Genetic association of HLA-A*2601 with ocular Behçet's disease in Japanese patients

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ABSTRACT

Objective. *Behçet's disease (BD) is known to be associated with HLA-B*51, especially HLA-B*5101, in many different ethnic groups. Recently, several HLA-A or -B alleles have been proposed as possible candidate genes for BD in addition to HLA-B*5101. To investigate those associations, we studied HLA-A and -B alleles in Japanese ocular BD patients and the association of possible susceptibility HLA genes with visual prognosis.*

Methods. *Eighty-eight Japanese BD patients with uveitis and 104 healthy controls were enrolled for analyses of HLA-A and B alleles. Statistical analysis was performed with Fisher's exact test and odds ratio (OR). Association of the possible susceptible HLA gene and visual prognosis was also examined.*

Results. *The phenotype frequency (PF) of HLA-A*2601 was significantly higher in the patients (37.5%) than the controls (14.4%) (pc=0.00529, OR=3.56), especially in patients without HLA-B*5101 (57.4% vs. 14.1%, pc=4.58x10^{-6}, OR=8.21). In contrast, the PF of HLA-A*2601 was not increased in patients with HLA-B*5101 (14.6% vs. 15.8%). Also, the PF in patients possessing HLA-A*2601 or HLA-B*5101 was increased up to 77.3%. Interestingly, the PF of HLA-A*2601 was significantly associated with poor visual prognosis corresponding to visual acuity of 0.1 or less in the worse eye (p=0.0262).*

Conclusion. *Our results indicate that HLA-A*2601 is possibly associated with ocular BD, independent of HLA-B*5101, indicating that HLA-A*2601 is an additional susceptibility allele candidate of ocular BD in Japan. HLA-A*2601 would also be a possible marker for poor visual prognosis.*

Introduction

Behçet's disease (BD) is a chronic systemic inflammatory disorder characterised by four major features; recurrent oral aphthae, genital ulcers, ocular disorders, and skin lesions. In addition, the disease is occasionally accompanied by inflammation in other tissues, such as the joints, vascular system, gastrointestinal tract, and central nervous system, as well as epididymis. BD is more prevalent in countries along the Silk Road from Japan to the Middle East, and both genetic and environmental factors appear to trigger the disease (1), though its etiology remains uncertain.

Approximately 30 years ago, a strong association was found between BD and HLA genes, especially HLA-B*51, indicating HLA-B as a strong candidate locus responsible for BD development (2). Later, HLA-B*51, especially one of its subtypes HLA-B*5101, was shown to be significantly associated with BD in many ethnic groups, including Japanese (3-7). More recently, the major histocompatibility complex (MHC) class I chain-related gene A (MICA), centromerically located only 46 kb from the HLA-B gene on chromosome 6, was proposed as a candidate gene for BD susceptibility (8). However, further analyses showed that the significant increase in frequency of MICA-A6 in BD patients could be explained by linkage disequilibrium with HLA-B51 (9). Thus, on the assumption that the actual pathogenic gene involved in BD development is HLA-B itself, the major disease susceptibility allele has been so far considered to be HLA-B51 (9). However, 40-50% of BD patients are HLA-B51-negative, which may be explained by the influence of other genetic factors and/or various external environmental or infectious agents.

On the other hand, Matsuki *et al.* showed a slight increase in frequency

of HLA-A*26 in BD (10), while Chung *et al*. observed an increase in that of HLA-A*26 in Taiwanese patients with ocular BD (11). Recently, Itoh *et al*. reported that the frequencies of HLA-A*2602 and HLA-B*3901 had increasing trends in Japanese patients without HLA-B*51 as compared to a control group without HLA-B*51 (12). Thus, a secondary type of HLA seems to influence on the onset of BD, but has not been clearly determined.

In this study, to confirm the association of HLA-A and B alleles, except for HLA-B*5101, with BD, we studied HLA-A and -B polymorphisms in Japanese patients suffering from ocular BD. In addition, we investigated the association of possible susceptibility HLA alleles with visual prognosis.

Patients and methods

Subjects

Eighty-eight Japanese patients with BD (71 men and 17 women, 51.7±13.2 years old) who suffered from uveitis, fulfilled the International Study Group (ISG) diagnostic criteria for BD (13) and were followed up more than 1 year were enrolled in this study.

