



Low producer MBL genotypes are associated with susceptibility to systemic lupus erythematosus in Odisha, India

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ARTICLE INFO

Article history:

Received 2 June 2012

Accepted 10 September 2012

Available online 19 September 2012

ABSTRACT

Variants of MBL gene have been associated with autoimmune disorders. The aim of this study was to explore whether common polymorphisms in MBL gene are associated with susceptibility to systemic lupus erythematosus (SLE) and its clinical manifestations in a cohort from eastern India. A total of 108 female SLE patients and 105 age, sex, and ethnically matched healthy controls were enrolled in the study. *MBL2* codon and promoter polymorphisms were genotyped by AS-PCR and dARMS PCR, respectively. Plasma level of MBL was quantified by ELISA. Higher frequency of BB genotype and minor allele (B) was observed in patients of SLE compared to healthy controls (BB genotype: $P = 0.0002$; OR = 5.75, 95% CI = 2.09–15.76, B allele: $P < 0.0001$; OR = 2.78, 95% CI = 1.66–4.64). *MBL2* codon 54, H-550L, Y-221X polymorphisms and combined MBL genotypes contributed to plasma MBL levels. Prevalence of MBL low producer genotype (LXA/LYB, LYB/LYB and LXB/LXB) was significantly higher in SLE patients compared to healthy control. ($P = 0.005$; OR = 3.09, 95% CI = 1.38–6.91). On analysis of clinical manifestations, MBL low producer genotype was significantly associated with autoimmune haemolytic anaemia ($P = 0.006$; OR = 13.06). Results of the present study indicate *MBL2* variants as possible risk factors for development of SLE and clinical manifestation in eastern India.

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1. Introduction

Systemic lupus erythematosus (SLE) is a complex inflammatory autoimmune disorder that involves many organs and systems. SLE is characterized by defective mechanisms of immunological tolerance, allowing autoantibody production against autoantigens, immune complex formation, deposition and tissue damage [1]. The etiology of SLE is unclear. However, various factors such as gender, ethnicity, environmental variables and genetic factors have been attributed to development of the disease [2].

Mannose binding lectin (MBL) is a liver derived protein which plays an important role in innate immunity. It binds to sugars on the surface of pathogenic micro-organisms, triggers complement activation and opsonization of antigens [3]. MBL deficiency results in abnormal clearance of apoptotic cells, over expression of auto-

antigens and generation of autoantibodies [1]. Plasma levels of MBL have been associated with single nucleotide polymorphisms (SNPs) at promoter and coding region of *MBL2* gene [4]. *MBL2* gene is located at chromosome 10 (10q11.2–21) and comprises of four exons. Three functional SNPs in exon 1 at codons 52 (rs5030737; C > T; Arg > Cys), 54 (rs1800450; G > A; Gly > Asp), and 57 (rs1800451; G > A; Gly > Glu), are known as D, B, and C variants, respectively, whereas A is the common non-mutated allele. In addition, two other functional polymorphisms in the promoter region of the *MBL2* gene, one located at positions –550 (rs11003125; G > C; H/L polymorphism) and another –221 (rs7096206; C > G; X/Y polymorphism) are also known to play an important role in determining plasma MBL levels [5]. Combined genotypes of exon1 and promoter polymorphisms have been shown to determine plasma concentration of MBL [6].

Although association of common SNPs in *MBL2* gene and SLE has been extensively studied in various geographical areas, none has been reported in the Indian population. There are limited reports defining the functional relevance of MBL variants in SLE. The present case control study based in a tertiary care hospital investigates the association of *MBL2* polymorphisms in SLE in a

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defined geographical area in India, endemic for parasitic diseases. Functional relevance of *MBL2* variants was also investigated by assessing plasma MBL levels in defined sub-types.

