Association of the wild-type A/A genotype of MBL2 codon 54 with asthma in a North Indian population

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Abstract. *Background:* High serum MBL level as well as polymorphisms in the mannose-binding lectin 2 (MBL2) gene resulting in MBL deficiency are involved in the mechanism of a number of non-infectious diseases such as asthma, conferring either risk or protection in different population studies. MBL being the first reactant of the MBL pathway is also a major determinant of the fate of the anaphylatoxins such as C3a and C5a, which are also pro-inflammatory mediators. The MBL2 gene polymorphisms thus control the serum levels of MBL as well as C3a and C5a.

Objective: This is the first case-control study conducted in India, investigating the role of MBL2 codon 54 A/B polymorphism in asthma pathogenesis.

Methods: A case-control study was performed with a total of 992 adult subjects, including 410 adult asthmatics and 582 healthy controls from regions of North India. The MBL2 codon 54 A/B polymorphism was genotyped by PCR-RFLP.

Results: Statistical analysis for the codon 54 polymorphism revealed that the wild (A) allele was significantly associated with asthma with OR = 1.9, 95% CI (1.4–2.4), and $p < 0.001$.

Conclusion: The MBL2 codon 54 A/B polymorphism is significantly associated with asthma and its phenotypic traits as the wild (A/A) genotype confers a significant risk towards the disease in the studied North Indian population.

Keywords: Asthma, mannose-binding lectin 2 (MBL2), anaphylatoxins, C3a, C5a, MBL2 codon 54 A/B polymorphism, North Indian population

1. Introduction

Asthma is a complex genetic inflammatory disorder of the lungs, characterized by acute bronchial hyper responsiveness (BHR), shortness of breath (SOB), chest tightness, cough and sputum production in response to a variety of external stimuli [36], with or without atopy [26].

Mannose-binding lectin (MBL), an active component of the innate immune system, is a Ca-dependent

serum protein synthesized by the hepatocytes in the liver [6]. MBL is a “pattern recognition molecule” encoded by the MBL2 gene located at 10q11.2-q21 [17,23] and is a trimer of three identical polypeptide chains, each possessing a cysteine rich region, a collagen like region, a neck and a ‘carbohydrate recognition domain’ (CRD), which enables the lectin protein to recognize the mannose and N-acetyl glucosamine moieties on the surface of a variety of pathogens including viruses, bacteria, fungi as well as protozoans [21].

While the MBL may be implicated in the microorganism destruction by the formation of a ‘membrane attack complex’ (MAC), it more importantly displays an opsonin effect by tagging the pathogen surface for recognition and ingestion by phagocytes via the

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mannose-binding lectin (MBL) pathway, which is one of the major pathways of the complement system [7]. Once the MBL tags the carbohydrate residue on the pathogen, it complexes with the MBL-associated serine proteases (MASPs) to form an enzyme that cleaves C4 into C4a and C4b. C4a is released while C4b binds to the enzyme. C2 then assembles on C4b and is cleaved into C2a and C2b. C2b is released while C2a binds to the enzyme to form a C4b2a domain which functions as 'C3 convertase' that cleaves C3 molecules into C3a and C3b, where C3a is released while C3b associates with C3 convertase to form 'C5 convertase' which then cleaves C5 to release C5a [18].

The anaphylatoxins C3a and C5a liberated as MBL pathway byproducts are potent pro-inflammatory mediators capable of causing smooth muscle contraction. Also, C3a and C5a are chemoattractants which recruit a variety of inflammatory cells such as neutrophils, eosinophils, monocytes and T-lymphocytes and result in tissue damage [30]. Moreover, C3a and C5a molecules have been established as potential effectors in asthma and allergic inflammatory disorders [13].

Serum MBL levels are influenced by polymorphisms in the MBL2 gene, where the three low producing SNPs in exon 1, at codon 52 resulting in substitutions of CGT → TGT (Arg → Cys), codon 54 GGC → GAC (Gly → Asp) and codon 57 GGA → GAA (Gly → Glu), alter the structural assembly of the MBL protein giving rise to A/D, A/B and A/C polymorphisms respectively [14, 22, 31].

Both MBL deficiencies as well as over secretions by the liver in blood have been associated with increased risk, severity, infections and autoimmune disorders [9, 24].