For comparisons, 104 Japanese healthy volunteers (80 men and 24 women, 50.2±10.6 years old), who were unrelated to each other or to the patients, and matched to the patient group in regard to sex and age, were also enrolled. All patients and control subjects gave informed consent to participate in the study, according to the guidelines of the Declaration of Helsinki.

HLA-A and -B typing

Genomic DNA was isolated from the fresh or frozen peripheral blood samples using DNA isolation kit (Blood and Cell Culture DNA Maxi kit, Qiagen KK, Tokyo, Japan) according to the manufacturer's instructions. DNA typing of HLA-A and -B alleles was performed using a sequence-specific oligonucleotide method. Briefly, generic amplifications of the HLA-A and -B genes were performed using biotin-labelled primers at the 50 end, with a GeneAmp PCR System 9700 (Applied Biosystems, Forester City, CA). Following amplification, the PCR products were hybridised with sequence-specific oligonucleotide probes using a commercially available reverse line blot assay kit (INNO-LiPA HLA-A update and INNO-LIPA HLA-B update Plus, Innogenetix, Dartford, England) (14). The results were analysed using the clinical trial version of LiRAS (Innogenetics) interpretation software for LiPA HLA (15). Reconfirmation of HLA typing was performed using a MICRO SSP Allele Apecific HLA class I DNA Typing Tray (One Lambda Inc., Canoga Park, CA, USA), according to the manufacturer's instructions.

HLA typing and visual prognosis

Since all of the BD patients in this study suffered from uveitis, we also examined the relationships between the major HLA alleles related to BD and visual prognosis of patients with ocular disease. In this study, BCVA was determined using a standard Landolt visual acuity (VA) chart. Visual prognosis of each patient was determined by best corrected visual acuity (BCVA) in the worse eye at the last remission during the observation period. The cut-off visual acuity was set at 0.1 (16, 17), which is commonly used in studies of visual prognosis in BD to indicate a loss of useful vision. A result of ≤0.1 was used to indicate poor visual prognosis.

Treatment regimens for uveitis

All of the BD patients in this study received some kind of treatment for ocular attacks of ocular BD. The treatment regimens for recurrent uveitis due to Behçet's disease in our hospital were as follows; at the exacerbation phase of the uveitis, local corticosteroid therapy (corticosteroid eyedrops, subconjunctival injection of corticosteroid) is administrated. If severe uveoretinitis is occurred including macular lesion, short term oral corticosteroid (30-40 mg/day) is properly administrated. On the other hand, the continuous therapy for the prevention of ocular attacks consists of oral colchicine (about 1mg/day) as the first choice and cyclosporine (5 mg/kg/day or less) as the second choice (18). If uveoretinitis could not be controlled by combination therapy of colchicine and cyclosporine, oral corticosteroid (about 10–20 mg/day), or infliximab in recent cases, should be considered and administrated.

Statistical analysis

Phenotype frequencies (PFs) were estimated by direct counting. The significance of the distribution of alleles between the patients with BD and healthy controls was first analysed by Fisher's exact probability test, then the primary *p*-values were corrected (*pc*-values) using Bonferroni correction by multiplying the primary *p*-value by the number of HLA alleles being tested. A *pc*-value of less than 0.05 was considered to be statistically significant. The strength of association was estimated by calculating the odds ratio (OR) with the 95% confidence interval (95%CI). The relationships between susceptible HLA alleles in the BD patients and clinical manifestations including visual prognosis were statistically examined by Fisher's exact probability test or Mann-Whitney's U-test. All statistical analyses were performed with SPSS for Windows version 12.0 (SPSS Inc., Chicago IL). This study was approved by the ethics committee of The University of Tokyo Hospital.

