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

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Background: Studies from our group have shown a protective role of pulmonary surfactant protein A (SP-A) against lung allergy and infections caused by *Aspergillus fumigatus*.
Objective: Present study investigated the association of polymorphisms in the collagen region of *SP-A1* and *SP-A2* (genes encoding SP-A) with allergic bronchopulmonary aspergillosis (ABPA) and its clinical markers.
Methods: Genomic DNA was extracted from blood samples of patients with ABPA and age-matched, unrelated control subjects. The polymorphisms were detected by means of PCR amplification and sequencing of the collagen region of *SP-A1* and *SP-A2*.
Results: Two exonic (SP-A2 G1649C and SP-A2 A1660G, 10 patients and 11 control subjects) and 2 intronic (SP-A2 T1492C, 8 patients and 8 control subjects; SP-A1 C1416T, 5 patients and 7 control subjects) polymorphisms in the collagen region of *SP-A2* and *SP-A1* showed significant association with patients with ABPA. A significantly higher frequency of the *AGA* allele (A1660G) of *SP-A2* was observed in patients with ABPA in comparison with control subjects ($P = .0156$, odds ratio [OR] = 4.78, 95% CI = 1.23 < OR < 18.52). This polymorphism, when existing along with a nonredundant polymorphism, SP-A2 G1649C (Ala91Pro) resulted in a stronger association with ABPA (A1660G and G1649C: $P = .0079$, OR = 10.4, 95% CI = 1.62 < OR < 66.90). Patients with ABPA with *GCT* and *AGG* alleles showed significantly high levels of total IgE and percentage eosinophilia versus patients with ABPA with *CCT* and *AGA* alleles.
Conclusion: The results indicated that SP-A2 G1649C and SP-A2 A1660G, polymorphisms in the collagen region of *SP-A2*, might be one of the contributing factors to genetic predisposition and severity of clinical markers of ABPA. (J Allergy Clin Immunol 2003;111:1001-7.)

Key words: *SP-A1, SP-A2, collagen, ABPA, single nucleotide polymorphism, association, IgE, eosinophilia*

Pulmonary surfactant, a complex surface active material found in the fluid lining the alveolar surface of the lungs, is composed of lipids (dipalmitoyl phosphatidylcholine) and proteins (human lung surfactant protein A [SP-A], SP-B, SP-C, and SP-D). A pathophysiologic role for surfactant was first appreciated in premature infants with respiratory distress syndrome and hyaline membrane disease, which is routinely treated with exogenous surfactant replacement. Biochemical surfactant abnormalities of varying degrees have been described in obstructive lung diseases (asthma, bronchiolitis, chronic obstructive pulmonary disease, and after lung transplantation), adult respiratory distress syndrome, pulmonary edema, other diseases specific to infants (chronic lung disease of prematurity and SP-B deficiency), interstitial lung diseases (sarcoidosis, idiopathic pulmonary fibrosis, and hypersensitivity pneumonitis), pulmonary alveolar proteinosis, and infectious and suppurative lung diseases (cystic fibrosis, pneumonia, and HIV).[1]

Allergic bronchopulmonary aspergillosis (ABPA), an allergic and inflammatory disorder of the airways, is caused by the pathogenic fungi *Aspergillus fumigatus*. A series of investigations by our group established that SP-A has an important role in strengthening the host defense against *A fumigatus* and its allergic disorders.[2,3] SP-A binds to various glycosylated allergens-antigens in 3-week culture filtrate of *A fumigatus* and the purified glycoprotein allergens gp55 and gp45. SP-A inhibits the ability of *A fumigatus*–specific IgE antibodies to bind these allergens and blocks *A fumigatus* allergen–induced histamine release from sensitized basophils of patients with ABPA.[2]

Macrophage- and neutrophil-mediated killing of *A fumigatus* conidia was enhanced in the presence of physiologic concentrations of SP-A.[4] Therapeutic administration of SP-A in a murine model of ABPA suppressed specific IgE lev-

