Association analysis identifies *TLR7* and *TLR8* as novel risk genes in asthma and related disorders

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ABSTRACT

Background: Toll-like receptors (TLRs) are structurally and functionally related and play important roles in the innate and adaptive immune system. By genome scanning, evidence of linkage between chromosome Xp22 and asthma and related atopic disorders has previously been obtained. Xp22 harbours the *TLR7* and *TLR8* genes.

Methods: The involvement of *TLR7* and *TLR8* in the aetiology of asthma and related disorders was investigated by a family based association analysis of two independently ascertained family samples comprising 540 and 424 individuals from 135 and 100 families, respectively. Ten affected individuals from families showing evidence of linkage to Xp22 were screened for sequence variations in *TLR7* and *8*, and nine single nucleotide polymorphisms (SNPs) identified were tested for association.

Results: In both samples, significant associations were observed for single SNPs and haplotypes of both *TLR7* and *8* in all four phenotypes investigated: asthma, rhinitis, atopic dermatitis and increased specific IgE. The most significant association was seen for rs2407992 (*TLR8*) in asthma ($p = 0.00023$, sample A and B combined, recessive model). In *TLR7*, rs179008 showed the strongest association. Both rs179008 and rs2407992 are of putative functional significance, potentially affecting *TLR7* processing and *TLR8* splicing, respectively. Haplotypes comprising the major alleles of these two SNPs were overtransmitted to the affected offspring (eg, $p = 0.00012$ in asthma, combined sample, additive model).

Conclusion: The results provide strong evidence that *TLR7* and *8* may confer susceptibility to asthma and related atopic disorders and highlight these receptors as interesting targets for individualised, causally directed treatment.

Allergic disorders such as asthma, rhinitis and atopic dermatitis constitute a high incidence global health problem that affects an increasing number of individuals who are often faced with a considerable loss in quality of life. When possible, the typical countermeasure is to avoid contact with known allergens. When this is not possible, however, the only option is medical treatment, which currently aims at modulating general immunological/inflammatory responses, often at the price of adverse side effects. A more focused treatment is dependent on a more detailed knowledge of the aetiology of the diseases.

The diseases are complex and depend on multiple poorly defined environmental as well as genetic factors. The genetic component, which is poly- or oligogenic in nature, is considerable, as evidenced by several family and twin studies.[1–5]

Several genome scans have been conducted, suggesting a number of susceptibility loci for allergic disease.[6] We have obtained evidence of linkage to chromosome Xp22 for atopic phenotypes, including asthma, atopic dermatitis and rhinitis.[7–9] Xp22 harbours two members of the Toll-like receptor family (*TLR7* and *8*), which play a central role in the immune response. TLRs are key receptors among germline encoded pattern recognising receptors that play a major role in the recognition of microbial pathogens and initiation of the innate and adaptive immune response. They have emerged relatively late on the stage of identified immunological players as their role in pathogen sensing was not really appreciated until 1998, when the gene at the locus responsible for recognising lipopolysaccharide (a cell wall component of gram negative bacteria) was cloned and found to be identical to *TLR4*.[10] Since then, a plethora of microbial derived ligands, collectively known as pathogen associated molecular patterns, as well as a handful of host derived ligands, have been found to trigger TLR signalling through one or more of the 10 TLRs (TLR1–10) so far identified in humans.

TLRs are expressed in various immune cells, including antigen presenting cells and cells that are likely to experience first encounter with incoming pathogens (eg, epithelial cells). Antigen presenting cells and particularly dendritic cells are important in the initial pathogen sensing and initiation of the immune response. Dendritic cells play a critical role in the crossroad between innate and adaptive immunity by controlling signals that direct naive T helper (Th) cells to differentiate into either Th1 or Th2 cells. Whether dendritic cells promote a Th1 or Th2 state, characterised by distinct cytokine profiles, depends to a large extent on which and how TLRs are triggered, which again depends on the type of infection.[11 12] In type I allergy and atopy, the Th1/Th2 balance is skewed towards a Th2 state characterised by an IgE hyper-production, and the potential of TLRs for decisive regulation of immune responses has brought considerable focus on these receptors in the search for an aetiological understanding of allergy and atopy.[13] In the specific context of asthma and related allergies, attention has been given to TLR2 and TLR4 as receptors of peptidoglycan from gram positive bacteria and lipopolysaccharide, respectively,[14] whereas comparably little attention has been given to TLR7 and TLR8.

