ORIGINAL ARTICLE

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Modifier effect of the Toll-like receptor 4 D299G polymorphism in children with cystic fibrosis

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Abstract

Introduction: Clinical phenotype varies amongst cystic fibrosis (CF) patients with identical CF transmembrane regulator (CFTR) genotype, suggesting genetic modifiers exist. One potential modifier is the Toll-like receptor 4 (TLR4) gene. TLR4 binds lipopolysaccharide (LPS), a constituent of *Pseudomonas aeruginosa* (PA), activating innate immunity and promoting inflammation. TLR4 polymorphisms are associated with LPS-hyporesponsiveness and may be protective in CF due to decreased inflammation.
Materials and Methods: DNA was extracted from blood of recruited CF subjects, and PCR performed to establish TLR4 D299G genotype. Case-notes were reviewed to obtain clinical data. Subjects possessing the TLR4 299G allele were compared with age, sex, and CFTR genotype-matched wild-type (299DD) subjects and also with all controls.
Results: 100 subjects (mean age 8.9 years) were studied, with 11 299DG heterozygotes identified. On case-matched analyses, no statistically significant differences between groups were found for mean±SEM rates of change of %predicted FEV_1/year (0.9±2.3 (DD) vs. –3.9±2.8 (DG), p=0.22), %predicted FEV_1 (76±8 vs. 74±11), p=0.91), or z scores for height (–0.47±0.26 vs. –0.24±0.19, p=0.48) and weight (–0.01±0.22 vs. –0.29±0.27, p=0.44). Median±SE survival age at first PA isolation was also not significantly different (3.5±2.1 vs. 6.5±2.4 years, p=0.29). No statistically significant differences were noted when 299DG heterozygotes were compared with all controls.
Conclusions: Potential reasons for absence of modifier effect include the basolateral location of TLR4 receptors on respiratory epithelium, or because inflammatory response to PA in the CF airway is so overwhelming that even a blunted response (as suggested for the 299G allele) results in increased inflammation and lung damage.

Key words: cystic fibrosis, genotype-phenotype correlation, modifier genes, Toll-like receptor 4.

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INTRODUCTION

The course and progression of disease is variable in cystic fibrosis (CF) patients, including those with identical CF transmembrane regulator (CFTR) genotype [26, 44]. This suggests that modulating factors exist [15, 33, 41, 48]. Environmental [21] and socioeconomic [7, 42] factors can influence the clinical course in CF and recent research has focused on the role of modifier genes [3–5, 10, 11, 14, 16–19, 22, 26, 27, 32, 35]. Such genes may be important in helping us to better understand the pathophysiology of disease progression in CF and may additionally offer a future role for therapeutic intervention [20].

Previous studies have identified potential CF modifier genes. Mannose-binding lectin (MBL) polymorphisms have been shown to affect lung function in CF patients [18, 19], and *in vitro* evidence shows variation in *B. cepacia* binding [10]. Some evidence suggests polymorphisms associated with increased tumor necrosis factor (TNF)-α production may confer a more severe pulmonary phenotype [48], whilst patients with high-expressing transforming growth factor-β1 alleles are reported to have more severe pulmonary disease [16]

and a more rapid decline in lung function [3]. No correlation between α-1 antitrypsin genotype and lung phenotype has been found, but earlier infection with *Pseudomonas aeruginosa* (PA) has been suggested [14, 17, 32, 35]. Other genes shown to influence PA infection have been the HLA complex [5] and the nitric oxide synthase gene [22], whilst the evidence on the role of the angiotensin-converting enzyme genotype in CF is conflicting [4, 27].

A potential class of modifier genes in CF is the Toll-like receptor (TLR) gene family (TLR1-10). TLRs are a family of transmembrane proteins which play an important part in the innate immune response by detecting infection and activating inflammatory responses [38]. TLRs are one of a number of so-called pattern-recognition receptors [34], whereby recognition of microbial products (or pathogen-associated molecular patterns) [1] by TLRs on dendritic cells leads to maturation of dendritic cells and the activation of antigen-specific adaptive immune responses. Several microbial molecules, such as lipoproteins and lipopolysaccharide (LPS), are known to activate or specifically bind to TLRs as "opportunistic ligands", but direct evidence of LPS binding is described for TLR4 only [39]. TLR4 binds LPS (a constitutive element of the PA cell wall) [37] and activates intracellular signaling, including nuclear factor (NF)-κB, via an enzymatic cascade, resulting in increased pro-inflammatory cytokine expression [37].

