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Distinct alleles of mannose-binding lectin (MBL) and surfactant proteins A (SP-A) in patients with chronic cavitary pulmonary aspergillosis and allergic bronchopulmonary aspergillosis

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Abstract

Background: Distinct host immune status predisposes to different forms of pulmonary aspergillosis.
Methods: Patients with chronic cavitary pulmonary aspergillosis (CCPA; n=15) or allergic bronchopulmonary aspergillosis (ABPA; n=7) of Caucasian origin were screened for single nucleotide polymorphisms (SNPs) in the collagen region of surfactant proteins A1 (*SP-A1*) and A2 (*SP-A2*) and mannose-binding lectin (*MBL*).
Results: The T allele at T1492C and G allele at G1649C of *SP-A2* were observed at slightly higher frequencies in ABPA patients (86% and 93%) than in controls (63% and 83%), and the C alleles at position 1492 and 1649 were found in higher frequencies in CCPA patients (33% and 25%) than in ABPA patients (14% and 7%) (all $p>0.05$). However, the CC genotype at position 1649 of *SP-A2* was significantly associated with CCPA ($\chi^2=7.94$; $p_{corr}\leq 0.05$). Similarly, ABPA patients showed a higher frequency of the TT genotype (71%) at 1492 of *SP-A2* than controls (43%) and CCPA patients (41%) ($p>0.05$). In the case of *MBL*, the T allele (OR=3.1, range 1.2–8.9; $p\leq 0.02$) and CT genotype ($\chi^2=6.54$; $p_{corr}\leq 0.05$) at position 868 (codon 52) were significantly associated with CCPA, but not with ABPA. Further analysis of genotype combinations at position 1649 of *SP-A2* and at 868 of *MBL* between patient groups showed that both CC/CC and CC/CT *SP-A2/MBL* were found only in CCPA patients, while GG/CT *SP-A2/MBL* was significantly higher in CCPA patients in comparison to ABPA patients ($p\leq 0.05$). SNPs analysed in *SP-A1* did not differ between cases and controls.
Conclusions: Distinct alleles, genotypes and genotype combinations of *SP-A2* and *MBL* may contribute to differential susceptibility of the host to CCPA or ABPA.
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Keywords: allergic bronchopulmonary aspergillosis; *Aspergillus fumigatus*; chronic cavitary pulmonary aspergillosis; mannose-binding lectin; surfactant protein A.

Introduction

Aspergillosis is a group of ecogenetic diseases caused by an opportunistic fungal pathogen *Aspergillus fumigatus* (*A. fumigatus*) and a number of less common species. It includes a variety of allergic, invasive and sub-invasive disorders. Invasive pulmonary aspergillosis (IPA) is often a fatal disease, characterised by tissue destruction that affects highly immunocompromised patients (1). Allergic bronchopulmonary aspergillosis (ABPA), which is characterised by hypersensitivity reactions, often occurs in immunocompetent hosts with asthma or cystic fibrosis (1). Besides these two extremes are various subacute and chronic forms of pulmonary aspergillosis (CPA), such as chronic necrotising pulmonary aspergillosis (CNPA) and chronic cavitary pulmonary aspergillosis (CCPA) (1). CCPA is characterised by multiple pulmonary cavities that expand over time with surrounding extracavitary shadowing, with elevated inflammatory markers and *Aspergillus* antibodies (2). It is distinguished histologically from subacute invasive pulmonary aspergillosis or CNPA by the lack of visible hyphal invasion of tissue (2), and the slow progression of infection, which takes place over months or years in mildly or non-immunosuppressed patients (2).