In murine models, MBL insufficiency has shown to cause hyperresponsiveness during fungal asthma [5]. MBL2 variant alleles have also been associated with susceptibility towards asthma in children with pneumonia infection [3]. A Turkish study has suggested implication of MBL in asthmatic children [4] and these findings are also supported by a Chinese study on asthmatic children where MBL2 codon 54 SNP has been associated with asthma [34]. Two Indian studies have demonstrated the role of MBL 1011G/A, SP-D 341 T/C and MBL 1011G/A polymorphisms in atopic asthma, respectively [11, 32].

Although a few studies have reported no association between MBL2 polymorphisms and atopic asthma in adults in a Finnish population [15] and in a Japanese study [37], increased MBL serum levels have been implicated in a wide range of non-infectious autoim-

mune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Type-I diabetes, Crohn's disease, cystic fibrosis, sepsis, lung injury, myocardial ischemia-reperfusion as well as asthma [8, 16, 20, 27, 28, 35]. Increased MBL levels have also been associated with infectious diseases such as tuberculosis (TB) and leprosy [2, 29], and it has been suggested that low MBL levels confer protection against TB [12].

Moreover, it has been observed that post allergen challenge, the anaphylatoxin C3a, which is otherwise produced as a byproduct of the MBL pathway, is present in elevated levels in the bronchoalveolar lavage (BAL) fluid of asthma patients [1, 10, 25, 38] as well as in the plasma [38], highlighting the role of C3a in MBL pathway. In two separate studies conducted in the USA, it has been observed that the deletion of C3a receptor in murine models of asthma protects against lung damage observed during allergen challenge during asthma attack, thereby signifying the role of C3a in asthma [1, 33]. Another study has suggested that the complement system not only plays an important role in innate immunity, but if uncontrolled, contributes to amplified inflammation [19].

The present study hypothesizes that the functional MBL2 codon 54 wild A allele results in high serum MBL level that leads to a greater availability of MBL for participation in the lectin pathway and hence result in an enhanced complement activation which cause asthma. On the other hand, the MBL2 exon 1 codon 54 A/B polymorphism results in low MBL production, resulting in a lesser participation in the MBL pathway by lowering the production of the first metabolite of the cascade itself, thereby resulting in a reduced production of anaphylatoxins such as C3a and C5a, which are otherwise mediators of inflammation and asthma.

2. Methods

Ethical Clearance for conducting the study on human blood samples was granted by the "Ethics Committee, PGIMER, Chandigarh". The study was conducted strictly in accordance with the ethical guidelines for bio-medical research on human subjects proposed by the "Central Ethics Committee on Human Research (CECHR) ICMR-2000" and of those contained in the "Declaration of Helsinki". The selection of asthma patients was based on physician's diagnosis. However, only the patients fulfilling the criteria of GINA (Global Initiative for Asthma) guidelines for diagnosis of bronchial asthma were recruited in the study.

This is the first case-control study conducted in India evaluating the role of MBL2 exon1 codon 54 A/B polymorphism in asthma pathogenesis by recruiting a total of 992 adult subjects. The patients were recruited from different states of North India such as Punjab, Haryana, Chandigarh, Uttar Pradesh, Himachal Pradesh, Uttaranchal, Jammu and Kashmir, Rajasthan and New Delhi. A total of 410 asthma patients visiting the Out Patient Department (OPD), Pulmonary Medicine, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, were enrolled in the study on the basis of physicians' diagnosis and spirometry test results. Out of the total, 323 subjects were asthma patients with allergic rhinitis. No ABPA (Allergic Bronchopulmonary Aspergillosis) patients were taken in the study. Informed Consent was duly obtained in written from the asthma patients participating in the study, and a detailed proforma of the asthma patients with a complete questionnaire regarding the clinical symptoms of the disease, i.e. wheeze/whistling, cough, shortness of breath (SOB), allergy, early morning or night symptoms, along with spirometry tests, etc., was assessed. Complete information of the patient regarding name, age, sex, history of the disease, occupation, etc., was taken into account (Table 1). Asthma patients with history of any other pulmonary ailment such as tuberculosis, Chronic Obstructive Pulmonary Disease (COPD), bronchitis, etc., were excluded from the study.

A total of 582 normal, healthy controls, with no history of asthma or allergic diseases or any other comorbid illness were inducted in the study. Some of the healthy volunteers were blood donors at various blood donation camps, educational institutes, employee groups. Care was taken that the control subjects did not have any of the patient conditions in the past. Any subject having a first degree relative with asthma or allergy has not been recruited as a control in the present study. Not only the respiratory or allergic skin disorders, any subject with other diseases such as diabetes, high blood pressure, etc., or with drinking and smoking habits have also not been included as controls in the study. Each control was first enquired for all of the above conditions at the time of taking their written informed consent and before the collection of blood samples.

