Genetic Background of Japanese Patients with Antineutrophil Cytoplasmic Antibody-Associated Vasculitis: Association of HLA-DRB1*0901 with Microscopic Polyangiitis

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ABSTRACT. ***Objective.*** To examine association of 8 candidate genes with susceptibility to antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) in Japanese patients. Little is known on the genetic background of AAV in Japanese patients mainly because of the difficulty in collecting a sufficient number of samples for the genetics study.

Methods. Sixty-nine patients, including 50 with microscopic polyangiitis (MPA), were recruited in a multicenter study. Among them, 64 patients were positive for myeloperoxidase (MPO)-ANCA. Associations of *HLA-DRB1*, tumor necrosis factor-α promoter (*TNF*), TNF receptor 2 (*TNFR2*), Fcγ receptor IIa (*FCGR2A*), IIb (*FCGR2B*), IIIa (*FCGR3A*), IIIb (*FCGR3B*), and CTLA-4 (*CTLA4*) polymorphisms were examined in a case-control analysis.

Results. A significant association of HLA-DRB1*0901 with MPA (p = 0.0037, OR 2.44, 95% CI 1.33–4.46), as well as with MPO-ANCApositivity (p = 0.0014, OR 2.44, 95% CI 1.41–4.22), was detected. There was no difference in the *TNF* promoter haplotype frequencies between patients with MPA and controls, excluding the possibility that the association of DRB1*0901 was secondarily caused by linkage disequilibrium with *TNF*. No association was observed for *TNFR2*, *FCGR*, or *CTLA4* with MPA, nor with the presence of MPO-ANCA, although the combined genotype *FCGR2A-131H/H* and *3A-176F/F* was increased in patients with MPA (p = 0.025).

Conclusion. There was an association of HLA-DRB1*0901 with MPA and MPO-ANCA positive vasculitis in Japanese patients. (J Rheumatol 2003;30:1534–40)

Key Indexing Terms:
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Microscopic polyangiitis (MPA), Wegener's granulomatosis (WG), and Churg-Strauss syndrome (CSS) all involve necrotizing vasculitis of small-to-medium-size vessels characteristically associated with antineutrophil cytoplasmic antibody (ANCA)[1], collectively referred to as ANCA-associated vasculitis (AAV). Although the etiology of AAV is unclear, presence of multiplex families[2] and difference in disease prevalence among populations[3] suggest involvement of genetic predisposition in the pathogenesis. To date, little is known about the actual genetic polymorphisms that confer susceptibility to AAV, and only a few genes have been consistently shown to be associated with AAV in 2 or more populations[4].

Association of HLA genes with AAV[5,6], WG[7-10], and MPO-ANCA associated glomerulonephritis[11] has been reported. Although the results are variable, 2 or more groups have found positive association of DR4[5,6] and DR9[9-11], and negative association of DR13[6,8]. Tumor necrosis factor (TNF-α) is functionally relevant in AAV[12], and the gene coding for TNF-α (*TNF*) is in linkage disequilibrium with HLA-DR. Thus, one plausible hypothesis is that the func-

tional polymorphisms in *TNF* are primarily important in the susceptibility, and the association of HLA-DR is caused by linkage disequilibrium with *TNF*. One study from Germany demonstrated the association of a microsatellite marker located in the proximity of *TNF* region with proteinase 3 (PR3)-ANCA positive patients[6], but a single nucleotide polymorphism (SNP) at TNF-α promoter –308 was not associated[6,13]. These results seem to suggest possible association of *TNF* polymorphisms other than –308, which has not yet been investigated.

There are 2 major receptors for TNF-α, TNF receptor 1 (TNFR1, TNFRSF1A) and TNFR2 (TNFRSF1B). *TNFR2* has a couple of common SNP that result in amino acid substitutions, one of which, M196R, has been shown to be associated with systemic lupus erythematosus (SLE)[14,15] and rheumatoid arthritis (RA)[16]. In addition, mice overexpressing *TNFR2* have shown systemic inflammation resembling vasculitis[17].

