BRIEF REPORT

Polymorphisms in Toll-Like Receptor Genes and Susceptibility to Pulmonary Aspergillosis

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Toll-like receptors (TLRs) are important components of innate immunity. We investigated the association between polymorphisms in the *TLR2, TLR4,* and *TLR9* genes and susceptibility to noninvasive forms of pulmonary aspergillosis. A significant association was observed between allele G on Asp299Gly *(TLR4)* and chronic cavitary pulmonary aspergillosis (odds ratio [OR], 3.46; $P = .003$). Susceptibility to allergic bronchopulmonary aspergillosis was associated with allele C on T-1237C *(TLR9)* (OR, 2.49; $P = .043$). No particular polymorphism was associated with severe asthma with fungal sensitization. These findings reinforce the importance of innate immunity in the pathogenesis of different forms of aspergillosis.

Aspergillus fumigatus is the most prevalent airborne, filamentous fungal pathogen in humans. It is the main cause of aspergillosis, a condition that can manifest as invasive, allergic, and chronic noninvasive syndromes. Allergic bronchopulmonary aspergillosis (ABPA) is an allergic hypersensitivity to bronchial colonization by *Aspergillus* species, which affects mainly patients with asthma and patients with cystic fibrosis. A severe asthma phenotype associated with fungal sensitization (SAFS) can also develop. In contrast, chronic cavitary pulmonary aspergillosis (CCPA) is a subacute and slowly destructive form of pulmonary aspergillosis.

Although the immunological status of the host is probably the main determinant of the clinical presentation of aspergillosis, very little is known about this subject. Recently, considerable attention has been focused on innate immunity, the first line of defense that ultimately activates the adaptive immune system through specific signals. The latest evidence has focused on proteins that belong to the collectin family, including the lung surfactant protein (SP), mannose-binding lectin (MBL), and pentraxin 3 (PTX3). Allele variants in a gene encoding SP-A have been associated with increased susceptibility to both ABPA and CCPA [1]. Also, distinct MBL genotypes have been linked with CCPA [1, 2], and an intronic polymorphism in the MBL gene was found to underlie elevated levels of MBL and greater disease severity in ABPA patients [3]. On the other hand, PTX3 was shown to play a role in resistance to *Aspergillus,* and PTX3-null mice showed increased susceptibility to invasive pulmonary aspergillosis [4].

Toll-like receptors (TLRs) are important components of the innate immune system. TLRs are transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats (LRR) and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain, which activates common signaling pathways. TLRs mediate the recognition of microbial challenges and subsequent inflammatory responses through rapid changes in the expression of genes encoding cytokines and inflammatory molecules. More specifically, TLR-2 and TLR-4 have been implicated as important components of the initial host immune response to fungal pathogens, both yeasts and molds [5].

Several variations in TLR genes have been studied, including that of Asp299Gly (A+896G) in the *TLR4* gene. This results in an amino acid change in the LRR domain of TLR-4, impairing its recognition ability. This single-nucleotide polymorphism (SNP) has been correlated with diminished airway response to inhaled lipopolysaccharide in healthy individuals. On the other hand, the Arg753Gln (G+2258A) polymorphism, which affects the TIR domain of TLR-2, impairs signal transduction and its functional activity. Another SNP, T-1237C, located within the putative promoter of the *TLR9* gene has been implicated in chronic inflammatory diseases including asthma [6] and Crohn disease. Moreover, TLRs have also been associated with several pathological conditions affecting the lungs. Thus, due to the significance of innate immunity in host defense, genetic variations in the genes of this system could have a major impact on immune responses to *Aspergillus* infections.

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Table 1. Polymerase chain reaction primers used to analyze polymorphisms in Toll-like receptor *(TLR) 2, TLR4,* and *TLR9* genes by use of Bi-PASA genotyping.

