Mannose-Binding Lectin Serum Level and Gene Polymorphism in Systemic Lupus Erythematosus Egyptian Patients

Rehab A. Rabie[1], Nevin F. Ibrahim[2], Manar G. Gebriel[1]

Departments of [1]Medical Microbiology & Immunology and [2]Internal Medicine, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Systemic lupus erythematosus (SLE) is an autoimmune disease in which the complement system plays a role in its pathogenesis. Mannose-binding lectin (MBL) is a serum protein, being a component of innate immune system, it is responsible for lectin pathway of complement activation. The presence of several polymorphisms at the coding regions of the *MBL-2* gene, especially single point mutation at codon 54, leads to decreased level and /or functional deficits of MBL, which seems to be a risk factor for occurrence of autoimmune diseases, such as in SLE. So, this study was carried out to determine the role of the serum MBL concentration and the genetic polymorphisms of *MBL-2* gene exon 1 codon 54 in Egyptian patients with SLE. Forty-eight SLE patients and 48 matched healthy controls were investigated. MBL serum level was measured by ELISA technique. *MBL-2* polymorphism at exon 1 codon 54 was determined by PCR-RFLP. Our results revealed a significant reduction in MBL serum level among SLE patient group in comparison to the control group (*P* <0.001). *MBL-2* genotyping among SLE patients, revealed the wild type (A/A) in 52.1% and mutant types (A/B, B/B) in 47.9%. While among healthy controls, the wild type was detected in 81.2% and the mutant types in 18.8% with a statistically significant association between this polymorphism and SLE susceptibility (*P*=0.008). Comparison of MBL serum level among different genotypes within the patient group showed that the mutant allele had a suppressive effect on MBL serum level. In conclusion, carrying *MBL-2* exon-1 codon 54 variant allele B was shown to be a risk factor for SLE.

Systemic lupus erythematosus (SLE) is an autoimmune disease that may affect any organ of the body and is presented by variety of clinical and immunological manifestations. Both environmental and genetic components with female sex have a strong role in its pathogenesis with an irreversible break in immunological tolerance. It is characterized by formation of autoantibodies and immunocomplexes [1]. The complement system plays an important role in the pathogenesis of SLE. Its activation leads to tissue injury and genetic deficiencies of some of its components are associated with occurrence of SLE [2].

Mannose-binding lectin (MBL) is serum protein with a structure similar to complement C1q. It is secreted from hepatocyte as an acute phase protein. Its carbohydrate recognition domains bind to carbohydrate residues found on microorganisms that lead to activation of the complement system through the lectin pathway. So, it is considered an important component of innate immune system which helps phagocytosis of microorganisms by phagocytes [3]. There are two human *MBL* genes, *MBL-1* is a pseudogene, and *MBL-2* is the only functional gene, which located on chromosome 10. *MLB-2* gene contains four coding exons. Exon 1 encodes part of collagenous region, exon 2 encodes the rest of the collagenous region, exon 3 encodes a neck region of MBL, while exon four encodes carbohydrate binding domain [4].

Mannose-Binding Lectin-2 gene is highly polymorphic having three independent single mutations (SNPs) in exon 1, in

addition to promotor polymorphism. The normal structure of MBL exon 1 is called allele A, while the three *MBL* exon 1 variants due to point mutation are called allele B (point mutation in codon 54, glycine to aspartic acid), allele C (point mutation in codon 57, glycine to glutamic acid) and allele D (mutation in codon 52, arginine to cysteine). These variants result in decrease MBL concentration due to incorrect assembly of the mature MBL protein [5].

The prevalence of these genotypes varies widely according to the ethnic origin. The B variant mutation is the most common polymorphism in African populations [6]. Low serum MBL levels caused by SNPs described above seem to be a risk factor for the occurrence of autoimmune diseases, including SLE. Previous studies concluded that the presence of variant alleles B, C or D alleles had a 1.6 times increased risk of acquiring SLE [7]. MBL binds and initiates uptake of apoptotic cells by macrophages, so its deficiency leads to accumulation of tissue debris which acts as a source of autoantigen with formation of autoantibodies. MBL also has a role in innate immunity, so that its deficiency leads to increased infection with different pathogens which may have some role in the pathogenesis of SLE [8]. So that MBL may have a protective function against occurrence of autoimmune disease, including SLE.