Low affinity Fcγ receptor genes (*FCGR*), located on 1q23, are known to possess functional polymorphisms. FcγRIIa and IIIb are expressed in neutrophils, while IIIa is expressed in monocytes and NK cells, all of which transmit activation signals. FcγRIIb is expressed in B cells and monocytes, transmits inhibitory signals, and is involved in the regulation of cell activation[18]. Polymorphisms of *FCGR* genes have been repeatedly implicated in the susceptibility to SLE[19], and we recently reported a new SNP coding for a non-synonymous substitution in *FCGR2B* and its association with SLE[20]. In AAV, where activation of neutrophils and monocytes plays a crucial role, *FCGR* genes are considered strong candidates. Increase in the frequency of *FCGR3B*-NA1/1 homozygotes was reported in myeloperoxidase (MPO)-ANCA positive patients in the UK[21], while a combination of *FCGR2A*-131R/R and *3A*-176F/F genotypes, both associated with decreased immune complex clearance, has been shown to be associated with recurrence of WG in a Dutch population[22].

Finally, CTLA-4, a negative regulator expressed in activated T cells, has an AT repeat polymorphism within the 3' untranslated region (3'UTR). Recently, an association of the *CTLA4* polymorphism with WG was described[13] and confirmed[23]. This polymorphism has not been examined for association with MPA.

In the genetic study of complex diseases, it is important to compare the association among populations with different genetic backgrounds. Association of HLA-DR9 has been reported in the Japanese population in WG[9] and in MPO-ANCA-associated glomerulonephritis[11]. However, the sample sizes analyzed in these studies are fairly small (16 and 12, respectively), and the association needs to be confirmed in a larger-scale study. No studies have been done on other candidate genes in Japanese, mainly because of the difficulty in recruiting a sufficient number of patients. We describe the results of the first multicenter collaborative study on the association of the above candidate genes with Japanese patients with AAV.

MATERIALS AND METHODS

Patients and controls. This study was a project of the Research Committee on Intractable Vasculitides, Ministry of Health, Labour, and Welfare of Japan. Sixty-nine patients positive for ANCA who were diagnosed with systemic vasculitis were recruited from 15 clinical centers in central Japan.

The classification of vasculitides was essentially based on the Chapel Hill Conference definitions[24]. Since those definitions do not provide diagnostic or classification criteria, and American College of Rheumatology classification criteria do not include MPA, the diagnosis of MPA was made in accord with the Japanese criteria proposed by the Research Committee in 1998 (Table 1), although these criteria have not been generally agreed upon in the international scientific community. In most cases, the diagnosis was confirmed by biopsy.

The demographics of the patients are summarized in Table 2. ANCA was measured using an enzyme linked immunosorbent assay (ELISA) as described[25]. Briefly, the MPO-ANCA ELISA (Nissho Co., Osaka, Japan) used MPO extracted from human neutrophil cytoplasmic α granules by Wieslab (Lund, Sweden) as the solid-phase antigen. After the addition of diluted serum samples, bound antibody was detected using alkaline-phosphatase labeled anti-human IgG. PR3-ANCA ELISA (BioCarb Diagnostics, Lund, Sweden) was performed similarly, except that the PR3 extracted from human neutrophil cytoplasmic α granule was used as the solid-phase antigen. Five of the 69 samples (7.2%) were positive for both MPO-ANCA and PR3-ANCA. The presence of a small proportion of double positive samples has been previously noted in a European study[26].

The controls were healthy individuals, 167 men and 136 women between the ages of 21 and 61 (mean ± SD 35.3 ± 9.9), and included researchers, laboratory workers, and students recruited around Tokyo area. The control data for *HLA-DRB1*[27], *TNF*, *TNFR2*[28], *FCGR2A*, *2B*, *3A*, and *3B*[20] were adopted from our previous studies. Central Japan has been shown to be relatively homogeneous with respect to genetic background, allowing for a case-control approach[29]. This study was reviewed and approved by the Ethics Committees of the participating institutions.

Genotyping methods. Genotyping was performed using genomic DNA isolated from peripheral blood leukocytes. *HLA-DRB1* typing was done using a polymerase chain reaction (PCR)-microtiter plate hybridization (MPH) technique[30]. *TNF* promoter, *TNFR2*-M196R[31], and *FCGR2B*-I232T[20] were genotyped based on fluorescence resonance energy transfer (FRET) technology, using LightCycler™ (Roche Diagnostics, Mannheim, Germany). *FCGR2A*-H131R, *FCGR3A*-F176V, and *FCGR3B*-NA1/2 polymorphisms were genotyped using PCR-restriction fragment length polymorphism (RFLP), PCR-single strand conformation polymorphism (SSCP), and a PCR sequence-specific primer (SSP), respectively[20]. *CTLA4* 3'UTR $(AT)_n$ polymorphism was genotyped using PCR, sequenced, and then analyzed using Genescan™ software (Applied Biosystems, Foster City, CA, USA) as described[13], with slight modifications.

