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Polymorphisms of the *TGF-β1* gene are not associated with bronchial asthma in Caucasian children

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The chromosomal region 19q13 has been found in linkage to allergic diseases in several genome-wide linkage screens. One candidate gene within this region is the gene coding for *TGF-β1*. Transforming growth factor (TGF)-*β* acts as an anti-inflammatory cytokine suppressing allergic inflammation and hyper-reactivity. However, in ongoing inflammation of the lungs it can induce fibrosis and airway remodelling as seen in chronic asthma. Several polymorphisms within *TGF-β1* have been identified and one, −C509T, has been shown to be in association with elevated immunoglobulin E levels and severe bronchial asthma in different populations. However, other studies failed to confirm the association. The present study investigated two polymorphisms within the gene coding for *TGF-β1*, −C509T and G915C, and for their potential association with bronchial asthma in Caucasian children. Genotyping of these polymorphisms was performed by means of restriction fragment length polymorphisms in a population of 231 asthmatic children and a control population of 269 individuals. Statistical analyses made use of the Armitage's trend test. In addition haplotypes were calculated by ARLEQUIN. None of the two polymorphisms showed association with bronchial asthma. They were found to be in linkage disequilibrium. We conclude from our data that *TGF-β1* is unlikely to represent a major gene in the development of bronchial asthma in the Caucasian population.

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Bronchial asthma is a highly complex disease for both clinicians and geneticists. For doctors the difficulty lies mainly in finding clear cut-off definitions and tests to determine whether one person is asthmatic or not. For geneticist the challenge is to find the real diseases-causing genes in between a variety of already described candidate genes for asthma and so far unidentified genes. The first step in hunting genes are linkage studies, and hundreds of such studies have been performed for allergic diseases like bronchial asthma, including about 20 genome-wide linkage screens. Several of these screens have identified linkage to the chromosomal region 19q13 in different populations (1–4). However, only very few association studies of candidate genes within this region exist, including the genes coding for ILT3 (5) and *TGF-β1* (6).

TGF-β1 is constitutively produced in healthy human airways (7) and is able to regulate the generation of proinflammatory and immunoregulatory cytokines derived from different inflammatory airway cells (8, 9). It plays an important role in down-regulation of cellular immunity and thus allergic inflammation. It has been shown that blockade of *TGF-β1* promotes antigen-induced airway inflammation and airway reactivity (10).

In contrast to these anti-inflammatory effects the activation of transforming growth factor (TGF)-*β* in ongoing allergic inflammation may

contribute to disease severity by enhancing fibrosis and the development of irreversible airway remodelling seen in asthma (8, 11, 12). In fact, TGF-β RNA expression is significantly increased in eosinophils of patients with severe asthma (13). At least some of these effects are mediated by the effects of TGF-β on T cells (14). In addition, TGF-β stimulates the release of interleukin (IL)-8 and induces the expression of cyclo-oxygenase (COX)-2 in human airway smooth muscle cells (15). In interaction with IL-13 *TGF-β1* increases eotaxin production in human airways fibroblasts (16). As we have previously found association of IL-8 and IL-13 with asthma in our paediatric population (17, 18), TGF-β might present a promising candidate gene for bronchial asthma in children.

Several polymorphisms within the gene coding for *TGF-β1* have already been identified and tested for association with asthma and atopy. The first association study found association of −C509T with immunoglobulin E (IgE) levels. The strongest association was seen in combination with a polymorphism in the promotor region of IL-10 (19). Rosenwasser also found association of −C509T with heightened IgE levels (20).

The study by Pulleyn et al. found the polymorphism −C509T to be the most informative marker of the *TGF-β1* gene to search for association with asthma (21). They found this single nucleotide polymorphism (SNP) in association with asthma severity. The −C509T is significantly associated with plasma concentration of *TGF-β1* (22) and induces a YY-1 consensus-binding site within a negative regulatory region of *TGF-β1* transcription. However, using luciferase reporter gene assay no differences between wild type and mutation could be found (23).

In studying lung fibrosis and graft fibrosis after lung transplantation it was shown that G915C increases the production of *TGF-β1* and is associated with the development of fibrotic lung disease (24).

However, another study failed to find association of any polymorphisms of TGF-β with atopic phenotypes (25).