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Supported by the Institute of Genomics and Integrative Biology (Council of Scientific and Industrial Research). Dr Taruna Madan is a recipient of Young scientist grant of Indian National Science Academy. Ms Shweta Saxena is a recipient of Senior Research Fellowship of Council of Scientific and Industrial Research.
Received for publication October 11, 2002; revised December 26, 2002; accepted for publication January 13, 2003.
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0091-6749/2003 $30.00 + 0
doi:10.1067/mai.2003.1395

Abbreviations used
ABPA: Allergic bronchopulmonary aspergillosis
OR: Odds ratio
SNP: Single nucleotide polymorphism
SP-A: Human lung surfactant protein A
SP-A2 G1649C: Polymorphism G to C at position 1649 of *SP-A2*

els and peripheral and pulmonary eosinophilia and caused a marked shift from a pathogenic T_H2 to a protective T_H1 cytokine profile.[3] Mice deficient in SP-A had a significant increase in IL-13 levels (1.5-fold) and eosinophilia (1.5-fold) in comparison with wild-type mice, suggesting an inherent increase in pulmonary hypersensitivity in these mice (unpublished data). In vivo studies of the *SP-A* knockout mice indicated that SP-A plays an important role in pulmonary infections.[5-7] In view of the importance of SP-A in host defense against *A fumigatus*, structural and functional changes in SP-A arising from various genetic polymorphisms might affect the outcome of host-pathogen interactions in patients with aspergillosis.

SP-A is the major nonserum pulmonary surfactant–associated protein with 18 functional units, showing significant structural similarity to C1q.[8] The human genome contains 2 highly similar *SP-A* genes (*SP-A1* and *SP-A2*) coding for SP-A protein.[9] On the basis of sequence differences within the coding region, more than 30 genetic variants (alleles) of *SP-A1* and *SP-A2* have been reported that are biochemically and functionally different.[10,11]

Some of the allelic variants of *SP-A1* and *SP-A2* have been associated with respiratory diseases, such as respiratory distress syndrome, chronic obstructive pulmonary disease, and pulmonary tuberculosis.[12-16] A recent case-control association study showed that the frequency of certain alleles in the collagen region of *SP-A2* is increased in patients with pulmonary tuberculosis in an Indian population.[17] The present study determined the frequencies of allelic variants of *SP-A1* and *SP-A2* in patients with ABPA. Two intronic polymorphisms (SP-A1 C1416T and SP-A2 T1492C) and 2 exonic polymorphisms (SP-A2 G1649C and SP-A2 A1660G) showed higher frequencies in the patients with ABPA than in the control subjects. With an increase in the number of subjects, SP-A2 A1660G, a redundant polymorphism, showed a significant association with ABPA. This polymorphism, when existing in association with a nonredundant polymorphism, SP-A2 G1649C (Ala91Pro), resulted in a stronger association with ABPA and clinical markers for its severity.

METHODS

Study population

After approval of the institute's human ethics committee, Indian subjects (n = 45) were screened for single nucleotide polymorphisms (SNPs) in the collagen region of *SP-A1* and *SP-A2*. These subjects included patients with ABPA (n = 5 for *SP-A1*, n = 10 for *SP-A2*) and unrelated and age-matched healthy control subjects (n = 7 for *SP-A1*, n = 11 for *SP-A2*; from same ethnic origin [ie, North Indian population]) for the preliminary study. SNPs at 1649 and 1660 positions of *SP-A2* were further screened in a larger group of patients with ABPA (n = 22) and unrelated age-matched healthy control subjects (n = 23). The Rosenberg criteria were followed for clinical confirmation of ABPA in the patients.[18] The range of onset of asthma in patients with ABPA included in the study was 11 to 48 years (mean, 22.9 years; mode, 23 years; median, 23 years).

Genomic DNA isolation

Genomic DNA was extracted from PBMCs. Ten milliliters of blood was slowly layered over Histopaque (Sigma), and buffy coat (layer of lymphocytes) was removed. These cells were lysed, and genomic DNA was isolated and purified from lymphocyte preparation after standard phenol/chloroform (1:1) extraction protocol.[19] Purified genomic DNA samples were used as templates in PCR for amplification of DNA sequences.

