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Association of genetic variants of mannan-binding (MBL) lectin-2 gene, MBL levels and function in ulcerative colitis and Crohn's disease

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Abstract

Ulcerative colitis and Crohn's disease are the two major forms of inflammatory bowel disease (IBD). A series of reports have hypothesized interplay of genetic and environmental factors in the pathogenesis of IBD. Polymorphism in the mannan-binding lectin-2 (MBL-2) gene is known to affect the structural assembly and function thereby predisposing subjects to various diseases. The present study was designed to evaluate effect of MBL-2 gene polymorphism on MBL levels and function in IBD patients. Genomic DNA was isolated from blood samples collected from 157 ulcerative colitis, 42 Crohn's disease and 204 control subjects. Genotyping for different polymorphic sites at exon1 of MBL-2 gene was performed by refractory mutation system-PCR and amplification followed by restriction digestion (PCR-RFLP). Serum MBL concentration and C4 deposition levels were estimated using ELISA. Mannan-binding lectin-2 genotypic variants were calculated in IBD and healthy controls. The frequency of single nucleotide polymorphisms at codon 54 was significantly higher in ulcerative colitis patients than controls ($P < 0.0001$). Ulcerative colitis patients with 'codon 54'-variation showed low serum MBL concentrations coupled with altered MBL function compared to controls. In conclusion, single nucleotide polymorphism in the MBL-2 gene is an important risk factor significantly affecting MBL levels and function in the development of ulcerative colitis among Indians.

Keywords

Mannan binding lectin, genetic variants, deficiency, function, ulcerative colitis, Crohn's disease

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Introduction

Ulcerative colitis and Crohn's disease are the two major manifestations of inflammatory bowel disease (IBD). These variants of IBD differ significantly in the disease manifestation with ulcerative colitis affecting the colon and the rectum and Crohn's disease affecting any part of the gastrointestinal (GI) tract including the terminal ileum. Though multitudes of factors are implicated in the etiopathogenesis of IBD,[1,2] reported data suggest complex multifactorial interplay between host genetic, enteric microflora and immunoregulatory components to play a decisive role in disturbing the balance between cytokine levels and increased complement activation.[3] Many inflammatory genes have previously been reported to be directly associated with IBD while other non-inflammatory related genes are known increase the susceptibility of IBD.[4]

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Mannan-binding lectin (MBL) is a calcium-dependent C-type lectin which multivalently binds to a variety of sugar moieties expressed by various microorganisms. The MBL acts as an important first line of defense against infection through complement activation. The activation of complement by MBL represents a pathway which is independent of the classical and alternative pathway but bears close resemblance to the classical pathway.[5–7] Mannan-binding lectin forms clusters of trimers with carbohydrate-recognition domains (CRDs) resulting in dimers, trimers, tetramers or hexamers. However, functional MBL is formed by trimers of carbohydrate recognition domains which associate covalently to contain up to 18 CRDs. Mannan-binding lectin is associated with serine proteases (MASP-1, -2 and -3); in addition, MBL also associates with MAP19 (truncated form of MASP-2)/sMAp.[8] On binding to carbohydrate molecules on the microbial surface, these serine proteases activate the complement system by cleaving and activating C4 and C2.[9] MASP acts as a C3 convertase, creating a C3b fragment from C3. This C3b then attaches to the pathogen surface and binds to receptors on phagocytes leading to opsonization. Ample key pathogenic processes inherent to the GI tract such as infection, immunological damage, and carcinogenesis have been linked to MBL.[10] The relative sufficiency of MBL function for any given individual is largely determined by genetic polymorphisms within the MBL-2 gene located on chromosome-10q11.2-q21. Mutations in the exon-1 of the MBL-2 gene dislodge the assembly of basic structural units of MBL leading to reduced functional MBL levels.[11] Polymorphism in the MBL-2 gene increases the susceptibility of various disease such as asthma,[12] Legionnaire's disease,[13] peritonitis[14] and also increases the early onset of autoimmunity in common variable immunodeficiency.[15] In contrast, Rector *et al.*[3] reported functional mutations in the MBL to be a protective factor in the development of sporadic ulcerative colitis. Three single nucleotide polymorphisms (SNPs) within the first exon of MBL-2 viz., codons 52, 54 and 57 significantly alter MBL function and their frequencies differ considerably in different geographically isolated populations.[16,17] Therefore, the present study was designed to investigate the association of genetic polymorphism in the MBL-2 gene, serum MBL levels and activation of complement pathway among Indian ulcerative colitis and Crohn's disease patients.