Hence this study was carried out to determine the role of the serum MBL concentration and the genetic polymorphisms of *MBL-2* gene exon 1 codon 54 in Egyptian patients with SLE.

Patients and Methods

A case-control study was conducted from January 2019 to November 2019 in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University and the Internal Medicine Department, Faculty of Medicine, Zagazig University.

Patients

A total of 48 patients with SLE, who were followed up at the out-patient clinic of the Internal Medicine Department, Zagazig University Hospitals, were included in our study irrespective of their disease stage or severity. Patients with lupus were matched to 48 healthy control subjects on the basis of age and sex.

Systemic Lupus Erythematosus patients with diabetes mellitus, malignancies, other autoimmune disease (as rheumatoid arthritis or autoimmune thyroiditis) were excluded from our study.

All SLE patients fulfilled at least four of the revised American College of Rheumatology (ACR) criteria for SLE diagnosis [9]. SLE patients routinely performed some laboratory assessments; complete blood cell count, serum C3, C4, antinuclear antibodies (ANA), anti-dsDNA, anti-smith antibodies, urine analysis and 24 hrs. urinary collection with protein clearance. Also, they were submitted for abdominal sonography, chest X-ray, echocardiography and renal biopsy.

This study was approved by the institutional review board (IRB) – Faculty of Medicine, Zagazig University. A written informed consent was obtained from both patients and controls before enrolling into the study. We followed the ethical principles of the Declaration of Helsinki during the preparation for this study.

Blood sampling

Five ml venous blood samples were obtained from each participant and divided into two tubes, sterile Wassermann's tube to collect serum, and EDTA containing tube. The blood collected in Wassermann's tube was centrifuged at 3000 RPM for 10 min and the supernatant serum was collected and stored at -20º C until time for measurement serum MBL level. The EDTA blood was stored at -20º C until being used for direct blood PCR.

Measurement of serum MBL level

Serum MBL level was measured in SLE patients and in healthy controls by sandwich enzyme-linked immunosorbent assay according to the manufacturer's company protocol (Quantikine ® ELISA Human MBL; R&D Systems, Minneapolis, USA). Diluted serum samples and standards were added to

microtiter wells, pre-coated with a monoclonal antibody specific for human MBL, in duplicates of 100 ml per well. After incubation for 2 hours at room temperature with shaking, washing away any unbound substances was done. An enzyme-linked monoclonal antibody specific for human MBL was added to the well, followed by washing. Then a substrate solution was added to the wells and finally the color developed in proportional to the amount of MBL bound. The optical density of each well was read within 30 minutes, using a microplate reader set to 450 nm, and the values were detected from the standard curve. The concentration read from the standard curve was multiplied by the dilution factor.

Mannose-binding lectin-2 polymorphism

Genotypes of the *MBL-2* gene codon 54-exon 1-point mutations were identified by using polymerase chain reaction - restriction fragment length polymorphism (PCR-RELP) method.

The *MBL-2* exon 1 was amplified by direct blood PCR kit (Phusion™ Blood Direct PCR Master Mix; Thermo Scientific™, USA). It was designed to perform PCR directly from whole blood with no prior DNA extraction. The kit was composed of 2X Phusion Blood Direct PCR Master Mix, EDTA, MgCl2 solution, 100% DMSO, universal control primer mix, nuclease-free water and DNA ladder. *MBL-2* gene primers, used to amplify PCR product size 329 bp [10], were supplied from (ThermoFisher scientific, USA). their sequences are listed in table (1)