Results

Phenotype frequencies of the HLA-A and -B alleles with the *p*-value of less than 0.05 after comparison between the BD patients and controls were shown in Table I. Of the 14 HLA-A alleles detected in this study, the PF of HLA-A*2601 was significantly higher in the patients, even when corrected by multiple testing (*pc*=0.00529, OR=3.56, 95%CI=1.77–7.15), while the allele frequency of HLA-A*2601 was also increased (18.8% in BD patients and 7.7% in controls, *pc*=0.0266, OR=2.77, 95%CI=1.47–5.23). In contrast, the PF of HLA-A*3101 was not different between the patients and controls (17.0% *vs*. 17.3%), even though HLA-A*31 has been reported to be increased in BD patients (10). Furthermore, the PF of HLA-A*2602 in the patients (5.7%) was not significantly different than that in the controls (3.8%).

Of the 28 HLA-B alleles detected in this study, the frequency of HLA-B*5101

Table I. Phenotype frequencies of HLA-A and -B alleles in Japanese patients with Behçet's disease.

	BD	PF(%)	C	PF(%)	*p* (Fisher)	*pc*
n. (cases)	88		104			
A*2601	33	37.5	15	14.4	0.000378	0.00529*1
B*5101	41	46.6	19	18.3	3.95×10^{-5}	0.00111*2
B*5201	12	13.6	30	28.8	0.0139	0.388*3
A*2601 and B*5101	6	6.8	3	2.9	NS	
A*2601 and/or B*5101	68	77.3	31	29.8	3.65×10^{-11}	1.43×10^{-8}*4

The alleles with the *p*-value of less than 0.05 were shown.
BD: Behçet's disease, C: control, NS: not significant .
p-values are corrected by 14 (HLA-A, a 4-digit), 28 (HLA-B, a 4-digit) or 392 (HLA-A and -B, HLA-A and/or –B, a 4-digit).
*1: OR=3.56; 95%CI=1.77-7.15; *2: OR=3.91; 95%CI=2.04-7.48; *3: OR=0.3895; 95%CI=0.185-0.818, *4: OR=8.12; 95%CI=4.23-15.6.

was 46.6% in the patients, which was significantly higher than that (18.3%) in the healthy controls, even when corrected by multiple testing (*pc*=1.11 x 10^{-3}, OR=3.91, 95%CI=2.04–7.48) (Table I). The PF of HLA-B*5101- or HLA-A*2601-positive was 77.3% in the patients, which was significantly greater than that in the controls (29.8%) (*pc*=1.43×10^{-8}, OR=8.12, 95%CI= 4.23–15.6).

The HLA-B*5102 allele was not detected in either the patients or controls. On the other hand, the PF of HLA-B*5201 was lower in our patients than the controls, though the difference was not significant after being corrected by multiple testing (*p*=0.0139, *pc*=0.389, OR=0.389, 95%CI=0.185–0.818).

In Table II, the PFs of A*2601 allele in HLA-B*5101-positive and -negative groups were shown. There were no significant differences for HLA-A*2601 between HLA-B*5101-positive BD patients and positive controls. No differences were observed for other A*26 alleles including *2602, *2603 and 2605 (data not shown) between HLA-B*5101-positive BD patients and positive controls. On the other hand, the frequency of HLA-A*2601 was significantly increased in the HLA-B*5101-negative BD patients as compared with the HLA-B*5101-negative controls (57.4% *vs*. 14.1%, *pc*=4.58×10^{-6}, OR=8.21, 95%CI=3.54-19.0). The allele frequency of HLA-A*2601 was also significantly increased (28.7% in BD patients and 7.6% in controls, *pc*=1.39×10^{-4}, OR=4.87, 95%CI=2.37-10.0). The PF of HLA-A*2601 in the controls with HLA-B*5101 (15.8%) was nearly equal to that in those without HLA-B*5101 (14.1%). These results suggest that HLA-A*2601 is associated with BD independent from the presence of HLA-B*5101. On the other hand, the PF of HLA-A2602 was not significantly increased in the patients (5.7%) as compared to the controls (3.8%). Also, the PF of HLA-A*2602 was nearly equal between the patients and controls without HLA-B*5101 (4.3% *vs*. 4.7%).

We further analysed the PFs of B*39 alleles in the group positive for HLA-B*5101 or A*2601, and in the group of HLA-B*5101-negative and A*2601-negative. The frequency of HLA-B*39 was significantly increased in the patients without both HLA-A*2601 and B*5101 as compared with the controls without both (30.0% *vs*. 4.1%, *pc*=0.0464, OR=10.0, 95%CI=2.23–44.8), though the numbers of samples were very small (20 and 73 for BD and controls, respectively). In the group of HLA-B*5101-negative and A*2601-negative, 4 BD patients possessed HLA-B*3901 and 2 patients showed HLA-B*3902, while all 3 control subjects showed HLA-B*3901.