TLR7 and TLR8 are homologues and have TLR9 as their closest evolutionary relative.[15 16] They both have affinity towards single stranded RNA and similar signalling characteristics but differ

somewhat in tissue expression patterns.[15–19] TLR7 and TLR8 are not expressed on the cell surface but are found in endosomal compartments (like TLR3 and TLR9) and their activation requires endocytosis of the pathogens. In a study investigating TLR expression in activated monocyte-like THP-1 cells, it was reported that TLR7 and TLR8 had the strongest overall upregulation in expression in response to classic innate immunity cytokines as well as live bacteria.[20]

We here report the results from a family based association study investigating the positional and functional candidate genes *TLR7* and *8* in relation to asthma, rhinitis, atopic dermatitis and increased specific IgE (positive RAST test) in two independently ascertained samples from Denmark.

METHODS

Subjects

Two independently ascertained family samples (A and B) were investigated (table 1). Informed consent was obtained from each participant prior to inclusion in the study. Local ethics committee approval was obtained in all regions where families were recruited.

Sample A included 135 nuclear families with sib pairs who had asthma, who were enrolled from four clinical centres in eastern, western and central parts of Denmark, primarily on the basis of patient records. Criteria for inclusion were recurrent cough, wheezing and dyspnoea as well as a positive metacholine challenge measured using the methods described by Yan *et al*.[21] The affected siblings were between 15 and 45 years of age. Lung function measurements including forced expiratory volume in 1 s and forced vital capacity were performed on all subjects. Blood samples were analysed for allergen specific IgE (CAP phadiatop; Pharmacia Upjohn, Copenhagen, Denmark); a positive test was defined as IgE $\geqslant$0.35 kUA/l for at least one of the allergens. Rhinitis and atopic dermatitis phenotypes were recorded on the basis of questionnaires. Details about the sample have been reported previously.[22]

Sample B comprised 100 Danish atopic families, including a total of 424 individuals enrolled in the ITA project via four paediatric and one adult outpatient allergy clinic in western Denmark. Recruitment, examination and clinical information have been described previously.[7 23] In brief, inclusion criteria were a minimum of two atopic siblings with doctor diagnosed asthma, rhinitis or atopic dermatitis and a reported effect of appropriate medication. All subjects were examined clinically and questionnaire tested by the same doctor who diagnosed the patients according to standard criteria in consensus with a second doctor evaluating the questionnaires only. The most common diagnosis was asthma affecting 70% of the siblings (table 1). Mean age among the offspring was 10.8 years. Blood samples were taken for DNA extraction and serum measurement of specific IgE to 11 common allergens (CAP RAST FEIA; Pharmacia Upjohn); a positive test was defined as above.

Both samples had a male/female ratio not diverging significantly from random distribution.

Sequencing and genotyping

Blood samples were used for preparation of genomic DNA according to standard procedures. In order to increase the chance of identifying causal risk alleles, 10 patients from families showing evidence of linkage to Xp22 in previous studies[7–9] were selected for sequence analysis of *TLR7* and *8*. Of the 10 patients sequenced, seven were females (ie, 17 X chromosomes were analysed). The TLR genes were screened for sequence variation in the coding sequence and intron/exon boundaries as well as in the promoter region (approximately 1000 bp of the upstream region) by direct sequencing using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA). Following ethanol precipitation, the sequencing reactions were analysed on an ABI-310 sequenator (Applied Biosystems). Both strands were analysed. The single nucleotide polymorphisms (SNPs) identified were subsequently genotyped in the two family samples, using the SNaP-shot single base extension technology (Applied Biosystems). Standard PCR conditions were used for both sequencing and genotyping. Primers are available on request. The genotypes were scored independently by two experienced investigators and tested for Mendelian inheritance.

Statistical analysis

The program FBAT V.2.0.2c[24 25] was used for family based association analysis of single SNPs and haplotypes. In the haplotype analysis, p values were obtained for individual haplotypes as well as for the overall/global distribution of all haplotypes of a specific marker segment. A sliding window approach was applied analysing segments of neighbouring SNPs. Because of prior evidence of linkage observed in both samples,[7–9] the empirical variance (-e option in the FBAT program) was used in all calculations to account for genotype

Table 1 Data for the two family samples

	Asthma	Rhinitis	Atopic dermatitis	Positive RAST
Sample A (135 families)				
Families with one affected child	0	47	51	35
Families with two affected children	135	74	68	73
Total families	135	121	119	108
Total affected siblings (% of total)	270 (100)	195 (72)	187 (69)	181 (67)
Sex ratio M:F in *Phenotype* (total = 0.8)	0.8	0.6	0.7	0.8
Sample B (100 families)				
Families with one affected child	31	36	33	36
Families with two affected children	45	35	34	40
Families with three affected children	12	8	6	7
Total families	88	79	73	83
Total affected siblings (% of total)	157 (70)	130 (58)	119 (53)	137 (61)
Sex ratio M:F in *Phenotype* (total = 1.2)	1.4	1.2	1.2	1.2

Sample A: mean age of siblings 28 years, mean forced expiratory volume in 1 s 3.5 l. Sample B: mean age of siblings 10.8 years. RAST, radioallergosorbent test.