Therefore, the aim of our study was to test polymorphisms within the gene coding for TGF-β for association with bronchial asthma in our Caucasian population. In contrast to previous studies, which were conducted in adults, we used a paediatric study population. Furthermore, our study population was larger than the populations used before. We selected the two polymorphisms −C509T and G915C, because previous studies show the most common haplotypes of *TGF-β1* can be ascertained by typing these two SNPs, because of the strong linkage disequilibrium of all the SNPs.

Material and methods

Asthmatic population

A total of 321 children with suspected asthma (aged 5–18 yr) were recruited from the south-western part of Germany between July 2000 and January 2003. The probands were characterized at the University Children's Hospital, Freiburg, Germany using a standardized clinical protocol. Participants were asked in advance to discontinue any asthma or allergy medication before the clinical testing. An extended medical history was recorded including occurrence and duration of wheezing symptoms, previous and acute medications, severity of previous asthma attacks, previous allergic rhinitis or conjunctivitis, atopic dermatitis and any family history of allergic diseases.

Skin prick test

Skin prick tests to 17 common allergens and positive (histamine) and negative controls were performed. The following allergens were tested: house dust mites (*Dermatophagoides pteronyssinus* and *D. farinae*), different grass and tree pollens (grasses, rye, birch, hazel, mugwort), *Aspergillus fumigatus*, *Alternaria alternata*, *Cladosporium herbarum*, dog, cat, rabbit, duck and horse dander. The wheal response diameters were recorded after 15 min. The skin prick test was regarded as positive if the wheal of the test allergen was at least half the size of the wheal of the positive control (26).

Specific and total IgE

Specific IgE was detected by enzyme-linked immunosorbent assays (ELISA) against two mixtures of grass pollens, mite allergens (*D. pteronyssinus* and *D. farinae*, respectively), cat and dog dander, hazel and birch pollens (Magic Lite; Chiron Diagnostics, Fernwald, Germany). The cut-off point for a positive test was 1.43 ML units (27). Measurement of total serum IgE was carried out by an enzyme allergosorbent test (Phadezym; Pharmacia, Uppsala, Sweden).

Pulmonary function tests

Pulmonary function tests (bodypletysmography) were performed using standard protocols (28). In

addition, exercise-induced asthma was diagnosed by subjecting the probands to physical exercise for 6 min under standardized conditions. The first spirometry and peak flow measurement were performed after 2–3 min and the second after 5–6 min. After 10 more minutes the children inhaled with 100 μg salbutamol and a third spirometry and peak flow measurement were taken after 15 min.

To test for bronchial hyper-responsiveness inhalations with increasing, doubling concentrations of histamine (from 0.125 to 8 mg/dl) were performed. Testing was stopped after a 15% fall in forced expiratory volume in 1 s (FEV_1).

Asthma definition

About 231 of the 321 recruited children were diagnosed as positive for bronchial asthma. The diagnosis was based on a clear-cut history of asthmatic symptoms, i.e. recurrent episodes of wheezing, the use of anti-asthmatic medication on a regularly basis and a fall in FEV_1 by at least 15% in histamine testing or exercise provocation. The anti-asthmatic drugs included typical β-agonists like salbutamol and standard corticosteroids used in asthma treatment like budenoside.

Control population

About 269 randomly chosen probands were used as controls (aged 19–40 yr). They originate from the same area in the south-western part of Germany. No medical history was taken and no medical testing was performed on controls.

Genotyping

DNA was extracted from peripheral blood leucocytes following standard protocols and column purified (DNA midikit, Qiagen, Hilden, Germany). Genotyping was performed by restriction fragment length polymorphism (RFLP). The polymorphism −C509T was amplified using the primer pair 5′-GGG GAC AGT AAA TGT ATG G-3′ and 5′-TGA GAC ACA GGG GAG CC-3′ (annealing temperature 65°C). After polymerase chain reaction (PCR) the whole product was digested with 1 units of *Dde*I (New England Biolabs, Frankfurt, Germany) for 4 h and typed by resolving the fragments on a 2% agarose gel.

The polymorphism G915C was amplified using the primer pair 5′-CGC TGC TGT GGC TAC TGG T-3′ and 5′-CTC CGG TTC TGC ACT CTC C-3′ (annealing temperature 65°C). After PCR the whole product was digested with 2 unit of *Bgl*I (MBI, St Leon-Rot, Germany) for 6 h and typed by resolving the fragments on a 4% agarose gel.

