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Molecular Genetics (HLA) of Behçet's Disease

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Behçet's disease (BD) has been known to be strongly associated with the human leukocyte antigen (HLA) B51. This B51 association has been confirmed in many different ethnic groups between the Middle East and Japan, and it has been proposed that BD is prevalent in those ethnic groups along the old Silk Route. The hypothesis could be made that B51 molecules are primarily involved in BD development through specific antigen presentation. However, polymorphic analyses of the TNFB gene and Tau-a microsatellite between the HLA-B and TNF genes indicate that the pathogenic gene of BD is not the HLA-B51 gene itself but another gene located around the HLA-B gene. HLA-C genotyping by the PCR-SSP method also suggests that the BD pathogenic gene is not the HLA-C gene itself but other gene located near the HLA-B gene. Recently we sequenced a single contig of 236,822 bp from the MICA gene (58.2 kb centromeric of HLA-B) to 90.8 kb telomeric of HLA-C and identified 8 novel genes designated NOB1-8 (NOB: new organization associated with HLA-B). During the course of the genomic sequence analysis we clarified the genetic structure of the MICA (MHC class I chain-related gene A) gene and found a triplet repeat microsatellite polymorphism of $(GCT/AGC)_n$ in the transmebrane (TM) region. Furthermore, the microsatellite allele consisting of 6 repetitions of GCT/AGC (MICA A6 allele) was present at a significantly higher frequency in the BD patient group than in the control group and a significant fraction of B51-negative patients were positive for this MICA A6 allele. These results suggest the possibility of a primary association of BD with MICA rather than HLA-B.

Key Words: Behçet's disease, HLA-B51, HLA-C, microsatellite, tumor necrosis factor, NOB, MICA

Behçet's disease (BD) is a refractory systemic inflammatory disease characterized by 4 major symptoms consisting of oral aphthous ulcers, ocular lesions, skin lesions, and genital ulcerations. It is also occasionally characterized by inflammation in tissues and organs throughout the body including the vascular system, central nervous system, gastro-intestinal tract, lungs, kidneys, and joints. Although the etiology and pathogenesis of BD are still uncertain, the onset of BD is believed to be triggered by the involvement of some external environment factors in individuals with a particular genetic background. The human major histocompatibility complex (MHC) encodes highly polymorphic leukocyte antigens (HLA) responsible for antigen presentation to T cells (Mizuki *et al.* 1994b) and BD is known to be strongly associated with a particular HLA-B allele, HLA-B51 (Ohno *et al.* 1982; Mizuki *et al.* 1992a; Mizuki *et al.* 1992d). However, it has not yet been clarified if the HLA-B51 gene itself is the pathogenic gene related to BD or if it is some other gene in linkage disequilibrium with HLA-B51. We have been performing gene analysis in the HLA region and

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cloning unidentified genes to identify the pathogenic gene of this disease (Mizuki *et al.* 1997b). In this paper, we wish to introduce an overview of genetic predisposition underlying the development of BD on the basis of our recent analysis.

HLA genotyping of BD and its primary association with B51

We and others have presented evidence of an HLA association with BD, and HLA-B51, one of the split antigens of HLA-B5, was found to be the most strongly associated genetic marker (Ohno *et al.* 1982; Mizuki *et al.* 1992a; Mizuki *et al.* 1992d). This strong association has been confirmed in many different ethnic groups from the Middle East to the Far East, including Turkish, Greek, Italian, French, English, Tunisian, Saudi Arabian, Iranian, Kuwaiti, Israeli, Uygur (inhabiting the northwestern part of China), Han Chinese, Korean, Taiwanese and Japanese, and about 40～80% of patients with BD in these populations possess B5 (B51). In these ethnic groups, comparison with the frequency of B5 (B51) in healthy controls gives a P-value of less than 0.0001 (also significant in a corrected P-value analysis), suggesting a strong correlation between BD and B5 (B51). Therefore, BD exhibits the same HLA association in different ethnic groups, and it is an attractive hypothesis that BD was spread in Asian and Eurasian populations from Japan to the Middle East, along with the distribution of its associated HLA allele, HLA-B51, by old nomadic or Turkish tribes along the Silk Route (Ohno *et al.* 1982). In this respect, anthropological analyses in progress among many different ethnic groups along the Silk Route will be valuable in testing this hypothesis (Geng *et al.* 1995; Mizuki *et al.* 1996b; Mizuki *et al.* 1997d; Mizuki *et al.* in press).

Although the HLA-B51 antigen has thus far been known to be a genetic marker of BD in many different ethnic group, it remains uncertain whether the HLA-B51 gene is truly responsible for BD or whether some other nearby genes which are in linkage disequilibrium with B51 are responsible. Recently, the B51 antigen has been identified to comprise 9 alleles, B*5101-B*5109 (Bodmer *et al.* 1997). In order to investigate whether there is any correlation of one particular allele among them with BD, we performed HLA-B51 subtyping by both the lymphocyte cytotoxicity test and the PCR-SSP (polymerase chain reaction-sequence specific primers) method (Mizuki *et al.* 1993; Mizuki *et al.* 1994a; Mizuki *et al.* 1997c). As a result, among B51-positive Japanese subjects with BD, the frequency of HLA-B*5101 and HLA-B*5102 was 98.1% (53/54) and 1.9% (1/54), respectively. In comparison with B51-positive healthy controls (B*5101: 98.0%, [50/51]; B*5102: 2.0%, [1/51]), the BD patients revealed no significant differences (Mizuki *et al.* 1993; Mizuki *et al.* 1994a). We also obtained similar results from BD patients in Greek (Mizuki *et al.* 1997c), Italian (Mizuki *et al*, submitted), Saudi Arabian (Mizuki *et al.* submitted) and Uygur (Mizuki *et al*, in preparation) populations. Namely, because of a marked increase in the frequency of the B51 antigen in BD patients, the frequency of the alleles encoding the B51 antigen was aiso increased, but in the B51 antigen positive subjects, there were no significant differences in the frequency of each allele (subtype frequency) between patients and healthy controls. Therefore, amino acids which are commom to all B51-encoding alleles, but not to any particular B51-encoding alleles and do not reside on other B antigen genes, may be responsible for the predisposition to BD through specific antigen presentation.

We have previously noted that the frequency of the B52 antigen (B*5201 allele), which is a split antigen of the B5 antigen as is B51 and differs from B51 (B*5101 allele) in the amino acid sequence only at 2 positions, was not increased in BD patients. This suggests that 2 amino acids specific for B51, asparagine at position 63 and phenylalanine at position 67, may be concerned with the development of BD (Fig. 1) (Mizuki *et al.* 1992a; Mizuki *et al.* 1992d).