The TLR4 gene is located on chromosome 9q32-q33. Two allelic variations of interest in CF are described [2], both of which are single-nucleotide polymorphisms. The first is an amino-acid change of aspartic acid to glycine (D299G) and the second is a threonine to isoleucine substitution (T399I). Allele frequency for the 299G allele varies from 3.1–5.6% [29, 43] and is approximately 6% for the 399I allele [43]. There is strong linkage disequilibrium between these two single-nucleotide polymorphisms of TLR4, with studies showing cosegregation for the D299G and T399I polymorphisms in 84–99% of cases [29, 31]. With regard to the D299G polymorphism, wild-type subjects possess two D alleles (DD), those with two G alleles are homozygous (GG), whilst heterozygotes have both D and G alleles (DG). A blunted response to LPS has been shown in 299G heterozygotes [40], suggesting a dominant genetic effect.

Seventy-three percent of children with CF contract a PA lung infection by the age of 3 years [8], and the cause of death in almost all CF patients is the progressive lung inflammatory response associated with unremitting PA infection [13]. LPS produced by PA is recognized by airway cells, possibly via TLR4 pathways, and leads to upregulation of the innate immune response, including activation of NF-κB. NF-κB activation in turn leads to enhanced expression of cytokines such as interleukins (IL)-6 and -8 and TNF-α. IL-8 is a powerful neutrophil chemoattractant, perpetuating neutrophilic inflammation in the CF lung. The resultant inflammation and action of neutrophil enzymes such as elastase may result in ongoing lung damage.

As TLR4 299G is associated with LPS hyporesponsiveness (reduced cytokine expression), it is possible that subjects with CF possessing the 299G allele may have less parenchymal lung damage in response to LPS-producing organisms such as PA. We hypothesize that patients with CF who have at least one 299G allele (those with D299G or 299GG) may have less severe disease than those with the wild-type 299DD genotype.

MATERIALS AND METHODS

Subjects

One hundred subjects were recruited prospectively from consecutive children attending for CF annual review at Great Ormond Street Hospital for Children. Clinical information was recorded for each child including age, height z scores, weight z scores, lung function data (predicted FEV_1%, predicted FVC%), Chrispin-Norman chest radiograph scores [9], age of first PA acquisition, and rate of decline in lung function These data were obtained from the Great Ormond Street Hospital CF Database. The study was approved by the Hospital's ethics committee.

Laboratory methods

Two milliliters of whole blood (EDTA sample) was taken from each patient and stored at -20^0C. DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen Ltd., Crawley, UK) from 200 µl of whole blood using the manufacturer's instructions. DNA was then eluted into 200 µl of sterile water and stored at -20^0C for further analysis. PCR was performed to establish TLR4 genotype using forward (GAAATGAAGGAAACTTGGAAAAGTT) and reverse (CATTTGTCAAACAATTAAATAAGTCAATAGTA) oligonucleotide primers (SIGMA-genosys, Cambridge, UK). PCR reactions were carried out in a total volume of 20 µl using 30 ng of DNA. Denaturation was at 95°C, annealing at 55°C, and a final extension at 72°C, all for 1 min. Thirty-four cycles were run. Following PCR amplification, fragments were digested using a restriction enzyme (*Nco*I, New England Biolabs) and electrophoresed through an acrylamide MADGE (microtiter array diagonal gel electrophoresis) gel, allowing electrophoresis of 96 samples. Gels were run at 100 V for 1.5 h and the DNA was visualized by ethidium bromide staining.

Statistical methods

Student's *t*-tests were used to compare two groups of variables, whilst analysis of variance (ANOVA) was used when analyzing the effect of more than one confounding variable. Pearson's correlation coefficient was used to measure the association between variables and age. For those subjects yet to have a first PA isolate, survival analysis with Kaplan-Meier log-rank statistics was

used to compare the effects of TLR4 D299G polymorphism on age at first PA isolate. The rate of change of predicted FEV_1% $year^{-1}$ was calculated by regressing annual FEV_1 measurements in time from the baseline FEV_1 measurement for each subject. Only FEV_1 measurements made above the age of 5 years were included, and subjects were required to have a minimum of three annual FEV_1 measurements for a rate of change in FEV_1 to be measured. Hardy-Weinberg equilibrium was calculated according to a standard formula.

Data were analyzed in two ways. Subjects who had the 299G allele were compared with the remainder of the study population and with subjects matched for age, sex, and CFTR genotype with the wild-type 299DD genotype.

RESULTS

Patient characteristics are summarized in Table 1. Ages ranged from 0.5–16.8 years (mean±SD: 8.9±4.3), and 53% subjects were female. Seventy-four percent of subjects had previously had a PA isolate on cough swab or sputum sampling. Eighty-nine patients had wild-type TLR4 (299DD), with 11 heterozygous 299DG patients. None were homozygotes. The observed allele frequency was therefore 5.5%, and the study population was in Hardy-Weinberg equilibrium.