Target gene, single-nucleotide polymorphism	Primer sequence (5'→3')	Primer concentration, μmol/L	DNA fragment sizes, bp
TLR2			PQ,524;PW,399;MQ,152
Arg753Gln			
	P: CTCCAGGCCAAAAGGAAGC	0.1	
	Q: AAAGATCCCAACTAGACAAAGA	0.1	
	W: ggcggcgggggccTGTTCATTATCTTCC	0.1	
	M: gggccggggTTCTGCAAGCTGCA	0.1	
TLR4			PQ,644;PW,455;MQ,220
Asp299Gly			
	P: AGAACTTAATGTGGCTCACAAT	0.1	
	Q: GAAAAAGCATTCCCACCTTTG	0.4	
	W: ggcggcgggggccTTAAATAAGTCAATAATAT	0.4	
	M: gggccggggTACTACCTCGATGG	0.4	
TLR9			PQ,644;PW,395;MQ,275
T-1237C			
	P: TCATTCAGCCTTCACTCAGA	0.4	
	Q: CACATTCAGCCCCTAGAGGG	0.05	
	W: ggcggcgggggccTGCTGTTCCCTCTGCCTGA	0.05	
	M: gggccgggggccATGAGACTTGGGGGAGTTTC	0.05	

NOTE. P, left outer primer; Q, right outer primer; W, inner wild-type primer; M, inner mutant primer.

In the present population-based, case-control study, we aimed to explore the contribution made by polymorphisms in the *TLR2, TLR4,* and *TLR9* genes to susceptibility to different forms of aspergillosis, namely CCPA, ABPA, and SAFS.

Materials and Methods. The study population comprised 76 patients; males and females were equally distributed. Mean age (±SD) was 57.6 (± 11.8) years. The main diagnoses were CCPA ($n = 40$), ABPA ($n = 22$), and SAFS ($n = 14$). These individuals were recruited from a cohort of patients attending South Manchester University Hospitals NHS Trust for *Aspergillus*-related diseases. Eighty unrelated healthy individuals of identical ethnicity were included as controls. Informed written consent was obtained from all participants.

The clinical definitions were as follows. CCPA was diagnosed in the presence of the following symptoms and/or results: (1) chronic pulmonary or systemic symptoms with exclusion of other pulmonary pathogens, (2) radiological evidence of progressive pulmonary lesions with surrounding inflammation (with or without an intracavitary mass), (3) precipitating (IgG) antibody to *Aspergillus* in the serum, and (4) persistently elevated inflammatory markers in the serum.

The diagnostic criteria for ABPA included the following symptoms and/or results: (1) asthma, (2) total serum IgE levels ⩾1000 IU/mL, (3) elevated *A. fumigatus*–specific serum IgE levels, (4) precipitating antibodies to *A. fumigatus* in the serum (not always present in patients with long-standing ABPA), and (5) central bronchiectasis. The minor diagnostic criteria for ABPA were as follows: peripheral blood eosinophilia (often absent in patients on steroids), repeated detection of *Aspergillus* in sputum, expectoration of brown plugs or flecks, and history of recurrent pulmonary infiltrates (transient or fixed).

The diagnosis of SAFS was made on the basis of the following recently proposed criteria [7]: (1) severe asthma, (2) total IgE levels <1,000 IU/mL, and (3) positive skin-prick test and/or raised specific IgE to *A. fumigatus.* We included in this study only SAFS patients who reacted to *A. fumigatus.* In contrast to patients with ABPA, patients with SAFS usually do not give a history of productive cough containing mucus plugs and infrequently have positive sputum cultures for fungi.

Genotype determination of polymorphisms in *TLR2* (Arg753Gln; SNP id: rs5743708), *TLR4* (Asp299Gly; SNP id: rs4986790), and *TLR9* (T-1237C; SNP id: rs5743836) genes was performed by use of bidirectional PCR amplification of specific alleles (Bi-PASA), as described elsewhere [8]. Details about the primers used in this study are shown in table 1.

The Fisher exact test and Pearson χ^2 test were used to compare allele frequencies between patient groups and controls. Consistency of genotype frequencies with the Hardy–Weinberg equilibrium was tested using a χ^2 test on a contingency table of observed vs. predicted genotype frequencies. χ^2 values, odds ratios (ORs), and *P* values were calculated with 95% confidence intervals.

Results. TLR polymorphisms in the control population were characterized as follows. Of the 80 control patients studied, 5 of 80 (6.2%), 10 of 80 (12.5%), and 15 of 80 (18.8%) patients were heterozygous for Arg753Gln (G/A), Asp299Gly (A/G), and

Table 2. Distribution of genotypes and allele frequencies of the studied polymorphisms in Toll-like receptor *(TLR) 2, TLR4,* and *TLR9* genes in study and control patients.