Statistical analysis. The genotype frequency, allele (haplotype) carrier frequency, and allele frequency were compared between patients and healthy controls using the chi-square or Fisher's exact test. Fisher's exact test was used when one or more variables within 2 × 2 tables were less than 5. Pvalues < 0.05 were regarded as statistically significant. Corrections for multiple comparisons were performed by multiplying the p value by the number of alleles. All statistical analyses were done using StatView J5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Sixty-nine patients with ANCA-positive systemic vasculitis were recruited, among whom 64 were positive for MPO-ANCA (Table 2). Fifty patients, all positive for MPO-ANCA, were classified as having MPA, 8 as CSS, 8 as WG,

Table 1. Modified diagnostic criteria for microscopic polyangiitis (microscopic PN) proposed by the Research Committee on Intractable Vasculitides, the Ministry of Health, Labour and Welfare of Japan, in 1998. (Originals in Japanese; excerpted and translated by the authors.)

Major manifestations
Rapidly progressive glomerulonephritis
Pulmonary hemorrhage or interstitial pneumonitis
Organ symptoms other than kidney and lung: purpura, subcutaneous bleeding, gastrointestinal bleeding, mononeuritis multiplex, etc.
Major histopathological findings
Necrosis and perivascular inflammatory cell infiltration of arterioles, capillaries, and postcapillary venules.
Major laboratory data
MPO-ANCApositive
Positive CRP
Proteinuria, hematuria, elevation of serum BUN, and creatinine
Chest radiograph: infiltrative shadow (alveolar hemorrhage), interstitial pneumonitis
Diagnosis
Definite
Two or more of the major manifestations and positive histopathological findings
Both of the first 2 major manifestations and the presence of MPO-ANCA
Probable
All 3 major manifestations
One of the major manifestations and the presence of MPO-ANCA.

Table 2. Patient demographics.

Disease	n	Male: Female	Age, yrs, mean ± SD	MPO-ANCA n	PR3-ANCA n
Microscopic polyangiitis	50	19:31	66.1 ± 11.4	50	3
Churg-Strauss syndrome	8	6:2	62.1 ± 10.1	8	0
Wegener's granulomatosis	8	5:3	45.8 ± 15.8	3	7
Classical polyarteritis nodosa	3	3:0	62.0 ± 18.5	3	0

and 3 as classical polyarteritis nodosa (cPN). Based on consideration of the sample numbers appropriate for association studies, subsequent analyses were focused on MPA and MPO-ANCA positive patients.

HLA-DRB1. The carrier frequency of *HLA-DRB1* alleles is shown in Table 3. The frequency of HLA-DRB1*0901 carriers was significantly increased in patients with MPA ($p = 0.0037$). HLA-DRB1*1101 was also seen more frequently in patients with MPA ($p = 0.039$). An increase of DRB1*0901 was also observed in MPO-ANCA positive patients other than those with MPA (7 of 14 patients were DRB1*0901 carriers); the statistical significance of the HLA-DRB1*0901 association was therefore more remarkable in the MPO-ANCA positive group ($p = 0.0014$, $p_{corr} = 0.029$).

When this association was analyzed with respect to the presence or absence of renal and pulmonary diseases, DRB1*0901 carrier frequency was not substantially different from that of the total MPO-ANCA positive group (data not shown).

TNF, TNFR2, FCGR, and CTLA4. We examined the association of TNF-α promoter haplotypes. There was only one patient with the *TNF –308A* allele, as expected from the low frequency of this allele in the Japanese population[32]. We therefore focused on the haplotypes formed by the 3 SNP at –1031, –863, and –857. There were no significant differences in the frequency of TNF haplotypes in patients with MPA (Table 4). These results excluded the possibility that the association with DRB1*0901 was secondarily caused by linkage disequilibrium with the TNF-α promoter haplotype.

There were no significant differences in the frequencies of *TNFR2*, *FCGR2A*, *FCGR2B*, *FCGR3A*, and *FCGR3B* genotypes between patients with MPA and controls (Table 4). However, when the combinations of *FCGR* genotypes were analyzed, the combined genotype of *FCGR2A*-131H/H and *FCGR3A*-176F/F was significantly increased in MPA ($p = 0.025$) (Table 4).

A significant association with these genotypes was not found in the MPO-ANCApositive group (data not shown).