Both host and pathogen factors determine the clinical outcome of different diseases caused by *A. fumigatus* (1). Human lung surfactant protein A (SP-A) and the serum protein mannose-binding lectin (MBL), octadecameric proteins belonging to the collectin subfamily of lectins, have been identified as key players that strengthen the host's innate immunity by interacting with pathogens and with immune effector cells (3). Polymorphisms in *MBL*, *SP-A1* and *SP-A2* (two genes encoding functional SP-A protein) have been reported to be important genetic determinants of multifactorial pulmonary diseases (3). In our earlier studies, we showed an important role of both SP-A and MBL in host defence against *A. fumigatus* (4). We

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also found significant association of certain *SP-A2* and *MBL* SNPs with ABPA in the Indian population (4, 5). Crosdale et al. showed that 70% of patients with chronic pulmonary aspergillosis had an *MBL* genetic heterozygous defect, mostly at codon 52 (6). The present study aimed to confirm this association with *MBL* and compare associations of SNPs of *SP-A1* and *SP-A2* with CCPA and explore associations with ABPA as a pilot study.

Materials and methods

Study design

Two categories of patients were included in the present study. Category 1 comprised 15 patients (all whites) diagnosed with CCPA. All had chronic (from several months to >12 years) pulmonary and systemic symptoms. For enrolment in the study as CCPA patients, subjects had to have a confirmed diagnosis of CCPA, as determined according to published criteria (2). Other pathogens that could account for the symptoms (e.g., mycobacteria) were excluded by repeated culture. The most common clinical presentation was a chronic productive cough, weight loss, shortness of breath, and in some cases, haemoptysis. All CCPA patients had radiological evidence of a progressive pulmonary cavitating lesion with surrounding inflammation, with or without an intracavitary mass. In addition, all patients had precipitating (IgG) antibodies to *Aspergillus* sp. in serum, persistently elevated inflammatory markers (C-reactive protein, erythrocyte sedimentation rate, or plasma viscosity), and a respiratory sample with a culture positive for *A. fumigatus* (the latter was not necessary if *A. fumigatus* precipitins and the above clinical and radiological characteristics were present). Category 2 comprised seven patients with ABPA (all white) diagnosed according to Rosenberg's criteria (7). All the study subjects gave their informed written consent for participation in the study, which was approved by the local Ethics Committee. For association analysis of CCPA and ABPA patients, the allele frequencies of the patient groups were compared with allele frequencies of controls of similar ethnic origin [SNP frequency data for controls (n=47) of European descent for *SP-A1* and *SP-A2* were taken from Seattle SNPs, NHLBI Program for Genomic Applications, SeattleSNPs, Seattle, WA, USA, URL http://pga.gs.washington.edu; the SNP frequency data for controls (n=82 white individuals) for *MBL* were taken from a previous study (6)]. SNP data from Seattle SNPs, NHLBI Program for Genomic Applications were previously used for finding recombination hotspots in the human genome. Since the control data for *SP-A2* and *MBL* were taken from two different sources in the public domain, the genotype combination (G1649C *SP-A2*/C868T *MBL*) frequencies for controls could not be obtained. No data concerning colonisation by *Aspergillus* spp. or any form of aspergillosis were available for the control groups.

PCR amplification and sequencing

Human genomic DNA was isolated from blood using a modified salting out procedure (8). PCR conditions and primers used for amplification of exon 1 of *MBL* (gene position as per GenBank accession no. AF08058) and exon 2, 3 of *SP-A1* and exon 3, 4 of *SP-A2* were similar to those reported earlier (4, 5). PCR products were purified by polyethylene glycol (PEG) purification and sequenced using an ABI 3730 sequencer (Applied Biosystems Inc., Foster City, CA, USA). Sequence data obtained were analysed for the polymorphisms using Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST/) and Seqman software from DNAstar (Madison, WI, USA).

Statistical analysis

Allelic distribution (number of alleles) was compared using an online 2×2 table (home.clara.net/sisa/) and Fisher's exact probability test (d.f.=1). The differences in genotypes between cases and controls were evaluated by online χ^2 test (d.f.=2). Differences in genotype combinations of *SP-A2* and *MBL* between the two patient groups were calculated at 95% confidence interval (CI) using an online 2×2 table. Actual observations instead of percentage frequencies were used for all the tests. Bonferroni's corrections were applied and $p \leq 0.05$ was considered significant.