Table 1. Descriptive statistics for study population

	Wild-type (299DD)	Heterozygote (299DG)	p-value
Total number	89	11	
Sex (M/F)	40/49	7/4	
Mean age (SD)	8.8 (4.2)	9.5 (5.1)	0.79
CFTR genotype			
ΔF508/ΔF508	58	8	
ΔF508/Other	21	2	
other/other	7	0	
Missing data	3	1	
Numbers with at least one PA isolate on sputum/cough swab	66	8	

When the subjects with D299G genotype (n=11) were compared against the subjects (n=89) with wild-type 299DD genotype, no significant differences were seen for lung function (predicted FEV_1%, predicted FVC%), rate of decline in lung function (predicted FEV_1% $year^{-1}$), age at first PA acquisition (Fig. 1), Chrispin-Norman chest radiograph scores, or nutritional parameters (height and weight z scores; Table 2). No differences in outcome measures were seen when analyzed by comparison of the 11 subjects with D299G genotype with 11 controls matched closely for age, sex, and CFTR genotype (Table 3). Further matching for PA acquisition also revealed no statistically significant differences, although a slower decline in lung function was suggested in the wild-type group (p=0.08).

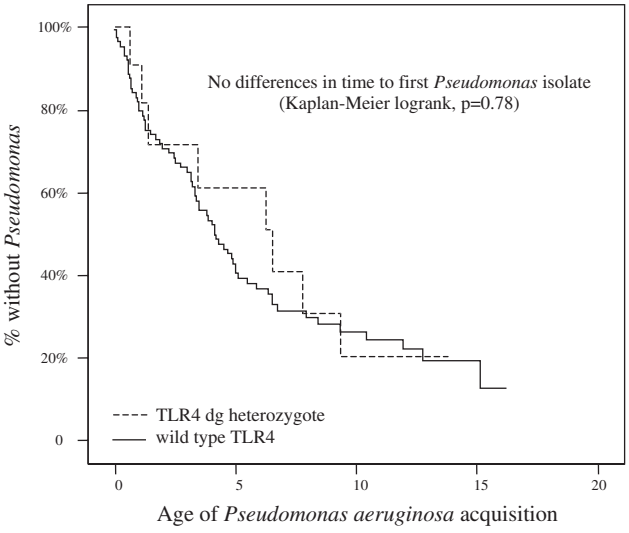

Fig. 1. Time to first *Pseudomonas aeruginosa* isolate by Kaplan-Meier survival analysis.

DISCUSSION

This study did not demonstrate an effect of TLR4 D299G polymorphism on lung function, height and weight z scores, Chrispin-Norman chest X-ray scores, or age at first PA acquisition when comparing against a heterogeneous control group or against cases matched for age, sex, and CFTR genotype. As with many genetic modifier studies in children with CF, relatively small numbers were enrolled in our study, reflecting the prevalence of this condition. A retrospective power calculation based on expected gene frequencies for the 299G allele indicates that 604 patients would have been needed to detect a 10% difference in FEV_1 at the 5% significance level with 80% power. However, whilst a small effect is nonetheless possible, it is unlikely that our study would have failed to detect a large effect for this polymorphism. Studies with similar numbers and gene frequencies have shown positive associations. For example, the association between MBL polymorphism and decreased lung function was suggested by 11 subjects with MBL variants matched with 11 controls amongst a total of 164 subjects [18]. In our study we also matched cases for sex, age, and CFTR genotype and, as with the population as a whole, no differences were detected. Indeed, no trends were suggested during this analysis.

It is possible that the TLR4 299G allele does have an effect on clinical phenotype, but that this may not become apparent until adulthood. Children with CF today remain relatively well (as in our population), making it less likely that a mild genetic modifying effect would be detected in childhood, as appears to be the case with MBL deficiency [12].

From previous studies, it is perhaps surprising that this polymorphism did not appear to have an effect on CF phenotype (if our results reflect the role of this poly-

Table 2. Comparative statistics across entire study population

	Wild-type (299DD)	Heterozygote (299DG)	p-value	Mean difference (95%CI)
Mean (SD)	n=75	n=8	0.55*	6 (–14, +26)
predicted FEV_1%	80 (27)	74 (30)		
Mean (SD)	n=75	n=8	0.52*	5 (–11, +21)
predicted FVC%	86 (22)	80 (24)		
Age at first PA acquisition	n=89	n=11	0.78**	
Median (SE survival)	4.2 (0.6)	6.5 (2.4)		
Mean (SD)	n=88	n=11	0.16*	–0.47 (–1.13, +0.19)
Height z score	–0.72 (1.07)	–0.24 (0.64)		
Mean (SD)	n=88	n=11	0.65*	–0.14 (–0.76, +0.47)
Weight z score	–0.43 (0.98)	–0.29 (0.90)		
Mean (SD)	n=89	n=11	0.16*	–2.4 (–5.9, +1.0)
Chrispin-Norman X-ray score	9.9 (5.3)	12.3 (6.5)		
Mean (SD)	n=58	n=6	0.12*	5.8 (–1.5, 13.1)
Rate of change in predicted FEV_1% $year^{-1}$	+1.9 (8.6)	–3.9 (6.9)		

* Unpaired t-test, ** Kaplan-Meier log-rank test.

