Mannose-binding lectin gene-2 polymorphisms and serum mannose-binding lectin levels in Behçet's disease

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ABSTRACT

Objective. *Behçet's disease (BD) is an autoimmune disease with an unknown etiology and mannose-binding lectin (MBL) is a pattern recognition receptor in the innate immune system, which is associated with some autoimmune diseases. We investigated MBL2 gene polymorphisms and serum MBL levels in BD patients and controls.*

Methods. *MBL2 gene polymorphisms in exon 1 (MBL2 54 Gly/Asp, (A/B)), promoter (MBL2 H/L (G-550C), MBL2 Y/X (G-221C)), and 5' UTR region (MBL2 P/Q (C+4T)) were investigated using polymerase chain reaction and restriction fragment length polymorphism in 119 BD patients and 252 healthy controls. Serum MBL levels were measured by enzyme linked immunosorbent assay in 49 BD patients and 102 sex-/genotype-matched controls.*

Results. *No significant difference was found between BD patients and controls in terms of MBL2 polymorphisms and MBL serum levels. However, the presence of genital ulcer and neurologic involvement were found to be associated with MBL2 54 allele A (OR=2.415, OR=6.632, respectively). Eye involvement was found to be related to the presence of the MBL2 54 AA or AB genotypes (OR=12.46), MBL2-G-550C allele H (OR=1.829). High serum MBL level (≥500 ng/ml) was associated with skin lesions (p=0.002).*

Conclusion. *The frequencies of the four MBL2 genetic polymorphisms examined were not different in BD patients and healthy controls. However, the presence of genital ulcer, eye involvement, and neuro-Behcet's disease were found to be associated with MBL2 polymorphisms that are associated with the production of high levels of MBL or functional MBL.*

Introduction

Behçet's disease (BD) is an autoimmune disease of unknown etiology, which involves the mucocutaneous, ocular, musculoskeletal, vascular, gastrointestinal, and central nervous systems. Genetic factors, infectious triggers, vascular damage, and immune system dysfunction are implicated in its pathogenesis (1). Mannose-binding lectin (MBL) is a serum protein produced by the liver and enhances opsonization or activates the complement pathway as a component of the innate immune system (2). Genetic polymorphisms in the promoter site and exon 1 of *MBL2* have been closely related with serum MBL levels or its function. Promoter nucleotide substitutions at *MBL2 G-550C* (H/L) and *MBL2 G-221C* (Y/X) modulate transcription, whereas several other promoter variants have no effect on MBL at the protein level (3, 4). Moreover, the variants in exon 1 of *MBL2* generate amino acid substitutions in its collagen-like region at codons *Gly54Asp* (B) and *Gly57Glu* (C) (5-7), and these substitutions interfere with the assembly of MBL trimers. Furthermore, the presence of the *Arg52Cys* (D) variant in exon 1 leads to aberrant intramolecular disulfide bonds, and results in variant MBL monomers with poor complement-fixing ability and possibly a higher turnover rate (8). In addition, a *C+4T* (P/Q) polymorphism in the 5' untranslated region (5'-UTR) of *MBL2* has also been reported in genetic studies (4, 5).

Genetic variations or low serum concentrations of MBL have been reported to be associated with increased risks of infectious diseases (9), and autoimmune diseases, such as, rheumatoid arthritis (10), systemic lupus erythematosus (11, 12), and Sjögren's syndrome (13). Recently, several reports have addressed the associations between MBL levels or polymorphisms and BD. One study in Turkey showed serum levels of MBL are lower in BD patients than in healthy controls, and that a severe disease course is more frequently observed in BD patients with

very lower serum MBL levels (14). A Japanese study also reported an association between BD and the genotype AB of *MBL2 54* (15). In a Korean genetic study, the haplotype HYPA was found to be associated with several organ involvements, early onset disease and poor response to therapy (16). However, the results of previous studies are inconsistent and the association between BD and MBL remains unclear. Therefore, we investigated *MBL2* gene polymorphisms and serum levels in BD patients and attempted to identify associations between them and clinical manifestations of the disease.