Results

Polymorphisms in *SP-A1, SP-A2* and *MBL* genes observed in CCPA and ABPA patients of Caucasian origin were similar to those reported earlier (5, 6, 9). Although none of the *SP-A1* and *SP-A2* alleles showed significant association with CCPA or ABPA in the present study owing to the small sample size, the odds ratios obtained were significantly high (>2.5) when allele frequencies at T1492C (intron 3) and G1649C (codon 91) of *SP-A2* were compared between ABPA patients vs. controls and CCPA patients vs. ABPA patients (Table 1). The T allele at T1492C and G allele at G1649C were observed at higher frequencies in ABPA patients than in controls, while C alleles at positions 1492 and 1649 of *SP-A2* were found at higher frequencies in CCPA patients than in ABPA patients (Table 1).

On comparison of the genotypes for *SP-A2* polymorphisms, the CC genotype at position 1649 of *SP-A2* was found to be significantly associated with CCPA ($\chi^2=7.94$, $p \leq 0.025$, $p_{corr} \leq 0.05$; Table 1). ABPA patients showed a higher frequency of the GG genotype at position 1649 of *SP-A2*, although CCPA and ABPA patients were not significantly different for genotypes at position 1649 of *SP-A2* ($\chi^2=3.52$, $p \leq 0.2$). ABPA patients showed a higher frequency of TT genotype (71.4%) at position 1492 of *SP-A2* than controls (43.4%) and CCPA patients (41.1%). There was no significant difference in genotypes at any of the *SP-A1* polymorphisms between controls and either of the patient groups (data not shown).

In the case of *MBL*, the T allele at position 868 (codon 52) was found to be significantly associated with CCPA (OR=3.26, range 1.2–8.9, $p_{corr}=0.02$) (Table 1). Comparison of the allele frequencies between CCPA and ABPA patients showed that the T allele at position 868 of *MBL* (OR=3.95, range 0.4–35.8, $p_{corr}=0.38$) occurred more frequently in CCPA patients than in ABPA patients (Table 1). Genotype comparisons showed that the CT genotype at position 868 of *MBL* was significantly associated with CCPA compared to controls ($\chi^2=6.54$, $p \leq 0.025$, $p_{corr} \leq 0.05$; Table 1). A comparison of genotype frequencies for C868T of *MBL* between the two patient

Table 1 Comparison of allele frequencies at C868T (codon 52) of *MBL*, and T1492C (intron 3) and G1649C (codon 91) of *SP-A2* in ABPA and CCPA patients and controls.

SNP	Study group	Genotype, n			Allele, n		CCPA vs. controls		ABPA vs. controls		CCPA vs. ABPA	
							p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)
MBL C868T		CC	CT	TT	C	T						
	CCPA (n=15)	8 (53.3%)	7 (46.6%)	0	23 (76.6%)	7 (23.4%)	0.02*	3.26 (1.2–8.93)	0.85	1.21 (0.1–9.9)	0.19	3.95 (0.4–35.8)
	ABPA (n=7)	6 (85.7%)	1 (14.3%)	0	13 (92.9%)	1 (7.1%)						
	Controls (n=82)	68 (82.9%)	14 (17.1%)	0	150 (91.5%)	14 (8.5%)						
SP-A2 T1492C		TT	CT	CC	T	C						
	CCPA (n=12)	5 (41.6%)	6 (50%)	1 (8.4%)	16 (66%)	8 (34%)	0.74	1.17 (0.4–3.02)	0.09	3.51 (0.7–16.6)	0.19	3.00 (0.5–16.7)
	ABPA (n=7)	5 (71.4%)	2 (28.6%)	0	12 (85.7%)	2 (14.3%)						
	Controls (n=46)	20 (43.4%)	18 (39.1%)	8 (17.3%)	58 (63%)	34 (37%)						
SP-A2 G1649C		GG	GC	CC	G	C						
	CCPA (n=12)	9 (75%)	0	3 (25%)	18 (75%)	6 (25%)	0.37	1.63 (0.5–4.7)	0.34	2.67 (0.3–21.8)	0.17	4.34 (0.5–40.5)
	ABPA (n=7)	6 (85.7%)	1 (12.3%)	0	13 (93%)	1 (7%)						
	Controls (n=47)	33 (70.2%)	12 (25.5%)	2 (4.2%)	78 (83%)	16 (17%)						