Polymerase chain reaction

To achieve specific amplification and economic use of DNA samples, we carried out 2 rounds of PCR amplification. The first round of PCR resulted in amplification of complete *SP-A1* and *SP-A2* by using full-gene primers (primers 1 and 2 for *SP-A1* and primers 3 and 2 for *SP-A2*). The amplification products of the first round of PCRs were diluted 1:50 times and were used as templates for the second round of nested PCRs of smaller fragments of the genes.[17,20] The PCR profile for full-gene primer pairs (for both *SP-A1* and *SP-A2*) was 95°C for 2 minutes; 33 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 3 minutes; and a final extension at 72°C for 5 minutes. Fifty microliters of PCR reaction mixture contained 5 μL of 10× buffer (Bangalore Genei), 8 μL of 2 mmol/L deoxyribonucleoside triphosphates (Bangalore Genei), 2 μL of 20 pmol/L of each primer, and 6 units of Taq DNA polymerase were used (Bangalore Genei).

The 3.3-kb PCR products obtained after amplifications of *SP-A1* and *SP-A2* were then diluted 1:50 in dH_2O and used as templates for amplifications of collagen domain coding regions of *SP-A1* (exon 2 and exon 3) and *SP-A2* (exon 3 and exon 4). Primers 6 and 7 were used to amplify *SP-A1* exons 2 and 3. Primers 4 and 5 were used to amplify *SP-A2* exons 3 and 4.[17] Another set of primers, 8 and 5, was used to amplify exon 4 of *SP-A2*, containing the polymorphic sites at the 91st and 94th codons.[17] The primers used in the present study and details of their locations are given in Table I.

Purification of PCR products and sequencing

The PCR products were electrophoresed on 1% agarose gel. Bands were cut out of agarose gel and purified with a Qiaquick gel extraction kit (Qiagen GmbH) and sequenced with gel terminator chemistry on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). The sequencing data obtained were confirmed twice by performing a complete repeat of the experimental procedure, which included amplification of stock genomic DNA, fragment purification, and sequencing of both DNA strands. Polymorphisms in the sequences obtained were identified with Basic Local Alignment Search Tool software located at the National Center for Biotechnology Information Web site.

Statistical analysis

Allele frequencies in patients with ABPA and control subjects were compared by using 2×2 tables and Fisher exact tests (for sample number <10 in a group). The χ^2, odds ratio (OR), and P values were calculated at 95% CI.

RESULTS

Characteristics of SNPs observed in the collagen region of *SP-A1* and *SP-A2* of 45 subjects in an Indian

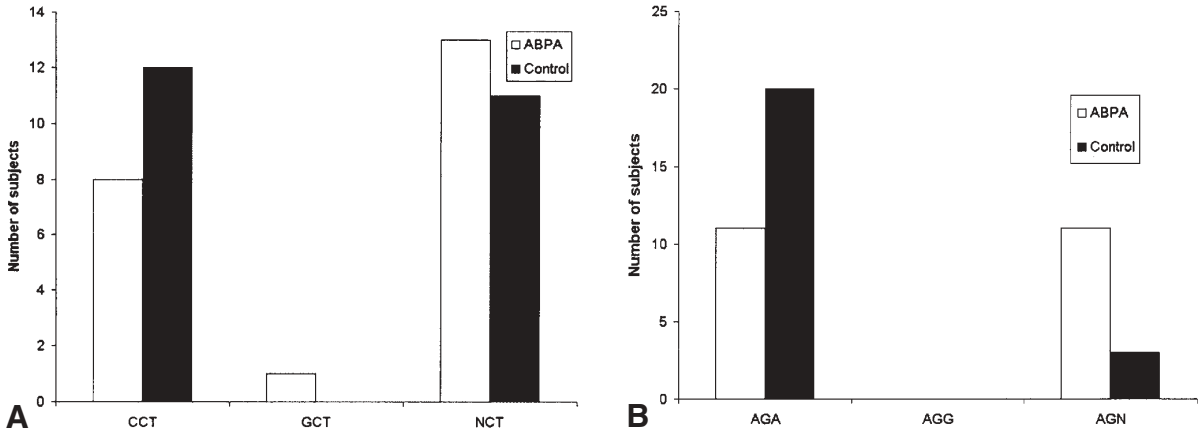

FIG 1. *SP-A2* genotypes observed in patients with ABPA and control subjects. **A,** SP-A2 G1649C (Ala91Pro): *GCT,* homozygous subjects for the *GCT* allele; *NCT,* heterozygous subject for *GCT/CCT* alleles; *CCT,* homozygous subjects for the *CCT* allele. **B,** SP-A2 A1660G (Arg94Arg): *AGA,* homozygous subjects for the *AGA* allele; *AGN,* heterozygous subjects for the *AGA/AGG* alleles; *AGG,* homozygous subjects for the *AGG* allele.