Subjects and methods

The study population consisted of 403 subjects, 157 ulcerative colitis patients, 42 Crohn's disease patients and 204 healthy controls. The subjects were in the age range of 18–80 years (41.10 ± 14.06 years) and included 265 males and 138 females. All the subjects with ulcerative colitis and Crohn's disease had a past history of more than 5 years whereas healthy controls included patients without any GI presentations. Diagnosis of IBD was done as per conventional clinical, endoscopic and histological criteria.[18,19] The MAYO score and the CDAI scores of all the subjects at the time participation were 8.40 ± 1.78 and 311.14 ± 54.10, respectively, indicating moderate-to-severe disease. Written and signed informed consent was obtained from all the patients participating in the study protocol which was approved by the Institutional Ethics Committee, Deccan College of Medical Sciences, Hyderabad, India.

For the present study, 3 ml venous blood samples were collected in K3-EDTA coated vacutainers from each patient undergoing colonoscopy. Genomic DNA was isolated from 200 µl anticoagulated whole blood using a commercially available kit (Bioserve Biotechnologies Pvt Ltd, Hyderabad, India). Amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) was used for genotyping 52nd codon whereas PCR-RFLP was used for 54th and 57th codon of exon-1 using previously described specific oligonucleotide primers.[3] Amplification for assessing SNP at 54 codon was performed as described previously[20] with minor modifications (annealing temperature 56°C). An internal control primer pair was included to amplify CC-chemokine receptor (CCR) in each reaction in order to ensure the efficiency of amplification of codon 52 by ARMS-PCR.[3] For codon 54 and 57 amplification, the respective amplicons were digested with 10 U of *BanI* and *MboII* (New England Biolabs, Ipswich, MA, USA), respectively, at 37°C for 120 min followed by enzyme inactivation at 65°C for 20 min. Amplicons and digested products were finally electrophoresed and analyzed on 1.5% agarose gel stained with ethidium bromide.

Serum MBL concentration levels were measured using a human MBL Mannan-binding ELISA kit (Antibody Shop, Gentofte, Denmark). Concentration calculations were done as per the manufacturer's instructions. Mannan-binding lectin pathway function was also indirectly monitored using C4 deposition ELISA which quantifies C4 deposited onto solid phase mannan surface as described previously.[11,21] After MBL binding to mannan, subsequent C4 deposition from added MBL-deficient human serum was measured using biotinylated anti-C4 antibody (Sigma Aldrich Inc., Castle Hill, Australia).

Statistical analysis

The genotypic distribution at codons 52, 54 and 57 of the MBL gene in patients and controls was performed in Table 1 using χ^2-test. For the analysis of serum MBL concentrations and C4 deposition (mean ranks), Kruskal–Wallis test was used followed by Mann–Whitney U-test for two variable comparison in Tables 2 and 3. $P < 0.05$ was considered significant.

Table 1. Genotypic frequencies at codons 52, 54 and 57 of the MBL gene in IBD and control subjects

	Genotypes, (*n* %)			
Codon	Wild-type	Heterozygous	Homozygous	*P* value (3 rows & 3 columns)
Codon52C/T	**CC**	**CT**	**TT**	–
Controls ($n=204$)	171 (83.82)	28 (13.72)	05 (2.45)	0.275
Ulcerative colitis ($n=157$)	120 (76.43)	30 (19.10)	7 (4.45)	–
Crohn's disease ($n=42$)	30 (71.42)	10 (23.80)	2 (4.76)	–
Codon54G/A	**GG**	**GA**	**AA**	–
Controls ($n=204$)	131 (64.21)	66 (32.35)	7 (3.43)	0.003
Ulcerative colitis ($n=157$)	69 (43.94)	75 (47.77)	13 (8.28)*	–
Crohn's disease ($n=42$)	21 (50.00)	19 (43.23)	2 (4.76)	–
Codon57G/A	**GG**	**GA**	**AA**	–
Controls ($n=204$)	188 (92.15)	16 (7.84)	–	0.605
Ulcerative colitis ($n=157$)	141 (89.80)	16 (10.19)	–	–
Crohn's disease ($n=42$)	37 (88.09)	5 (11.90)	–	–

*$P < 0.0001$ (ulcerative colitis vs controls).