OR, odds ratio; p-values and OR shown for allele comparisons only; CCPA, chronic cavitary pulmonary aspergillosis; ABPA, allergic bronchopulmonary aspergillosis. Control data taken from Crosdale et al. (6) for *MBL* and from Seattle SNPs, NHLBI Program for Genomic Applications (SeattleSNPs, Seattle, WA, USA) for *SP-A2*. The first base is the wild type, the second the substitution. *Statistically significant after Bonferroni correction.

groups showed that the CT genotype was higher in CCPA patients compared to ABPA patients ($\chi^2=2.1$, $p \leq 0.14$) (Table 1). The TT genotype was not observed in either of the patient groups or in controls.

Since the *SP-A2* and *MBL* genes are located in proximity on chromosome 10, we compared the frequencies of combinations of genotypes for position 868 of *MBL* and 1649 of *SP-A2* in the two patient groups. CC/CT and CC/CC *SP-A2/MBL* genotypes occurred only in the CCPA patients (n=3). The three CCPA patients with these genotype combinations had particularly destructive disease, unilateral in two and bilateral in one. GC/CC *SP-A2/MBL* genotype was observed in only one ABPA patient. The GG/CT *SP-A2/MBL* genotype occurred more frequently in CCPA patients (41.66%) than in ABPA patients (14.28%) ($p_{corr}=0.18$, OR=6.42, range 0.60–68.31). ABPA patients showed a higher frequency of GG/CC *SP-A2/MBL* genotype ($p_{corr}=0.02$, OR=9, range 1.28–63.02) than CCPA patients, with 70% of ABPA patients showing this genotype combination. GC/CT *SP-A2/MBL* genotype was not observed in either of the patient groups.

Discussion

Our observation of a significant association of the T allele and CT genotype at position 868 of *MBL* (codon 52) with CCPA only is in accordance with the findings of Crosdale et al., who reported a significant association of the T allele (50%) and CT genotype (50%) with CNPA (6). A T substitution in codon 52 of the *MBL* gene results in a Cys residue instead of Arg in the collagen domain, a dramatic reduction in higher-order oligomers, and low levels of functional MBL in serum, and may render the host less immunocompetent. These results thus provide additional data supporting the role of the T allele and CT genotype of MBL in predisposition to both forms of chronic pulmonary aspergillosis (subacute invasive aspergillosis), viz. CCPA and CNPA (6).

The higher frequencies of the T allele at position 1492 and the G allele at position 1649 of *SP-A2* in ABPA patients of Caucasian origin compared with controls are in agreement with our earlier observations in ABPA patients of Indian origin (5). In contrast, a higher frequency of C alleles at positions 1492 and 1649 and CC genotype at position 1649 of *SP-A2* in CCPA patients compared to ABPA patients suggests the predominance of distinct alleles of *SP-A2* in the two patient groups. The G1649C polymorphism is at the first base in codon 91 of SP-A2 where the G allele encodes an alanine residue and C allele encodes a proline residue. Codon 91 is part of the collagen domain of SP-A2, in which proline residues provide structural stability to the collagen triple-helical structure. It is interesting to note that the allele/genotype

encoding alanine is more frequent in ABPA patients, while the allele/genotype encoding proline is more frequent in CCPA patients than in controls.