Table 3. Inter-group analyses of 11 patients with TLR4 299G allele and 11 controls matched for age, sex, and CFTR genotype

	Wild-type (299DD)	Heterozygote (299DG)	p-value	Mean difference (95%CI)
Total	n=11	n=11	0.76*	
Mean age (SD)	9.9 (5.4)	9.2 (5.1)		
Sex (M/F)	7/4	7/4		
Mean (SD)	n=9	n=8	0.91*	1.4 (–25, +28)
predicted FEV_1%	76 (22)	74 (30)		
Mean (SD)	n=9	n=8	0.97*	0.4 (–21, +22)
predicted FVC%	81 (18)	80 (24)		
Age at first PA acquisition	n=11	n=11	0.29**	
Median (SE survival)	3.5 (2.1)	6.5 (2.4)		
Mean (SD)	n=11	n=11	0.48*	–0.23 (–0.90, +0.44)
Height z score	–0.47 (0.85)	–0.24 (0.64)		
Mean (SD)	n=11	n=11	0.44*	0.28 (–0.45, +1.00)
Weight z score	–0.01 (0.72)	–0.29 (0.90)		
Mean (SD)	n=11	n=11	0.16*	–1.2 (–6.7, +4.4)
Chrispin-Norman X-ray score	11.1 (5.9)	12.3 (6.5)		
Mean (SD)	n=6	n=6	0.22*	4.8 (–3.3, 12.9)
Rate of change of predicted FEV_1% $year^{-1}$	+0.9 (5.5)	–3.9 (6.9)		

* Unpaired t-test, ** Kaplan-Meier log-rank test.

morphism in the CF population). *In vitro* evidence supports the involvement of TLR4 in PA-induced activation of cytokines [6, 47], animal work has shown that TLR4 knockout mice do not respond to LPS [25, 28], and TLR4 polymorphisms in humans are associated with a blunted response to inhaled LPS [2]. A plausible explanation for a lack of effect, however, is that the inflammatory response to LPS in the CF airway is so overwhelming; even a blunted response results in lung damage. Work has shown that PA changes the configuration within the CF airway from a penta-acylated to a hexa-acylated LPS form [23]. Human TLR4 recognizes the change in configuration, and NF-κB activation is 100 times greater with the hexa-acylated form of LPS than with the penta-acylated form [23]. Therefore, in the CF airway, differential recognition of LPS leads to massive innate immune system activation. The consequence of this may be that, although possession of the TLR4 299G allele leads to a blunted response to LPS, the response is so overwhelming that even this 'dampened' response results in high levels of cytokine activation and subsequent parenchymal lung damage. In addition, LPS is not the only microbial signal, and other bacterial components, signaling through other receptors, may be equally important [45].

A further potential reason for the lack of effect of TLR D299G is that the cellular location of TLR4 may be important for function, as has been shown in other cells [46]. Although demonstrated to be present in airway epithelial cells, TLR4 lies on the basolateral, rather

than the epithelial surface [36]. It is suggested that TLR4 may have a limited role in epithelial responses to bacteria, and the failure of NF-κB activation in response to LPS by airway epithelial cells is cited as evidence for this lack of effect [36]. Epithelial TLR4 expression may therefore not be critical to luminal signaling (such as the presence of PA in the CF airway) and other cell types in the lung may fulfil this role. This may be especially important in CF, where TLR4 expression in the bronchial epithelium is reduced compared with control subjects [24].

As described above, D299G is not the only TLR4 polymorphism. Another TLR4 polymorphism, T399I, is described [2] that could potentially play a role in CF. However, in transfection experiments, only the D299G polymorphism was shown to interrupt LPS signaling via TLR4 [37]. Furthermore, there is strong linkage disequilibrium and cosegregation (84–99%) between the D299G and T399I polymorphisms [29, 31], suggesting that few (if any) of the wild-type group would possess the other polymorphism, suggesting that phenotypic differences are unlikely to have been underestimated.

In summary, no effect on clinical phenotype was seen for TLR4 genotype in CF in the 100 subjects we studied. Sub-analysis on cases matched for age, sex, and CFTR genotype similarly suggested no effect. Although relatively small numbers limited the study, no trends were noted, and we suggest that it is unlikely that the TLR4 D299G polymorphism alone is sufficient to play an important clinical role in CF. Further information on potential interactions with other modifier genes may, however, become apparent in the future when the results of large multi-center studies analyzing a number of candidate genes become available [30, 49].

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