The PCR reaction was performed in a total reaction mixture of 50 µl, containing 25 µl Blood Direct PCR Master Mix, 2.5 µl forward primer, 2.5 µl reverse primer, 5 µl whole blood and 15 µl nuclease free water. Universal control for the kit was used to amplify 237 bp fragment of mammalian genomic DNA in 50 µl total reaction mixture, formed of 25 µl 2X Phusion Blood Direct PCR Master Mix, 1 µl universal Control Primer Mix, 10 µl whole blood and 14 µl nuclease free water. The PCR was performed using thermal cycler as follow: initial lysis of cells for 5 min at 98º C, followed by 40 cycles including denaturation for 5 s at 98º C, annealing for 30 s at 58º C, and extension for 30 s at 72º C with a final extension at 72º C for 1 min. Analysis of the amplified products was done on 1.5% agarose gel electrophoresis. The obtained PCR products (329 bp product) were subsequently digested by restriction enzyme, *Ban*I (BshNI) according to the manufacture's protocol (BshNI; Thermo Scientific™, USA). This performed in a 30 µl total reaction mixture, formed of 10 µl PCR amplicon, 17 µl nuclease-free water, 2 µl 10X Fastdigest Green Buffer and 1 µl *BanI*. Following by incubation at 37º C for 5 minutes, then 10 µl of the digested product were loaded into 2% agarose gel and analyzed by electrophoresis after staining with ethidium bromide. The wild allele (A) was cleaved into two fragment, 245 and 84 bp, while the mutant allele (B) remained uncut (329 bp). The three determined patterns are shown in table 2 [10].

Statistical Analysis

The collected data were statistically analyzed using SPSS software (Statistical Package for the Social Sciences software version 25). Quantitative data were represented as mean value ± standard deviation (SD), median and range. Mann–Whitney test (nonparametric) and independent t-test (parametric) were used for comparing quantitative data. Kruskal-Wallis test was used for nonparametric multigroup comparison. Chi-square test (X^2) and odds ratio (OR) were used for comparing proportions. Results were considered statistically significant when *P* (probability) values were equal to or less than 0.05.

Table 1. Primer set used for amplification of *MBL-2* gene codon 54-exon 1

Primer sequence (5'-3')	Product size	Ref
F: GTAGGACAGAGGGCATGCTC R: CAGGCAGTTTCCTCTGGAAGG	329 bp	[10]

Table 2. *BanI* PCR-RFLP patterns of *MBL-2* gene codon 54-exon 1 [10]

Genotype	Restriction fragment length
A/A genotype (wild/wild)	245 bp & 84 bp
B/B genotype (mutant/mutant)	329 bp
A/B genotype (wild/mutant)	329 bp, 245 bp & 84 bp

Results

Forty-eight SLE patients (female/male = 46/2) with a mean age at time of diagnosis 23 ± 5.3 years, as well as forty-eight apparently healthy subjects (female/male = 45/3) were enrolled in this case/control study. These SLE patients fulfilled at least four of the revised American College of Rheumatology (ACR) criteria for SLE diagnosis. The median serum MBL concentration in SLE patients was (575

ng/ml) which was highly significantly lower than that of the control (2895 ng/ml) with a *P*-value <0.001 (Table 3).

Table 3. Serum levels of MBL in SLE patients and control groups.

MBL serum level (ng/ml)	SLE patients	Controls	*P*-value
Mean±SD	656.6±447.4	2674.2±631	
Median	575	2895	<0.001*
(Range)	(106 – 2100)	(990 – 3300)	

*Mann Whitney Test: statistical significance (*P* <0.05).

MBL-2 genotyping at codon 54 of exon 1 was investigated in both groups and revealed the wild type (A/A) in 52.1% (n=25) of SLE patients and mutant types (A/B, B/B) in 47.9% (n=23). While in healthy controls, the wild (A/A) type was detected in 81.2% (n=39) and the mutant (A/B, B/B) types were 18.8% (n=9) with a statistically significant association (*P*=0.008) between *MBL-2* gene polymorphism and SLE susceptibility (Table 4).

Table 4. Distribution of *MBL-2 gene* genotypes among SLE patients and healthy controls.