2. Materials and methods

2.1. Subjects

SLE is a chronic inflammatory disorder and 70–90% patients are female [7]. In genetic association studies, gender wise analysis has been recommended in several earlier reports [8–10]. In view of only two male patients in the cohort they were excluded to maintain uniformity of analysis. Therefore, we enrolled only female patients attending rheumatology clinic and/or admitted to SCB Medical College, Cuttack, Odisha, India, during 2007–2009. SLE diagnosis was based on the revised American College of Rheumatology (ACR) classification criteria [11]. After a detailed clinical examination and laboratory investigation, the clinical manifestations were categorized under photosensitivity rash, malar rash, discoid rash, oral ulcer, arthritis, myocarditis, serositis, nephritis, neuropsychiatric disease, pneumonitis and autoimmune hemolytic anemia (AIHA). The clinical profile of 108 SLE patients are summarized in Table 1. 105, age matched females of identical ethnicity were included as healthy controls (HC). None of the controls reported history of autoimmune disorder. About 5 ml blood in EDTA was collected; sera were separated and used for immunoassay. The residues were preserved with equal amount of 8 M urea at -20°C until use for isolation of DNA. The study was approved by the institutional ethical committee of SCB Medical College Cuttack, Odisha, India. Informed written consent was obtained from each patient.

2.2. Typing of the *MBL* gene

Genomic DNA was purified from stored blood using the QIAamp DNA isolation kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. *MBL* promoter polymorphisms (-550 H/L and -221 Y/X) and codon polymorphisms (52, 54 and 57) were typed by double amplification refractory system (dARMS) and allele specific polymerase chain reaction (AS-PCR) respectively. Most of the conditions were similar to a previous report [12]. However, for genotyping of codon polymorphism allele specific PCR was employed in place of multiplex PCR and modified in the thermocycling conditions as follows: 12 cycles of 10 s at 95°C followed

by 10 s at 62°C , followed by 15 cycles of 30 s at 95°C , 30 s at 62°C and 30 s at 72°C . The final extension was 7 min at 72°C . PCR products were visualized with 3% agarose gel electrophoresis. About 40% of the randomly selected samples were sequenced. Results were found to be 100% concordant with genotyping by PCR, which ensured absence of genotyping error.

2.3. *MBL* quantification

The plasma MBL was quantified by enzyme linked immunosorbent assay (ELISA) kit (R&D Systems) according to manufacturer's instructions.

2.4. Statistical analysis

Genotype and allele frequency were calculated by direct counting. SNPalyze software (Dynacom, Japan) was employed to calculate Hardy–Weinberg equilibrium. Fisher's test was used for comparison of genotype, allele frequencies and to test association of combined genotype with clinical and laboratory data. Odds ratios (ORs), 95% confidence intervals (95% CIs) were calculated by Graphpad prism 5.01. For analysis of Table 2, the allele genotypes with higher frequency were selected as reference (OR = 1) and the other ORs were calculated relative to that reference (Fisher's exact test, 2×2 contingency tables). For combined genotypes association with SLE (Table 3) the high producer combined genotypes group was considered as reference (OR = 1) and the other OR were calculated relative to that reference (Fisher's exact test, 2×2 contingency tables). Association of combined genotype with clinical and laboratory findings (Table 4) were performed by Fisher's exact test 2×3 contingency tables. *P* value less than 0.01 was taken as significant (Bonferroni correction for three SNPs $0.05/3 = 0.01$). Distribution of plasma MBL levels in genotype and combined genotypes were assessed by D'Agostino & Pearson omnibus normality test. Based on results of normality test, the association of genotype and combined genotypes with plasma MBL were analysed by analysis of variance (ANOVA) or Kruskal–Wallis test followed by an appropriate post test. Graphpad prism 5.01 software was used for these statistical analyses. Power analysis was performed using the statistical program G*Power 3.1 [13].