There are 6 (A,B,C,D,E and F) pockets in the HLA grooves of the class I antigen (class II antigen: 5), and the pocket B, the major one, is formed by these amino acid residues at positions 63 and 67 (Saper *et al.* 1991; Fremont. *et al.* 1992). In most class I alleles, the amino acids in antigen peptides for binding with HLA molecules (in general, amino acids at position 2 of nonapeptide) are determined and limited by the kinds of amino acids constituting the pocket B, so such limited amino acids within

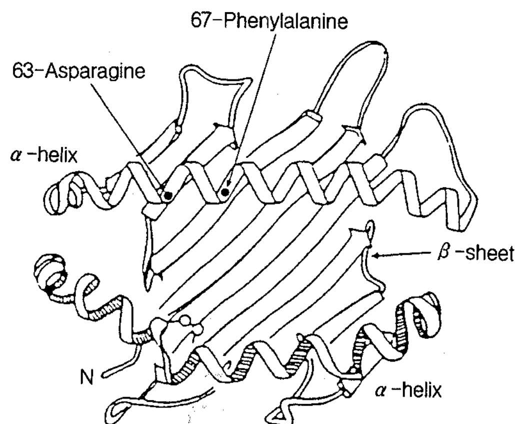

Fig. 1. Location of two amino acids differing in B51 and B52. The two amino acids specific for B51, 63-asparagine and 67-phenylalanine, constitute a part of the pocket B in the HLA groove (Mizuki et al. 1992a).

peptides are referred to as anchor residues. Thus, the peptides for binding with HLA molecules are dependent on amino acids constituting the pocket B of each HLA molecule and hence among HLA alleles there are completely different motifs for binding peptides in general (Rammensee *et al.* 1995). Namely, due to HLA polymorphism, peptides for binding vary greatly with different amino acids specific for each HLA allele, particularly those constituting the pocket B (Rammensee *et al.* 1995).

Actually, although B51 (B*5101-B*5103 alleles) and B52 (B*5201 allele) differ in the amino acid sequence only at 2 sites (at positions 63 and 67) in the α2 domain, the binding peptide motifs differ remarkably since these 2 sites constitute the pocket B (Table 1 and Fig. 1) (Falk *et al.* 1995; Rammensee *et al.* 1995). Thus, the hypothesis can be presented that the B51 molecule is primarily involved in BD development because of the presence of agretopes which have a high affinity for the B51 molecule in some BD-provoking extrinsic factors (bacteria, viruses, etc.) (Mizuki *et al.* 1992a; Mizuki *et al.* 1992d).

We also performed HLA class II (HLA-DRB1, -DQA1, -DQB1 and -DPB1) genotyping among Japanese BD patients (Mizuki *et al.* 1992a; Mizuki *et al.* 1992d) using the PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) technique (Mizuki *et al.* 1992c). No significant difference was revealed in any class II alleles between the patient and control groups in the corrected P-value test , but P-value analysis showed a significantly high frequency of DRB1*0802 and significantly low frequencies of DQA1*0103, DQB1*0601 and DQB1*0501. These relative increase and decreases of the HLA class II alleles could be explained by a linkage disequilibrium with HLA-B51 (Mizuki *et al.* 1992a; Mizuki *et al.* 1992d). Because the HLA class II genes are located far from the HLA-B gene, more than 1000kb apart, and genetic recombination should have taken place frequently for a long period, the class II association with BD has been presumably weakened by contrast with a tight association of HLA-B51 with BD. These results further confirmed that the susceptibility gene to BD should reside around the HLA-B gene region rather than within the class II region (Mizuki *et al.* 1992a; Mizuki *et al.* 1992d).

Table 1. Peptide binding motifs of HLA-B51 (B*5101 ~B*5103) and HLA-B52 (B*5201)

HLA	Positions								
	1	2	3	4	5	6	7	8	9
B*5101	×	**A** **P** **G**	×	×	×	×	×	×	**F** **I**
B*5102	×	**P** **A** **G**	Y	×	×	×	×	×	I V
B*5103	×	**A** **P** **G**	Y	×	×	×	×	×	V I F
B*5201	×	Q	F Y W	×	L I V	×	×	**I** **V**	**I** **V**

Boldface letters indicate anchor residue and thin face letters indicate auxiliary anchor residue. The letter "X" indicates that any amino acid can be bound (Mizuki *et al.* 1997b).

Analysis in HLA-B51 transgenic mice

Various symptoms of BD have been considered to result from neutrophil hyperactivity. Increased

chemotaxis, active oxygen overproduction, and increased endothelial cytotoxicity are primarily involved. In general, no marked changes have been observed in phagocytic and bactericidal functions. However, neutrophil hyperactivity is a common finding in various inflammatory diseases and they are not specific for BD. The question is, what triggers neutrophil hyperactivity? In other words, the determination of the first provocative cause of BD onset is essential for the elucidation of the disease onset mechanism.

It is interesting that increased neutrophil function has been reported both in BD patients and healthy individuals who were B51-positive (Kaneoka *et al.* 1993; Takeno *et al.* 1995), although no evidence was found for the direct involvement of the B51 antigen in neutrophil hyperactivity. Accordingly, in order to investigate direct involvement of B51 in neutrophil hyperactivity, Takeno *et al.* attempted to conduct studies using B51 transgenic mice produced by the human HLA-B51 gene-transfer into mice. (Takeno *et al.* 1995)

Increased H_2O_2 production by neutrophils after stimulation with formyl-methionine leucine phenylalanine (fMLP) has been reported in B51 transgenic mice (Takeno *et al.* 1995). Since H_2O_2 production by neutrophils was not increased after stimulation with fMLP in B35 transgenic mice, a high increase in H_2O_2 production by neutrophils in these B51 transgenic mice was not ascribable to the xenogenic MHC genes transfection, but was considered to be a specific phenomenon resulting from the expression of B51 molecules (Takeno *et al.* 1995). Taken this together with the increased neutrophil function in B51-positive individuals, they have presented the hypothesis that the B51 molecule is directly involved in the hyperactivity of neutrophils in BD through specific antigen presentation.

However, in this analysis, the type of exogenous antigen peptides and their association with antigen presentation, which play the central role in immune responses between HLA and T cells, are still in a black box. And so the question remains unanswered as to the type of pathogenic antigen expressed in mice presents some exogenous antigen peptides, which are unrelated to the development of BD to T cells, but which lead to increased H_2O_2 production due to the intense reactivity of the B51 antigen toward these exogenous antigens. This is no more than comparative analysis of reactivity between B51 and B35 transgenic animals toward some unidentified and perhaps not the same exogenous antigen peptides, which have nothing to do with BD-inducing exogenous molecules, using H_2O_2 production by neutrophils as an index.

To our knowledge, no evidence has been found for the direct participation of the B51 antigen in neutrophil hyperactivity, and there has also been no evidence to indicate a direct involvement of B51 in the development of BD. However, the primary association of B51 with BD cannot be rigorously excluded. Considering the HLA function, it is possible that the B51 antigen is directly involved in the disease onset. These studies using B51 transgenic mice appear to be imporant for the future, and the mechanism for the onset of BD could be clarified by more extensive analyses.