Patients and methods

Patients

A total 119 BD patients, as diagnosed using the International Study Group Criteria (17), and 252 healthy controls were enrolled at the Rheumatologic Clinic, Seoul National University Hospital. Clinical data including sex, age, age at disease onset, duration of disease, the presence of oral ulcer, genital ulcer, skin lesions (erythema nodosum like lesion, acneiform lesion), eye involvement (uveitis or retinitis), arthritis, neuro-Behçet's disease, and deep vein thrombosis, and the results of skin pathergy test and HLA-B51, were collected by reviewing medical records. After obtaining informed consent, blood samples were drawn for genotyping and for determining serum MBL levels. The serum MBL levels were measured in 49 BD patients and 102 sex-/genotype-matched controls randomly selected from among genotyped subjects.

Genotyping

Genomic DNA was extracted from peripheral blood using QIAamp Blood Mini Kits (Qiagen, Valencia, USA). The presence of polymorphism chosen from previous studies was determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis or PCR with sequence-specific primers. All restriction endonucleases for RFLP were purchased from New England Biolabs (Beverly, USA). *MBL2 G-550C* (H/L) status was determined by PCR using 5'-GGGGCTAGGCTGCTGAGGTTTC-3' as a forward primer and 5'-TTGCTTCCCCTTGGTGTTGTA-3' as a reverse primer and by RFLP using *Acc*I (13). *MBL2 C+4T* (P/Q) status was determined by PCR using 5'-CAGATTGTAGGACAGAGGGCGAGCT- 3' as a C-specific forward primer and 5'- CAGATTGTAGGACAGAGGGCAAGCT- 3' as a T-specific forward primer and 5'-CACCATACTCAGGAGAAGGA-3' as a reverse primer and by *Sac*I and *Hind*III digestion. The *MBL2 Arg52Cys* (D) status was determined by PCR using 5'-CATCAACGGCTTCCCAGGCAAAGACGCG-3' as a forward primer and 5'-AGGATCCAGGCACTTTCCTCTGGAAGG-3' as a reverse primer and by *Mlu*I digestion (5). The presences of the *MBL2 Gly54Asp* (B) and *Gly57Glu* (C) polymorphisms were determined by PCR using 5'-GTAGGACAGAGGGCATGCTC-3' as a forward primer and 5'-CAGGCAGTTTCCTCTGGAAGG-3' as a reverse primer and by *Ban*I and *Mbo*II RFLP (18). *MBL2 G-221C* variant (X/Y) status was determined using sequence-specific primers, *i.e.* 5'-CCTGCCAGAAAGTAGAGAGG-3' as a forward primer and 5'-CTGGAAGACTATAAACATGCTTTCC-3' as a G-specific reverse primer and 5'-GGAAGACTATAAACATGCTTTCG- 3' as a C-specific reverse primer (19).

Measurement of serum MBL

Blood samples were centrifuged at 3000 rpm for 10 minutes immediately after being drawn. Serum was separated and stored at -70°C until required for assay. Serum MBL levels were measured using MBL oligomer ELISA kits (KIT 029, Bioproto Diagnostics, Gentofte, Denmark). Briefly, diluted serum samples were incubated in microwells precoated with a specific antibody against the MBL carbohydrate-binding domain. Unbound components were removed by washing. Wells were incubated with biotinylated antibody to MBL, washed, incubated with horseradish peroxidase-conjugated streptavidin, and then with a chromogenic substrate. The reaction was stopped with sulfuric acid, and optical density was read at 450 nm using an ELISA reader.