3. Results

3.1. Mutation at codon 52 and 57 are very rare in studied population

One hundred five HC and 108 SLE patients were genotyped to find an association between SLE and *MBL* polymorphisms. Out of the total 213 subjects, only three individuals were heterozygous for codon 52 and one heterozygous mutant for codon 57, suggesting rarity of these polymorphisms. As highlighted in Table 2, among 105 healthy individuals, fifteen were heterozygous and five were homozygous for codon 54 polymorphism. The prevalence of HL and LL genotypes of *MBL* (H-550L) polymorphism was found to be 25% and 67% respectively. The distribution of YX and XX genotypes of *MBL* promoter (Y-221X) was 30% and 17% respectively, in healthy individuals. In both HC and SLE patients, genotypes of *MBL* 54 (A/B) and *MBL* (Y-221X) polymorphisms deviated from Hardy–Weinberg equilibrium (HWE) [HC: *MBL* 54 (A/B): $\chi^2 = 10.67$, $P = 0.001$; *MBL* (Y-221X): $\chi^2 = 9.00$, $P = 0.002$; SLE: *MBL* 54 (A/B): $\chi^2 = 67.42$, $P < 0.0001$; *MBL* (Y-221X): $\chi^2 = 38.28$, $P < 0.0001$]. The pair wise linkage disequilibrium (LD) analysis of all the three loci showed that these are in linkage disequilibrium (LD) with $|D'| = 1$, $r^2 = 0.11$, Akaike's information criterion (AIC) = 34.61 for *MBL* (H-550L) and *MBL* (Y-221X) pair;

Table 1
Clinical characteristics of SLE patients and healthy controls.

Clinical profiles	SLE (<i>n</i> = 108)	Healthy control (<i>n</i> = 105)
Sex (male/ female)	0/108	0/105
Age in years (mean \pm SD)	27.73 \pm 8.481	30.24 \pm 6.48
Duration of disease months (mean \pm SD)	2.303 \pm 1.880	–
ACR criteria		
Photosensitivity rash	32 (30)	–
Malar rash	43 (40)	–
Discoid rash	18 (17)	–
Oral ulcer	49 (45)	–
Arthritis	51 (47)	–
NPSLE	22 (20)	–
Myocarditis	14 (13)	–
AIHA	9 (8)	–
Serositis	20 (19)	–
Nephritis	50 (46)	–
Pneumonitis	9 (8)	–

Note. Data are no. (%) of participants unless otherwise specified. NPSLE, neuropsychiatric systemic lupus erythematosus; AIHA, autoimmune hemolytic anemia.

Table 2

Frequency of mannose binding lectin (MBL) genotype and allele found among healthy controls (HC), and Indian SLE patients.

MBL2 Polymorphisms	Genotype or Allele	SLE (n = 108)	HC (n = 105)	P value	OR (95% CI)
MBL 54 (A/B) Co-dominant inheritance model	Genotype				
	AA	74 (69)	85 (81)	1	Ref
	AB	9 (8)	15 (14)	0.51	1.45 (0.59–3.51)
Recessive inheritance model	BB	25 (23)	5 (5)	0.0002	5.75 (2.09–15.76)
	AA+AB	83 (77)	100 (95)	1	Ref
	BB	25 (23)	5 (5)	0.0001	0.16 (0.06–0.45)
H-550L	Allele				
	A	157 (73)	185 (88)	1	Ref
	B	59 (27)	25 (12)	<0.0001	2.78 (1.66–4.64)
	Genotype				
	LL	78 (72)	70 (67)	1	Ref
	HL	28 (26)	27 (25)	0.87	1.07 (0.57–1.99)
Y-221X	HH	2 (2)	8 (8)	0.05	4.45 (0.91–21.71)
	Allele				
	L	184 (85)	167 (80)	1	Ref
	H	32 (15)	43 (20)	0.12	1.48 (0.89–2.44)
	Genotype				
	YY	64 (59)	56 (53)	1	Ref
Y-221X	YX	19 (18)	32 (30)	0.06	1.92 (0.98–3.76)
	XX	25 (23)	17 (17)	0.58	0.77 (0.38–1.58)
	Allele				
	Y	147 (68)	144 (69)	1	Ref
	X	69 (32)	66 (31)	0.91	0.97 (0.64–1.46)

Note. Data are no. (%) of participants unless otherwise specified. HC, healthy control; OR, odds ratio; CI, confidence interval.