The genotyping was performed by two investigators unaware of the phenotype.

Statistical analysis

Statistical analysis was performed using the Armitage's trend test with the DE FINETTI program. The Armitage's trend test is a statistical method that does not assume Hardy–Weinberg Equilibrium (HWE) to test for association between a SNP and the disease of interest. As deviation from HWE has been shown to inflate the chance of false-positive association (29) the Armitage's trend test should be more useful than the usual chi-square test. The extent of linkage disequilibrium between SNPs has been calculated using the ARLEQUIN program. HWE was calculated with both programs – DE FINETTI and ARLEQUIN.

Approval

The collection of serum and the subsequent DNA material and the experimental procedures were approved by the Ethical Commission of the University of Freiburg. A statement of informed consent was signed by all participants or in the case of children signed by their parents.

Results

Genotyping was performed in the population of 231 asthmatic children and in the control population of 269 individuals.

The allelic frequency of −C509T and G915C are shown in Table 1. Both polymorphisms were in HWE as calculated by the DE FINETTI program (see Table 1) and by the ARLEQUIN program (data not shown). Also linkage disequilibrium existed between the polymorphisms as calculated by the ARLEQUIN program (data not shown).

Table 1. Genotype distribution and Hardy–Weinberg Equilibrium for −C509T and G915C

	Homozygous wild type	Heterozygous	Homozygous mutation	HWE
−C509T				
Controls	121 (0.45)	111 (0.41)	37 (0.14)	0.16
Asthmatics	94 (0.41)	108 (0.47)	29 (0.13)	0.81
G915C				
Controls	230 (0.86)	37 (0.14)	2 (0.01)	0.66
Asthmatic	192 (0.83)	37 (0.16)	2 (0.01)	0.69

Values expressed in parentheses are percentage.

Table 2. Allelic frequency and association analysis of the polymorphisms by the Armitage's trend test

	Mutation controls (%)	Mutation asthmatics (%)	Armitage's trend (p-value)
−C509T	34.4	35.9	0.62
G915C	7.6	8.9	0.48

The results of the association studies are included in Table 2. None of the polymorphisms was in association with bronchial asthma.

Discussion

Several polymorphisms have been identified in *TGF-β1*, i.e. −G800A, −C509T, 72insC, T869C and G915C (21–23). For the following reasons our study focused on two of those polymorphisms, −C509T within the promotor region and G915C in exon 1 leading to a substitution of amino acid 25 in the signal peptide from Arginine to Proline: independent studies have shown that both polymorphisms are in association with elevated TGF-β concentrations in serum (20, 22). −509T was found to be the most informative marker for association studies with asthma in one study (21). In fact, −C509T is the only SNP in *TGF-β1* found to be associated with atopic phenotypes, i.e. elevated IgE levels and asthma. G915C has been found to be in association with fibrotic lung disease and might therefore be also important in the development of bronchial asthma (22).

Previously published association studies of *TGF-β1* investigated either populations of atopic or asthmatics adults. In contrast, we used a paediatric population. Outcome studies of paediatric asthma show that in the transition period from childhood to adulthood asthma symptoms decrease and thereby asthma seems to be resolved in about 50% of asthmatic children (30). By using adult asthmatics for genetic studies one might preferably identify genes for persisting bronchial asthma and they might be quite different from genes for the onset of asthma in children. This difference might be especially important in studying TGF-β, because this gene product plays an important role in long ongoing inflammatory processes and chronic airway remodelling. It might well be that in acute, short-lasting paediatric asthma this gene and its polymorphisms are less important.

Furthermore, association was only found with the phenotype severe asthma, but not with asthma in general (21). For this subgroup the study population was quite small, so the results might reflect a type I error. On the contrary, we did not classify the children in severe or mild asthmatics and thus could not test for association with the phenotype of severe asthma.

Also the lack of association in our study could reflect a type II error. However, our study population is larger than the cohorts used in the other studies of *TGF-β1*. Thus, we would expect to see at least a tendency towards an association with asthma if the gene had a major impact on the development of asthma in our population.

One should also bear in mind that the other studies investigated populations of different ethnic background, i.e. a Czech population, an English population and an American population.

We conclude from our data that *TGF-β1* does not play a major role in the development of bronchial asthma in our Caucasian population.

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