Unlike CCPA patients, no significant association was observed between SNP C868T of *MBL* and ABPA patients of Caucasian origin. This is similar to our earlier observations in ABPA patients of Indian origin (9), which instead showed a significant association with the A allele of the novel SNP A1011G of *MBL*. This SNP was, however, not observed in this study (9).

A significantly higher frequency of the mutant T allele of codon 52 in CCPA patients and CT genotype in CCPA patients, and of the wild-type C allele and CC genotype in ABPA patients suggests that distinct alleles at codon 52 of *MBL* may predispose to different clinical entities of aspergillosis. Also, varied plasma levels of MBL contributed by the mutant allele and wild-type allele may influence differential susceptibility of subjects to CCPA or ABPA. It is important to note that we observed significantly higher MBL blood levels in ABPA patients (mean 3.45, SD 1.43 μg/mL) of Indian origin compared to controls (mean 2.08, SD 0.505 μg/mL) (9).

The occurrence of distinct genotype combinations of *SP-A2* G1649C and *MBL* C868T in CCPA and ABPA patients suggests that these combinations, along with other genetic factors, may confer distinct immune status to the host and may thus be partially responsible for the pathogenesis of these diverse clinical entities caused by the same fungus. Recently we also reported some distinctive cytokine SNPs in these patient groups. Both ABPA and CCPA patients appear to be higher producers of interleukin (IL)-15, a Th2-promoting cytokine, and lower producers of TNF-α, a central cytokine in protective responses, compared with controls (10). Conversely CCPA occurs in patients who are genetically lower producers of both IL-10 and transforming growth factor β1 (TGF-β1) (11).

This study implies that the presence of the T allele and CT genotype at position 868 of *MBL*, the CC genotype at position 1649 of *SP-A2* and its combination with the CC or CT genotype of position 868 of *MBL* increase susceptibility specifically to CCPA in the Caucasian population. Although we could not attain statistical significance for some of our results owing to the small sample size, the inferences drawn may be validated in larger study groups from different populations and the inclusion of patients with chronic cavitary lung diseases/allergic diseases not due to *Aspergillus* as control groups.

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References

1. Latge JP. The pathobiology of Aspergillus fumigatus. Trends Microbiol 2001;9:382–9.
2. Denning D, Riniotis K, Dobrashian R, Sambatakou H. Chronic cavitary and fibrosing pulmonary and pleural aspergillosis: case series, proposed nomenclature change, and review. Clin Infect Dis 2003;3:265–80.
3. Van de Wetering JK, Van Golde LM, Batenburg JJ. Collectins: players of the innate immune system. Eur J Biochem 2004;271:1229–49.
4. Madan T, Kaur S, Saxena S, Singh M, Kishore U, Thiel S, et al. Role of collectins in innate immunity against aspergillosis. Med Mycol 2005;43(Suppl 1):S155–63.
5. Saxena S, Madan T, Shah A, Muralidhar K, Sarma PU. Association of polymorphisms in the collagen region of SP-A2 with increased levels of total IgE antibodies and eosinophilia in patients with allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol 2003;111:1001–7.
6. Crosdale DJ, Poulton KV, Ollier WE, Thomson W, Denning DW. Mannose-binding lectin gene polymorphisms as susceptibility factor for chronic necrotizing pulmonary aspergillosis. J Infect Dis 2001;184:653–6.
7. Rosenberg M, Patterson R, Mintzer R, Cooper BJ, Roberts M, Harris KE. Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. Ann Intern Med 1977;86:405–14.
8. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;16:1215.
9. Kaur S, Gupta VK, Shah A, Thiel S, Sarma PU, Madan T. Plasma mannan-binding lectin levels and activity are increased in allergic patients. J Allergy Clin Immunol 2005;116:1381–3.
10. Sambatakou H, Pravica V, Hutchinson I, Denning DW. Cytokine profiling of pulmonary aspergillosis. Int J Immunogenet 2006:33;297–302.

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