Since all of the BD patients in this study suffered from uveitis, the relationships between their visual prognosis and HLA- B*5101 or A*2601 were examined. The backgrounds of the patients with and without HLA- B*5101 and A*2601 are shown in Tables III and IV, respectively. There were no significant differences in regard to sex, age of ocular onset, uniocular/binocular ratio, iridocyclitis/panuveitis ratio, period between ocular onset and initial visit, or number of patients receiving or not receiving immunosuppressants (colchicine, prednisolone, cyclosporine, cyclophosphamide, infliximab, tacrolimus, methotrexate, azathioprine) between the HLA-B*5101-positive and -negative patients, or between those who were HLA-A*2601-positive and -negative. Visual acuity from the initial visit to the last visit in the affected eye became significantly worse in the HLA-A*2601-positive patients as compared to those who were HLA-A *2601-negative (*p*=0.0492 and 0.0222, Table IV). HLA-A*2601-positive patients were significantly associated with poor visual prognosis (BCVA in the worse eye at final observation ≤0.1) (*p*=0.0262, Fisher's exact probability test), where-

Table II. Phenotype frequencies of HLA-A*2601 allele in subjects with and without B*5101 in Japanese patients with Behçet's disease.

	Subjects with B*5101						Subjects without B*5101					
	BD	PF(%)	C	PF(%)	*p* (Fisher)	*pc*	BD	PF(%)	C	PF(%)	*p* (Fisher)	*pc*
n. (cases)	41		19				47		85			
A*2601	6	14.6	3	15.8	NS		27	57.4	12	14.1	3.27×10^{-7}	4.58×10^{-6}*

BD: Behçet's disease; C: control; NS: not significant.
p-values are corrected by 14 (HLA-A, a 4-digit).
*OR=8.21; 95%CI=3.54-19.0.

Table III. Background of the patients with and without HLA-B*5101.

	HLA-B5101 negative	HLA-B5101 positive	*p*-value
No. of patients	47	41	n.s.
Male / female*	36:11	35:6	n.s.
Observation length**	16.0 ± 8.2	15.7 ± 7.6	n.s.
Ages of ocular onset**	36.1 ± 9.6	32.4 ± 11.2	n.s.
Uniocular / binocular*	4:43	3:38	n.s.
Iridocyclitis / panuveitis*	2:45	4:37	n.s.
Periods between ocular onset and initial visit**	5.0 ± 7.5	5.2 ± 6.4	n.s.
Visual acuity at the initial visit**	0.271 ± 0.151	0.247 ± 0.121	n.s.
Visual acuity at the last visit**	0.119 ± 0.061	0.148 ± 0.062	n.s.
With:without colchicine*	36:11	33:8	n.s.
With:without oral predonisolone*	22:25	20:21	n.s.
With:without cyclosporine*	19:28	21:20	n.s.
With:without cyclophosphamide*	7:40	6:35	n.s.
With:without infliximab*	4:43	2:39	n.s.
With:without tacrolimus*	1:46	2:39	n.s.
With:without methotrexate*	2:45	1:40	n.s.
With:without azathioprine*	2:45	1:40	n.s.

*p-values were calculated by the Fisher's exact probability test method.
**p-values were calculated by the Mann-Whitney's U-test method.

Table IV. Background of the patients with and without HLA-A*260.1.