Table 3

Combined functional genotypes distribution of MBL2 (promoter and exon1) polymorphisms in SLE patients and healthy controls.

MBL2 combined Genotypes	SLE (n = 108)	HC (n = 105)	P value	OR (95% CI)
<i>High MBL producer</i>				
HYA/HYA, HYA/LYA, HYA/LXA, LYA/LYA, LYA/LXA	61 (56)	70 (67)	Ref	1
<i>Intermediate MBL producer</i>				
HYA/LYB, LYA/LYB, LXA/LXA	20 (19)	25 (23)	0.86	1.08 (0.55–2.15)
<i>Low MBL producer</i>				
LXA/LYB, LYB/LYB, LXB/LXB	27 (25)	10 (10)	0.005	3.09 (1.38–6.91)

Note. Data are no. (%) of participants unless otherwise specified. HC, healthy control; OR, odds ratio; CI, confidence interval.

Table 4

Distribution of MBL expression genotype in different clinical categories of SLE.

Clinical Phenotype	Combined genotypes		
	High (n = 61)	Inter (n = 20)	Low (n = 27)
Photosensitivity rash	17 (28)	6 (30)	9 (33)
Malar rash	26 (43)	5 (25)	12 (44)
Discoid rash	8 (13)	3 (15)	7 (26)
Oral ulcer	25 (41)	9 (45)	15 (56)
Arthritis	30 (49)	8 (40)	13 (48)
NPSLE	11 (18)	4 (20)	7 (26)
Myocarditis	8 (13)	2 (10)	4 (15)
AIHA	0	1 (5) ^a	11 (41) ^a
Serositis	13 (21)	3 (15)	4 (15)
Nephritis	29 (48)	9 (45)	12 (44)
Pneumonitis	6 (10)	2 (10)	1 (4)

Note. Data are no. (%) of participants unless otherwise specified. NPSLE, neuropsychiatric systemic lupus erythematosus; AIHA, autoimmune hemolytic anemia; OR, odds ratio; CI, confidence interval.

^a P = 0.0006.

|D'| = 1, r^2 = 0.03, AIC = 10.29 for MBL (H-550L) and MBL 54 (A/B) pair; and |D'| = 0.59, r^2 = 0.02, AIC = 3.54 for MBL (Y-221X) and MBL 54 (A/B) pair.

Post hoc power of the study was computed to detect the probability of an association between MBL2 polymorphisms and SLE at the 0.05 level of significance, assuming small effect size (w = 0.20) [14]. This analysis revealed that the current study has 83% power to detect the association.

3.2. Genotype-phenotype relationship of MBL polymorphisms

Out of 108 SLE patients, plasma samples of 59 were available for quantification of plasma MBL by ELISA. Fig. 1 shows association of MBL polymorphisms (A: H-550L; B: Y-221X; and C: codon 54 A/B) with plasma MBL levels. A significant association of MBL codon 54 polymorphism (A/B) and plasma MBL levels was observed: homozygous mutant (BB) displayed lower levels of plasma MBL than wild type subjects (AA) (Fig. 1A). YY genotype of Y-221X polymorphism displayed higher plasma MBL compared to XY genotype (Fig. 1B). In addition, promoter polymorphism (H-550L) also showed significant association with plasma MBL: carriers of HL genotype displayed higher MBL than LL genotype (Fig. 1C). The functional analysis of HH genotype could not be done in view of very low number of samples in SLE patients. In an independent study of 113 healthy controls from Odisha, India we observed significant association of MBL2 polymorphism with plasma MBL levels (unpublished results).