TABLE I. Primers used for PCR amplification

Oligo (n)	Location	S/AS	Nucleotide position	Sequence
SP-A1FF (1)	Intron A	S	469-488	5′ ACTCCATGACTGACCACCTT 3′
SP-A1,2FR (2)	Intron E	AS	3845-3865	5′ TGCCACAGAGACCTCAGAGT 3′
SP-A2FF (3)	Intron 1	S	472-491	5′ ATCACTGACTGTGAGAGGGT 3′
SP-A2E3,4F (4)	Exon 3	S	1078-1097	5′ GCTGTGCCCTCTGGCCCTTA 3′
SP-A2E3,4R (5)	Intron 4	AS	1980-2009	5′ TGCCTCGTCCGCATTCACCCT TCAGACTGC 3′
SP-A1E2,3F (6)	Intron A	S	995-1014	5′ GATGGGGTCACGGCCATCCC 3′
SP-A1E2,3R (7)	Intron C	AS	1962-1991	5′ TGCCTCGTCCGCATTTCACCC TTCAGACAGC 3′
SP-A2E4CF (8)	Exon 3	S	1602-1631	5′ TGCCTGGAGCCCCTGGTGTCC CTGGAGAGC 3′

S, Sense; *AS,* antisense.

population (patients with ABPA = 22, control subjects = 23) are shown in Table II. Frequencies of these SNPs in patients with ABPA and control subjects observed in the preliminary study are shown in Table III. Statistical analysis showed that 4 of these SNPs have significant differences in their frequencies observed in patients with ABPA versus control subjects (SP-A1 C1416T: P = .1176, OR = ∞; SP-A2 T1492C: P = .034, OR = 4.84; SP-A2 G1649C: P = .0314, OR = 4.156; SP-A2 A1660G: P = .0584, OR = 7.00). Similar P values were obtained for all the polymorphisms by both 2 × 2 table and Fisher exact analysis. The two SNPs with significant ORs observed in the coding region of *SP-A2* (SP-A2 G1649C and SP-A2 G1660A) were further screened in a larger group of patients with ABPA (n = 22) and control subjects (n = 23).

Distribution of genotypes in patients with ABPA and control subjects with respect to SP-A2 G1649C (*CCT* homozygotes, *C/GCT* heterozygotes, and *GCT* homozygotes) and SP-A2 G1660A (*AGA* homozygotes and *AGA/G* heterozygotes) are shown in Fig 1. There is only one patient with ABPA homozygous for the *GCT* allele, whereas none of the patients or control subjects were homozygous for the *AGG* allele. Statistical analysis showed that A1660G is significantly associated with ABPA (P = .0156, OR = 4.7778; Table IV). Because both the SNPs (1649 and 1660) are very close to each other, association of simultaneous occurrence of the *GCT* and *AGG* alleles with ABPA was analyzed. The co-occurrence of the *GCT* and *AGG* alleles showed a stronger association with ABPA (Fig 2). Eighty percent of the subjects carrying both the alleles are patients with ABPA (P = .0079, OR = 10.4), whereas only 50% (C1649G) and 60% (A1660G) of patients carry these 2 alleles separately. This is suggestive of an additive effect of these alleles of *SP-A2* on disease outcome.