CTLA4. Finally, the $(AT)_n$ repeat polymorphism within *CTLA4* 3'UTR was examined. The frequency of the 86 bp allele, which accounts for the majority of *CTLA4* alleles in Caucasians, was substantially low in our Japanese group (Table 5). No significant association was observed with the MPA (Table 5) or MPO-ANCA positive groups (data not shown).

Table 3. Frequency of *HLA-DRB1* allele carriers in Japanese patients with microscopic polyangiitis (MPA) and controls.

DRB1	MPA (n = 50) n (%)	MPO-ANCA(n = 64) n (%)	Controls (n = 265) n (%)
0101	5 (10.0)	7 (10.9)	26 (9.8)
0401	3 (6.0)	3 (4.7)	6 (2.3)
0403	2 (4.0)	3 (4.7)	13 (4.9)
0405	8 (16.0)	10 (15.6)	65 (24.5)
0406	2 (4.0)	3 (4.7)	19 (7.2)
0407	3 (6.0)	4 (6.3)	6 (2.3)
0410	2 (4.0)	2 (3.1)	5 (1.9)
0802	4 (8.0)	5 (7.8)	18 (6.8)
0803	9 (18.0)	13 (20.3)	37 (14.0)
0901	25 (50.0)*	32 (50.0)**	77 (29.1)
1101	4 (8.0)†	5 (7.8)‡	5 (1.9)
1201	1 (2.0)	1 (1.6)	19 (7.2)
1202	1 (2.0)	1 (1.6)	11 (4.2)
1302	5 (10.0)	7 (10.9)	51 (19.2)
1401	1 (2.0)	1 (1.6)	13 (4.9)
1403	2 (4.0)	3 (4.7)	10 (3.8)
1405	0 (0)	0 (0)	17 (6.4)
1406	1 (2.0)	1 (1.6)	7 (2.6)
1501	8 (16.0)	11 (17.2)	29 (10.9)
1502	9 (18.0)	9 (14.1)	54 (20.4)
1602	0 (0)	0 (0)	8 (3.0)
others	1 (2.0)	2 (3.1)	13 (4.9)

* $p = 0.0037$, $p_c = 0.074$, OR: 2.44, 95% CI: 1.33–4.46. ** $p = 0.0014$, $p_c = 0.029$, OR: 2.44, 95% CI: 1.41–4.22. † $p = 0.039$, $p_c = 0.79$, OR: 4.52, 95% CI: 0.97–21.1. ‡ $p = 0.027$, $p_c = 0.57$, OR: 4.41, 95% CI: 1.36–14.2.

DISCUSSION

Our most interesting finding was the association of HLA-DRB1*0901 with MPA. HLA-DRB1*0901 is one of the most frequent alleles in the general Asian population (5–17%), but is rare in other populations (0–3%)[33]. Such a difference may be related to the epidemiological difference of vasculitis among populations. The incidence of MPA was reported to be lower than that of WG in a UK population[34], while the prevalence of MPA seems to be higher than WG in Japan (unpublished observations).

Because all patients with MPA were positive for MPO-ANCA, it was difficult to distinguish whether DRB1*0901 is associated with the clinical entity of MPA, or with MPO-ANCA production. Patients with vasculitis other than MPA who are positive for MPO-ANCAalso showed a high carrier frequency of DRB1*0901, so DRB1*0901 was considered more likely to be associated with MPO-ANCA positivity. However, a previous study in Japan indicated an association of HLA-DR9 with PR3-ANCA positive WG[9]. Further studies with a larger number of WG patients are necessary to make a further assessment.

If DRB1*0901 is associated with MPO-ANCA production, it is conceivable that DRB1*0901 might present a specific antigenic peptide from MPO and activate specific T cells. Analysis of the T cell receptor in patients with DRB1*0901 is under way. Alternatively, it is possible that the association of DRB1*0901 is a secondary one caused by linkage disequilibrium with another gene of primary significance. HLA-DRB1*0901 is also known for an association with several autoimmune diseases in Japan, including juvenile onset myasthenia gravis[35], type I diabetes mellitus[36], and RA[37], and antiphospholipid antibody production in patients with SLE[38]. Association of a variety of autoimmune diseases with DRB1*0901 might support the latter hypothesis, because it is unlikely that DRB1*0901 could bind peptides from a variety of unrelated autoantigens in a specific manner.

TNF-α was a strong candidate for such a primarily responsible gene. We have reported that a specific haplotype of TNF-α, comprising –1031C, –863A, and –857C, is in linkage disequilibrium with DRB1*0901[39]. This haplotype was suggested to have high promoter activity[32], and an association with Crohn's disease in Japanese was reported[28]. However, in our present study, we did not find an association of the TNF-α promoter with MPA, indicating that the association with DRB1*0901 was not caused by linkage disequilibrium with TNF-α.