Table 2 Single nucleotide polymorphisms (SNPs) investigated

Gene (cytogenetic position)	SNP number in this study	rs Number (major/minor allele)	Position	Position in gene, subst	MAF	
					A	B
TLR7 (Xp22)	1	rs179008 (A/T)	12813580	Ex3, Q11>L	T: 0.24	0.21
	2	rs5743781 (C/T)*	12814891	Ex3, A448>V	T: 0.012	0.003
	3	rs864058 (C/T)	12815951	Ex3, syn	T: 0.074	0.11
TLR8 (Xp22)	4	rs5741883 (C/T)	12834142	Promoter	T: 0.25	0.20
	5	rs3764879 (C/G)	12834618	Promoter	G: 0.24	0.24
	6	rs3764880 (A/G)	12834747	Ex1, M1>V	G: 0.21	0.22
	7	rs5744077 (A/G)*	12847108	Ex2, M10>V	G: 0.002	0
	8	rs2159377 (C/T)	12847434	Ex2, syn	T: 0.19	0.15
	9	rs2407992 (G/C)	12849033	Ex2, syn	C: 0.42	0.38

Position according to UCSC (http://genome.ucsc.edu/, Mar. 06 assembly).
*SNPs included from dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).
MAF, minor allele frequency; TLR, Toll-like receptor.

correlation among affected sib pairs that result from linkage, thus testing the null hypothesis "linkage but no association". The data were analysed using an additive model, which does not assume any specific mode of inheritance and is in general the most sensitive test.[24] The additive model is equivalent to the classic transmission disequilibrium test.[26] However, power calculations using PBAT[27 28] revealed that a recessive model would have the strongest power in our samples. For example, analysis of SNP1 in sample A would have a power of 0.899 under the additive model whereas the recessive model resulted in a power of 1 (assuming a significance level of 0.05, a population prevalence of the disease of 0.1 and a genetic attributable fraction of 0.2). Therefore, we also analysed the data assuming a recessive mode of inheritance. As the majority of tests performed were not independent, the p values reported are not corrected for multiple testing. However, the most significant single SNP and haplotype results survive a Bonferroni correction.

The degree of linkage disequilibrium (LD) between the SNPs in our samples was calculated using the program Haploview, which creates a maximal set of unrelated individuals, using family data only for parent/offspring phasing.[29] This program was also used to assess the LD pattern of the X chromosomal region of interest in the CEPH population (CEU) using SNP genotype data from the HapMap project (http://www.hapmap.org/).