HLA-C genotyping by PCR-SSP

The HLA-C gene is roughly estimated to be located about 90 kb telomeric of the HLA-B gene by pulsed-field gel electrophoresis analysis (Bronson *et al.* 1991). Recently, the distance between the HLA-C and HLA-B genes was exactly determined to be 81,247-bp long by DNA sequencing analysis adopting the shotgun strategy (Mizuki *et al.* 1997a). Regardless of this close localization of the HLA-C gene to the HLA-B gene, HLA-C analysis among BD patients has not been performed sufficiently.

HLA class I (HLA-A,-B, and -C) typing has been performed serologically. Serologic typing of HLA-C antigens is more difficult than other class I antigens and so-called HLA-Cw blank antigens, which cannot be serologically defined, are still present at the gene frequency of 30-50% in all human races (Baur *et al.* 1984; Aizawa, 1986). The most likely cause of serologically undefined HLA-C alleles (HLA-Cw blank) is the lack of suitable antisera coupled with low cell-surface expression (approximately 10% of the level of either HLA-A or -B), as well as a lack of knowledge of the haplotypes, which leads to potentially HLA-Cw blank-detecting sera being wrongly classified as HLA-B specific. Therefore, in HLA-C typing, untypeable so-called blank samples were frequent and correlation between the HLA-C

antigen and HLA-associated diseases could be established only with limited HLA-C antigens.

Actually, in HLA-C typing among BD patients, all the C antigens linked with the B51 antigen were untypeable due to no reaction against any serum used and no significant differentiation between patients and controls was clearly determined. After reanalyzing the HLA-C serologic typing data of the BD patients, it was found that one of the two C antigens possibly associated with B51-positive patients was blank and that another, or identical blank, was also common in the B51-negative patients. Therefore, the presence of HLA-Cw blank antigen(s) in most of BD patients suggested that this blank antigen(s) carries a particular HLA-C allele(s), which is correlated more strongly with BD than B51, and is primarily involved in BD development. Therefore, in order to identify the alleles of these serologically blank C antigens, we carried out genetic typing for the HLA-C gene by the PCR-SSP method (Mizuki *et al.* 1996a).

The frequency of the HLA-Cw*14 allele was 48.9% in BD patients, which was significantly higher compared to 24.0% in healthy controls (χ^2=12.53, R.R.=3.0). The frequency of HLA-Cw*15 was also significantly higher in the BD group (17.8% patients vs. 7.3% controls, χ^2=4.71, R.R.=2.7) (Table 2) (Mizuki *et al.* 1996a). These HLA-C alleles are in linkage disequilibrium with the B51 antigen and hence may have increased in association with B51. Accordingly, the HLA-C antigen frequencies were compared between B51-positive and

Table 2. Phenotype frequencies of HLA-C alleles among Behçet's disease patients (Mizuki *et al.* 1996a)

Alleles	Controls (N=96)	Patients (N=90)	χ^2	P-value	R.R
Cw*01	36 (37.5%)	21 (23.3%)	4.39	0.0398	0.51
Cw*02	0 (0 %)	0 (0 %)			
Cw*0302	0 (0 %)	4 (4.4%)			
Cw*0303	16 (16.7%)	16 (17.8%)			
Cw*0304	24 (25.0%)	7 (7.8%)	9.92	0.0027	0.25
Cw*04	9 (9.4%)	8 (8.9%)			
Cw*0501	1 (1.0%)	0 (0 %)			
Cw*06	0 (0 %)	1 (1.1%)			
Cw*07	19 (19.8%)	12 (13.3%)			
Cw*0704	0 (0 %)	0 (0 %)			
Cw*08	30 (31.3%)	26 (28.9%)			
Cw*12	14 (14.6%)	11 (12.2%)			
Cw*1203	0 (0 %)	0 (0 %)			
Cw*14	23 (24.0%)	44 (48.9%)	12.53	0.0005	3.0
Cw*15	7 (7.3%)	16 (17.8%)	4.71	0.0434	2.7
Cw*1504	0 (0 %)	0 (0 %)			
Cw*1505	0 (0 %)	0 (0 %)			
Cw*16	0 (0 %)	0 (0 %)			
Cw*1701	0 (0 %)	0 (0 %)			

Table 3. HLA-B antigen frequencies among Behçet's disease patients with HLA-Cw*14 (Mizuki *et al.* 1996a)

Antigen	Controls (gene No=28)	Patients (gene No=50)	χ^2	P-value	R.R
B51	10 (35.7%)	41 (82.0%)	15.01	0.0001	8.2
B44	16 (57.1%)	7 (14.0%)	14.06	0.0001	0.12
other HLA-B	2 (7.1%)	2 (4.0%)			

-negative individuals, and there was no HLA-C allele showing a significant difference between the BD and healthy control groups (data not shown) (Mizuki *et al.* 1996a). Analysis focusing on the linkage to HLA-Cw*14 revealed that, in the healthy controls, HLA-Cw*14 was associated with B44 and B51 at the frequencies of 57.1% and 35.7%, respectively, while in the BD group, the association of Cw*14 with B44 was merely 14.0% and that the association with B51 was exceedingly common at 82.0% (Table 3) (Mizuki *et al.* 1996a). If HLA-Cw*14 is a real pathogenic gene of BD, both B44 and B51 in linkage disequilibrium with this allele should be equally increased in association with the increase in Cw*14 and the linkage frequency should be almost equal to that in the Cw*14-positive healthy controls. However, in the patient group, Cw*14 was linked exclusively with B51, but not with B44 with which there was a strong linkage in the healthy controls. These data suggest that the pathogenic gene of BD is not the HLA-C gene (HLA-Cw*14 and/or Cw*15), but the HLA-B51 gene itself or some other gene located near the HLA-B locus (Mizuki *et al.* 1996a).

Anaysis of Tau-a microsatellite polymorphism

As mentioned above, HLA-B51 or some other nearby gene in linkage disequilibrium with HLA-B51 has been considered as a possible candidate gene for the control of susceptibility to BD. In the previous study, we have shown that the NcoI 10.5 kb fragment of the TNFβ (tumor necrosis factor β) gene located only 260 kb centromeric of the HLA-B gene is assosiated significantly with BD as a linkage distortion between the TNFβ and HLA-B genes (Mizuki *et al.* 1992b). However, since diallelic polymorphism does not provide an informative marker to map in detail the disease susceptibility gene, it is still uncertain whether the HLA-B51 or some other nearby gene is the pathogenic gene of BD. Then, which is more likely to be the pathogenic gene of BD, HLA-B or some other nearby gene? To answer this question, we investigated the genetic polymorphism of the Tau-a microsatellite existing only 10 kb telomeric of the TNF genes between the HLA-B and TNF genes in BD patients and healthy controls (Mizuki *et al.* 1995).