Gene, single-nucleotide polymorphism	Study group (n)	No. (% frequency)					χ^2	*P*	OR (95% CI)
		Genotype			Allele				
TLR2, Arg753Gln		**G/G**	**G/A**	**A/A**	**G**	**A**			
	Controls (80)	75 (93.8)	5 (6.2)	0 (0.0)	155 (96.9)	5 (3.1)			
	ABPA (22)	22 (100)	0 (0.0)	0 (0.0)	44 (100.0)	0 (0.0)	1.41	0.587	1.032 (1.004–1.061)
	SAFS (14)	12 (85.7)	2 (14.3)	0 (0.0)	26 (92.9)	2 (7.1)	1.07	0.280	2.385 (0.439–12.944)
	CCPA (40)	38 (95.0)	2 (5.0)	0 (0.0)	78 (97.5)	2 (2.5)	0.07	1.000	0.795 (0.151–4.190)
TLR4, Asp299Gly		**A/A**	**A/G**	**G/G**	**A**	**G**			
	Controls (80)	70 (87.5)	10 (12.5)	0 (0.0)	150 (93.8)	10 (6.3)			
	ABPA (22)	21 (95.5)	1 (4.5)	0 (0.0)	43 (97.7)	1 (2.3)	1.07	0.463	0.349 (0.043–2.802)
	SAFS (14)	12 (85.7)	2 (14.3)	0 (0.0)	26 (92.9)	2 (7.1)	0.03	0.695	1.154 (0.239–5.570)
	CCPA (40)	25 (62.5)	**15 (37.5)**	0 (0.0)	65 (81.3)	**15 (18.8)**	8.93	0.003	3.462 (1.477–8.110)
TLR9, T-1237C		**T/T**	**T/C**	**C/C**	**T**	**C**			
	Controls (80)	65 (81.2)	15 (18.8)	0 (0.0)	145 (90.6)	15 (9.4)			
	ABPA (22)	14 (63.6)	7 (31.8)	1 (4.54)	35 (79.5)	**9 (20.5)**	4.08	0.043	2.486 (1.005–6.145)
	SAFS (14)	13 (92.9)	1 (7.1)	0 (0.0)	27 (96.4)	1 (3.6)	0.03	0.474	0.358 (0.045–2.825)
	CCPA (40)	33 (82.5)	7 (17.5)	0 (0.0)	73 (91.3)	7 (8.8)	0.02	0.874	0.927 (0.362–2.373)

NOTE. ABPA, allergic bronchopulmonary aspergillosis; CCPA, chronic cavitary pulmonary aspergillosis; CI, confidence interval; OR, odds ratio; SAFS, severe asthma with fungal sensitization. χ^2 and *P* values were calculated regarding allele frequencies.

T-1237C (T/C) polymorphisms, respectively. In addition, no homozygous mutation was observed in controls (i.e., A/A for Arg753Gln, G/G for Asp299Gly, or C/C for T-1237C). The distribution of genotypes did not deviate from those predicted by the Hardy-Weinberg equilibrium.

There was no significant difference between patients with ABPA and control patients with respect to allele frequencies or genotype distribution of the *TLR2* and *TLR4* polymorphisms (table 2). The G/A genotype in *TLR2* was not detected in any patient with ABPA. Although patients with ABPA had a lower frequency of the G allele in *TLR4,* compared with control patients (2.3% vs. 6.3%), this difference did not reach statistical significance ($P = .463$). Patients with ABPA had a significantly higher frequency of allele C for the T-1237C SNP in *TLR9* than control patients (20.5% vs. 9.4%; $P = .043$; OR, 2.49).

The frequency of allele A for the *TLR2* polymorphism among patients with SAFS was more than twice that observed among control patients (7.1% vs. 3.1%), although this difference was not statistically significant ($P = .280$). No difference was observed regarding the *TLR4* variation between patients with SAFS and control patients; both presented similar allele frequencies. Patients with SAFS had a lower frequency of allele C in the *TLR9* gene, although this difference was nonsignificant in comparison with control patients (3.6% vs. 9.4%; $P = .474$). When patients with SAFS and ABPA were considered together and compared to control patients, no difference in allele frequency in the TLR genes was observed (data not shown).