Statistical analysis

The clinical characteristics of BD patients and controls were compared using the *t*-test or the χ^2 test for continuous and categorical variables, respectively. Serum levels of MBL in BD patients and controls or for genotypes were compared using the analysis of variance (ANOVA). Testing for compliance with Hardy-Weinberg equilibrium was performed using the χ^2 test. Estimations or analyses of alleles, genotypes, or haplotypes and calculations of odds ratios (OR) were performed using UNPHASED, version 3.0.12 (MRC Biostatistics Unit, Cambridge, UK) and differences in the haplotype frequencies of the BD patient and control groups were analyzed by permutation testing (1,000 random permutations). If the cell count was zero, it was replaced with 0.5 to produce an approximate OR. *P*-values of <0.05 were considered statistically significant. For multiple comparisons, probability values (*p*-values) were corrected (p_c) for the numbers of comparisons made, and results were considered significant when p_c values were <0.05. Data were analyzed using SPSS software, version 12.0K (SPSS Inc, Chicago, IL).

Results

Demographic and clinical characteristics in BD patients and controls

One hundred and nineteen BD patients (males: 51.3%) and 252 healthy controls (males: 24.6%) were studied. The mean age of BD patients (mean ± SD) was 41.7±10.1 years and that of the controls was 40.1±15.4 years. HLA-B51 was found more frequently in BD patients than in controls (37.0% *vs.* 21.8%, *p*=0.018, the χ^2 test). The clinical characteristics of BD patients are shown in Table I.

MBL2 genetic polymorphism and serum levels of MBL in BD patients and controls

The *Arg52Cys* and *Gly57Glu* genetic polymorphisms in exon 1 of *MBL2* were not detected in patients or control, and thus, were excluded from further analysis. Allele and genotype frequencies of the *MBL2* polymorphism were similar

Table I. Clinical characteristics of Behçet's disease patients.

Characteristics	n=119
Age (year, mean ± SD)	41.7 ± 10.1
Sex	61 / 58
(Male / Female)	(51.3%/48.7%)
Age of onset (year, mean ± SD)	28.5 ± 11.0
Disease duration (year, mean ± SD)	13.2 ± 8.3
HLA-B51 positivity	44 (37.0%)
Oral ulcer	119 (100%)
Genital ulcer	92 (77.3%)
Ocular lesion	45 (37.8%)
Anterior uveitis	43 (36.1%)
Retinitis	3 (2.5%)
Skin lesion	96 (80.7%)
Erythema nodosum like lesion	77 (64.7%)
Acneiform lesion	47 (39.5%)
Pathergy test	66 (55.5%)
Deep vein thrombosis	20 (16.8%)
Neurologic involvement	6 (5%)
Arthritis	57 (47.9%)

in patients and controls (Table II). Because haplotype HYPA was reported to be associated with clinical manifestations in a recent study (16), the frequencies of haplotypes of H/L-Y/X-P/Q-A/B were estimated and compared for patients and controls. However, no difference was found for any haplotype (p_c=0.6174, by permutation test, 1,000 random simulations). Moreover, no significant difference was found between *MBL2* polymorphism frequencies in patients and controls after stratifying by sex or positivity for HLA-B51 (data not shown). Furthermore, serum MBL levels were not significantly different in patients and controls {median (range): 2,660 (0-25,772) *vs.* 1,185 (0-11,416), respectively, p=0.111 by the Mann-Whitney U test}. The proportion of individuals with very low MBL (<100 ng/ml) was not different between patients with BD and controls. However, those with high serum MBL level (≥500 ng/ml) were significantly more frequent in BD compared to controls (82.0% *vs.* 62.7%, p=0.037, by the χ^2 test).

Association between serum MBL levels and genetic polymorphisms

MBL2 54 and *MBL2* H/L were found to be associated with serum MBL levels in BD patients and in healthy controls, as was expected (Fig. 1). Genotype AA of *MBL2 54* in exon 1 was found to be related to a higher MBL level than genotype AB or BB (p=0.003 for BD patients and p=0.001 for controls respectively, by the ANOVA). Moreover, genotype HH of the *MBL2* promoter site polymorphism was found to be associated with higher MBL serum levels than genotypes HL or LL (p=0.006 for BD patients and p=0.031 for controls respectively, by the ANOVA). However, no significant association was found in the *MBL2* Y/X or *MBL2* P/Q variants and serum MBL levels.