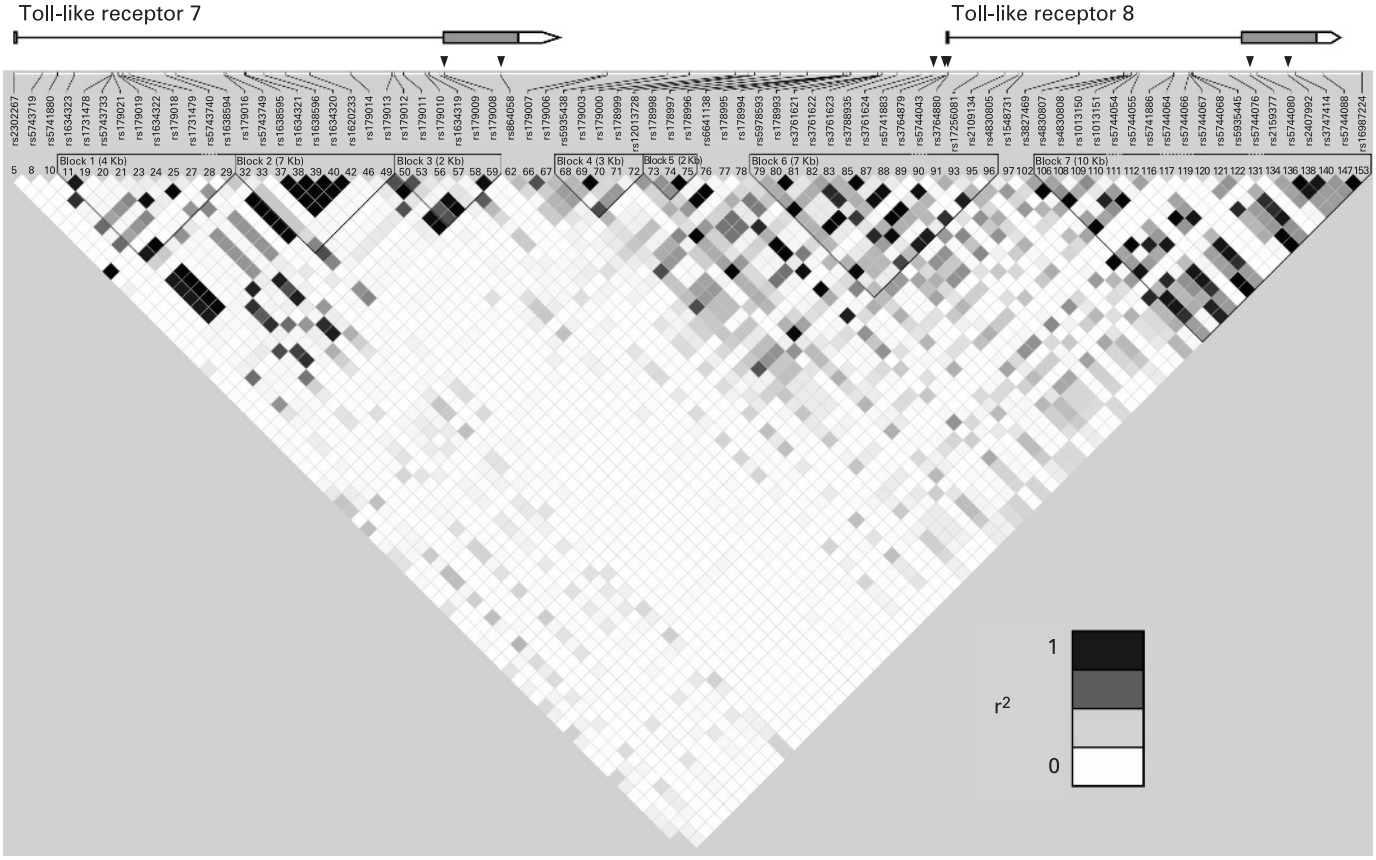

Figure 1 Linkage disequilibrium (LD) in the Toll-like receptor (*TLR*)*7–TLR8* region based on the CEU population (HapMap data) represented as r^2. LD blocks are calculated from D′.[34] Arrows mark positions of *TLR7* and *TLR8* single nucleotide polymorphisms (SNPs) used in this study (rs5743781 and rs5744077 are not included as they are not polymorphic in the CEU population).

Table 3 Linkage disequilibrium (r^2) between *TLR7* and *TLR8* SNPs for sample A and B

Gene	SNP		S1	S2	S3	S4	S5	S6	S7	S8	S9
TLR7	rs179008	(S1)		•	0.03	•	•	•	•	0.01	0.02
TLR7	rs5743781	(S2)	•		•	•	•	•	•	•	•
TLR7	rs864058	(S3)	•	•		•	•	0.01	•	0.03	0.02
TLR8	rs5741883	(S4)	0.05	•	0.01		0.06	0.04	•	0.01	0.03
TLR8	rs3764879	(S5)	•	•	0.01	0.08		0.7	•	0.3	0.1
TLR8	rs3764880	(S6)	•	•	•	0.07	0.9		•	0.2	0.08
TLR8	rs5744077	(S7)	•	•	•	•	•	•		•	•
TLR8	rs2159377	(S8)	0.03	•	0.03	0.01	0.4	0.3	•		0.2
TLR8	rs2407992	(S9)	•	•	0.01	0.01	0.2	0.2	•	0.3	

Sample A in top right corner of table, sample B in bottom left corner.
•$r^2<0.01$.
SNP, single nucleotide polymorphism; TLR, Toll-like receptor.

In silico analysis

The program MatInspector (www.genomatix.de)[30 31] was used to analyse potential transcription factor binding sites around SNPs located in the promoter regions. To assess the possible effect of synonymous SNPs, we used FastSNP.[32] The SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/)[33] was used to predict and analyse changes in the signal sequences for SNPs located in the N terminal end of the protein.

RESULTS

Sequencing of 10 individuals from families showing evidence of linkage to Xp22 revealed seven SNPs in the *TLR7* and *TLR8* genes (table 2). In addition, two non-synonymous SNPs from the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) were added to the study. However, these SNPs were very rare in our samples (table 2). For all SNPs, the genotype distribution was found to be in Hardy–Weinberg equilibrium among the parents in both samples.

