

LETTER TO THE EDITOR

ECZEMA AND FOOD ALLERGY IN AN ITALIAN PEDIATRIC COHORT: NO ASSOCIATION WITH TLR-2 AND TLR-4 POLYMORPHISMS

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Recent studies have indicated that Toll-like receptor polymorphisms or their impaired signalling, specifically TLR-2 and TLR-4, were correlated with a higher risk for allergy. The purpose of this study is to evaluate the associations of TLR-2 and TLR-4 single nucleotide polymorphisms (SNP) and atopic traits in a cohort of 159 Italian allergic children (102 affected by eczema and 57 by IgE-mediated food allergy) and 147 healthy controls recruited in Rome, Italy. DNA was isolated from the peripheral blood and TLR-2 R753Q/TLR-4 D299G polymorphisms were determined by TaqMan MGB probes using Real-Time PCR technique. In the control group, the TLR-2 polymorphism R753Q had a prevalence of 2.5% while the frequency of the TLR-4 D299G was 12%. None of the 159 allergic patients showed the R753Q SNP. By contrast, 7/57 patients with food allergy (12%) and 6/102 subjects with eczema (6%) carried the TLR-4 mutation. In our cohort, no evidence of correlation between TLR-2 or TLR-4 polymorphism and eczema and food allergy incidence and/or severity was found. Further studies are needed to clarify the possible role of TLR-2 and TLR-4 polymorphism in allergic disease, in Italian children.

In recent years, many studies have greatly increased our understanding of the immunologic mechanisms involved in the pathogenesis of allergic disease (antigen presentation, effectors and regulatory T cells, histamine, G protein-coupled receptors, IgE, airway remodelling). The hygiene hypothesis, that is still a complex question, suggests that lack of microbial stimulation in early infancy may lead to allergy. Actually, it was observed that infants colonised in the first week(s) of life with *Staphylococcus aureus* have a lower risk of developing food allergies (1). Reduced early microbial exposure has become a leading cause to explain the increasing rate of allergic disease, thus

generating intense interest in the interaction between the microbial environment and key microbial recognition pathways, namely Toll-like receptor (TLR) function. TLRs were found on many cells involved in immediate host defence, including neutrophils, natural killer cells, antigen-presenting cells, which play a critical role in adaptive T-cell responses, and also CD4⁺ CD25⁺ T cells, which are important in the prevention of hyper-reactive immune responses. It has been suggested that exposure to specific infections drives the maturing immune system in infancy/childhood towards a protective Th1 phenotype rather than the Th2 cell phenotype that is associated with atopy. Therefore,

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it is possible that a defective expression or genetic alterations of TLR receptor might contribute to an unbalanced level of Th2 and Th1 immune response. Several studies have indicated that polymorphisms or the impaired signalling of TLRs were correlated with a increased risk for allergy in adults and/or children. TLR-4 D299G and TLR-2 R753Q polymorphisms have been associated with asthma and/or atopic eczema (2-4), and their defective signalling led to allergic sensitization to food protein in mice (5-6). Newborns with a high risk of allergy have also been noted to have altered generation of putative regulatory T-cell populations after LPS stimulation, presumably through TLR-4 pathways. Also, TLR-2 mutation has been associated with a higher risk of asthma in European children (2), with atopic dermatitis having severe phenotype (3). By contrast, recent papers demonstrated that polymorphisms in TLR-2 and TLR-4 were not associated with asthma or eczema (7-8).

The purpose of this study is to evaluate the association of TLR-2 R753Q (rs5743708) and TLR-4 D299G (rs4986790) single nucleotide polymorphisms (SNP) and atopic traits in a cohort of 159 Italian allergic children.

MATERIALS AND METHODS

Patients

One hundred and fifty-nine Italian allergic children (102 with eczema and 57 with food allergy) and 147 healthy controls, matched for age and sex, selected consecutively during the period January 2008-May 2009 from S. Pietro Hospital-Fatebenefratelli, Rome, Italy, were enrolled in this study. Eczema was diagnosed following the criteria of Hanifin and Rajka and the clinical severity was assessed by the SCORAD index. One hundred and two (102) subjects, suffered from eczema (64 male; age range months: 3-206, median 54), showed a mild to severe dermatitis phenotype (SCORAD score: median 46 points, range 8-96 points). Fifty-three of 102 children showed IgE mediated eczema (total IgE > 100 UI/ml) (51.9%), 24/102 suffered from asthma as secondary pathology, (23.5%) and 17/102 presented food allergy (16.6%). Regarding food allergy, all 57 children were positive to IgE-mediated provocation test to cow's milk (29 male; age range in months: 6-198, median 58, 57/57 with skin prick tests and prick by prick positive to cow's milk allergens). Thirty-eight of 57 had mainly gastrointestinal symptoms and insufficient growth (66.6%), 19 of 57 developed eczema and/or urticaria.