	HLA-A2601 negative	HLA-A2601 positive	*p*-value
No. of patients	55	33	n.s.
Male:female*	44:11	27:6	n.s.
Observation length**	16.2 ± 7.7	15.4 ± 8.3	n.s.
Ages of ocular onset**	35.0 ± 10.9	33.5 ± 9.8	n.s.
Uniocular:binocular*	5:50	2:31	n.s.
Iridocyclitis:panuveitis*	6:49	0:33	n.s.
Periods between ocular onset and initial visit**	5.1 ± 6.5	5.2 ± 7.7	n.s.
Visual acuity at the initial visit**	0.316 ± 0.153	0.187 ± 0.118	0.0492
Visual acuity at the last visit†	0.175 ± 0.062	0.0825 ± 0.065	0.0022
With:without colchicine*	43:12	26:7	n.s.
With:without oral predonisolone*	24:31	18:15	n.s.
With:without cyclosporine*	26:29	14:19	n.s.
With:without cyclophosphamide*	8:47	5:28	n.s.
With:without infliximab*	3:52	3:30	n.s.
With:without tacrolimus*	2:53	1:32	n.s.
With:without methotrexate*	1:54	2:31	n.s.
With:without azathioprine*	2:53	1:32	n.s.

*p-values were calculated by the Fisher's exact probability test method.
**p-values were calculated by the Mann-Whitney's U-test method.

as HLA-A*5101-positive patients were not (p=0.521) (Table V).

Discussion

In the present study, 14 HLA-A alleles and 28 HLA-B alleles were detected. The allele nomenclature, allele frequencies, and PFs in the control group were essentially the same as those previously described for bone marrow donors registry (19, 20). Our results fitted to Hardy-Weinberg equilibrium.

In this study, the PF of HLA-A*2601 was significantly increased, whereas that of HLA-B*5201 showed a decreasing tendency in the patient group. The HLA-A*2601 allele was more significantly increased in the BD after excluding the subjects possessing HLA-B*5101, though the numbers of cases examined was rather low. The PF of HLA-A*2601 was not increased in the patients who possessed HLA-B*5101 as compared with the controls with HLA-B*5101. In the controls, the PF of HLA-A*2601 in those positive for HLA-B*5101 was nearly equal to that of those without HLA-B*5101. These results suggest that HLA-A*2601 is possibly associated with BD independent from HLA-B*5101, thus indicating HLA-A*2601 as an additional susceptibility allele in Japanese patients with ocular BD. The results also suggest that there is no linkage between HLA-A*2601 and HLA-B*5101. In addition, the frequency of HLA-A*2601 or HLA-B*5101 was highly increased in the BD patients as compared to the control (77.3%, pc=1.43X10^{-8}). This result suggests that the combination of HLA-A*2601 or HLA-B*5101 could be one of disease markers for ocular BD, though 23% of patients without both these alleles would be missed.

There would be three possibilities in which these two different HLA alleles contribute to the pathogenesis of BD. At first, there is a possibility that HLA-B*5101 and HLA-A*2601 would recognise the same antigenic peptide. Second, HLA-B*5101 and HLA-A*2601 would recognise the different antigenic peptide, and the peptide that HLA-A*2601 would recognise might induce ocular involvement. It is well-known that Vogt-Koyanagi-Harada disease and acute anterior uveitis are associated with specific HLA alleles. There is the third possibility that true susceptible gene for BD would exist near HLA-B*5101 and HLA-A*2601. In any case, further analyses must be necessary to clarify the mechanisms of HLA-B*5101 and HLA-A*2601 on the contribution of BD.

A previous study found weak increases in the incidences of HLA-A*2602 and B*3901 in Japanese BD patients without HLA-B*51, and suggested that those two alleles might also have some secondary influence on the onset of BD (12). In that report, no significant increase in the incidence of HLA-A*2601 was indicated. In the present study, the phenotype of the HLA-A*2602 allele was not as prevalent in the entire patient group, which was nearly the same as the controls (5.7% *vs*. 3.8%), nor

Table V. Relationship between poor visual prognoses and the major HLA alleles in Japanese patients with Behçet's disease.

Final VA in worse eye	0.15 or more	0.1 or less
HLA-B5101+ (n=40)	24 (60%)	16 (40%)
HLA-B5101- (n=48)	25 (52%)	23 (48%)
Fisher's exact probability test: p=0.521		
Final VA in worse eye	0.15 or more	0.1 or less
HLA-A2601+ (n=33)	13 (39%)	20 (61%)
HLA-A2601- (n=55)	36 (65%)	19 (35%)

Fisher's exact probability test: p=0.0262; OR=0.343; 95%CI=0.141-0.837.

was it high in patients without HLA-B*5101 (4.3% *vs*. 4.7%). We did not find an association of HLA-A*2602 in the present BD patients. These results clearly suggest that HLA-A*2601, but not HLA-A*2602, contributes to BD, at least in Japanese patients with ocular disease.