A microsatellite generally consists of repetitive sequences of 2 or 3 bases and all eukaryotic DNA contains a family of such repetitive sequences as junk DNA. The number of repetitive sequences varies among individuals and hence microsatellites can be used as informative polymorphic markers for genetic mapping. In this study, the Tau-a micro-

Table 4. Polymorphism of the Tau-a microsatellite between TNF and HLA-B genes in Behçet's disease (Mizuki *et al.* 1995)

Microsatellite		Control (N=40)	Patient (N=60)	χ^2	P-value	R.R
PCR product	(GT) repeat					
93	4	0 (0 %)	1 (1.7%)			
95	5	0 (0 %)	5 (8.3%)			
97	6	10 (25.0%)	18 (30.0%)			
99	7	4 (10.0%)	4 (6.7%)			
101	8	0 (0 %)	2 (3.3%)			
103	9	1 (2.5%)	6 (10.0%)			
105	10	9 (22.5%)	16 (26.7%)			
107	11	19 (47.5%)	4 (6.7%)	22.596	P<0.0001	0.1
109	12	1 (2.5%)	0 (0 %)			
111	13	3 (7.5%)	6 (10.0%)			
113	14	2 (5.0%)	18 (30.0%)	9.375	P<0.01	8.1
115	15	11 (27.5%)	10 (16.7%)			
117	16	4 (10.0%)	4 (6.7%)			
119	17	3 (7.5%)	11 (18.3%)			
121	18	7 (17.5%)	5 (8.3%)			
123	19	1 (2.5%)	0 (0 %)			

satellite consisting of 11 repetitions of GT was decreased remarkably (6.7% patients vs 47.5% controls, χ^2=22.596, R.R.=0.1) and conversely that of 14 repetitions was increased significantly in the patient group (30.0% patients vs 5.0% controls, χ^2 =9.375, R.R.=8.1) (Table 4) (Mizuki *et al.* 1995). Therefore, there is no doubt that specific haplotypes exist in BD patients which differ from those in healthy controls. However, it is not certain whether these results simply reflect linkage disequilibrium with HLA-B51 or whether these are some other genes around the Tau-a microsatellite or HLA-B region that may be associated primarily with BD. Therefore, we reanalyzed the genetic polymorphism of the Tau-a microsatellite by dividing patients into the B51 antigen-positive and -negative groups to investigate any linkage between allelic polymorphism of the Tau-a microsatellite and HLA-B antigens (Table 5).

As a result, it was found that there were 6, 10, 14, 15 and 17 repetitive sequences in the Tau-a microsatellite associated with the B51 antigen. If B51 is the pathogenic gene responsible for BD, a similar increase in the frequency of each Tau-a allele associated with B51 should have been found in the patient group, and the distribition of Tau-a alleles associated with B51 should have been almost the same in the B51-positive patient and control groups. However, among the Tau-a microsatellite alleles linked to B51, only the Tau-a allele consisting of 14 repetitions was significantly more common in the BD group than in the control group (Table 5). Furthermore, the Tau-a microsatellite consisting of 14 GT repeats was still found to be significantly more frequent in the B51-negative BD group than in the B51-negative healthy control group (data not shown). Therefore, the linkage of B51 with this microsatellite differed significantly between the patients with BD and healthy controls. These results suggest that the pathogenic gene of BD is not HLA-B51 itself but some other gene located on the haplotype linked firmly to the Tau-a microsatellite allele and having 14 repeats (Mizuki *et al.* 1995). However, since the frequency of the Tau-a microsatellite allele consisting if 14 repeats is 30% in the BD patient group, it is reasonable to assume that the pathogenic gene of BD is located around the HLA-B locus rather than the TNF gene region. The difference in the distribution of Tau-a alleles on the B51 haplotypes was observed in the haplotypes which had been recombined in the region between the HLA-B and TNF genes, and different Tau-a alleles belonged to the haplotypes which had recombined with the B51 haplotype. Furthermore,

Table 5. Polymorphism of the Tau-a microsatellite between TNF and HLA-B genes in Behçet's disease patients with HLA-B51 (Mizuki *et al.* 1995)

Microsatellite		Control (N=27)	Patient (N=35)	χ^2	P-value	R.R
PCR product	(GT) repeat					
93	4	0 (0 %)	1 (2.9%)			
95	5	0 (0 %)	1 (2.9%)			
97	6	9 (33.3%)	10 (28.6%)			
99	7	1 (3.7%)	3 (8.6%)			
101	8	1 (3.7%)	2 (5.7%)			
103	9	2 (7.4%)	3 (8.6%)			
105	10	9 (33.3%)	5 (14.3%)	3.163	P<0.1	0.3
107	11	4 (14.8%)	1 (2.9%)	2.939	P<0.1	0.2
109	12	0 (0 %)	0 (0 %)			
111	13	5 (18.5%)	5 (14.3%)			
113	14	3 (11.1%)	12 (34.3%)	4.463	P<0.05	4.2
115	15	4 (14.8%)	8 (22.9%)			
117	16	0 (0 %)	3 (8.6%)			
119	17	10 (37.0%)	9 (25.7%)			
121	18	3 (11.1%)	3 (8.6%)			

different Tau-a alleles associated with B51 can also be explained by the microsatellite slippage mutation which occurs significantly more frequently than conventional mutation within the genes (Miwa, 1994).

Genomic sequence determination of the HLA class I region and identification of novel genes designated NOB genes

The HLA region, located on chromosome 6p21.3, encompasses a 4,000 kb (4Mb) segment (Fig. 2). Currently 19 HLA or HLA-like expressed genes, more than 80 non-HLA expressed genes and 25 pseudogenes have been localized within the HLA region. On average, there in one gene detected at least every 20～30 kb in the HLA region. It is hard to predict whether or not the gene density observed in the HLA region is remarkably high since other regions in the human genome have not yet been characterized in sufficient detail. It is notable that many non-HLA genes involved or uninvolved in the immune response are located in the HLA region, although the function of most of these genes still remains uncertain. These are fewer genes so far identified in the class I region than in the class II or class III regions, especially in the gene-dense region of approximately 700 kb between the INT3 and BAT1 genes where more than 40 expressed genes have been identified (Campbell and Trowsdale, 1993). This is mainly because the class I region has not been studied so extensively, but it seems likely that the number of genes within the class I region will continue to increase as more sophisticated means of detecting coding sequences become available. As mentioned in the previous section, it is probable that the pathogenic gene responsible for BD is not the HLA-B51 gene itself, but some other gene around HLA-B. Therefore, in order to clarify the genomic structure of the uncharacterized class I region and to identify new genes involved in the development of BD, the genomic sequence of 7 contiguous cosmid clones isolated from a YAC (Y109) clone covering the 237 kb segment around the HLA-B and -C genes was determined (Fig. 2).