Linkage disequilibrium

The nine SNPs investigated spanned a 35 kb region containing the *TLR7* and *TLR8* genes located head to tail (fig 1). A relatively low degree of LD was found except for one pair (SNP5–SNP6 in the *TLR8* gene) displaying r^2 values of 0.7–0.9 (table 3), which is consistent with the low LD seen across the region in the CEU population (fig 1).

Association analysis

Association with asthma and related phenotypes was found for SNPs and haplotypes in both independent samples. SNP1 and 9 corresponding to rs179008 (*TLR7*) and rs2407992 (*TLR8*), respectively, showed the strongest and most consistent association (table 4 and supplementary table 1 online), both showing association with all tested phenotypes. In each sample, significant associations were observed for SNP9 (*TLR8*) in asthma whereas SNP1 showed significant associations only in sample B (and merely a trend towards association in sample A). Overall, the most significant results were found for SNP9 (*TLR8*) in asthma ($p = 0.00023$) and atopic dermatitis ($p = 0.00066$) in the two samples combined under a recessive model.

Because *TLR7* and *TLR8* are located on the X chromosome, we analysed boys and girls separately as well as combined. This revealed some differences in the association pattern. Association

Table 4 Association analysis of *TLR7* and *TLR8* SNPs

	Asthma		Rhinitis		Atopic dermatitis		Positive RAST	
	Additive	**Recessive**	**Additive**	**Recessive**	**Additive**	**Recessive**	**Additive**	**Recessive**
Sample A								
SNP1 (*TLR7*)							0.066	
A^{OT}								
SNP9 (*TLR8*)	0.054	**0.047**	0.074	**0.019**		0.058		
G^{OT}	**0.018 (G)**	**0.0071 (G)**		**0.016 (G)**		**0.046 (G)**		**0.025 (G)**
Sample B								
SNP1 (*TLR7*)	**0.012 (B)**	0.056	**0.013**	**0.0071**			**0.027**	**0.0090**
A^{OT}		**0.012 (B)**	**0.0054 (B)**	**0.0054 (B)**	**0.020 (B)**	**0.020 (B)**	**0.011 (B)**	**0.011 (B)**
SNP9 (*TLR8*)	**0.00069**	**0.00053**			0.066	0.052	0.083	0.061
G^{OT}	**0.0035 (B)**	**0.0035 (B)**						
	0.046 (G)	0.059 **(G)**						
Sample A+B								
SNP1 (*TLR7*)	0.069	**0.018**	**0.025**	**0.0053**		0.074	**0.0047**	**0.0044**
A^{OT}	**0.0092 (B)**	**0.0092 (B)**	**0.0039 (B)**	**0.0039 (B)**	**0.024 (B)**	**0.024 (B)**	**0.0052 (B)**	**0.0052 (B)**
SNP9 (*TLR8*)	**0.00025**	**0.00023**	**0.033**	**0.012**	**0.021**	**0.00066**	0.063	**0.014**
G^{OT}	**0.035 (B)**	**0.035 (B)**		**0.037 (G)**	0.069 **(G)**	**0.016 (G)**		**0.0077 (G)**
	0.0020 (G)	**0.0010 (G)**						

Only p values <0.1 are included in the table. p Values $\leqslant 0.05$ are indicated in bold type.
B and G, boys and girls, respectively (values with no letter represent the total); OT, overtransmitted allele; RAST, radioallergosorbent test; SNP, single nucleotide polymorphism; TLR, Toll-like receptor.

with SNP1 was strongest in boys while association with SNP9 seemed more or less the same in boys and girls, although the girls produced the strongest signal in sample A. Evidence of association was also seen for rs5741883 (SNP4 in *TLR8*) among girls with rhinitis in sample A ($p = 0.033$, recessive).

The haplotype analysis assessing adjacent SNPs in a sliding window fashion revealed several significant results some of which are shown in supplementary table 2A and 2B (available online). Overall, the results supported the single SNP analysis. Moreover, significant associations were seen in sample A between *TLR7* and increased specific IgE (RAST positive) that were not revealed in the single SNP analysis, thereby providing *TLR7* association in both samples for this phenotype. Likewise, significant associations were seen in both samples for identical individual haplotypes encompassing SNPs from both *TLR7* and *8* (eg, SNP1–4 haplotypes in rhinitis and positive RAST). Specific haplotype analysis of SNP1 and 9 showed association in all phenotypes. The haplotype comprising the two major alleles (TG) was overtransmitted, most significantly in asthma ($p = 0.00012$, combined sample, additive model).

