

EDITORIAL

Toll-like receptor polymorphisms and allergic disease: interpreting the evidence from genetic studies

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Gene–environment interaction is likely to impact on the development of allergic disease. Thus, genetic approaches have been applied to the study of multifactorial, allergic conditions such as asthma, atopy, allergic rhinitis and atopic dermatitis, as shown in Fig. 1. The Toll-like receptors (TLRs) have emerged as a key mediator of the innate immune response. TLRs are pattern-recognition receptors that bind bacterial ligands (and endogenous ligands) then act via signalling pathways to mediate innate immune responses, with bridging to adaptive immunity. Therefore, studies of polymorphisms in TLR genes have the potential to advance our understanding of the response to endotoxin, the so-called ‘hygiene hypothesis’ and the pathogenesis of allergic diseases.

The study by Noguchi et al. [1] in this month’s issue is a well-designed candidate gene study of TLR polymorphisms. In a Japanese asthmatic population, these investigators screened the *TLR2*, *TLR3*, *TLR4* and *TLR9* genes, and detected certain polymorphisms occurring at a frequency of 4% or higher in the general population. Using family-based association studies and case-control analyses, they detected no associations between either single polymorphisms or combinations of polymorphisms (haplotypes), and either the risk of developing asthma or elevated total serum IgE levels. A polymorphism in the promoter region of *TLR2*, consisting of either an insertion or deletion of DNA sequence, was shown in *in vitro* studies to alter the expression of the *TLR2* gene [1]. This systematic study of candidate genes in innate immunity, in relation to specific allergic phenotypes in this particular population, provides us with an opportunity to review the interpretation of candidate gene studies in allergic diseases.

Critical appraisal of genetic association studies has been described in detail elsewhere [2–5]. These reviewers emphasize that genetic studies should adhere to rigorous epidemiological standards, as well as incorporating study designs that adequately assess the variation present in the candidate genes of interest. A number of attributes of such ‘genetic epidemiology’ studies need to be considered when interpreting the relevance of each new study. This study used both family and case-control analysis [1]. The use of family-based cohorts for genetic association analysis has also been previously employed in studies of TLR polymorphisms and asthma, particularly *TLR4* [6, 7]. By considering the trio of parents and affected child, family-based studies remove the risk of confounding by ethnicity or undetected population admixture [2]. This confounding by population stratification is an association between an allele with subgroups of the population. However, family-based studies are less powerful than case-control studies [8], and more recently, population stratification has been shown to be less of concern than first thought [9].

Although there are also concerns about how to perform and interpret genetic case-control studies [2, 5, 10], such studies are more feasible and powerful, and have been used to examine *TLR4* polymorphisms in asthma [7, 11]. The selection of cases and matched controls from the same population is important, and subjects should be accurately phenotyped so as to avoid misclassification. The use of anonymous controls in the study by Noguchi and colleagues presumes that these subjects are free of the disease of interest; any misclassification would tend to underestimate a true association.

Prioritization of polymorphisms for study remains a critical issue [5, 12]. In Mendelian, single gene disorders, mutations occurring in coding regions have most commonly been discovered as the cause of disease, especially mutations that lead to premature truncation of the protein, or radically alter the expressed amino acid in the protein. Coding region polymorphisms have been the focus of genetic studies of multifactorial disease, but polymorphisms in non-protein coding regions may also be biologically important (e.g. promoter, 3′ untranslated region, intronic regions near intron/exon boundaries where splicing occurs). Noguchi and colleagues sequenced the coding and promoter regions to identify common polymorphisms in 32 asthmatics from their population. Similarly, Raby et al. [6] sequenced 90 asthmatics, to identify novel polymorphisms in the promoter, coding and adjacent intronic regions of the *TLR4* gene, and Lazarus et al. [13] sequenced exons and untranslated regions of the *TLR9* gene.

Combinations of polymorphisms (haplotypes) should be considered, in order to more accurately define the causal polymorphism [5]. For example, a single polymorphism at a certain location in the gene sequence may be in linkage disequilibrium with nearby polymorphisms; i.e. the gene form or allele at this locus is more commonly inherited with other adjacent polymorphisms. This means that the association with a phenotype may not actually be due to the effect of this polymorphism, but could be the effect of nearby polymorphisms that are in linkage disequilibrium with the first allele studied. Noguchi and colleagues ascertained the common haplotypes within their population, and tested for association with phenotypes.