MBL-2 genotype	Patients (n=48) No. (%)	Controls (n=48) No. (%)	Total (n=96) No. (%)	*P* value
Wild (A/A)	25 (52.1%)	39 (81.2%)	64 (66.7%)	
Mutant (A/B)	18 (37.5%)	8 (16.7%)	26 (27%)	0.008*
Mutant (B/B)	5 (10.4%)	1 (2.1%)	6 (6.3%)	

*Chi-square test: $P \leq 0.05$ is statistically significant.

Analyzing MBL serum level within different genotypes between both SLE patients and the healthy controls, revealed that wild A/A genotype was associated with a higher level of circulating MBL, with its level in controls (median=2900 ng/ml) was significantly higher compared with SLE patients (median=740 ng/ml) and a *P*-value <0.001. Also, there was a reduction in serum MBL level in mutant genotypes in both SLE patients and control group, with increasing the rate of reduction in homozygous (B/B) mutant type than heterozygous (A/B) mutant type. Moreover, there was a significant reduction in serum MBL level in mutant genotypes in SLE patients compared to the same genotype in control group (*P*<0.001) (Table 5).

Table 5. Serum levels of MBL in SLE patients and control groups according to genotypes.

Genotypes	MBL level (ng/ml)				*P* value
	SLE Patients		Control		
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
Wild (A/A)	856 ± 483.3	740 (310 - 2100)	2792.6 ± 509.9	2900 (1300 - 3300)	<0.001*
Mutant (A/B)	522.2 ± 261.3	500 (220 - 1280)	2293.8 ± 818.8	2610 (990 - 3100)	<0.001*
Mutant (B/B)	143.2 ± 43	130 (106 - 210)	143.2 ± 43	1100 (1100)	<0.001**

* Mann Whitney Test, **Independent t Test: statistical significance (*P* <0.05).

Comparing MBL serum level of different genotypes within the case group, we found that the mutant types (A/B, B/B) had a suppressive effect on MBL serum levels; (median=500 and 130 ng/ml) respectively. A significant difference of MBL concentrations between A/A and A/B genotypes (P=0.008) was detected, also a significant difference of MBL concentrations between A/A and B/B genotypes (P= 0.001), and no significant difference detected between A/B and B/B (P=0.06) (Table 6).

Table 6. Comparison of MBL serum level among different genotypes of case group.

Patient group(n=48)	MBL level (ng/ml)			*P*-value	
	A/A (n=25)	A/B (n=18)	B/B (n=5)		
Mean ± SD	856 ± 483.3	522.2 ± 261.3	143.2 ± 43		0.008** 1
Median	740	500	130	0.001*	0.001** 2
(range)	(310 - 2100)	(220 - 1280)	(106 - 210)		0.06** 3

Test of significance, Kruskal-Wallis test. *Significant difference (P<0.05 is significant); **1 P value of MBL concentrations between A/A and A/B genotypes, **2 P value of MBL concentrations between A/A and B/B genotypes; **3 P value of MBL concentrations between A/B and B/B genotypes.

Among SLE patients, vasculitis and lupus nephritis were more frequently observed in patients with mutant genotypes (39.1% vs 8%, P = 0.01, OR = 7.4) and (60.8% vs 24%, P = 0.009, OR = 4.9) respectively. Regarding the complement level, SLE patients with mutant alleles were significantly less likely to produce C3 (47.8% vs 24%) and C4 (47.8% vs 20%) with P values of 0.08 and 0.04 and OR of 2.9 and 3.6, respectively. Regarding other clinical and laboratory features of SLE patients, no significant associations were found among wild and mutant *MBL-2* genotypes, but they were more likely to occur among mutant genotype cases (Table 7).

Table 7. Clinical and laboratory data of SLE patients among different *MBL-2* genotypes.