For each of the clinical markers of ABPA, the *GCT* and *AGG* alleles were observed to be associated with severity (Table V). Total IgE and eosinophilia were significantly higher in the group of patients with ABPA carrying the *GCT* allele, the *AGG* allele, or both (IgE = 19,625 IU/mL, eosinophilia = 18.1%) in comparison with the patient group carrying the *CCT* and *AGA* alleles (IgE = 5133 IU/mL, eosinophilia = 9.33%; P = .000 and .040, respectively). The percent predicted FEV_1 in the group of patients with ABPA carrying the *GCT* allele, the

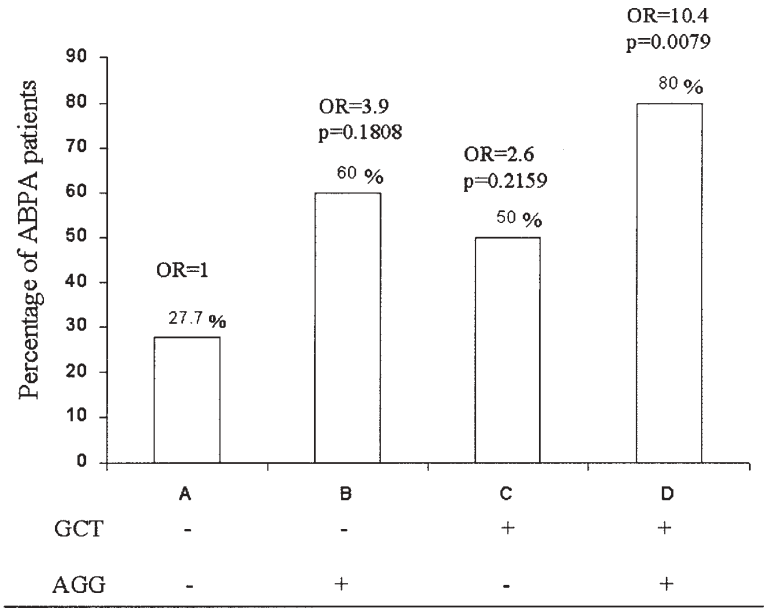

FIG 2. Interaction between the *SP-A2* alleles *GCT* (91st codon) and *ACG* (94th codon) in patients with ABPA versus control subjects. *P* values and ORs (95% CI) are in comparison with the *CCT/AGA* genotype. *A,* Group of patients with ABPA carrying *CCT* and *AGA* alleles; *B,* group of patients with ABPA carrying *CCT* and *AGG* alleles; *C,* group of patients with ABPA carrying *GCT* and *AGA* alleles; *D,* group of patients with ABPA carrying *GCT* and *AGG* alleles.

TABLE II. Characteristics of polymorphisms observed in collagen region of human *SP-A1* and *SP-A2* in Indian population

	Nucleotide		Amino acid	
Gene location	**Position**	**Change**	**Position**	**Change**
SP-A1				
Exon 2	1162	C/T	39th	His to His
Exon 2	1193	C/G	50th	Leu to Val
Intron B	1416	C/T	—	—
SP-A2				
Intron 3	1382	C/G	—	—
Intron 3	1492	T/C	—	—
Exon 4	1649	G/C	91st	Ala to Pro
Exon 4	1660	A/G	94th	Arg to Arg

Nucleotide position of *SP-A1* is according to accession no. M 30838.
Nucleotide position of *SP-A2* is according to accession no. M 68519.

AGG allele, or both (56.0%) was lower than that for the group of patients with ABPA carrying the *CCT* and *AGA* alleles (68%). However, the difference between 2 patient populations was not statistically significant ($P = .150$) with respect to FEV_1.

DISCUSSION

Increased incidence of ABPA in genetically predisposed atopic patients, asthmatic patients, and patients with cystic fibrosis indicates a multifactorial genetic basis for the disease.[21,22] A plausible genetic contribution to ABPA is also suggested by its familial occurrence.[23-26] In recent years, polymorphisms of several candidate genes, such as that for cystic fibrosis transmembrane regulatory protein (*CFTR*) and *HLA-DR*, have been correlated with susceptibility to ABPA.[27-32] In a recent study the frequency of *CFTR* mutations was observed to be significantly higher in patients with ABPA (6/21 [28.5%] patients) than in control subjects with asthma (2/43 [4.6%] subjects, $P = .01$).[32] The *HLA-DR* alleles *DR2*, *DR5*, and possibly *DR4* or *DR7* contribute to susceptibility of the subjects to ABPA, whereas individuals with the *HLA-DQ2* allele are resistant to develop ABPA.[28-31]