Allergy tests

The *in vivo* diagnosis was made with biologically standardized extracts of *Dermatophagoides pteronissynus* and *farinae*, mixed grass pollen (*Lolium perenne*, *Dactylis glomerata*, *Phleum pratense* and *Festuca pratensis*), cat and dog epithelium, *Parietaria judaica*, tree mix (*Betula alba*, *Cupressus sempervirens* and *Olea europea*) and the most common food allergens (cow milk, egg, peanut, cod fish, wheat and soybean). Wheals of greater than 3 mm in diameter were considered positive if skin reactivity to a positive and negative control was as expected.

In all patients and controls, total and specific serum IgE for inhalant and food allergens were tested with UniCap IgE Pharmacia (Upjohn, Uppsala, Sweden) according to the manufacture's instructions. The diagnostic gold standard, the provocation test to cow's milk, was performed as previously described (9) in all 57 children affected by food allergy.

Genotyping method

DNA was isolated from the peripheral blood of all subjects by Qiamp Blood Kit (Qiagen, Hilden, Germany). Determination of the TLR-2 R753Q and TLR-4 D299G polymorphism was performed by TaqMan MGB probes (Applied Biosystem) using a real-time PCR allelic discrimination (iCycler iQ™ Real-Time Detection System version 3.021, Biorad). Primers and hybridization probes were designed with Beacon Designer (Biorad) (Table I).

Allelic discrimination PCR reaction was performed in a final volume of 25 µL containing 1 µL of DNA solution, 12.5 µL of TaqMan Universal PCR Master Mix, 0.62 µL of 40X Assay Mix (unlabeled PCR primers and TaqMan MGB probes), and distilled water. In every assay, controls for the wild type and mutations were included. Reaction mixtures were loaded into 96-well plates. The conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation (92°C for 15 s), annealing and extension in one step (60°C for 1 min). The fluorescent yield for the two different dyes was measured and presented in a two-dimensional graph.

Statistics

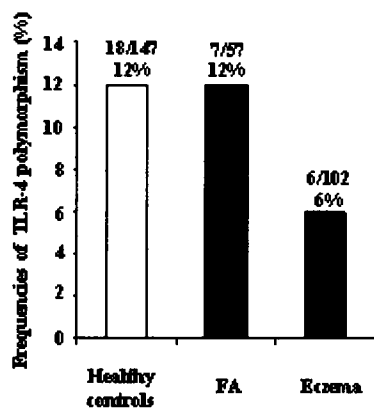
TLR polymorphism frequencies of allergic patients and controls were compared using the 2 tailed chi-square test. Results were considered statistically significant when the probability of findings was less than 5% ($p < 0.05$).

RESULTS

Considering TLR-2 R753Q, a prevalence of 2.5% was found in healthy controls, while none of the allergic patients displayed the mutation. The

Table 1. Primers and probes for allelic discrimination.

Primers for sequencing		
	Forward	Reverse
TLR-4 D299G	5'-CCATTGAAGAATTCCGATTAGCATA-3' 5'-AGTTTGACAAATCTGCTCTAG-3'	5'-CACTCACCAGGGAAAATGAAGAA-3' 5'-CAATAGTCACACTCACCAG-3'
TLR-2 R753Q	5'-TGGAGAACTTCAATCCC-3' 5'-TGATGAGAACAAATGATGCTGCC-3'	5'-TTTCAGAAAGCACAAAGA-3' 5'-TCCTTCCCGCTGAGCCTC-3'
TaqMam® Probe sequences		
TLR4	MUT^b 5'-CTCGATGGTATTATTG-3' WT^a 5'-CCTCGATGATATTATT-3'	
TLR2	MUT^b 5'-TGTGTCTTCATAAGTGGGACTTCATTC-3' WT^a 5'-TGTGTCTTCATAAGCGGGACTTCATTC-3'	