Table 2. Mannan-binding lectin levels in ulcerative colitis, Crohn's disease and control subjects with codon 54 polymorphisms of MBL-2

	Serum MBL levels ng/ml (mean ranks)			
	Codon 54 genotype	Controls ($n=204$)	Ulcerative colitis ($n=157$)	Crohn's disease ($n=42$)	*P*-value
	GG	122.80 ($n=131$)	91.16 ($n=69$)	102.57 ($n=21$)	0.003
	GA	90.27 ($n=66$)	74.51 ($n=75$)	70.21 ($n=19$)	0.077
	AA	11.50 ($n=7$)	11.58 ($n=13$)	11.00 ($n=2$)	0.993

Codon 54 genotype	Controls ($n=204$)	Ulcerative colitis ($n=157$)	*P*-value	Controls ($n=204$)	Crohn's disease ($n=42$)	*P*-value
GG	110.59 ($n=131$)	81.35 ($n=69$)	0.001	78.21 ($n=131$)	65.81 ($n=21$)	0.231
GA	78.65 ($n=66$)	64.27 ($n=75$)	0.037	45.12 ($n=66$)	35.63 ($n=19$)	0.140
AA	10.50 ($n=7$)	10.50 ($n=13$)	1.0	5.00 ($n=7$)	5.00 ($n=2$)	1.0

Table 3. C4 deposition levels in ulcerative colitis, Crohn's disease and control subjects with codon 54 polymorphisms of MBL-2

	UC4 deposition levels/μl (mean ranks)			
	Codon 54 genotype	Controls ($n=204$)	Ulcerative colitis ($n=157$)	Crohn's disease ($n=42$)	*P*-value
	GG	120.79 ($n=131$)	95.33 ($n=69$)	101.43 ($n=21$)	0.021
	GA	95.03 ($n=66$)	70.70 ($n=75$)	68.71 ($n=19$)	0.004
	AA	12.07 ($n=7$)	11.19 ($n=13$)	11.50 ($n=2$)	0.958

Codon 54 genotype	Controls ($n=204$)	Ulcerative colitis ($n=157$)	*P*-value	Controls ($n=204$)	Crohn's disease ($n=42$)	*P*-value
GG	108.53 ($n=131$)	85.25 ($n=69$)	0.007	78.26 ($n=131$)	65.52 ($n=21$)	0.218
GA	82.50 ($n=66$)	60.88 ($n=75$)	0.002	46.03 ($n=66$)	32.47 ($n=19$)	0.035
AA	11.07 ($n=7$)	10.19 ($n=13$)	0.747	5.00 ($n=7$)	5.00 ($n=2$)	1.0

Correlation between C4 deposition levels and serum MBL levels was performed by calculating Pearson's correlation coefficient using ORIGIN Pro v8.0.

Results

Distribution of MBL-2 genotypes among IBD patients and healthy controls

Three genotypic variants one each at codons 52 and 54 and two variants at codon 57 were observed (Figure 1, Table 1), respectively. Among the three codon SNPs only codon 54 was found to be statistically significant ($P = 0.003$). In patients with ulcerative colitis, 69 (43.94%) of 157 had G/G genotype, 75 (47.77%) had heterozygous variants at codon 54 (G/A), and 13 (8.28%) subjects showed homozygous variant at codon 54 (A/A). In Crohn's disease, 21 (50%) of 42 had G/G genotype, 19 (43.23%) were found to show heterozygous variants at codon 54 (G/A), and two (4.76%) subjects showed homozygous variant at codon 54 (A/A). The frequency of SNPs at codon 54 was significantly higher in patients with ulcerative colitis than in healthy controls ($P < 0.0001$). However, there was no significant difference observed for the frequency SNPs at codon 54 between Crohn's disease patients and controls (Table 1).

Polymorphism at codon 54 and serum MBL levels

Serum MBL among the subjects who carry the G/G genotype at codon 54 showed sufficient levels ($\geq$1000 ng/ml), subjects with heterozygous G/A genotype showed reduced levels ($\leq$1000 ng/ml) whereas subjects with homozygous A/A genotype showed deficiency levels (as defined $\leq$100 ng/ml) in both controls and patient groups. The cut-off values for MBL levels in our study is based on those reported by Minchinton *et al.*[11] and Eisen *et al.*[13] Comparison of serum MBL levels in IBD and control subjects revealed a significant difference with G/G genotype ($P = 0.003$; Table 2). When we compared ulcerative colitis versus control and Crohn's disease versus control G/G, G/A and A/A genotype carrying MBL levels individually, G/G and G/A in ulcerative colitis showed significance $P = 0.001$ and $P = 0.037$, respectively (Table 2).