No significant association was found in *FCGR* genotypes for MPA or MPO-ANCApositivity. However, the number of individuals with combined *FCGR2A*-131H/H and *FCGR3A*-176F/F genotypes was significantly increased in patients with MPA. These alleles were not in significant linkage disequilibrium with each other in Japanese[20]. FcγRIIa constitutes the main receptor for IgG in neutrophils,

Table 4. TNFα promoter haplotype, *TNFR2 (TNFRSF1B)*-M196R and *FCGR* genotype frequencies in Japanese patients with MPA.

	MPA (n = 50) n (%)	Controls (n = 265) n (%)
TNFα promoter (carrier frequency)		
U01*	47 (94.0)	232 (87.5)
U02	15 (30.0)	83 (31.3)
U03	9 (18.0)	69 (26.0)
U04	2 (4.0)	14 (5.3)
TNFR2		
196M/M	42 (84.0)	205 (78.5)
196M/R	8 (16.0)	54 (20.4)
196R/R	0 (0)	3 (1.1)
FCGR2A		
131H/H	36 (72.0)	197 (65.0)
131H/R	14 (28.0)	95 (31.4)
131R/R	0 (0)	11 (3.6)
FCGR2B		
232I/I	32 (64.0)	183 (60.4)
232I/T	16 (32.0)	104 (34.3)
232T/T	2 (4.0)	16 (5.3)
FCGR3A		
176F/F	30 (60.0)	145 (47.8)
176 F/V	18 (36.0)	132 (43.6)
176V/V	2 (4.0)	26 (8.6)
FCGR3B		
NA1/1	21 (42.0)	116 (38.3)
NA1/2	26 (52.0)	145 (47.8)
NA2/2	3 (6.0)	42 (13.9)
Genotype combination		
FCGR2A H/H and *3A* F/F	23 (46.0)**	91 (30.0)

* *TNFA*-U01–U04 indicate haplotypes formed by 3 SNPat positions–1031, -863, and -857 within the promoter region of TNFα gene. U01: TCC, U02: TCT, U03: CAC, U04: CCC. ** p = 0.025 (MPA vs control, chi-square analysis from 2 × 2 contingency table). There were no other significant differences.

Table 5. Frequency of *CTLA4* 3′ untranslated region $(AT)_n$ alleles in Japanese patients with MPA. There were no significant differences between patients and controls.

Allele (bp)	MPA (2n = 98) n (%)	Controls (2n = 222) n (%)
86	27 (27.6)	50 (22.5)
100	1 (1.0)	2 (1.9)
102	16 (16.3)	40 (18.0)
104	34 (34.7)	70 (31.5)
106	4 (4.1)	21 (9.5)
108	7 (7.1)	19 (8.6)
110	0 (0)	4 (1.8)
116	0 (0)	1 (0.5)
118	1 (1.0)	3 (1.4)
122	4 (4.1)	4 (1.8)
124	0 (0)	0 (0)
126	1 (1.0)	5 (2.3)
128	3 (3.1)	1 (0.5)
130	0 (0)	2 (0.9)

while FcγRIIIa is mainly expressed in monocytes, macrophages, and NK cells. FcγRIIa-131H has a higher affinity for human IgG2 and IgG3 than does 131R. On the other hand, FcγRIIIa-176F exhibits a lower affinity for human IgG1 and IgG3 than does 176V[18]. It can be speculated that immune complexes that failed to be efficiently cleared though the low affinity allele of FcγRIIIa on macrophages might in turn stimulate neutrophils through the high affinity allele of FcγRIIa. It is interesting that this haplotype formed by *FCGR2A*-131H and *FCGR3A*-176F has recently been shown to be associated with SLE in non-Caucasians[40].

A significant association with MPA was not observed for the frequency of the *CTLA4* 3'UTR $(AT)_n$ allele, recently shown to be associated with WG in Caucasians[13,23]. This could be due to the difference between MPA and WG, and/or between the genetic background of Japanese and Caucasians. The frequency of the common 86 bp allele was found to be markedly lower in the general Japanese population compared with Caucasians[13,23], representing one of the differences in the genetic background of the Japanese population and Caucasians.

We demonstrated an association of HLA-DRB1*0901 with microscopic polyangiitis in Japanese patients in a multicenter collaborative study. The mechanism of this association requires further study.

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