In conclusion, replication of allelic association for SNPs (*TLR8*) or individual haplotypes (*TLR7*/8) was obtained for all four phenotypes investigated in the two samples.

In silico analyses

SNP1 in *TLR7* (rs179008) is a non-synonymous change, altering a Gln residue to a Leu residue in the signal peptide sequence of *TLR7* at amino acid position 11. SignalP 3.0 identified distinct N, H and C regions along the signal sequence (H for hydrophobic) and the most probable cleavage site between residue 26 and 27. According to this prediction, the Gln 11 constitutes the last amino acid in the N region within the signal sequence. Changing Gln11 to Leu shortens the N region and extends the H region, indicating that this substitution polymorphism may affect the processing of TLR7.

SNP4 in *TLR8* (rs5741883) is located in the promoter region where it affects potential transcription factor binding sites according to the MatInspector program. In the presence of the C allele, a binding sequence for the aryl hydrocarbon receptor (AhR)/AhR nuclear translocator (ARNT) heterodimer is formed. The AhR is implicated in mediating responses to various xenobiotics, such as dioxin and polychlorinated biphenyls, which have been reported to alter the cellular and humoral immune responses.[35] In the presence of the T allele, a binding sequence for the Atp1a1 regulatory element binding factor 6 is formed. This transcription factor has been shown to regulate the expression of interleukin 2,[36] which has recently been associated with allergic diseases and atopy.[37]

The synonymous *TLR8* SNP9 (rs2407992) was analysed using FastSNP. According to this, there is a medium to high risk of rs2407992 affecting the splicing by abolishing a splice enhancer domain.

DISCUSSION

In this study, we have examined the possible role of *TLR7* and *TLR8* on the X chromosome in the aetiology of asthma and related disorders by conducting a family based association study of two Danish samples involving 540 and 424 individuals, respectively. In the two independently ascertained family samples, replicated association was obtained for SNPs or haplotypes of *TLR7* and *TLR8*, suggesting these genes as novel disease genes for asthma and related disorders.

The general pattern of allelic and haplotypic transmission distortion was the same for the two samples and the four phenotypes investigated. However, some difference regarding the degree/significance of the transmission distortions was evident (table 4 and supplementary table 2A, B online). This could be because of sample heterogeneity originating from demographic and clinical differences between sample A and B. The siblings in sample A were older than 15 years whereas the probands in sample B were primarily children. Other inclusion criteria and diagnostics were not completely the same either. Thus even though the diagnoses are the same, the susceptibility alleles might not be of equal significance in the two samples.

Furthermore, some gender differences were observed. In particular, SNP1 and *TLR7* haplotypes showed most significant association in boys, suggesting a predominantly recessive effect of these variants.

The region harbouring *TLR7* and *8* is characterised by a very low degree of LD (fig 1, table 3), indicating that the associations observed represent separate independent signals in *TLR7* and *8*, respectively. For instance, the two SNPs showing the most significant results, SNP1 (*TLR7*) and SNP9 (*TLR8*), display an intermarker r^2 of around 0.01. Furthermore, LD analysis of data provided by the HapMap project shows that there is practically no LD between the *TLR7* and *8* genes and flanking genes, making the possibility that the signals observed are caused by LD to neighbouring disease genes highly unlikely.

SNP1 (rs179008, *TLR7)* is non-synonymous changing a Gln to a Leu in the signal sequence, thereby extending the H region and shortening the N region, as mentioned. In general, there is a high degree of degeneracy in endoplasmic reticulum (ER) signal sequences, which at first glance may reflect a high versatility of the translocation and ER import machinery. However, a number of studies suggest that correct processing of proteins at the ER membrane depends on protein specific sequence specificity within the signal sequences. Thus mutation of the signal sequence has been shown to affect secretion and translocation efficiencies,[38 39] post translational processing events such as glycosylation[38] and even membrane integration and topology.[40 41] Thus even though our theoretical knowledge is not yet sufficient to accurately predict the consequences of the rs179008 variation, it is likely that it somehow affects the processing of *TLR7* at the ER membrane.

Likewise, the in silico analyses suggested functional impacts of SNP4 and SNP9 on *TLR8* expression and splicing, respectively. These results support the causal relationship and should be scrutinised experimentally. Alternatively, the SNPs might be LD proxies for causal SNPs that were not detected in the sequence screening. However, analysing HapMap SNPs, no (common) SNPs in tight LD with the associated SNPs of the present study seem to be of likely functional significance (data not shown).