^a TaqMan MGB probe labelled with FAM fluorochrome^b TaqMan MGB probe labelled with VIC fluorochrome**Fig. 1.** Frequencies of TLR-4 polymorphism in 159 allergic children with food allergy (FA) and eczema. Significant differences between allergic patients and the healthy control group were not observed.

frequency of the TLR-4 polymorphism was 12% in the healthy control group (18 of 147) and 8% (13 of 159) in allergic children, including 7 of the 57 patients

with food allergy (12%) and 6 of 102 subjects with eczema (6%) ($p=ns$ for both, Fig. 1). Furthermore, in eczema, no correlation between SNP, IgE level and severe dermatitis phenotype was found. All 6/6 patients with TLR-4 mutations exhibited a phenotype not IgE-associated, characterized by mild eczema (SCORAD score: range, 13-29 points).

DISCUSSION

Our study carried out on an Italian allergic pediatric cohort, suggests that neither TLR-2 nor TLR-4 polymorphisms are associated with atopic diseases. These findings are in agreement with other studies performed in ethnically diverse subjects affected by asthma and eczema, in which no TLR-2 or TLR-4 SNPs were correlated with allergy (7-8). By contrast, other reports demonstrated a possible role of TLR polymorphisms in intensification of susceptibility to infections with various pathogens, defining a subgroup of patients with severe allergy

(3). Since the allergic diseases appear to be strongly influenced by genetic factors, in addition to environmental causes, such results cannot be generalized to diverse allergies in correlation with various populations. Indeed, genetic association studies are strongly influenced by the ethnic and geographic contest in which they are carried out, and consistent differences could be present even in relatively homogeneous areas such as Europe (10). In this contest, association studies in different geographic areas could contribute to better understanding the real role of a given polymorphism in a specific area, and negative studies, such as the present one, may have the role of avoiding over-estimation of positive data.

To our knowledge, the role of TLR-2 and TLR-4 allele variant in human cow's milk allergy is poorly investigated. In food allergy, the mucosal immune system plays an important role and TLR status may influence the development of the intestinal microflora. The multiple signaling through different TLRs in the gastrointestinal mucosa may prevent a dominant role by a single TLR in susceptibility to allergic sensitization to food proteins. Even if the lack of TLR-4 expression is associated with a major susceptibility to food allergy (5), Berin et al. demonstrated that in mice TLR-4 may influence allergic responses positively or negatively according to individual genetic background and the nature of the antigens (6). Our data do not indicate correlation between TLR-2 or TLR-4 polymorphism and cow's milk allergy in our cohort of 57 children.

In eczema, microorganisms and TLRs, that recognize specific molecular signatures on microbes, are likely to play a more important role than in food allergy. Since common features of eczema are cutaneous colonization with *Staphylococcus aureus*, defective host immune defence against microbial pathogens (dysfunctional epithelial barrier, reduced production of antimicrobial peptides and defects in TLR signalling) and high IgE levels, a strict correlation between polymorphism and pathology could be postulated, as reported by other studies (3). By contrast, in our subset of pediatric patients, no association between TLR-2 and TLR-4 polymorphism and eczema prevalence and/or severity emerged. In fact, in our eczema children, TLR-4 polymorphism occurred only in 6% of allergic patients suffering from mild and non-IgE-mediated eczema. One

of the possible explanation could be found in the different mutation rate in the control group. In fact, Ahmad-Nejad et al. (3), who demonstrated association between TLR4 polymorphism and eczema, observed a SNP frequency of 11% in 78 patients versus a mutation rate of 2.5% in 39 healthy subjects. The low frequency of control group differs with other studies and our current data, which report frequencies of about 6-14% of TLR-4 D299G non-synonymous polymorphism in Indo-European individuals (11).

In conclusion, our data show for the first time that polymorphisms in TLR2 or TLR4 are not associated with the frequency of food allergy, and no further association between TLRs mutation and atopy were found. We acknowledge that a potential limitation of the present study could be represented by the relatively limited number of subject analyzed. Indeed, the possible association of TLR polymorphism and allergic diseases in Italian children need to be further investigated before conclusions can be drawn.

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