Previous reports were inconsistent in demonstrating association of individual MBL variants (promoter and/or codon polymorphism) with plasma levels of MBL [15–17]. It is presumed that the total MBL level is regulated by combined effect of these polymorphisms. We analyzed plasma MBL levels in different genotype combinations (H-550L, Y-221X and codon 54 A/B) and the results are shown in Fig. 2. According to previous reports [12,18], combined genotypes were grouped as high (HYA/HYA, HYA/LYA,

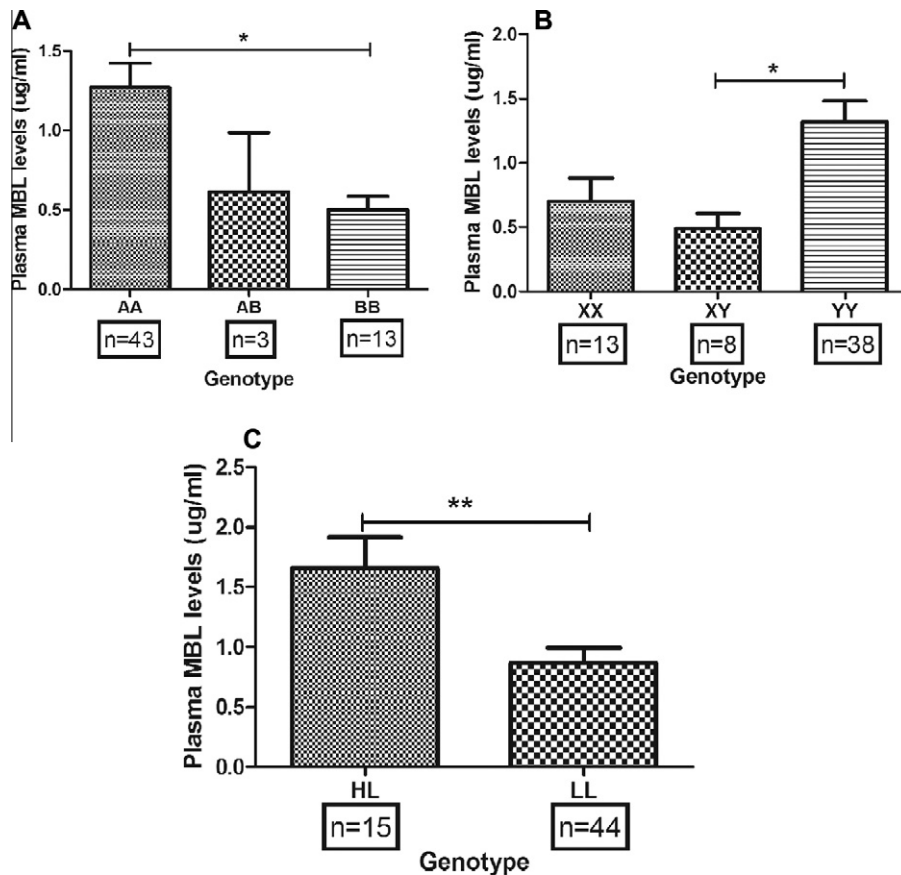


Fig. 1. Association between *MBL2* polymorphisms and plasma MBL levels in SLE patients. Plasma concentrations (mean \pm standard deviation) of MBL were measured, using a commercial kit. Based on availability, plasma of 59 SLE patients were quantified and correlated with their respective genotypes (A: codon 54 A/B, B: Y-221X and C: H-550L). Mean plasma levels of MBL among genotypes were compared by unpaired *t* test (C) or ANOVA followed by turkey's multiple comparison post test (A and B). *P* value less than 0.05 was considered as significant. **P* < 0.05, ***P* < 0.01.