Interestingly, two recent studies have found rs179008 (SNP1, *TLR7)* associated with age related macular degeneration and chronic hepatitis C virus infection, respectively, thus indicating that this variant may be involved in susceptibility to a broad range of inflammatory phenotypes.[42 43] Furthermore, an SNP in intron 1 of *TLR7* has been reported to protect against advanced inflammation and fibrosis in male patients with chronic HCV infection.[44]

Our results strongly suggest that *TLR7* and *TLR8* are novel risk genes in asthma and related atopic disorders. Several non-genetic lines of evidence support the involvement of these two TLRs in immune related diseases and suggest these two TLRs as potential drug targets. Of particular interest, it has recently

been shown in murine models that the synthetic TLR7/TLR8 ligand resiquimod (R-848, S-28463) is effective in preventing the development of the asthmatic phenotype as well as inhibiting an inflammatory reaction in response to allergens when the asthmatic phenotype has been established.[45–47] Considering the results of the present study, treatment with TLR7/8 ligands should be causally directed and holds promise for genetically based individualised treatment of these common disorders.

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REFERENCES

1. **Duffy DL,** Martin NG, Battistutta D, *et al*. Genetics of asthma and hay fever in Australian twins. *Am Rev Respir Dis* 1990;**142**:1351–8.
2. **Larsen FS,** Holm NV, Henningsen K. Atopic dermatitis. A genetic-epidemiologic study in a population-based twin sample. *J Am Acad Dermatol* 1986;**15**:487–94.
3. **Hanson B,** McGue M, Roitman-Johnson B, *et al*. Atopic disease and immunoglobulin E in twins reared apart and together. *Am J Hum Genet* 1991;**48**:873–9.
4. **Palmer LJ,** Burton PR, James AL, *et al*. Familial aggregation and heritability of asthma-associated quantitative traits in a population-based sample of nuclear families. *Eur J Hum Genet* 2000;**8**:853–60.
5. **Skadhauge LR,** Christensen K, Kyvik KO, *et al*. Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. *Eur Respir J* 1999;**13**:8–14.
6. **Blumenthal MN.** The role of genetics in the development of asthma and atopy. *Curr Opin Allergy Clin Immunol* 2005;**5**:141–5.
7. **Haagerup A,** Borglum AD, Binderup HG, *et al*. Fine-scale mapping of type I allergy candidate loci suggests central susceptibility genes on chromosomes 3q, 4q and Xp. *Allergy* 2004;**59**:88–94.
8. **Haagerup A,** Bjerke T, Schiotz PO, *et al*. Atopic dermatitis—a total genome-scan for susceptibility genes. *Acta Derm Venereol* 2004;**84**:346–52.
9. **Brasch-Andersen C,** Vestbo J, Haagerup A, *et al*. Replication study support suggestive evidence for linkage to chromosome Xp using 136 sib pair families with asthma. *Proc Am Thor Soc* 2005;**2**:A620.
10. **Poltorak A,** He X, Smirnova I, *et al*. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 1998;**282**:2085–8.
11. **Takeda K,** Akira S. Toll-like receptors in innate immunity. *Int Immunol* 2005;**17**:1–14.
12. **Iwasaki A,** Medzhitov R. Toll-like receptor control of the adaptive immune responses. *Nat Immunol* 2004;**5**:987–95.
13. **Yang IA,** Fong KM, Holgate ST, *et al*. The role of Toll-like receptors and related receptors of the innate immune system in asthma. *Curr Opin Allergy Clin Immunol* 2006;**6**:23–8.
14. **von Mutius E.** Asthma and allergies in rural areas of Europe. *Proc Am Thorac Soc* 2007;**4**:212–16.
15. **Chuang TH,** Ulevitch RJ. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw* 2000;**11**:372–8.
16. **Du X,** Poltorak A, Wei Y, *et al*. Three novel mammalian toll-like receptors: gene structure, expression, and evolution. *Eur Cytokine Netw* 2000;**11**:362–71.
17. **Heil F,** Hemmi H, Hochrein H, *et al*. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004;**303**:1526–9.
18. **Lund JM,** Alexopoulou L, Sato A, *et al*. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A* 2004;**101**:5598–603.
19. **Diebold SS,** Kaisho T, Hemmi H, *et al*. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004;**303**:1529–31.
20. **Zarember KA,** Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002;**168**:554–61.
21. **Yan K,** Salome C, Woolcock AJ. Rapid method for measurement of bronchial responsiveness. *Thorax* 1983;**38**:760–5.