Blood samples were collected in EDTA coated vials, and stored at -80°C until genomic DNA extraction was done. Genomic DNA was isolated from the thawed blood samples by the Sodium Saline Citrate Buffer Method, and checked for DNA on 0.8% agarose gel by electrophoresis.

Table 1
Characteristics of the study population

	Asthma patients 410 (%)	Controls 582 (%)
Sex		
Males	183 (44.6)	351 (60.3)
Females	227 (55.4)	231 (39.7)
Age	38.1 ± 16.2	41.9 ± 16.6
Allergic rhinitis	323 (78.8)	0
No rhinitis	87 (21.2)	582
Allergic to atleast 2 provoking factors	366 (89.3)	0
Non-allergic	44 (10.7)	582
Non-Smoker	345 (84.1)	582
Ever-Smoker	65 (15.9)	0
Spirometry data* ($n = 190$)		
FVC observed	2.56 ± 0.96	0
FVC predicted	3.19 ± 0.73	0
FEV ₁ observed	1.94 ± 0.82	0
FEV ₁ Predicted	2.68 ± 0.77	0
FEV ₁ /FVC observed	75.00 ± 13.71	0
FEV ₁ /FVC predicted	83.12 ± 5.84	0

FVC, Forced Vital Capacity; FEV₁, Forced Expiratory Volume in 1 second.

*Spirometry test was conducted for 190 asthma patients.

The amplification of the MBL2 codon 54 was done with forward 5'-AGTCGACCCAGATTGTAGGACA GAG-3' and reverse 5'-AGGATCCAGGCAGTTTCC TCTGGAAGG-3' primers [14]. PCR was carried out in a thermal cycler, in a total volume of 25 μl containing: 10X PCR Buffer, 3 mM MgCl₂, 1 mg/ml nuclease free BSA, 50 pmol of each primer, 10 mM of each dNTP, 0.125 U Taq polymerase and 2 μl genomic DNA. The PCR conditions were: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 60°C for 1 min, 72°C for 1 min, and final extension step at 72°C for 10 min. A 349-bp PCR product was observed as a parent band by electrophoresis on 2% agarose gels stained with ethidium bromide and visualized by UV transillumination. Alleles A and B were detected using 5U Ban I restriction digestion of the 349-bp fragment. Ban I cleaves the wild allele A into two fragments of 260 bp and 89 bp while leaving the mutant allele B uncut as 349 bp, while heterozygous A/B genotype is observed as 349, 260 and 89 bp bands in a single lane (Fig. 1).

The European Molecular Genetics Quality Network (EMQN) good practice guidelines have been followed. A few PCR vials with all the PCR contents except the DNA, were also included per PCR batch as "negative controls". No contamination was observed and there were no "false positives". To minimize the risk of contamination, sterilized and autoclaved solutions and equipment were used during DNA isolation. The ingredients for PCR were well stored at -20°C and were

Table 2
Distribution of MBL2 codon 54 genotypes and allele frequencies in asthma patients and controls

Genotype frequencies	Asthma patients 410 (%)	Controls 582 (%)	OR	(95% CI)	p-value
Genotype Frequencies					
B/B	5 (1.2)	25 (4.3)		Ref (1.0)	
A/A	308 (75.1)	353 (60.7)	2.0	(1.5–2.6)	0.0001
A/B	97 (23.7)	204 (35.1)	0.6	(0.4–0.8)	0.0001
A/A+A/B	405 (98.8)	557 (95.8)	3.6	(1.3–10.9)	0.0053
Allele Frequencies					
B	107 (13.0)	254 (21.8)		Ref (1.0)	
A	713 (87.0)	910 (78.2)	1.9	(1.4–2.4)	0.0001

A/A, Homozygous Wild; A/B, Heterozygous; B/B, Homozygous Mutant; OR, Odds Ratio.

Fig. 1. PCR-RFLP products of MBL2 codon 54 A/B polymorphism on 2% Agarose gel. Lanes 1, 2, 3, 4, 5, 8, 9, 10, 12, 13, 15, 16: Homozygous wild A/A genotype (260 and 89 bp), Lanes 6, 11, 14: Heterozygous A/B genotype (349, 260 and 89 bp), Lane 7: Homozygous mutant B/B genotype (349 bp), Lane 17: 100 bp Ladder.

thawed just before use. The patients and controls were not genotyped in separate batches but were randomly analyzed. Retyping of samples was done at random to check for the homology of results.