A YAC library constructed from the B cell line, CGM1 was used for screening and a YAC clone, Y109, was isolated from the library using HLA class I specific primers. It was found to span 600 kb, including the HLA-B and -C genes. This YAC clone was subcloned into pWE15 cosmid vector and then 315 isolated cosmid clones were aligned into a contig. Chromosomal location was assigned by both fluorescent *in situ* hybridization (FISH) and human and hamster somatic cell hybrid analysis using representative cosmid clones as probes. As a result, Y109 turned out to be a chimeric YAC clone, in which about 240 kb of the insert was derived from the chromosome 6p21.3 region and the remaining 360 kb insert from the chromosome 7p36 region (Nizuki *et al.* 1996c). Thus, in order to determine the genomic sequence of the cosmid contig derived from the chromosome 6p21.3 region, a shot-gun strategy was adopted. Namely, 7 contiguous cosmid clones covering the 237 kb segment around HLA-B and -C loci were selected from the contig (Fig. 2). To construct a shotgun library, each of the cosmid clones was sonicated directly, repaired with Klenow fragment, size-fractionated and ligated into a blunt-ended Sma I site of the dephosphorylated pUE 19 plasmid vector. Approximately 500 recombinant plasmid clones per each cosmid were randomly selected and subjected to cycle sequencing, followed by electrophoresis and analyses with ABI 373S automated sequencers.

As a result, we sequenced a single contig of a 236,822-bp-long region between the MICA gene 58.2 kb centromeric of HLA-B to 90.8 kb beyond HLA-C by the redundancy of 6.4 (Accession No. D84349) (Mizuki *et al.* 1997a). This region was confirmed to contain the 4 previously known genes, MICA, HLA-17 (HLA class I pseudogene), HLA-B and HLA-C. The numbers of A (adenine), C (cytosine), G (guanine) and T (thymine) were 66,997, 52,352, 52,463 and 65,010, respectively, indicating 44.3% GC content on average in this area, which corresponds to the isochore H1, although the HLA class I region in gross belongs to the GC-richest isochore H3 (53% GC on average) (Fukagawa *et al.* 1995). Homology searches of the nucleotide sequences against the most recent DNA database, GenBank release 90, were carried out using FASTA and BLAST. The 237 kb sequence was also analyzed for coding probability and exon-intron structure, using the coding recognition module of Grail, the gene finding program Grail II

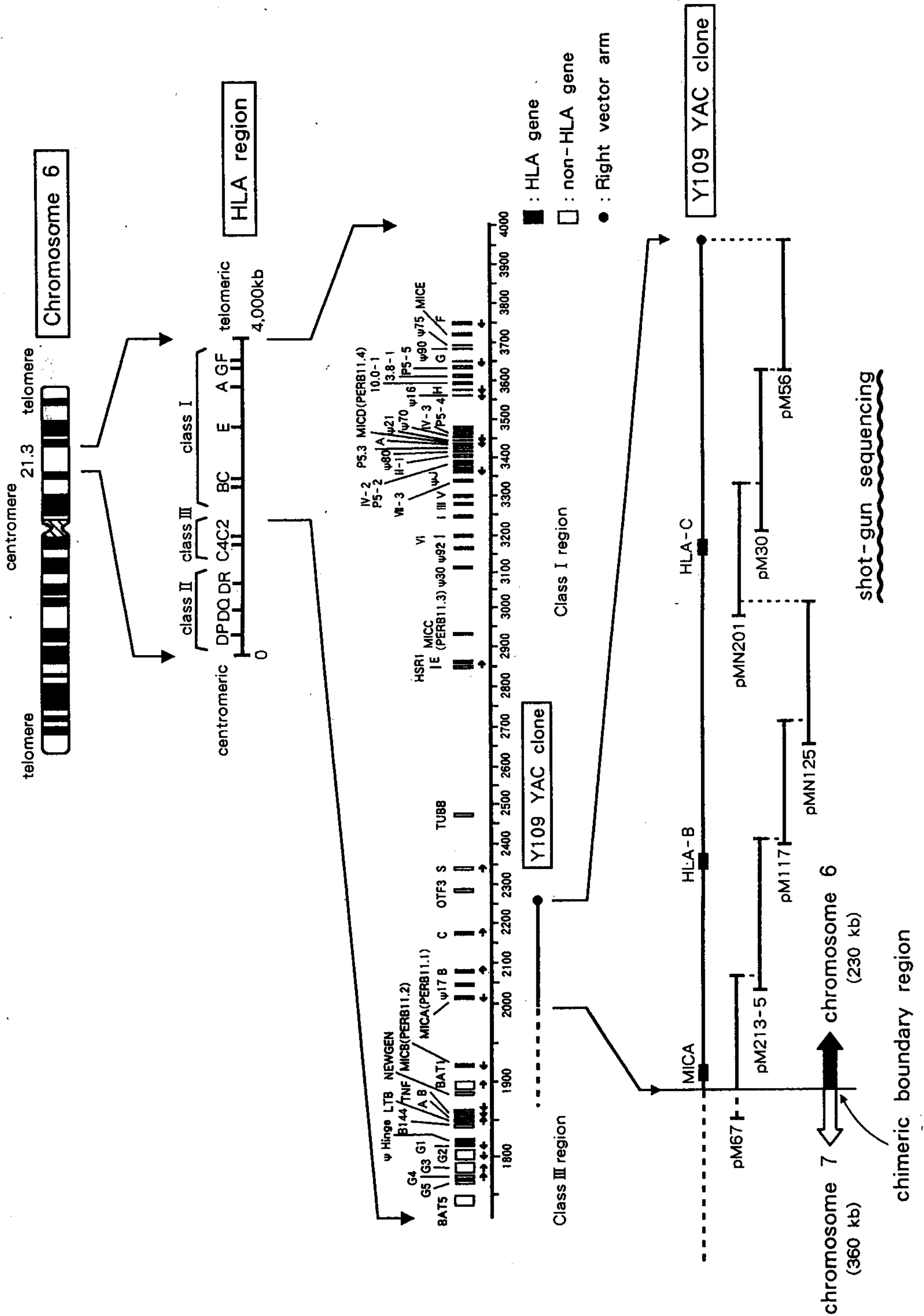

Fig. 2. Chromosomal mapping of the Y109 YAC clone. Seven contiguous cosmid clones, pM67, pM213-5, pM117, pMN125, pMN201, pM30, pM56, covering the 237 kb segment around the HLA-B and -C genes in the HLA class I region on the short arm of chromosome 6 (6p21.3) were subjected to DNA sequencing.

(Uberbacker and Mural, 1991). Then, as for the regions where coding sequences were predicted by homology search and/or Grail II analysis, we have performed RT-PCR and Northern blot analyses. As a result, 8 novel genes were found in this area. These novel genes were designated NOB (new organization associated with HLA-B) genes (Fig. 3) (Mizuki *et al.* 1997a, Inoko *et al.* 1997).