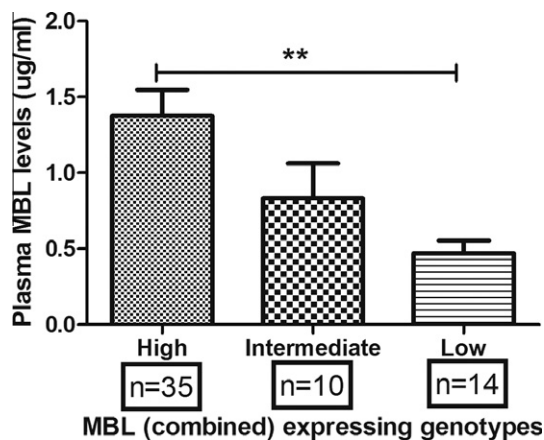


Fig. 2. Relationship between combined *MBL2* polymorphisms and plasma MBL. Plasma MBL levels of 59 SLE patients were quantified by ELISA, using a commercial kit (R & D Systems). According to a previous report [12], combined genotypes were grouped in to higher (HYA/HYA, HYA/LYA, HYA/LXA, LYA/LYA and LYA/LXA) intermediate (HYA/LYB, LYA/LYB and LXA/LXA) and lower expressing (LXA/LYB, LYB/LYB and LXB/LXB) genotypes. High expressing genotypes displayed significantly higher levels of plasma MBL compared to low expressing genotypes. Mean plasma levels of MBL among genotypes were compared ANOVA and followed by turkey's multiple comparison post test. *P* value less than 0.05 was considered as significant. ***P* < 0.01.

HYA/LXA, LYA/LYA and LYA/LXA), intermediate (HYA/LYB, LYA/LYB and LXA/LXA) and low (LXA/LYB, LYB/LYB and LXB/LXB) expressing types and plasma MBL levels were compared. As shown in Fig. 2,

high expressing genotypes displayed higher plasma MBL levels when compared to lower expressing genotypes.

3.3. BB genotype and B allele are associated with SLE

Association of MBL (codon 54 A/B, –550 H/L and –221Y/X) polymorphisms with development of SLE was analysed by various inheritance models. Of all the models, the co-dominant and recessive models gave the most parsimonious fit for MBL (codon 54 A/B) polymorphism. As depicted in Table 2, the prevalence of allele B and BB genotype was significantly higher in the SLE patients compared to healthy control (allele B: *P* < 0.0001; OR = 2.78, 95% CI = 1.66 to 4.64, genotype BB (co-dominant model): *P* = 0.0002, OR = 5.75, 95% CI = 2.09 to 15.76; genotype BB (recessive model): *P* = 0.0001, OR = 0.16, 95% CI = 0.06–0.45). On the other hand, all inheritance model failed to demonstrate association of MBL promoter polymorphisms (–550 and –221) with SLE.

3.4. Low producer genotypes are associated with SLE

Earlier we demonstrated the effect of combined MBL genotypes on plasma MBL levels. To determine whether these functional genotypes are associated with SLE, we analyzed the combined genotypes (H-550L, Y-221X and codon 54 A/B) in both patients and healthy controls. Results are shown in Table 3. The frequency of low producer genotypes was significantly higher in SLE patients compared to healthy controls (*P* = 0.005; OR = 3.09, 95% CI = 1.38–6.91).

3.5. Association of MBL expression genotype and SLE clinical manifestations

Distribution of MBL genotypes in various clinical categories of SLE was analysed and shown in Table 4. Patients with low expression genotype had AIHA more frequently than the patients with intermediate expression genotype ($P = 0.006$, OR = 13.06, 95% CI = 1.51–112.5). Distribution of MBL expression genotype was not significantly associated with other clinical phenotypes.

4. Discussion

Mbl has evolved as an important candidate gene for association studies because of its important function in innate immune system [3]. MBL binds to the sugar molecules of apoptotic cell and induces phagocytosis by macrophages [5]. Individuals having lower levels of plasma MBL are unable to clear apoptotic cells effectively [19,20]. Defective clearance of apoptotic material leads to prolonged exposure of autoantigens to the immune system and antibody generation [4]. Plasma MBL level has been correlated with common SNPs at *MBL2* gene [15–17,21] and associated with SLE in various populations [6,15,22–28]. However, there are no reports on Indian SLE patients.