MBL2 polymorphism and clinical manifestations of BD

Because the *MBL2 54* mutation in exon 1 and the *MBL2* H/L variant have been reported to affect serum MBL, we investigated the possibilities of associations between these polymorphisms and the clinical manifestations of BD. The clinical variables examined were; age at disease onset, presence of genital ulcer, skin lesions, eye involvement, deep vein thrombosis, arthritis, neurologic involvement and pathergy test findings. Among BD patients, the *MBL2 54* allele A were found to be more common in those with genital ulcer (for allele A, 66.3% *vs.* 40.7%, OR=2.415, 95% confidence interval (CI)=1.248-4.686, p=0.010, by likelihood ratio test). In addition, the AA or AB genotypes were more frequent in patients with eye involvement (100% *vs.* 88%, OR=12.46, p_c=0.008 by likelihood ratio test), and allele H was also more common in BD patients with eye involvement (58.9% *vs.* 43.9%, OR=1.829, 95% CI=1.076-3.109, p=0.025 by likelihood ratio test). Moreover, *MBL2 54* allele A was more common in patients with neurologic involvement (100% *vs.* 75.2%, OR=6.632, p=0.010 by likelihood ratio test, all genotypes were AA).

The frequency of haplotype HYPA in various clinical manifestations was not different compared to the overall frequency of other haplotypes except HYPA. The high serum MBL level (≥500 ng/ml) was associated with skin lesions (87.0% *vs.* 25.0%, p=0.002, by χ^2 test).

Table II. Allele and genotype frequencies of *MBL2* polymorphisms in Behçet's disease (BD) patients and healthy controls.

Loci*	Allele or genotype	BD (n=119)	Controls (n=252)	p_c-value†	OR (95% CI)‡
MBL2 H/L	H (*G*)	118 (49.6%)	259 (51.4%)		–
	L (*C*)	120 (50.4%)	245 (48.6%)	> 0.05	1.075 (0.790 - 1.463)
	HH (*GG*)	25 (21.0%)	62 (24.6%)		–
	HL (*GC*)	68 (57.1%)	135 (53.6%)		1.249 (0.722 - 2.161)
	LL (*CC*)	26 (21.8%)	55 (21.8%)	>0.05	1.172 (0.607 - 2.264)
MBL2 Y/X	Y (*G*)	215 (90.3%)	458 (90.9%)		–
	X (*C*)	23 (9.7%)	46 (9.1%)	>0.05	1.065 (0.629 - 1.803)
	YY (*GG*)	97 (81.5%)	206 (81.7%)		–
	YX (*GC*)	21 (17.6%)	46 (18.3%)	>0.05	0.970 (0.548 - 1.714)
	XX (*CC*)	1 (0.8%)	0 (0%)		–
MBL2 P/Q	P (*C*)	219 (92.0%)	459 (91.1%)		–
	Q (*T*)	19 (8.0%)	45 (8.9%)	>0.05	0.895 (0.506 - 1.549)
	PP (*CC*)	100 (84.0%)	207 (82.1%)		–
	PQ (*CT*)	19 (16.0%)	45 (17.9%)	>0.05	0.874 (0.486 - 1.572)
	QQ (*TT*)	0 (0%)	0 (0%)		–
MBL2 A/B	A (*G*)	182 (76.5%)	373 (74.0%)		–
	B (*A*)	56 (23.5%)	182 (26.0%)	>0.05	0.876 (0.612 - 1.225)
	AA (*GG*)	72 (60.5%)	140 (55.6%)		–
	AB (*GA*)	38 (31.9%)	93 (36.9%)		0.795 (0.495 - 1.274)
	BB (*AA*)	9 (7.6%)	19 (7.5%)	>0.05	0.921 (0.397 - 2.140)

*H/L: *MBL2 G-550C*, Y/X: *G-221C*, P/Q: *C+4T* in 5' UTR, A/B: *Gly54Asp*; †p_c: corrected p-value by Bonferroni methods; ‡OR: odds ratio relative to reference alleles or genotypes, 95% CI: 95% confidence interval.