NOB1 is a novel housekeeping gene with a transcript of 10 kb. A partial cDNA clone, termed NOB1.1 (Accession No. D83543), was isolated from a skin fibroblast cDNA library and showed 100% nucleotide identity with the genomic sequence. NOB2 is a pancreas specific gene with a transcript of 1.1 kb. NOB3 is a skeletal muscle specific gene with multiple transcripts of 7, 1.4, 1.2 and 0.9 kb. However, it could not be ruled out whether these species of transcripts resulted from alternative splicing from a single gene or if they were derived from independent genes homologous to each other constituting a multicopy gene family. NOB4 is a placenta specific gene with a transcript of 6 kb. Although it is not certain whether these genes are expressed in eyes or not, each of them was considered to be a possible candidate gene of BD on the basis of its chromosomal localization. NOB5 is a skeletal muscle specific gene with a transcript of 7 kb and may be a pseudogene originated from a partial duplication of NOB3. The three remaining genes, NOB6, NOB7 and NOB8, were identified by homology search analysis. NOB6 is a new member of the P5 multicopy genes and designated as P5-8 (Accession No. D83771). NOB7 is a dihydrofolate reductase pseudogene and designated as DHFRP (Accession No. D83769). NOB8 is a ribosomal protein L3 homologous gene and designated as RPL3-Hom (Acession No. D83770). It will be necessary to isolate and characterize cDNA clones of NOB1～4 and other new genes remaining unidentified and also to investigated their causal relation with the deveolpment of HLA-B or -C associated diseases including BD. Genomic sequence determination is still in progress as to the telomeric direction and we have already sequenced more than 1.3 Mb in the HLA class I region.

Analysis of the MICA and MICB genes

Recently, a highly divergent MHC class I chain-related gene family, MIC, was identified within the class I region. The MIC gene contains 5 copies of homologues (MICA, MICB, MICC, MICD, and MICE) in the genome and they are spread across the 2 Mb MHC class I region. Among 5 MIC genes,

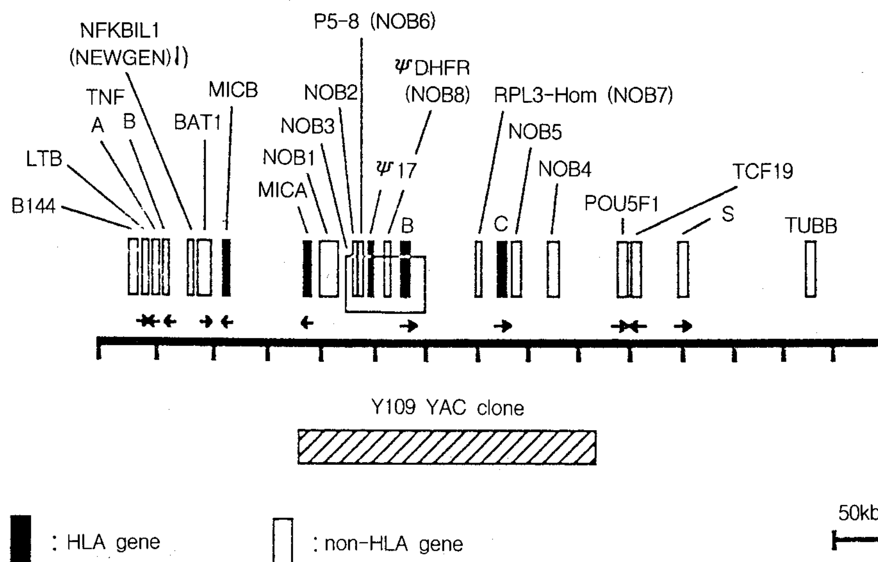

Fig. 3. Localization of the NOB genes in the HLA class I region (Inoko et al. 1997).

2 genes, MICA and MICB, are functional genes and located between the HLA-B and TNF genes. The MICA and MICB, are functional genes and located between the HLA-B and TNF genes. The MICA and MICB genes are localized 46.4 kb and 141.2 kb centromeric of the HLA-B gene, respectively, and their orientations are opposite to that of the HLA-B gene (Mizuki *et al.* 1997a; Shiina *et al.* in press). The full-length MICB cDNA sequence is 2,376 bp (Bahram and Spies, 1996a), much longer than that of MICA, which is 1,382 bp (Bahram *et al.* 1994). However, the length of open reading frame (ORF) is equal for each encoding a polypeptide of 383 amino acids. The larger size of a MICB transcript is due to the presence of the longer 3' untranslated region. The MICA and MICB genes show 91% nucleotide and 83% amino acid identities in their coding regions, where they share a low level (26~31%) of amino acid identity in the extracellular domains with MHC class I sequences of human, mouse and various other species of mammalians and non-mammalians, including reptiles, chickens, and frogs (Bahram *et al.* 1994; Leelayuwat *et al.* 1994). MIC genes are conserved in most if not all mammalians and therefore represent a second lineage of mammalian MHC class I genes.

MICA and MICB are transcribed in fibroblast and epithelial cell lines but not in T-or B-lymphocytes in contrast to MHC class I genes which are ubiquitously expressed. Expression levels of MICA and MICB are not affected by type I and II interferons (Bahram *et al.* 1994), known to markedly upregulate the level of typical MHC class I gene expression, but are responsive to cell-stress due to heat shock response elements in their promoters similar to those of HSP70 genes (Bahram and Spies, 1996b; Groh *et al.* 1997). MICA and MICB molecules include 8 and 5 sites for potential N-linked glycosylation, respectively, and lack all of the residues implicated in the binding of CD8, making it difficult to present any ligands to cytotoxic T cells. It has been indicated that several unique structural similarities are shared by MICA and H-2T22^b and H-2T10^b, which can be recognized by $\gamma\delta$ T cells (Ito *et al.* 1990; Schild *et al.* 1994). MICA and MICB are absent from the mouse genome, mirrored by the reciprocal lack of murine non-classical H-2 loci (class Ib) such as H-2Q, M and T in other mammalian species. Thus, MIC genes are predicted to be evolutionary counterparts of some of these murine class Ib loci (Bahram and Spies, 1996b; Fodil *et al.* 1996). Since some of these murine class Ib molecules can selectively present bacterial peptides recognized by $\gamma\delta$ T cells (Schild *et al.* 1994; Kaliyaperumal *et al.* 1995), a similar role for MIC molecules in other species was hypothesized (Bahram and Spies, 1996b; Fodil *et al.* 1996). MICA encodes a cell surface glycoprotein that is not associated with β_2-microglobulin and is conformationally stable independent of conventional class I peptide ligands (Groh *et al.* 1996). Therefore, MICA and MICB may have been adapted for some specialized function, presumably in the early evolutionary process of MHC class I genes.