Deposition of C4 in subjects with polymorphisms at codon 54

Deposition of C4 levels were significantly deficient in subjects with homozygous (0.02–0.10 U/μl), low (0.05–0.79 U/μl) in heterozygous and sufficient in wild-type (0.15–2.05 U/μl) both among the patient groups and controls. The cut-off values for C4 deposition reported in the present study are based on those previously reported by Minchinton *et al.*[11] and Eisen *et al.*[13] However, comparison of C4 deposition levels in IBD and control subjects revealed a significant difference with G/G genotype ($P = 0.021$) and G/A genotype ($P = 0.004$; Table 3). When we compared ulcerative colitis versus control and Crohn's disease versus control with different genotypes carrying MBL levels individually, G/G and G/A in ulcerative colitis showed significance $P = 0.007$ and $P = 0.002$, respectively; in Crohn's disease, only G/A showed a significant difference ($P = 0.035$) with control (Table 3).

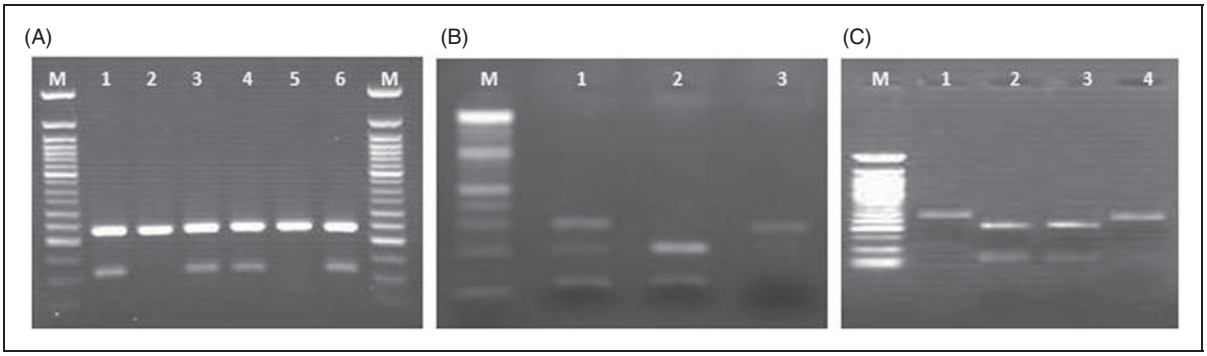

Figure 1. (A) Genotyping of codon 52 by ARMS-PCR. Amplification reaction was repeated twice, once using wild-type forward primer (lanes 1, 3 and 5) and once with mutated forward primer (lanes 2, 4 and 6). The MBL amplification product (110 bp) showing wild-type (*i.e.* codon 52 mutation is absent, lanes 1 and 2), mutated type (*i.e.* the mutation is present on both alleles, lanes 5 and 6), or heterozygote (*i.e.* containing wild-type allele and mutated allele lanes 3 and 4, respectively). The 238-bp band is the product CCR5 gene (used as an internal positive control) for amplification. 'M'-50 bp molecular mass DNA marker (New England Biolabs, Ipswich, Germany). (B) PCR-RFLP patterns of codon 54 after restriction digestion with *Ban-I* enzyme. Lane 1: three bands (298, 195 and 103 bp) represent heterozygote. Lane 2: two bands (195 and 103 bp) of wild-type. Lane 3: undigested homozygote (298 bp). M: 100 bp molecular mass DNA marker (New England Biolabs). (C) PCR-RFLP patterns of codon 57 after restriction digestion with *Mbo-II* enzyme. Lanes 1 and 4: undigested product (328 bp) of wild-type. Lanes 2 and 3: two bands (274 and 54 bp) represent mutated type. M: 50 bp molecular mass DNA marker (New England Biolabs).

Correlation between serum MBL levels and C4 deposition assay

The correlation between the MBL levels and C4 deposition was performed; serum MBL levels were linearly affecting the function of MBL (C4 deposition) levels. Correlation coefficient for the MBL levels and C4-deposition assay among the ulcerative colitis and controls were found to be $r = 0.961$ ($P < 0.001$) and $r = 0.868$ ($P < 0.001$), respectively (Figure 2).