In the present study we investigated the hypothesis that genetic polymorphisms in *SP-A1* and *SP-A2* might influence the host's susceptibility to ABPA. In the Indian population 7 polymorphic sites (including exonic and intronic polymorphisms) were observed in the collagen region of *SP-A1* and *SP-A2*.[17] Of 9 polymorphisms reported for the collagen region of *SP-A2*, the Indian population exhibited polymorphisms at only 2 sites (GenBank accession no. NM_005411). In *SP-A1* 2 polymorphic sites were observed in the collagen region, one of which is reported (total reported 3, GenBank accession no. XM_039059), whereas the other is a novel polymorphic site.[17] Four of these SNPs (2 exonic and 2 intronic polymorphisms) showed high frequencies in patients with ABPA versus control subjects. Interestingly, in a previous study we observed strong associations of 3 of these SNPs with patients with pulmonary tuberculosis of Indian origin.[17]

Although significance of the intronic polymorphisms in the collagen region of *SP-A1* and *SP-A2* is not known, it might affect binding of transcription factors and splic-

TABLE III. Allelic frequencies of SNPs observed in the collagen region of *SP-A1* and *SP-A2* in patients with ABPA and control subjects

	Allele frequencies				
Allele	**Patients with ABPA**	**Control subjects**	**OR (95% CI)**	**χ^2 Test**	***P* value**
SP-A1	n = 5	n = 7			
1162 C/T					
C	8	10	1.600	0.229	.6325
T	2	4	0.2310 < OR < 11.0823		
1193 C/G					
C	6	8	1.125	0.020	.8886
G	4	6	0.2161 < OR < 5.8554		
1416 C/T					
C	10	11	∞	2.449	.1176
T	0	3	NaN < OR < ∞		
SP-A2	n = 8	n = 8			
1382 C/G					
C	15	15	1.000	0	NS
G	1	1	0.0571 < OR < 17.5098		
1492 T/C					
T	11	5	4.840	4.500	.0338
C	5	11	1.0853 < OR < 21.5838		
1649 G/C	n = 10	n = 11			
G	11	5	4.156	4.627	.0314
C	9	17	1.0984 < OR < 15.7211		
1660 A/G	n = 10	n = 11			
G	5	1	7.000	3.58	.0584
A	15	21	0.7400 < OR < 66.2145		

Similar *P* values were observed by means of both 2 × 2 and Fisher exact analysis.
NS, Not significant.

TABLE IV. Two by two statistical analysis of frequencies of G1649C and A1660G of *SP-A2* in patients with ABPA (n = 22) versus control subjects (n = 23)

	Allele frequencies				
Allele	**Patients with ABPA**	**Control subjects**	**OR (95% CI)**	**χ^2 Test**	***P* value**
SP-A2	n = 22	n = 23			
1649 G/C					
G	15	9	2.1264	2.426	.1193
C	29	37	0.8152 < OR < 5.5468		
1660 A/G					
G	11	3	4.7778	5.846	.0156
A	33	43	1.2326 < OR < 18.5189		

ing. At this point of time, it is also not clear whether these intronic polymorphisms are in linkage disequilibrium with other functional polymorphisms.

Association of SP-A2 G1649C (alanine to proline, 91st codon) with patients with ABPA was higher (P = .1193, OR = 2.1264, 95% CI = 0.8152 < OR < 5.5468) compared with its association with patients with pulmonary tuberculosis (P = .1320, OR = 1.576, 95% CI = 0.8696 < OR < 2.8564).[17] Proline is an important component of repetitive subunit Gly-X-Pro in the collagen region of SP-A and is known to provide stability to triple helical collagenous structures.[33] However, the precise effect of this change of amino acid on functionality of SP-A is not yet known. SP-A2 A1660G, a redundant polymorphism (arginine to arginine, 94th codon) showing association with ABPA in the present study, was also associated with pulmonary tuberculosis.[17] The association of SP-A2 A1660G was significantly higher with patients with pulmonary tuberculosis (P = .000, OR = 8.9412, 95% CI = 3.3136 < OR < 24.126) compared with that in patients with ABPA (P = .0156, OR = 4.7778, 95% CI = 1.2326 < OR < 18.5189). This polymorphism lies in close vicinity of the 3′ splice site (exon 4/intron4 at 1670) and might affect the process of splicing. Homozygotes for the *GCT* and *AGG* alleles have not been observed in the control subjects. The absence of *AGG* homozygotes and the presence of a small number of *GCT* homozygotes observed in the patients is suggestive of loss of a vital function associated with homozygosity of these alleles.