1) Triplet repeat polymorphism in the TM region of the MICA gene

As described previously, we characterized the Y109 YAC clone including a 237-kb segment around the HLA-B and -C genes. Subcloning the YAC clone into the pWE15 cosmid vector and aligning it into a contig, we isolated overlapping cosmid clones containing the MICA gene. Thus, the nucleotide sequence of the MICA gene was obtained by the shotgun sequencing procedure. The exon-intron organization with 6 exons separated by introns was grossly similar to that of MHC class I genes (Bahram *et al.* 1996; Mizuki *et al.* 1997a)

Comparison of the coding sequence of the MICA gene in the cosmid clone with the MICA cDNA sequence previously reported (Bahram *et al.* 1994). revealed a 4-nucleotide insertion which caused a frame shift mutation resulting in a premature termination by the generation of a stop codon (TAA) in the TM region of the MICA genomic sequence (see the allele A5.1 in Fig. 4). Because this truncated MICA molecule is not rich in hydrophobic amino acid residues in the TM region, it may not reside on the cellular membrane, but rather be secreted as soluble antigen (Mizuki *et al.* 1997e). These facts prompted us to screen for other genetic polymorphisms in the MICA TM region using 68 HLA-homozygous B-cell lines provided by the 10th International Histocompatibility Workshop. Consequently, additional triplet repeat (GCT/AGC) poly-

morphisms were identified in the MICA TM region. In the 68 B-cell lines examined, 5 distinct alleles in the TM region of the MICA gene were detected. As shown in Fig. 4, the alleles consist of 4, 5, 6 and 9 repetitions of GCT/AGC (alanine) in addition to the above mentioned one with 5 repetitions of GCT/AGC with one additional nucleotide insertion (G/C). They are designated A4, A5, A6, A9 and A5.1, respectively (Fig. 4) (Mizuki *et al.* 1997e).

Microsatellietes are not generally identified within genes. However, on the other hand, it is well known that several human hereditary disease-associated genes, including myotonic dystrophy (Brook *et al.* 1992; Mahadevan *et al.* 1992), Kennedy's disease (La Spada *et al.* 1993), spinocerebellar ataxia type I (Orr *et al.* 1993), Huntington's disease (The Huntington's Disease Collaborative Reserch Group, 1993) and dentatorubralpallidoluysian atrophy (Koide *et al.* 1994; Nagafuchi *et al.* 1994) contain CAG/ CTG triplet repeats. Four of these genes contain CAG/ CTG which encode polyglutamine repeats in their coding regions. The development of these diseases is associated with the massive expansion of CAG/CTG repeats, whereby the severity of the disease is increased and the age of onset is lowered in successive generations. In this context, it is of great interest to explore the possibility that any microsatellite polymorphism existing in the TM region of the MICA gene leads to the development of hereditary diseases including BD and ankylosing spondylitis.

Therefore, to address the possibility that the MICA gene, rather than HLA-B51, is responsible for determining the genetic predisposition to BD, triplet repeat polymorphism in the TM region of the MICA gene was investigated in 77 Japanese patients with BD and 103 controls. As seen in Table 6, the phenotype frequency of the A6 allele was significantly increased in patients with BD compared to healthy controls (P=0.00011, Pc=0.00055). Forty-four out of 77 patients with BD were B51-positive (57.1%) and all of them possessed the A6 allele. Furthermore, 13 B51-negative patients possessed the A6 allele and, in total, 57 out of 77 patients carried A6 (74.0%) (Pc=0.00055), indicating that the association of the A6 allele in the MICA gene with BD is stronger than that of HLA-B51 (Pc<0.001) (Mizuki *et al.* 1997e). By now, more than 20 alleles have been recognized in the putative peptide binding regions (exons 2 and 3) of the MICA gene (Fodil *et al.* 1996; S. Bahram, personal communication). Therefore, the conspicuously high frequency of the A6 microsatellite allele in BD patients may reflect linkage disequilibrium with a certain allelic form of the MICA in the peptide binding region.

Although the immunopathogenic mechanism underlying the development of BD remains uncertain, its various characteristic symptoms have been attributed to neutrophil hyperactivity and abnormality or hyperactivity of $\gamma\delta$ T cells (Koide *et al.* 1993; Yamashita *et al.* 1993), which possibly recognize MICA-bound ligands (Bahram and Spies, 1996b; Fodil *et al.* 1996). Obstructive angiitis in organs such as eyes (choroid and retina), oral mucosa, genitals and the skin, all of which are likely to be exposed to infectious microorganisms, is the first step in the inflammation characteridtic of BD. The MICA gene is expressed specifically in fibroblast and epithelial cells, again precisely where the inflammation of BD exclusively takes place. Based on the chromosomal localization of the MICA gene, its predicted function, and the strong association ot the microsatellite allele, A6, in the TM region, the MICA gene is one of strong candidates for susceptibility to BD.

2) Allelic variants of the MICA and MICB genes

In light of the high degree of polymorphism in typical MHC class I genes, it was of great importance to assess the extent of allelic variation within the MIC locus. In 20 homozygous-typing cell lines (HTCLs) studied, 27 variations, among which 22 were nonsynonymous, were identified in the coding sequence of MICA (exons 2, 3 and 4), establishing the identified of 16 MICA alleles (MICA001～MICA016) (Fodil *et al.* 1996). In subsequent analysis, 4 new alleles were identified, amounting to a total of 20 MICA alleles to date (S. Bahram, personal communication). A tentative superimposition of MICA polymorphic residues upon the HLA-A2 three-dimensional structure indicates that most MICA variants tend to map to the periphery of the putative antigen binding cleft (Fig. 5). Thus, the putative ligand binding site may be invariant facilitating presentation of a conserved