SLE patients Clinical, lab data	Wild genotype (n=25) No. (%)	Mutant genotype (n=23) No. (%)	*P*-value	OR (95% CI)
Arthritis	12 (48%)	16 (69.5%)	NS	2.5 (0.8 - 8)
Vasculitis	2 (8%)	9 (39.1%)	0.01*	7.4 (1.4 - 39.3)
Cardiovascular	7 (28%)	8 (34.7%)	NS	1.3 (0.4 - 4.6)
Lupus nephritis	6 (24%)	14 (60.8%)	0.009*	4.9 (1.4 - 17)
Pulmonary	5 (20%)	8 (34.7%)	NS	2.1 (0.6 - 7.8)
Dermatologic	3 (12%)	6 (26.1%)	NS	2.6 (0.5 - 11.9)
Neuropsychiatric	6 (24%)	10 (43.5%)	NS	2.4 (0.7 - 8.4)
ANA	13 (52%)	15 (65.2%)	NS	1.7 (0.5 - 5.5)
Anti-ds DNA	13 (52%)	15 (65.2%)	NS	1.7 (0.5 - 5.5)
Anti-smith	3 (12%)	5 (21.7%)	SN	2 (0.4 - 9.7)
Low C3	6 (24%)	11 (47.8%)	NS	2.9 (0.8 - 9.9)
Low C4	5 (20%)	11 (47.8%)	0.04*	3.6 (1 - 13.1)
Hematological cytopenia	13 (52%)	17 (73.9%)	NS	2.6 (0.7 - 8.8)

*Chi square test: $P \leq 0.05$ is statistically significant.

Discussion

When some classical pathway components of the complement are deficient, the abnormal clearance of immune complexes and apoptotic cells may participate in the occurrence of SLE. Also, MBL can bind to apoptotic cells and initiate their uptake through the lectin pathway, thus, abnormal clearance of apoptotic cells due to MBL deficiency may also contribute to the occurrence of SLE and provide a source of autoantigens [11].

There is a growing interest on the role of MBL in the occurrence of autoimmune diseases, so we aimed in this case/control study to investigate the serum level of MBL and to determine whether the polymorphism of *MBL-2* gene is associated with the occurrence SLE.

Also, we aimed to investigate the possible association of *MBL*-2 polymorphism with the clinical manifestations and laboratory presentations of SLE patients.

A total 48 SLE patients (female/male = 46/2) with a mean age at time of diagnosis (23±5.3) as well as 48 apparently healthy subjects (female/male = 45/3) were enrolled in this case/control study.

Initially, we estimated the serum level of MBL in our SLE patients and healthy controls and we found that the median serum MBL concentration in SLE patients was (575 ng/ml) which was highly significantly lower than that of the controls (2895 ng/ml) with a *P* value <0.001. Our results were supported by another study carried out in Egypt by Okasha *et al* [12] who declared that serum MBL of the SLE patients was significantly lower than that of the healthy controls, Also, Losada *et al* [13] in Spain declared that SLE patients showed more severe MBL deficiency compared to controls. However, Perazzio *et al* [14] observed only higher frequency of mild and moderate MBL deficiency in Brazilian SLE patients compared to controls.

We selected the B variant allele (codon 54 mutation) to investigate rather than others, because this allele is the most common mutation found in Africa, and also other previous studies reported that B allele in particular is associated with significant reduction in MBL serum level and thus susceptibility of autoimmune diseases [15]. When we investigated the *MBL-2* mutation in both groups, we found the wild type (A/A) in 52.1 % of SLE patients and mutant types (A/B, B/B) in 47.9%. While in healthy controls, the wild (A/A) type was detected in 81.2% and the mutant (A/B, B/B) types were 18.8% which was statistically significant (*P*=0.008).

Our data comes in accordance with the results of Losada López *et al* [13] who found the wild type more frequently in the healthy controls compared with SLE patients in Caucasians and suggested that low MBL levels in the *MBL-2* variant could be a risk factor for the development of SLE. Other supportive results were declared by Piao *et al* and Øhlenschlæger *et al* [16, 17] in North American patients and Danish patients with SLE respectively. Moreover, a Meta-analysis study carried out by Lee & his co-worker [18] on different ethnic groups (Africans, Asian and Europeans) confirmed that the *MBL-2* gene polymorphism (codon 54) was associated with occurrence of SLE and its prevalence was ethnicity dependant.