The genotypic as well as the allelic distribution of the MBL2 codon 54 A/B polymorphism, between the asthmatics and control subjects, were analyzed statistically using Fisher's exact test. The data was analyzed with SPSS 17.0 software and Epi Info version 3.4.3. Statistical significance was assumed for $p < 0.05$.

3. Results

In the present study, a total of 992 subjects, including 410 adult asthma patients and 582 adult healthy controls were genotyped for the MBL2 codon 54 A/B polymorphism. Very interesting results were observed for the polymorphism in the current study.

Statistical analysis of the results indicated that the mutant B allele was more prevalent among the con-

trols (21.8%) than in asthma patients (13.0%) while the overall genotypic distribution of the wild allele (A) was higher among asthma patients (87.0%) as compared to the controls (78.2%), conferring a significant risk towards asthma as there was a positive association between asthma and the A allele with OR = 1.9, 95% CI (1.4–2.4) and $p < 0.001$ (Table 2).

The genotypic frequencies revealed that the homozygous mutant genotype (B/B) was more prevalent in control subjects (4.3%) than in asthma patients (1.2%). The heterozygous genotype (A/B) was more prevalent among the controls (35.1%) as compared to the asthma patients (23.7%) with OR = 0.6, 95% CI (0.4–0.8) and $p < 0.001$. However, it was observed that the homozygous wild genotype (A/A) was more prevalent in asthma patients (75.1%) than in the controls (60.7%) with OR = 2.0, 95% CI (1.5–2.6) and $p < 0.001$. Furthermore, it was observed that asthma patients with homozygous wild genotype A/A or at least one copy of the wild allele (A/A+A/B), were predisposed to the disease with OR = 3.6, 95% CI (1.3–10.9) and $p = 0.005$ (Table 2).

Table 3
Phenotypic characteristics of the study population and MBL2 codon 54 A/B polymorphism

Phenotypic traits	n	A/A	A/B	B/B	A	B	OR	(95% CI)	p-value
Controls									
Males	351	201	134	16	536	166		Ref (1.00)	
Females	231	152	70	9	374	88		Ref (1.00)	
Asthmatics									
Sex									
Males	183	135	46	2	316	50	2.1	(1.4–3.2)	0.001
Females	227	173	51	3	397	57	1.7	(1.1–2.6)	0.018
Occurrence									
Seasonal	282	216	64	2	316	50		Ref (1.00)	
Throughout	128	92	33	3	217	39	0.8	(0.5–1.3)	0.367
Severity									
Wheeze on exertion	216	155	58	3	368	64		Ref (1.00)	
Wheeze at rest	194	153	39	2	345	43	1.5	(0.9–2.4)	0.121
Family History									
Family History (Nil)	285	219	62	4	500	70		Ref (1.00)	
Family History (+ve)	125	89	35	1	213	37	0.8	(0.5–1.2)	0.274
Smoking Status									
Non Smoker	345	253	88	4	594	96		Ref (1.00)	
Ever Smoker	65	55	9	1	119	11	2.0	(0.9–4.4)	0.076
Cough									
Cough (Nil)	74	57	16	1	130	18		Ref (1.00)	
Cough (+ve)	336	251	81	4	583	89	0.9	(0.5–1.7)	0.786
Sputum Production									
Sputum (Nil)	95	69	26	0	164	26		Ref (1.00)	
Sputum (+ve)	315	239	71	5	549	81	1.2	(0.7–2.1)	0.613
Pattern of Daily Symptoms									
Morning/Night SOB	312	229	79	4	537	87		Ref (1.00)	
Anytime SOB	98	79	18	1	176	20	1.5	(0.8–2.8)	0.191
Rhinitis									
Rhinitis (Nil)	87	62	22	3	146	28		Ref (1.00)	
Males	36	27	8	1	62	10		Ref (1.00)	
Females	51	35	14	2	84	18		Ref (1.00)	
Rhinitis(+ve)	323	246	75	2	567	79	1.3	(0.7–2.3)	0.424
Males	147	108	38	1	254	40	0.9	(0.4–2.3)	0.980
Females	176	138	37	1	313	39	1.7	(0.8–3.5)	0.208
Allergy									
Allergy (Nil)	44	30	14	0	74	14		Ref (1.00)	
Males	19	15	4	0	34	4		Ref (1.00)	
Females	25	15	10	0	40	10		Ref (1.00)	
Allergic (+ve)	366	278	83	5	639	93	1.5	(0.7–3.0)	0.345
Males	164	120	42	2	282	46	0.7	(0.2–2.5)	0.408
Females	202	158	41	3	357	47	2.4	(0.9–6.2)	0.076

A/A, Homozygous Wild; A/B, Heterozygous; B/B, Homozygous Mutant; OR, Odds Ratio; SOB, Shortness of Breath.