Although our findings indicate that the contribution of HLA-A*2601 to ocular BD is independent from HLA-B*5101, the contribution of HLA-A*2601 in BD patients without ocular involvement was not examined in this study. In addition, the contributions of HLA-A*2601 to other clinical features were not investigated. Further analysis must be necessary for confirming the contribution of HLA-A*2601 in BD.

Previously, an increasing trend of HLA-A*31 and a decreasing trend of HLA-A*3303 in BD patients have been reported (10, 12). However, in the present study, the PF of these alleles were not significantly different from that in the controls (data not shown).

Our findings reconfirmed that HLA-B*5101 is a susceptiblity allele for ocular BD. In previous reports, increasing (22, 23) and decreasing (10) trends for the PF of HLA-B52 were reported. In the present study, a decreasing trend for the PF of HLA-B*5201 in BD patients as compared to the controls was observed (13.6% *vs*. 28.8%). That trend was observed in both patients with HLA-B*5101 (12.2% *vs*. 31.6% in controls) and in those without HLA-B*5101 (14.9% *vs*. 28.2%). However, those decreases were not statistically significant and may have been related, at least in part, to the trend of weak negative-linkage of A*2601 to B*5201 observed in control group (p=0.221, OR=0.335, 95%CI=0.071-1.586).

Many reports provided the risk factors for the prognosis of visual acuity of ocular BD, indicating that male gender (16, 23, 24), early onset age (16, 23, 24), involvement of central nervous system (24, 25), vascular thrombosis (24, 25), skin lesion (25), posterior segment type uveitis (23, 25), current (time-updated) overall activity of ocular disease and increased numbers of ocular attacks per year (26). We previously examined patients with ocular BD initially diagnosed between 1980 and 1999 and reported that both the percentage of those with poor visual prognosis less than 0.1 and numbers of ocular attacks in patients who had been diagnosed in the 1990s were significantly reduced, as compared to those in the 1980s (18).

There are several reports regarding to the relationship between HLA-B*51 and visual acuity in BD patients, though no significant association between them has been found (17, 27, 28). In addition, to the best of our knowledge, there are no reports regarding an association between HLA-A*26 and visual acuity in BD patients. To clarify the relationships between major HLA alleles and visual prognosis, we examined the backgrounds of BD patients and visual acuity at the first and last visits, and compared the results between HLA-A*2601-positive and negative patients, and between B*5101-positive and negative patients. There were no significant differences for sex, age of ocular onset, uniocular/binocular ratio, iridocyclitis/panuveitis ratio, period between ocular onset and initial visit, and the numbers of patients receiving or not receiving immunosuppressants between those who did and did not possess HLA- B*5101 (Table III) or HLA-A*2601 (Table IV). However, visual acuity at both the initial and last visit in the affected eye was significantly worse in the HLA-A*2601-positive group than in the HLA-A *2601-negative group (Table IV), and the difference in visual acuity between those groups was increased at the last visit (p=0.0022 and p=0.0492 respectively) (Table 4). Moreover, HLA-A*2601 was significant associated with poor visual prognosis (BCVA ≤0.1 in the worse eye at the final observation) (p=0.0262, Table V). In contrast, no significant associations were observed between the phenotypes of HLA-A*5101 and visual acuity at the first and the last visits (Table III), or with poor visual prognosis (p=0.521, Table V). These results suggest that HLA-A*2601 would be a possible marker for poor visual prognosis and ocular disease severity. However, since the numbers of patients and controls in our study were not large, additional analyses with increased numbers are necessary for clarifying the relationship between HLA-A*2601 and poor visual prognosis.

In conclusion, our results showed that HLA-A*2601 is possibly associated with BD independent from HLA-B*5101, indicating that HLA-A*2601 is an additional susceptibility allele in Japanese patients with ocular BD. In addition, HLA-B39 may also be a susceptibility allele for ocular BD. Finally, HLA-A*2601 would be a possible marker for poor visual prognosis and ocular disease severity.

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