The choice of candidate gene itself requires knowledge of the biological relevance of the molecule. TLRs bind bacterial ligands, and thus would be considered to be of relevance to asthma, especially in relation to early life exposure to bacteria having a potentially protective effect against the development of atopy and asthma (the ‘hygiene hypothesis’). *TLR4* has been the TLR most commonly studied in asthma and atopy so far. In addition, *CD14* polymorphisms have been examined in relation to atopy, IgE levels and asthma in a number of

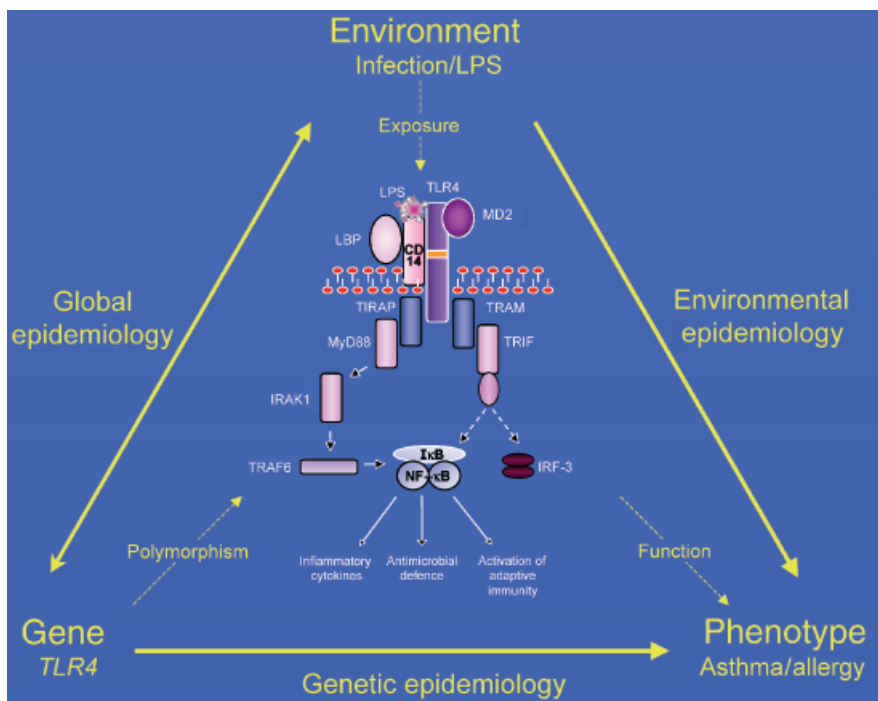


Fig. 1. Approach to understanding gene-environment interaction in asthma and allergy. To understand the biological importance of Toll-like receptors in asthma and allergy, studies should examine interactions between gene and phenotype (genetic epidemiology), environment and phenotype (environmental epidemiology), and all three factors (global epidemiology). Supporting evidence from these studies would help to identify causal pathways that lead to the development of asthma and allergy, in genetically susceptible individuals exposed to environmental risk factors.

studies [14–17]. CD14 binds LPS but has no transmembrane domain and requires TLR4 for signal transduction. Other cofactors and adapter proteins are also critical to the function of the LPS receptor (e.g. MD2, LPS-binding protein). Hence the possibility of gene-gene interaction should be explored within a pathway, to systematically assess the effects of genetic variation on activation or suppression of the pathway.

The phenotypes studied should be biologically and clinically relevant. In the study by Noguchi and colleagues, the presence or absence of asthma was the main outcome, and total serum IgE was also considered; however, no associations were observed. In a candidate gene study that we have performed in UK families [7], other asthma or atopy-related phenotypes were also measured. We found no association of the *TLR4* Asp299Gly polymorphism with asthma, total serum IgE or asthma severity score, but we observed a statistically significant increase in atopy severity score with this polymorphism [7]. The atopy severity score was derived by principal component analysis from the size and extent of positive skin prick tests and specific IgE levels to common aeroallergens. Thus this composite score appeared to be more sensitive to small to moderate genetic effects.

In any negative association study, the power to detect a pre-defined difference should be estimated. Noguchi and colleagues show that they had adequate power for their family-based analysis (137 families, 466 members), although they did not provide similar estimates for the relatively small case-control study they performed as replication (133 asthmatics, 190 controls). Power calculations depend on various assumptions, and often multiple comparisons are not taken into account. There are now power calculations available that also consider gene-gene and gene-environment interaction [18,19]. Large sample sizes would ensure that important effects are detected, and would reduce bias from misclassification of cases or genotypes. Adequately powered studies are

key to successful replication of genetic association studies. When no associations are found, it is possible that true, unidentified associations exist between the polymorphisms and other unmeasured phenotypes, which are intermediate between the gene and the final development of disease, e.g. TLR density, or downstream pro-inflammatory response to bacteria [20].