Discussion

In the present study, no polymorphism of the *MBL2* gene was found to be associated with the presence of BD, but genetic polymorphisms of MBL2 were related to several of its clinical mani-

Table III. Estimated haplotype frequencies in Behçet's disease patients and healthy controls (adjusted *p*-value from permutation test = 0.623).

Haplotype*	Behçet's disease (n=119)	Controls (n=252)	OR† (95% CI‡)
HYPA	116 (49.6%)	251 (51.7%)	-
LYPA	26.27 (11.2%)	45.43 (9.3%)	1.251 (0.736, 2.127)
LYQA	16.73 (7.2%)	31.57 (6.4%)	1.147 (0.607, 2.167)
LXPA	23 (9.8%)	38 (7.8%)	1.31 (0.746, 2.299)
LYPB	49.73 (21.3%)	107.6 (22.1%)	1 (0.669, 1.496)
LYQB	2.267 (1.0%)	12.43 (2.6%)	0.395 (0.087, 1.786)

*Rare alleles with frequencies of less than 2% were excluded from the analysis; H/L: *MBL2 G-550C*, Y/X: *G-221C*, P/Q: *C+4T* in 5' UTR, A/B: *Gly54Asp*; †OR: odds ratio relative to reference alleles or genotypes; ‡95% CI: 95% confidence interval.

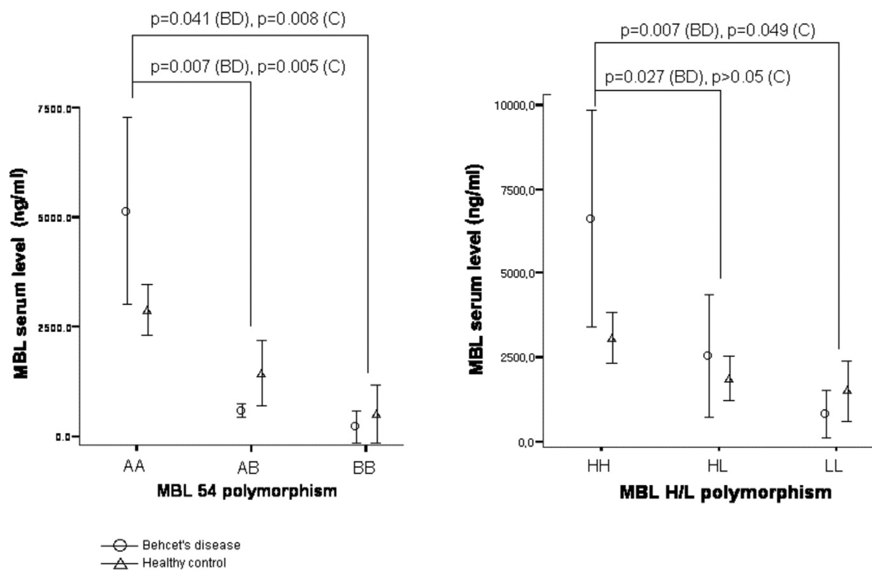

Fig. 1. Relationships between serum MBL levels and the *MBL2* 54 and *MBL2* H/L polymorphisms in patients with Behçet's disease (n=49) and controls (n=98). Data are presented using mean (❍, △) and standard deviation (horizontal bar). All *p*-values were calculated using ANOVA (*MBL2* 54; *p*=0.003 for BD patients and *p*=0.001 for controls, *MBL2* H/L (*p*=0.006 for BD patients and *p*=0.031 for controls respectively, by the ANOVA) and post-hoc Tukey's test (*p*-values are presented in Figures. BD: Behçet's disease, C: control).

festations including; the presence of genital ulcer, eye involvement, and neurologic involvement. Of the genetic variants of *MBL2* exon 1 or of the promoter site, polymorphisms known to produce functional MBL or high levels of MBL were more common in patients with genital ulcer, eye involvement or neurologic involvement. High serum MBL levels (≥500 ng/ml) were detected in a significantly higher proportion of patients with BD compared to controls and were associated with skin manifestations. The associations between genetic polymorphisms and serum MBL concentration found in the present study concurred with previously published results (3).

