 Int J Hum Genet, 6(3): 229-232 (2006)

Screening of MBL and SP-D Genes in Indian Population for SNPs and Their Association with Atopic Asthma

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KEYWORDS Mannan-binding lectin; surfactant protein D; collagen; intron; exon

ABSTRACT Mannan-binding lectin (MBL) an acute phase serum protein and surfactant protein D (SP-D) are the collectins that besides their role as innate immune molecules have been suggested to play an important role in asthma. To screen single nucleotide polymorphisms in the gene encoding MBL and SP-D in patients with atopic asthma. PCR amplification of collagen encoding region for MBL and –NH2 terminus encoding region of SP-D from DNA samples of asthmatic subjects, followed by their sequencing. In the MBL gene the SNP's were studied in 5 controls and 20 patients in exon 1 and intron 1 of the gene. (Alleles 816 A/G, 868 C/T, 875 G/A, 884 G/A and 1011 G/A) while exon 1 of lung surfactant protein D was studied in 5 patients and 5 normal subjects. Of the five SNPs in MBL, G1011A, a novel SNP at position 1011 in the intron 1 of MBL was observed in 60% of asthma patients, while it was not observed in the controls. In case of SP-D out of the two reported SNPs (T341C and G282A in exon1 of SP-D) only T341C was observed in the present study. Present pilot study shows G allele at G1011A in MBL and C allele at T341C in SP-D to be significantly associated with atopic asthma and may serve as marker to identify individuals at risk to atopic asthma, however a greater sample size is required. Further, the present study shows Indians to be genetically distinct w.r.t. exon 1 of SP-D.

INTRODUCTION

Mannan-binding Lectin (MBL) and Surfactant protein D (SP-D) belong to the collectin subgroup of lectin family whose other members include SP-A, CL-43 and Bovine conglutinin. They are characterized by presence of collagenous domain and carbohydrate recognition domain (CRD). They have been shown to be important innate immune molecules (Gjerstorff et al. 2004).

Allergic asthma is a Th2 type pulmonary disorder characterized by generalized reversible obstruction of airflow (Jack A Elias et al. 2003). It's early diagnosis which is based on a history of recurrent wheeze, cough, or shortness of breath, reversible airway obstruction demonstrated by pulmonary-function testing and in cases where questions exist, a methacholine challenge demonstrating airway hyperresponsiveness is important in wake of the studies showing asthma causing progressive lung impairment and increasing asthma associated morbidity over last few decades (5000/Year in US and 100000/year through out world) (Spyros Papiris et al 2002).

Asthma is an ecogenetic disorder with interplay between environmental and genetic factors governing its pathogenesis and susceptibility (CSGA.1997) Hundreds of genetic association studies on asthma-related phenotypes have been conducted in different populations. Variants in 70 candidate genes have been reported to be associated with asthma or related traits (Blumenthal 2005).

Lung SP-A, SP-D and serum MBL are suggested to be involved in asthma pathogenesis. For example, both SP-A and SP-D have been proposed to play a protective role in asthma (Cheng et al. 2000; Wang et al. 2001) while serum MBL has been shown to be responsible for maintenance of pulmonary hypersensitivity in murine model of asthma (Cory et al. 2004). SP-D deficient mice show enlarged terminal airways and emphysema. Signs of obstructive airway disease in SP-D knockout mice, however, probably reflect the result of an imbalanced chronic lung inflammation with pathological airway remodeling

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rather than an impact of a lack of SP-D on biophysical surfactant function in the airways (Wert et al. 2000). In view of the important role played by MBL and SP-D in asthma pathogenesis, we have carried out a pilot study involving association of asthma with single nucleotide polymorphisms (SNP's) in the exon 1of MBL and SP-D in Indian population.

MATERIAL AND METHODS

Subjects: Blood samples from 20 confirmed bronchial asthma patients (male & female) with age ranging between 20 years to 60 years and 5 healthy volunteers who were attending the Allergy Clinic at Mahavir Hospital & Research Centre were studied. The Institute's Human Ethics Committee approved the study and informed consent was obtained from all participants.