22. **Vestbo J,** Thomas W. Asthma and genes in a Danish population: outline of an on-going study. The ASTHMAGEN study group. *Eur Respir Rev* 2000;**10**:396–9.
23. **Haagerup A,** Bjerke T, Schoitz PO, *et al*. Allergic rhinitis—a total genome-scan for susceptibility genes suggests a locus on chromosome 4q24–q27. *Eur J Hum Genet* 2001;**9**:945–52.
24. **Horvath S,** Xu X, Laird NM. The family based association test method: strategies for studying general genotype–phenotype associations. *Eur J Hum Genet* 2001;**9**:301–6.
25. **Horvath S,** Xu X, Lake SL, *et al*. Family-based tests for associating haplotypes with general phenotype data: application to asthma genetics. *Genet Epidemiol* 2004;**26**:61–9.
26. **Spielman RS,** Ewens WJ. The TDT and other family-based tests for linkage disequilibrium and association. *Am J Hum Genet* 1996;**59**:983–9.
27. **Lange C,** Lyon H, DeMeo D, *et al*. A new powerful non-parametric two-stage approach for testing multiple phenotypes in family-based association studies. *Hum Hered* 2003;**56**:10–17.
28. **Lange C,** DeMeo D, Silverman EK, *et al*. Using the noninformative families in family-based association tests: a powerful new testing strategy. *Am J Hum Genet* 2003;**73**:801–11.
29. **Barrett JC,** Fry B, Maller J, *et al*. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005;**21**:263–5.
30. **Quandt K,** Frech K, Karas H, *et al*. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995;**23**:4878–84.
31. **Cartharius K,** Frech K, Grote K, *et al*. MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* 2005;**21**:2933–42.
32. **Yuan HY,** Chiou JJ, Tseng WH, *et al*. FASTSNP: an always up-to-date and extendable service for SNP function analysis and prioritization. *Nucleic Acids Res* 2006;**34**:W635–41.
33. **Bendtsen JD,** Nielsen H, von Heijne G, *et al*. Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 2004;**340**:783–95.
34. **Gabriel SB,** Schaffner SF, Nguyen H, *et al*. The structure of haplotype blocks in the human genome. *Science* 2002;**296**:2225–9.
35. **Blanco GA,** Cooper EL. Immune systems, geographic information systems (GIS), environment and health impacts. *J Toxicol Environ Health B Crit Rev* 2004;**7**:465–80.
36. **Ikeda K,** Kawakami K. DNA binding through distinct domains of zinc-finger-homeodomain protein AREB6 has different effects on gene transcription. *Eur J Biochem* 1995;**233**:73–82.
37. **Christensen U,** Haagerup A, Binderup HG, *et al*. Family based association analysis of the IL2 and IL15 genes in allergic disorders. *Eur J Hum Genet* 2006;**14**:227–35.
38. **Rutkowski DT,** Ott CM, Polansky JR, *et al*. Signal sequences initiate the pathway of maturation in the endoplasmic reticulum lumen. *J Biol Chem* 2003;**278**:30365–72.
39. **Holden P,** Keene DR, Lunstrum GP, *et al*. Secretion of cartilage oligomeric matrix protein is affected by the signal peptide. *J Biol Chem* 2005;**280**:17172–9.
40. **Rutkowski DT,** Lingappa VR, Hegde RS. Substrate-specific regulation of the ribosome- translocon junction by N-terminal signal sequences. *Proc Natl Acad Sci U S A* 2001;**98**:7823–8.
41. **Ott CM,** Lingappa VR. Signal sequences influence membrane integration of the prion protein. *Biochemistry* 2004;**43**:11973–82.
42. **Schott E,** Witt H, Neumann K, *et al*. Association of TLR7 single nucleotide polymorphisms with chronic HCV-infection and response to interferon-a-based therapy. *J Viral Hepat* 2008;**15**:71–8.
43. **Edwards AO,** Chen D, Fridley BL, *et al*. Toll-like receptor polymorphisms and age-related macular degeneration. *Invest Ophthalmol Vis Sci* 2008;**49**:1652–9.
44. **Schott E,** Witt H, Neumann K, *et al*. A Toll-like receptor 7 single nucleotide polymorphism protects from advanced inflammation and fibrosis in male patients with chronic HCV-infection. *J Hepatol* 2007;**47**:203–11.
45. **Moisan J,** Camateros P, Thuraisingam T, *et al*. TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway. *Am J Physiol Lung Cell Mol Physiol* 2006;**290**:L987–95.
46. **Camateros P,** Tamaoka M, Hassan M, *et al*. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am J Respir Crit Care Med* 2007;**175**:1241–9.
47. **Sel S,** Wegmann M, Sel S, *et al*. Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. *J Immunol* 2007;**178**:7805–13.