Previous studies on MBL in Behçet's disease have reported disparate results. Inanc *et al*. found serum MBL was lower in BD, and that low serum MBL was related to a severe disease course, and in men, with vascular involvement (14). Wang *et al*. found the AB genotype in exon 1 of *MBL2* 54 was more common in BD patients (n=30) than in healthy controls (n=30) (15). From the results of these studies, the polymorphism producing dysfunctional MBL might be associated with BD susceptibility. However, in a recent genetic study involving 282 BD patients and 271 normal controls, the low MBL producing genotype LL of *MBL2* H/L was found to be present at low frequency in BD. Furthermore, the HYPA haplotype containing the H and A allele, which is associated with high levels of MBL or functional MBL, was found to be associated with BD occurrence and some clinical manifestations, such as, early onset disease, poor response to therapy, and the presence of ocular lesion or vasculitis (16). These conflicting results could be explained by different genetic backgrounds, patient inclusion criteria (two Japanese criteria or those of the International Study Group for Behçet's Disease), or heterogeneity of clinical manifestations. However, polymorphisms of *MBL2* have been consistently reported to be closely related with MBL functionality or serum level. Thus, genetic background difference may not entirely explain disparities in results.

Association between *MBL2* polymorphism and BD may depend on disease manifestations. Furthermore, the clinical manifestations of BD are diverse and cluster of these manifestations may reflect meaningful BD subsets (20, 21). Uveitis may develop frequently in patients with genital ulcer and oral ulcer whereas deep vein thrombosis may not. Neurologic disease may involve another cluster of patients. In the present study, genital ulcer, eye manifestations, and neuro-Behçet's disease were found to be more frequent in patients with high-producing, wild-type genetic variants, whereas deep vein thrombosis and arthritis were not. Notably, Inanc *et al*. found that vascular involvement in male BD patients was associated with a depressed serum MBL level (14).

MBL is known to participate in the immunopathogenesis of autoimmune diseases. In particular, it has been demonstrated to facilitate the clearance of apoptotic cells, and defects in this process have been shown to cause autoimmunity (22). Alternatively, increases in serum MBL can facilitate an immune response via the lectin pathway of complement activation (23). Moreover, MBL deficiencies leave the individual susceptible to infections (24), which can initiate inflammation in autoimmune diseases (25). The role of infectious agents including *Streptococcus* and herpes simplex virus in pathogenesis of BD has been studied

(14, 26). In our study, however, the *MBL2* polymorphism related to the deficiency or dysfunction of MBL was not associated with BD susceptibility or manifestations whereas the polymorphism of functional MBL was related with several manifestations. These results are similar to previous Korean study (16). In systemic lupus erythematosus, *MBL2* alleles associated with dysfunctional MBL have been demonstrated to predispose disease developments (27, 28). In late stage of rheumatoid arthritis, however, the severity of inflammation was associated with MBL activity, suggesting that MBL acts as a double-edged sword in autoimmune diseases (29).

A number of limitations of the present study require consideration. First, the study population was not large enough to analyze the association between MBL and detailed clinical manifestations, such as, different types of skin manifestations or vascular involvement. Second, serum MBL levels were determined in about a half of the study subjects, which showed high serum MBL level (≥500 ng/ml) was associated with skin manifestation whereas genetic polymorphisms were not. The serum level, however, may fluctuate during the disease course and for other reason. Therefore, we thought genetic variants could reflect the average level of MBL in BD more exactly than cross-sectional result of serum MBL level. Prospective studies in which MBL levels are measured repeatedly are required to answer questions related to these issues.

In conclusion, no significant difference was found between BD patients and healthy controls in terms of *MBL2* genetic polymorphisms or haplotype frequencies. However, the presence of genital ulcer, eye involvement, and neurologic involvement in BD patients were found to be associated with the *MBL2 54* and *MBL2* H/L polymorphisms that produce high levels of MBL or functional MBL. These results suggest that particular clinical manifestations of BD have different genetic backgrounds related to genetic polymorphisms of *MBL2*.

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