Various studies have described the distribution of *MBL-2* gene polymorphisms in healthy Indian population. Prevalence of codon 52 (D) and 57 (C) mutants are rare corroborating an earlier report from North India population. [29] An independent community based study by us in Odisha, India, (unpublished observations) also observed the absence of these polymorphisms. However, in Southern India, prevalence of MBL codon 52 (D) and 57 (C) variants was 8–16% and 12% respectively [30,31]. The heterogeneity in distribution of these polymorphisms in Indian population could be attributed to local selection pressure by various infectious agents [32–34]. In the present study, distribution of genotypes of MBL 54(A/B) and MBL(Y-550X) polymorphisms deviated from HWE. Disagreement of genotypes distribution from HWE has been attributed to population stratifications and/or selection pressure [35]. Since all the patients and controls enrolled for this study were from a common ethnicity, deviation of HWE is probably due to selection pressure. The studied population is endemic to parasitic diseases like filariasis [36,37] and malaria [34,38]. Previous studies by our group showed significant association of *MBL2* polymorphisms with filariasis and malaria (unpublished observations), providing credence to the hypothesis that infectious diseases exert selection pressure on human population [32,39,40]. LXB genotype has been reported occasionally and termed as very rare in different populations [5]. However, in the present study we observed presence of LXB genotype in both patients and controls. These discrepancies could be population specific. Higher prevalence of LXB genotype in the studied population is possibly due to its protective nature from certain infectious diseases like filariasis and *P. falciparum* malaria.

Functional SNPs in a gene may affect the protein levels. Polymorphisms at amino acid coding region may result in deformed proteins, which degrade leading to its lower levels. Similarly, variants at promoter regions affect binding of transcription factor and possibly reduce transcripts levels [41]. This study revealed an association of BB genotypes with lower plasma MBL. It corroborates with the previous observations in Danish and Australian population [16,21,42]. The present study also revealed a significant association of promoter region polymorphisms (H-550L and Y-221X) with plasma MBL levels. The role of BB genotype on susceptibility to SLE has been described in Chinese [27], Japanese [6] and Spanish [22] populations. This association also appears to hold true for Indians as well. Although the promoter variants were associated

with SLE in Caucasians [22], Asians [26,27] and Africans [43], we failed to establish that association in our cohort.

The present study further highlights the necessity for combined analysis of several linked SNPs in genetic association studies. Although *MBL2* gene polymorphisms (54 A/B, –550 H/L and –221 Y/X) were associated with plasma MBL levels individually, only homozygous mutants for codon 54 (BB) resulted in predisposition to development of SLE. Further, combined analysis of *MBL2* polymorphisms also revealed significant association between MBL low producer genotypes and low plasma MBL levels as a risk factor for SLE and AIHA possibly due to poor immune complex clearance.

Association of MBL expression genotype with different clinical categories in SLE has been defined with regard to nephritis [22,24]. Interestingly, we observed a strong association of AIHA with low producer MBL genotype and not with other clinical manifestations. AIHA is characterized by destruction of red blood cells by circulating auto antibodies. The exact mechanism correlating MBL levels with AIHA is not understood. Earlier studies have shown that MBL binds to apoptotic material and mediates its clearance by macrophages [44,45]. Thus subjects with low serum MBL levels may promote generation of auto antibodies some of which target red blood cells.

5. Conclusion

The study confirmed the association of *MBL2* gene polymorphism with susceptibility to SLE in Indian females. BB genotype and minor allele B are significantly associated. Additionally, low MBL producing genotypes (LXA/LYB, LYB/LYB and LXB/LXB) are associated with SLE and autoimmune hemolytic anemia (AIHA).

Disclosure statement

The authors declare no conflicts of interest.

Acknowledgments

We would like to thank all patients and controls included in this study. We also thank to Mr. Subrat K. Mohanty for collection of blood from patients and healthy controls. The work was supported by intra-mural grants from Department of Biotechnology, Govt. of India to Institute of Life Sciences, Bhubaneswar.

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