TABLE V. Clinical markers of ABPA severity split by *SP-A2* 91st and 94th codon polymorphisms

	Patients with ABPA with genotype: 91st codon (*GCT/NCT*), 94th codon (*AGN*), or both; n = 18, mean (SD)	Patients with ABPA with genotype: 91st codon (*CCT*) and 94th codon (*AGA*); n = 5, mean (SD)	*P* value (Student *t* test)
FEV_1 (%)	56.0 (7.93)	68 (12.2)	.150
Total IgE (IU/mL)	19,625 (14,500)	5133 (1140)	.000
Eosinophilia (%)	18.1 (7.52)	9.3 (5.79)	.040

It is interesting to note that the 91st codon of *SP-A1* codes for proline and nucleotide at 1660 is invariably G in all the control subjects and the patients of Indian origin screened in the present study, although polymorphisms at the 91st and 94th codons of *SP-A1* have been reported in other populations (GenBank accession no. XM_039059). In view of variable contributions of *SP-A1* and *SP-A2* to SP-A protein (ratio of *SP-A1*:*SP-A2* varies from 0.94 to 6.80 among individuals), the combined effect of polymorphisms in *SP-A1* and *SP-A2* on protein function might vary among individuals.[11]

A marked increase in total IgE antibodies and peripheral eosinophilia and a decrease in lung performance (percent FEV_1) in the group of patients with ABPA carrying the *AGG* allele, the *GCT* allele, or both suggested that these alleles might be contributing to the severity of the disease. In view of association of *SP-A2* polymorphisms with disease severity, it is important to note that *SP-A2* is more responsive to glucocorticoids (prescribed to patients with ABPA as first-line treatment) than *SP-A1*, and patients with ABPA respond variably to the glucocorticoid therapy.[34-36]

Association of the same alleles of *SP-A2* with susceptibility of subjects to ABPA and pulmonary tuberculosis suggest that these alleles lead to loss of an important function related to pulmonary defense mechanism of the host. Although ABPA and pulmonary tuberculosis are clinically distinct entities, they share the same site of infection (upper lobes are affected with both conditions especially posterior segments) and have similarities in their radiologic picture.[37-40]

Patients with pulmonary edema (a physiologic lung disease), however, did not show a significant association with either of these polymorphisms alone or with both polymorphisms together (unpublished data from our laboratory; frequency of *GCT* allele: patients with pulmonary edema patients [4/14], control subjects [5/14]; frequency of *CCT* allele: patients [10/14], control subjects [9/14]; frequency of the *AGA* allele: patients [13/14], control subjects [13/14]; frequency of the *AGG* allele: patients [1/14], control subjects [1/14]).

The collagen region comprising both the SP-A2 G1649C and SP-A2 A1660G polymorphisms interacts with receptors on alveolar macrophages, an important line of defense against both ABPA and pulmonary tuberculosis. Weikert et al[41] demonstrated that the collagen-like domain of human SP-A binds to the specific phagocyte surface receptor SPR210 during phagocytosis of human SP-A–coated *Mycobacterium bovis* BCG. These polymorphisms might result in altered conformation or affinity of SP-A, affecting its binding to the receptors on macrophages, and might lead to their reduced activation during host-pathogen interactions. Investigations on interactions of recombinantly expressed allelic variants of *SP-A2* with *A fumigatus* might facilitate understanding on the role of *SP-A2* polymorphisms in pulmonary host defense.

The polymorphisms SP-A2 G1649C and SP-A2 A1660G of the collagen region of *SP-A2* might be one of the genetic factors that determine susceptibility to ABPA and could be useful as a potential diagnostic marker for predisposition to aspergillosis.

We thank the Functional Genomics Unit, Institute for Genomics and Integrative Biology, for the sequencing of PCR products. We also thank Dr Deepak, Vallabhbhai Patel Chest Institute, for his help in collection of samples from patients with ABPA.

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