DNA Extraction: Genomic DNA was isolated from peripheral venous blood using modified salting out procedure (Miller et al. 1988). Briefly nuclei were isolated from 10 ml blood by addition of ice-cold cell lysis buffer followed by centrifugation at 3100 rpm for 15 minutes. To the pelleted nuclei 12 ml of nucleus lysis buffer, 0.8 ml of 10% SDS and 50uL of proteinase K (20 mg/ml) were added. Proteins were then precipitated using 4 ml of 6M NaCl and finally pelleted by spinning at 2500 rpm for 15 minutes. After addition of two volumes of 100% ethanol to the supernatant the tube was inverted several times until the DNA precipitate was visible, which was then removed with a plastic spatula or pipette tip and transferred to an eppendorf tube containing 100 - 200 ul Tris buffer.

Polymerase Chain Reaction: All PCR amplification reactions used 50 ng of the template DNA, 1.5 mM $MgCl_2$, 20.0 pico mole of forward and reverse primers, 0.2 mM dNTP (Amersham, UK), 1.5 units of Taq polymerase (Bangalore genei, India) in 50 uL of reaction.

List of the primers and their respective amplification conditions have been given below.

Primers used for amplification of MBL exon 1

Exon 1

FP: 5'TGC ACC CAG ATT GTA GGA C 3' 19 MER

RP: 5' AGC TGA ATC TCT GTT TTG A 3' 19 MER

Conditions:

Initial denaturation: 94^0C

25 Cycles: 94^0C for 45 sec, 52^0C for 45 sec 72^0C for 45 sec

Final Extension: 72^0C for 7min

Size of the PCR product: 417 bp approx

Primers used for amplification of SPD exon 1

Exon 1 SP-D (L05483)

FP-5'TGAGCCAAGTCCCTAAACCAT 3'

RP-5'CTCTCCCATCCCGTCCATC3'

Conditions:

Initial denaturation: 94^0C

35 Cycles: 95^0C for 45 sec, 63.5^0C for 45 sec 72^0C for 45 sec

Final Extension: 72^0C for 10min

Purification of the PCR Products: PCR products were PEG purified. For PEG purification briefly two volumes of PEG/NaOAc (3M, Ph 4.8) mixture was added to the PCR product, and incubated at -20^0C for 15 minutes followed by centrifugation at 3200 rpm for 50 minutes at 4^0 C. Supernatant was decanted off and the pelleted DNA was given two 70% ethanol washes. Samples were air dried and resuspended in 10 uL of autoclaved MilliQ water and quantitated on 1 % agarose gel.

Cycle Sequencing: Purified PCR products thus obtained were subjected to cycle sequencing reaction based on dideoxy termination reaction. Briefly 35-50 ng of the purified PCR product was amplified using 1pM of the forward primer and 4.0uL of ready reaction mix in a 10uLreaction. Autoclaved MilliQ water was used to make up the volume wherever required. Amplified product was precipitated using 150mM EDTA and 3M NaOAc followed by two 70% ethanol washes and air-dried. Samples were loaded on capillary-based ABI 3730 sequencer as per the standard procedures. Cycle sequencing program consisted of 25 cycles of denaturation at 96^oC for 10 seconds, annealing at 50^0C for 5 seconds, extension at 60^0C for 4 minutes and final incubation at 4^0C. Sequence data obtained was analyzed for the polymorphisms using Basic Local Alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST/)

Statistical Analysis: Statistical analysis and frequencies of various alleles were calculated using online two by two table (home.clara.net/sisa/). Chi-square, Odds ratio and p values were calculated at 95% confidence interval (CI).

RESULTS AND DISCUSSION

Single nucleotide polymorphisms and their characteristics observed in exon1 of MBL and

SP-D have been given in (Table 1).

A comparison of allele frequencies between asthma patients and control subjects for the SNP's observed in MBL and SP-D revealed that A allele at G1011A in intron 1 of MBL and C allele at T341C of SP-D is significantly associated with atopic asthma in Indian population (Table 2).

Although knowledge about collectin molecules as modulators of the allergic inflammatory reaction in asthma is still limited, the idea that these proteins play a role in asthma has attracted increasing attention.