Replication adds weight to the consistency of the initial findings. Meta-analyses of results should be performed where possible, in order to pool sample sizes and increase the power to detect differences, provided that the individual studies meet pre-defined criteria and quality. Meta-analyses of gene associations for multifactorial disease have shown that perhaps one-third of initial positive findings are replicated; therefore a substantial number of false positive studies are reported, which may be reduced through larger studies and other genetic epidemiological considerations [21]. Similarly a negative study such as this one by Noguchi and colleagues may also fail to be replicated, for various reasons including different phenotypes, inadequate power, ethnic differences or differences in environmental exposures between otherwise identical cohorts. Noguchi and colleagues did examine for any potential relationships between different racial groups. To avoid publication bias, it would be useful to have an on-line repository of genetic association studies listing all positive and negative results, such as the Asthma and Allergy Gene Database [22]. Replication was also performed in the study by Raby et al. [6] for the negative associations with *TLR4* polymorphisms in two different cohorts of families with asthma.

The study by Noguchi and colleagues adhered to the principle of 'common variant, common disease' by examining only polymorphisms with a frequency of 4% or higher. It is possible that rarer polymorphisms or mutations may influence the susceptibility or progression to disease. The *TLR4*

Asp299Gly polymorphism is present in between 3% and 7% of Caucasian populations, but the frequency in this Japanese population was below 4% and therefore this polymorphism was not genotyped. In a study of meningococcal disease, rare coding mutations other than the *TLR4* Gly299 allele were collectively found more commonly in patients with meningococcal disease [23].

Demonstrating the functionality of polymorphisms remains a challenge. Few functional studies have been performed in relation to the *TLR4* gene. The primary study performed by Arbour et al. showed that the *TLR4* Asp299Gly (and Thr399Ile) polymorphisms were associated with hypo-responsiveness to inhaled endotoxin challenge, decreased density of TLR4 in airway epithelium and decreased production of inflammatory cytokines in response to endotoxin [20]. In another study, carriers of the *TLR4* Gly299 allele had reduced circulating levels of acute-phase reactants and cytokines in the blood [24]. However, monocytes from subjects carrying the *TLR4* polymorphisms showed no difference in response to LPS *in vitro*, possibly reflecting tissue specificity, differences in LPS used or other factors relating to the microenvironment (e.g. circulating LPS-binding protein, serum CD14 and TLR4 expression at the time of the experiment) [25]. Other studies have demonstrated associations of the *TLR4* polymorphisms with infection [26, 27], premature births [28], transplant rejection [29, 30] and vascular disease [24, 31]. However, not all studies have found associations of disease with these *TLR4* polymorphisms [32–35]. Noguchi and colleagues elected to study in detail an insertion/deletion polymorphism in the promoter region of *TLR2* that they identified. Transfection studies with either the sequence inserted or deleted showed a functional difference in the activity of the promoter towards controlling gene expression. This suggests that, although there was no association with the particular phenotypes studied in this population, it may be that this polymorphism could have functional effects in other settings, e.g. response to Gram-positive bacteria by TLR2. Associations are just that; causality depends on a whole spectrum of evidence that points towards a consistent mechanism [4]. Demonstrating that a certain polymorphism leads to functional effects, especially of relevance to the intermediate steps leading to disease, adds weight to an epidemiological association.

The biology of the TLRs appears to be of central importance in the host response to the environment, and therefore identifying genetic variants that enhance or perturb these systems is an important goal. Genetic studies are likely to contribute to understanding the pathogenesis of allergic disease, identify individuals at risk of allergic disease and worsened severity, and lead to the development of novel therapeutic strategies. The recent report of gene-environment interaction in relation to the *TLR4* gene and endotoxin exposure, by Werner et al. [11], shows the way forward in this field. They demonstrated that subjects with the usual (wild-type) *TLR4* alleles had an increased prevalence of asthma with elevated endotoxin levels in house dust, whereas those individuals who carried the hypo-responsive *TLR4* alleles had an attenuated risk of asthma with increasing endotoxin exposure. Thus, in addition to rigorous study design (adequate power, relevant genes in a pathway, haplotypes of polymorphisms within each gene, relevant phenotypes), genetic studies should ideally include environmental measures

to detect gene-environment interaction, as performed for *TLR4* [11] and *CD14* [17], and intermediate phenotypes to demonstrate the functionality of the polymorphisms in their population.

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