On the other hand, lack of significant association between *MBL-2* gene variants and SLE disease was detected by Momot *et al* [19], in a study carried out in Germany, but generally they pointed out higher frequency of the mutant genotypes in SLE

patients. This diversity in results may originate from differences in ethnic groups of the study populations.

When we compared MBL serum level of different genotypes within the case group, we found that the mutant alleles (A/B, B/B) had a suppressive effect on MBL serum levels; (median=500 and 130 ng/ml) for A/B and B/B respectively (P=0.001). A significant difference of MBL concentrations between the wild A/A and the mutant A/B & B/B genotypes (P=0.008 & 0.001) respectively was detected, and a P value of 0.06 was detected between A/B and B/B which was near but failed to reach the significant level. And analyzing the MBL level among different genotypes within the case and the control groups revealed that the wild genotype was associated with a significant higher level of circulating MBL in controls compared with the cases (P<0.001).

Our results were consistent with those reported by Okasha *et al* [12] who declared that individuals with variant alleles had a decreased MBL serum concentrations compared with individuals with wild MBL genotype. And added that those with homozygous mutations had a lower or even almost undetectable MBL serum concentration. Moreover, results reported by Crosdale *et al* in Caucasoid [20] supported our results. The mutation occurs in *MBL*-2 coding regions may result in decreased synthesis of this protein or production of dysfunctional one that is incapable of oligomerization [16].

When we compared our wild and mutant SLE MBL genotypes, the mutant group showed lower C3 and C4 serum level than the wild group (P= 0.08 and 0.04). This result confirms the fact that the MBL protein works to activate the complement system, so its deficiency may result in decreased activation and reduced level of the complement system, that leads to inadequate clearance of immune complex with immune complex deposition causing organ damage [21].

Mannose-binding lectin deficiency also may predispose to accumulation of apoptotic cells which is considered an important source of autoantigens and so resulting in abnormal immune system activation, and autoantibody production in SLE patients [22].

In our study, we were interested in clarifying such effect, so we studied different autoantibodies in different MBL genotypes. We detected that ANA, anti-dsDNA and anti-Smith antibodies were more frequently found in patients with MBL-mutant genotypes compared with patients with the wild type but unfortunately our results were not significant (P=0.35, 0.35 and 0.36) respectively. But our finding was supported by the results of Piao and his colleagues [16] who declared that in their SLE patients, anti-Smith antibody and dsDNA were more likely to be produced by the variant MBL alleles and added that such results supported the link between MBL deficiency and SLE and also suggested an important effect of genetic factors and autoantibody production in those individuals. Adding to the effect of MBL deficiency on the immune complex deposition in different tissues, we found that vasculitis and nephritis more frequently and significantly found in patients with mutant alleles (P=0.01 & 0.009 and OR=7.4 & 4.9) so SLE patients carrying MBL-mutant genotype have an increased risk of development of vacuities and lupus nephritis than those carrying MBL-wild genotype. Front *et al* [23] found that the cardiovascular disease and vasculitis in SLE patients with MBL- mutant genotype was 3.3 times higher

than in patients with wild type, and added that this explained the decrease in immune complex clearance and complement activation in those patients with immune complex deposition in blood vessels and renal tissues. Also results by Pradhan & his co-workers [24] and Tanha & his co-workers [25], among Indian and Danish patients with SLE respectively, pointed out the increased renal involvement in the mutant alleles. They reported that patients with MBL-mutant genotype had 2.6 times higher risk of developing nephritis, and MBL serum levels below 100 ng/ml were associated with 2 times increased risk of developing nephritis.

In conclusion MBL deficiency and gene polymorphism codon 54 influence the susceptibility to SLE in Egyptian patients. MBL is considered a disease modifier in those patients and combination of the *MBL-2* gene polymorphism with other genetic and environmental factors should be considered. Further studies with a complete genotype profile of the gene (*MBL-2* exon 1 and promoter) and MBL levels are needed with a larger sample of population to confirm that SLE patients are candidates for MBL therapy.

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