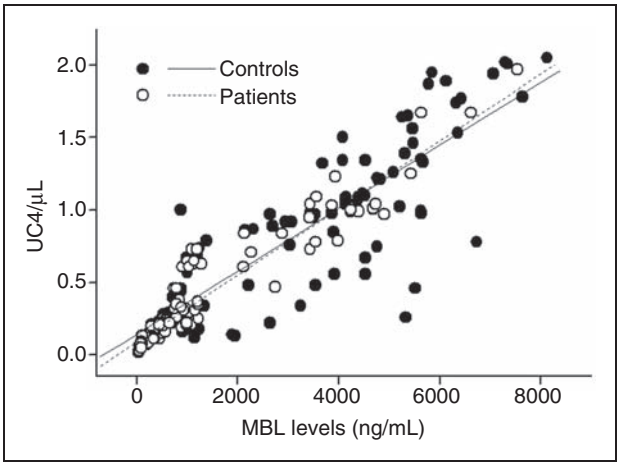

Figure 2. X–Y plots comparing nanogram per millilitre in MBL assay and corresponding C4-deposition results given in units of C4 deposited per microlitre (dashed line and white spots for ulcerative colitis patients and dark solid line and filled black spots for controls). Correlation coefficient for result of the C4-deposition assay and MBL levels assay was 0.961 ($P < 0.001$) in ulcerative colitis and 0.868 ($P < 0.001$) in controls.

Discussion

The effect of MBL-2 gene polymorphism in IBD cases has been little investigated with the majority of studies showing lack of association between them. The present study reports the influence of genetic variants of exon 1 of MBL-2 on serum MBL levels and function in subjects with ulcerative colitis and Crohn's disease. We investigated all the three reported genetic polymorphisms at codons 52, 54 and 57, serum MBL levels and C4-deposition levels.

The present study found significantly more point mutations at codon 54 in ulcerative colitis patients than in controls ($P < 0.0001$). Comparison between Crohn's disease versus controls did not achieve statistical significance. Interestingly, genetic polymorphisms in codons 52 and 57 were not found to be significantly associated with either disease forms, similar to the results of the other studies done on various disorders.[20,22] In view of the enigmatic relationship that co-exists between MBL-2 and inflammatory bowel disease, the experimental evidence of the present study suggests that SNPs at exon-1 of MBL-2 may be one of the risk factors for variable MBL levels and functions in patients with ulcerative colitis. The results of our study agree with those obtained by Wang *et al.*,[23] who reported a significantly high frequency of MBL-2 polymorphism among ulcerative colitis patients. However, our data are in contrast to the report of Rector *et al.*,[3] who demonstrated a protective effect of MBL mutations in the genesis of sporadic ulcerative colitis cases. The reason for this discrepant result may be primarily attributed to the study design, as the latter investigated the frequency of MBL mutations in IBD patients.

Most of the MBL-2 polymorphism studies on ulcerative colitis and Crohn's disease conducted in the past have provided very limited data of its association with IBD.[3,23–25] The major reason for this may be attributed mainly to the study design which lacked one of the three essential components: (i) MBL genotype; (ii) circulating serum MBL levels; and (iii) MBL function. This study, on the contrary, evaluated the polymorphisms of MBL-2 gene and its effect on circulating MBL levels and function. Since genetic polymorphism involving the promoter region in other disease conditions had already established their crucial role in disease outcome,[15,26] the major focus of the present study was on the exon-1 polymorphisms and its relevance in IBD. Our study found that mutations in the MBL-2 gene (especially at codon 54) led to the decrease in MBL levels and functions as seen by the deficient C4 deposition levels among the ulcerative colitis patients. Further, when we assessed the levels of MBL and C4 deposition in all the subjects with codon 54 polymorphism, the results demonstrated strong positive correlation with $r = 0.961$ ($P = 0.001$) and $r = 0.868$ ($P = 0.001$) in ulcerative colitis subjects and controls, respectively. Our overall analysis illustrated existence of strong positive correlation between MBL levels and C4 levels, *i.e.* increased MBL levels result in increased C4 deposition and functioning and vice versa. However, no such association could be established in Crohn's disease subjects owing to the limited number of study subjects with Crohn's disease and remains the major limitation of our study. Our results are supported by the findings of van Till *et al.*[27] and Eisen *et al.*,[13] who reported development of abdominal yeast infection in secondary peritonitis patients and increased predisposition of Legionnaires' disease among patients with functional MBL deficiency, respectively.

Conclusions

The present study demonstrated that serum MBL levels and function were significantly reduced in ulcerative colitis patients compared to healthy controls with SNP at codon 54 of MBL-2 gene. Our findings suggest that functional mutation in the MBL-2 gene may be

one of the mitigating factors responsible for the development of ulcerative colitis. However, future studies with a larger number of Crohn's disease patients are warranted to replicate the results of the present study.

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