The human MBL constitutes an important component of innate immunity. Low levels of MBL in the serum have been associated with a wide variety of clinical manifestations. MBL gene consists of four exons. Three major variants, which are located at codons 52, 54 and 57 of exon 1, are associated with a low serum concentration of MBL (Lipscombe et al. 1992). As per SNP database of NCBI (http://www.ncbi.nlm.nih.gov/) exon 1 of SP-D has been reported to have two SNP's namely T341C and G282A. There are also reports regarding occurrence of T/C polymorphism leading to methionine to threonine change at codon 11 of exon 1 of SP-D (Lahti et al. 2002; Liu et al. 2003; Leth-Larsen et al. 2005) However only T341C polymorphism leading to Met31Thr change was observed in the present study.

Hibberd et al. studied the association of 3 variants (in codons 52, 54, and 57 of exon 1 of the MBL gene) in relation to susceptibility to meningococcal disease. They found homozygosity or compound heterozygosity for these 3 alleles in 1.5 to 2.7% of controls and in 7.7 to 8.3% of subjects with meningococcal disease (Hibberd et al. 1999).

In Gambian population. Lipscombe et al. found a GGA-to-GAA transition converting codon 57 of exon 1 from glycine to glutamic acid, gly54-to-asp mutation led to the substitution of carboxylic acid for an axial glycine in the translated protein, which would lead to the mutation that is expected to disrupt the secondary structure of the collagenous triple helix of the 96-kD MBL subunits. The substitution of adenine for guanine in codon 57 led to the introduction of an MboII restriction site between bases 250 and 251. However in the

Table 1: Characteristics of SNPs observed in exon 1 and intron 1 of Mannan Binding Lectin (MBL) and SP-D in Indian subjects.

Gene location	*Nucleotide Gene/Change*	*Amino acid*	
		Position	*Change*
Exon 1	MBLA816G	34th	Ala to Ala
Exon 1	MBLC868T	52nd	Arg to Cys
Exon 1	MBLG875A G/A	54th	Gly to Asp
Exon 1	MBLG884A	57th	Gly to Glu
Intron 1	MBLG1011A	-	-
Exon 1	SP-D T341C	31st	Met to Thr

Table 2: Allelic frequencies of SNPs observed in exon 1 and intron 1 of MBL and SP-D in Asthma and control subjects.

Allele	*Allele frequencies*		*OR (95% CI)*	*Chi-squareTest*	*P-Value*
	Asthma Patients	*Controls*			
816 A/G	N=20	N=5			
A	5	24	1.5		
G	5	16	0.373<O.R.<6.0325	0.255	0.5665
868 C/T					
C	10	39			
T	0	1	NaN<O.R.<Infinity	0.255	0.6135
875 G/A					
G	8	35	1.75	0.374	0.5409
A	2	5	0.2862<O.R.<10.7021		
884 G/A					
G	8	33	1.1786		
A	2	7	0.2046<O.R.<6.7894	0.034	0.8539
1011 G/A					
G	7	36	3.8571	2.658	0.1030
A	3	4	0.7033<O.R.<21.1538		
341 T/C					
T	N=5	N=5			
C	5(50%)	3(30%)	2.33	.0833	0.003
	5(50%)	7(70%)	1.30<O.R.<4.16		

present study, we observed a significant association of a novel intronic SNP G1011A of MBL with atopic asthma region and there was no association of the reported SNPs in the exon 1 at the codons 52, 54, 57 with asthma patients.

It has recently been demonstrated that SP-D inhibit allergen-induced proliferation of lymphocytes and histamine release from whole blood in response to the house dust mite allergen Dermatophagoides pteronyssinus in a dose-dependent manner and suggest that lung collectins may be important molecules in asthma pathogenesis, both during the acute asthma attack characterized by histamine release and in the chronic airway inflammation by modulating lymphocyte proliferation (Madan et al. 1997; Wang et al. 1996, 1998).

In the present study a significant allelic association was observed in exonic 1 region of SP-D in small group of patients when compared with normals. The change in the exonic 1 region may be as one of the cause, which is associated with the imbalance. Comprehensive studies need to be performed in these patients to distinguish the allelic association in exon 1 region of SP-D.

The present preliminary study proposes that both MBL and SP-D form good candidate genes polymorphisms in which confer susceptibility to atopic asthma.

ACKNOWLEDGEMENT

We thank Dr. Nafees Sultana and Ms. R. Eswari for the clinical and technical help rendered during the study.

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