRT2-expressing cells including ECs, indicating that anti-FLRT2 autoantibody may exhibit direct pathogenicity [9].

5.2. ICAM-1. As AECAs can be detected in patients with collagen diseases, especially SLE, RA, and Takayasu arteritis [9], we further attempted to identify the autoantigens using SARF. One sample (X10-3) from an RA patient showed strong AECA activity (Figure 2(a)), and we selected this serum sample as the prototype of AECA for subsequent cell sorting. Using SARF, HUVEC cDNA-expressing YB2/0 cells were stained with X10-3 IgG and fluorescein isothiocyanate-(FITC-) conjugated secondary antibody, and cells with strong