Patients with CCPA demonstrated a statistically significant higher frequency of allele G in the *TLR4* gene, compared with controls (18.8% vs. 6.3%; OR, 3.46; $P = .003$). The frequency of SNPs in the *TLR2* and *TLR9* genes was similar for patients with CCPA and control patients.

Discussion. TLR-4 is among the major receptors involved in the recognition of pathogenic fungi and the initiation of the inflammatory response. Wang et al. [9] proposed TLR-4 as the main receptor for *Aspergillus* hyphae, because monoclonal antibodies directed against CD-14 and TLR-4 partially inhibited TNF-α release from human monocytes stimulated by *A. fumigatus* hyphae. Accordingly, TLR-4–deficient mice have shown increased susceptibility to *A. fumigatus* infection. Netea et al. [10] showed that macrophages from TLR-4–deficient mice produced less TNF-α and IL-1 than did macrophages from control mice upon stimulation with *A. fumigatus* conidia but not with hyphae. These data suggest that TLR-4–mediated signals might be lost during *Aspergillus* germination, therefore suggesting that this phenotypic switching might be a mechanism to escape the immune system. Interestingly, we found the Asp299Gly polymorphism *(TLR4)* to be highly associated with CCPA (OR, 3.46; $P < .01$). This SNP had not been previously observed to be associated with acute invasive aspergillosis [11]. Because its presence does not ultimately affect signal transduction and cytokine production in mononuclear cells challenged with *A. fumigatus,* a putative mechanism for disease association would be an abnormal TLR-4 extracellular domain, which hampered its function by disrupting microbial recognition. Patients with CCPA appear to have multiple defective immune responses, of which this may be one in a minority of patients. On the other hand, allele G on Asp299Gly *(TLR4)* was slightly less frequent in ABPA patients. This is perhaps unexpected, as it has been shown to be protective in hyperinflammatory states, such as atherosclerosis, by dimin-

ishing the levels of proinflammatory cytokines [12]. The significance, if any, of this observation requires a study involving a larger number of patients with ABPA for clarification.

We observed no association between *TLR2* polymorphisms and susceptibility to CCPA. Similar findings were previously described for patients with invasive aspergillosis [11]. Interestingly, the *TLR2* SNP was not present in any patient with ABPA, which could argue for a protective role against ABPA. It is curious in this regard that TLR-2–deficient mice are highly susceptible to experimental ABPA (L. Romani, unpublished observations). Ultimately, the numbers in our study population were too small to show any significant difference, suggesting at maximum a weak effect. For the same reason, one can speculate that allele A in *TLR2* might increase the risk for SAFS.

Also interesting was the association of ABPA with the *TLR9* SNP (OR, 2.49; $P < .05$). TLR-9 is a receptor that detects unmethylated CpG motifs prevalent in bacterial and viral DNA. As *A. nidulans* has been shown to be virtually devoid of genomic cytosine methylation [13] (which is probably also true for *A. fumigatus*), *TLR9* activation by CpG motifs may occur after lysis of fungal cells during *Aspergillus* infections. However, the importance of *TLR9* in aspergillosis is still obscure. Although both conidia and hyphae of *A. fumigatus* seem to signal through TLR-9 on murine neutrophils [14], TLR-9–deficient mice showed paradoxically greater conidiocidal activity and hyphal damage than wild-type mice. In addition, TLR-9 has been associated with anti-allergic activities. Accordingly, *TLR9* SNP has been associated with an increased risk of asthma [6], and TLR-9 agonists are under development for patients with asthma and other allergic conditions. It remains to be elucidated if this constitutes an independent risk factor for ABPA, since all ABPA patients in our cohort were also diagnosed with asthma. In addition, work by Novak et al. [15] showed that the C allele of T-1237C decreases *TLR9* expression, which could also be proposed as a feature underlying susceptibility to ABPA .

Due to the limited number of patients in the study, the importance of the *TLR2* SNP in patients with aspergillosis cannot be underestimated, although its association with CCPA seems statistically improbable. No polymorphism was associated with the SAFS phenotype, reflecting either the limited number of patients, or more likely, different pathogenesis. Also, the importance of polymorphisms affecting other TLR genes (as well as other SNPs in the TLRs investigated here) cannot be excluded. Because multiple receptors appear to be involved in the recognition of different components of *Aspergillus,* a better understanding of the signaling processes involved in innate immunity is ultimately required.

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