However, categorizing the asthma patients on the basis of the phenotypic characteristics of the disease (Table 3), as obtained from their detailed proforma, no significant association was observed between the MBL2 codon 54 A/B polymorphism and asthma phenotypes (all $p > 0.05$).

4. Discussion

The present study is the first one to investigate the role of MBL2 codon 54 A/B polymorphism in asthma

propensity in a North Indian population and this research has revealed a highly protective effect of the polymorphism.

The results obtained from the current study supported the above hypothesis with the observations that both the allelic as well as the genotypic frequencies revealed a major role of the functional MBL2 gene in conferring risk towards asthma. In the overall scenario, the wild A allele was more prevalent in the asthma patients (87.0%) than in the controls (78.2%) in contrast to the mutant B allele which was more prevalent among the control subjects (21.8%) than in the asthma patients

(13.0%), indicating an important role of the functional MBL2 gene in asthma pathogenesis, which can well be attributed to the fact that a functional MBL2 gene enhances serum MBL levels. It was also observed that the asthma patients with homozygous wild genotype A/A or with at least one copy of the wild A allele (A/A+A/B) had a significantly increased risk of asthma and were highly predisposed to the disease (Table 2). However, no significant difference in the MBL2 codon 54 allele distributions was found for the phenotypic traits of asthma (all $p > 0.05$). Further categorizing the non-atopic asthma patients on the basis of gender, neither the non-atopic males nor the females were predisposed to asthma risk.

MBL deficiencies as well as over secretions by the liver in blood have been associated with increased risk, severity, infections and autoimmune disorders [9,24].

Furthermore, in an Indian study conducted on MBL SNPs (816 A/G, 868 C/T, 875 G/A, 884 G/A and 1011 G/A), and Surfactant protein-D (SP-D), it has been observed that MBL 1011 G/A and SP-D 341 T/C polymorphisms were significantly associated with greater risk of atopic asthma [11], while another Indian study has also observed that MBL 1011 G/A polymorphism resulted in an increased risk for asthma with rhinitis and Allergic Bronchopulmonary Aspergillosis (ABPA) [32].

However, the present study shows completely contrasting results, revealing the predisposing effect of the functional MBL2 codon 54 A allele as a risk factor for asthma pathogenesis and in contrast a highly protective role of MBL2 codon 54 A/B polymorphism in asthma. The results of the present study are supported by various studies wherein high MBL serum levels have been implicated in other non-infectious diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis, Type-I diabetes, Crohn's disease, cystic fibrosis, sepsis, lung injury, myocardial ischemia-reperfusion as well as asthma [8,16,20,27,28,35]. Apart from the involvement in non-infectious diseases, increased MBL levels have also been associated with infectious diseases such as tuberculosis and leprosy [2,29] and it has been suggested that low MBL levels confer protection against TB [12].

Moreover, it has been observed that post allergen challenge, the anaphylatoxin C3a, which is otherwise produced as a byproduct of the MBL pathway, is present in elevated levels in the bronchoalveolar lavage (BAL) fluid of asthma patients [1,10,25,38] as well as in the plasma [38], highlighting the role of C3a in MBL pathway. In two separate studies conducted in the USA, it

has been observed that the deletion of C3a receptor in murine models of asthma protects against lung damage observed during allergen challenge during asthma attack, thereby signifying the role of C3a in asthma [1, 33]. Another study has suggested that the complement system not only plays an important role in the innate immunity, but if uncontrolled, contributes to amplified inflammation [19].

Thus, the findings of the present study reveal a protective role of the MBL exon 1 codon 54 A/B polymorphism in asthma by offering checkpoints at the MBL as well as the anaphylatoxin production steps in the complement pathway and highlight the major role of the MBL2 codon 54 gene with the functional wild A allele in asthma pathogenesis and suggest that the enhanced serum MBL levels may predispose an individual to asthma by resulting in an increase in the production of pro-inflammatory anaphylatoxins such as C3a and C5a during the lectin complement pathway.

The role of MBL2 gene polymorphisms in asthma is a much debated scenario. However, the reason for the contrasting results of the studies on MBL2 gene polymorphisms association with asthma obtained in different populations globally can be attributed to the differences in the ethnicities as well as to the complex interplay of the multiple genes and environmental factors involved in the mechanism of asthma pathogenesis.

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