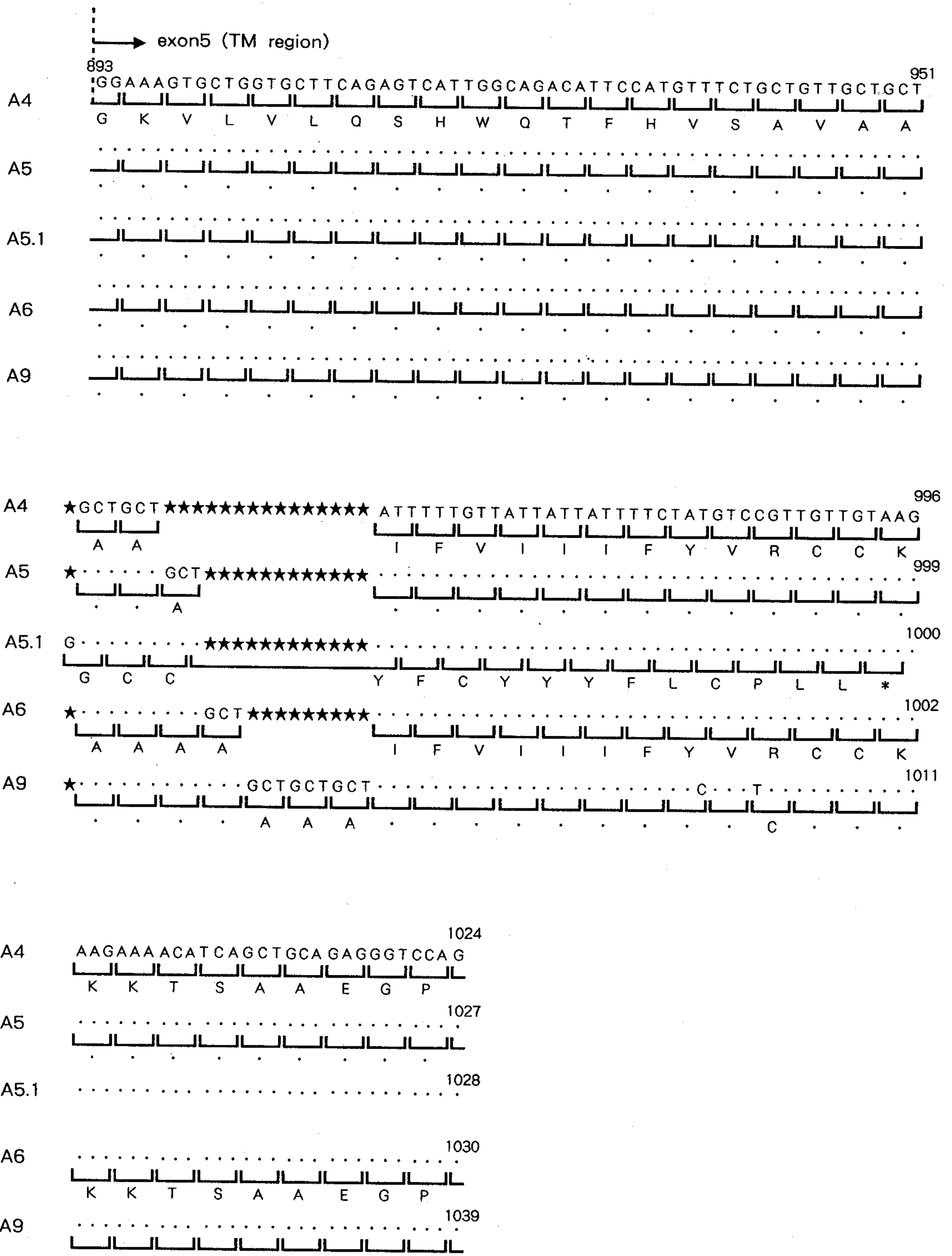

*Fig. 4. Microsatellite polymorphism in the TM region (exon 5) of the MICA gene. Alphabetical character under the bracket represents amino acid abbreviation. Dot shows the same nucleotide or amino acid as the upper one. Black star shows nucleotide deletion site, and *indicates stop codon (Mizuki et al. 1997e).*

Table 6. Phenotype freqeuencies of the microsatellite polymorphism in the TM region (exon 5) of the MICA gene in Behçet's disease (Mizuki *et al.* 1997e)

Microsatellite		Control (N=103)	Patient (N=77)	χ^2	P-value	R.R
PCR product	(GCT/AGC) repeat					
179	A4	31 (30.0%)	15 (19.5%)			
182	A5	54 (52.4%)	30 (39.0%)			
183	A5.1	17 (16.5%)	6 (7.8%)			
185	A6	47 (45.6%)	57 (74.0%)	14.562	0.00011	3.40
194	A9	32 (31.1%)	20 (26.0%)			

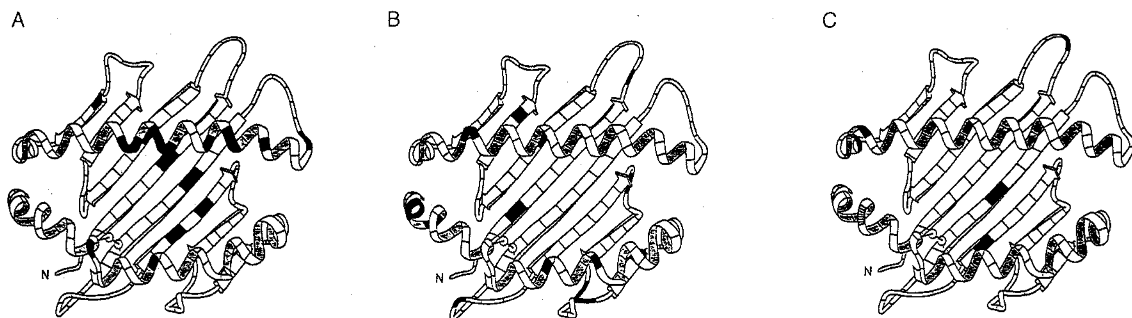

Fig. 5. Comparative view of A: HLA, B: MICA, and C: MICB variable residues depicted in black (Ando et al. 1997).

antigen by the MICA molecule. The unusual distribution of the MICA amino acid substitutions along the outer edge of the putative MICA ligand binding site is consistent with a possible lateral interaction of this molecule with the T-cell or NK antigen receptors.

Similarly, to elucidate the extent of MICB allelic variation, we sequenced exons 2 (α1), 3 (α2), 4 (α3), and 5 (TM) as well as introns 2 and 4 of this gene in 46 HLA homozygous B-cell lines. As a result, 7 non-synonymous, 2 synonymous, and 4 intronic nucleotide variations were observed, establishing the identification of 11 MICB alleles (Ando *et al.* 1997). Interestingly, one allele has a nonsense mutation resulting in a premature termination codon in the α2 domain. The 7 amino acid replacements define 6 alleles designated as MICA0101~MICB0106, and the premature termination characterizes one allele, MICB0107N (N:null) (Ando *et al.* 1997). Thus, MICB appesrs to have fewer alleles than MICA, not unlike the allelic ratio between the HLA-C and -B loci. Like MICA polymorphisms, most MICB variants tend to map to the periphery of the putative ligand binding cleft but not the residues presumably in direct contact with the peptides or the T-cell receptors (Fig. 5), possibly facilitating presentation of a conserved antigen by the MICB molecule.

Taken together, MICA was found to be a highly polymorphic member with more than 20 alleles, while MICB, with a total of 11 alleles, is characterized by relatively low polymorphism. We have already established the methods to differentiate these MICA and MICB alleles using the PCR-SSP, -RFLP and -SSCP (single strand conformation polymorphism) techniques. The MICA and MICB allele typing in BD patients is in progress to investigate whether any specific amino acid for BD exists in the putative peptide binding regions of the MICA or MICB genes.

CONCLUSIONS

The present investigations on the genetic predis-

position for BD was reviewed and discussed in relation to our recent data. Although the possibility of the primary association of B51 with BD cannot be excluded, it is probable that the pathogenic gene responsible for the development of BD is not the B51 gene itself but another gene located between the HLA-B and TNF genes. We have sequenced a single contig of the 236,822-bp -long region around the MICA, HLA-17, HLA-B, HLA-C and OTF genes and further sequencing is in progress. Eight novel genes provisionally designated NOB1 to NOB8 were identified in this area. Although some of them were considered to be possible candidate genes, the MICA was revealed to be the strongest candidate gene responsible for the development of BD.

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