**STAT6 as an asthma candidate gene: polymorphism-screening, association and haplotype analysis in a Caucasian sib-pair study**

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The human signal transducer and activator of transcription 6 (STAT6) gene represents one of the most promising candidate genes for asthma and other inflammatory diseases on the chromosomal region 12q13–q24. Therefore we screened all 23 exons, including parts of the neighbouring introns, as well as the promoter region for common polymorphisms and tested them for linkage/association with asthma and related traits (total serum IgE level, eosinophil cell count and SLOPE of the dose-response curve after bronchial challenge) in a Caucasian sib-pair study (108 families with at least two affected children). We could identify 13 single nucleotide polymorphisms (SNPs), which are all non-coding. A recently described dinucleotide (GT) repeat in exon 1 was also examined. Besides the confirmation of the four alleles described elsewhere we could identify a new one, named allele A5. Neither the SNPs nor the GT repeat showed linkage/association to asthma. Two intronic SNPs and one SNP in the 3′ untranslated region of the gene showed weak association to total IgE levels (P = 0.0200, 0.0260 and 0.0280, respectively), whereas a significant association was found between a SNP in intron 18 and an increase in total IgE levels (P = 0.0070). However, the most promising effect was seen between allele A4 of the GT repeat polymorphism and an increase in eosinophil cell count (P = 0.0010). From these findings we conclude that the human STAT6 gene is rather involved in the development of eosinophilia and changes in total IgE levels than contributing to the pathogenesis of asthma.

INTRODUCTION

Asthma has become the most common chronic childhood disease in developed nations, affecting more than 155 million individuals (1,2). Both, gene–gene as well as gene–environment interactions contribute to its overall phenotype (3). To find chromosomal regions that are linked and/or associated with asthma, atopy and other inflammatory diseases several genome-wide screens, as well as candidate-regions studies, have been performed (4–15) and are available on the internet (http://cooke.gsf.de/asthmagen) (16). Many of these studies, including our own, have shown linkage of asthma and related phenotypes, e.g. total serum IgE levels, eosinophil cell count, bronchial hyper-responsiveness (BHR), to the chromosomal region 12q13–q24 in different ethnic populations worldwide (4–6,8–13). This region harbours a number of candidate genes for asthma and allergy, including signal transducer and activator of transcription 6 (STAT6), integrin-β 7 (ITGB7), leukotriene A4 hydrolase (LTA4H), stem cell factor (SCF), interferon-γ (IFNG), insulin-like growth factor 1 (IGF1), β subunit of nuclear factor-Y (NFYB) and neuronal nitric oxide synthase (NOS1) (5,19).

Of these genes STAT6 represents one of the most promising candidate genes. The human STAT6 gene, which belongs to the STAT-family of transcription factors, maps to chromosome 12q13.3–q14.1, encompasses over 19 kb and contains 23 exons (20). The first two exons of the STAT6 gene are non-coding [5′ untranslated region (5′UTR); 20] (GenBank accession nos: AF067572–AF067575, XM006760). STAT6 is involved in the interleukin 4 (IL-4) and interleukin 13 (IL-13) signalling pathway (21–25), two cytokines that are related to allergic asthma and other inflammatory and allergic diseases (22–24,26).

IL-4 induces the proliferation of T-lymphocytes and is important for the differentiation of Th2-cells (27). Furthermore, IL-4 activation of B lymphocytes (B-cells) triggers class-switching to the IgE isotype (28). IL-13, which is secreted by activated T-cells, shares many biological functions with IL-4 (29). IL-13 has been shown to affect B-cells by inducing enhancement of the expression of MHC class II and CD23 and by induction of IgE class-switching (29). It is well known that elevated numbers of activated Th2-cells, mast cells and eosinophils, both in the bronchial mucosa and in bronchoalveolar lavage (BAL) fluid, are constant features of asthma (26) as well as increased serum IgE levels for the atopic form.

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Further evidence of the important role of STAT6 in these pathways came from STAT6−/− mice. Kuperman et al. (30) showed that STAT6−/− mice failed to develop airway hyperresponsiveness after allergen provocation. Furthermore, these animals produced no detectable levels of serum IgE, lacked a typical Th2-cytokine response and were protected from antigen-induced mucus production (30). Similar results have been found by Miyata et al. (26) and Akimoto et al. (31). Both authors could show that STAT6−/− mice were protected from bronchial eosinophilia and damage of the lung tissue.

In order to identify common polymorphisms, especially single nucleotide polymorphisms (SNPs) of the human STAT6 gene that might be associated with asthma and related phenotypes, we systematically screened all 23 exons and the promoter region of the gene for SNPs. Therefore we sequenced defined PCR fragments of the interesting regions. All identified SNPs, together with additional ones from the public SNP database (www.ncbi.nlm.nih.gov/SNP), were then genotyped in 108 sib-pair families using capillary-sequencing and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. A recently described dinucleotide polymorphism (GT repeat) in the first exon (exon 1) of the gene (32) was also tested within the study. For the qualitative trait asthma, individual SNPs as well as the dinucleotide polymorphism were analysed by a transmission/disequilibrium test (RC-TDT) to test for linkage in the presence of association within families (33). A quantitative TDT (QTDT) (34) was carried out for the quantitative, asthma associated traits. Additionally, we examined the distribution of the haplotypic combinations of the SNPs found using a modified expectation-maximization (EM) algorithm which includes nuclear family information (35).

RESULTS

SNP screening

To find SNPs in the human STAT6 gene we sequenced all 23 exons, including parts of the neighbouring introns, and the promoter region of the gene in 16 unrelated healthy and 16 asthmatic probands of a population-based study (36). By this approach 10 SNPs were identified. Two SNPs, 5′flankingSNP1 and 5′flankingSNP2, were found in the promoter region of the gene (5′flanking region; Table 1). 5′flankingSNP1 is located 77 bp upstream of a CCAAT-box, which represents a putative enhancer element, and 1672 bp upstream of a prominent transcriptional initiation site (20). The second variant of the promoter region, 5′flankingSNP2, is located 1116 bp upstream of the transcription initiation site and 474 bp downstream of the CCAAT-box. Of the remaining eight SNPs, three are intronic (in16SNP1, in17SNP1 and in18SNP1; Table 1) and five belong to the 3′UTR of exon 23 (3′UTRSNP1-5; Table 1). Four of the 3′UTR-SNPs have been entered to the public SNP database during the study (www.ncbi.nlm.nih.gov/SNP; Table 1). No SNP was found in the coding region of the gene.

SNP genotyping

For further association analysis all 10 SNPs found by sequence analysis, plus six additional ones, in2SNP1-3, in8SNP1-2 and in12SNP1, that were taken directly from the public SNP database (www.ncbi.nlm.nih.gov/SNP), were genotyped within our sib-pair study using MALDI-TOF mass spectrometry or sequencing (Table 1).

Whereas all of the SNPs found in the screening sample could be confirmed, only three of the six taken from the public SNP-database, in2SNP1, in2SNP2 and in2SNP3, were present (Table 1). All genotypes passed a paternity check (Mendel check) and were in Hardy–Weinberg equilibrium (HWE), except 3′UTRSNP2, which had a borderline P-value of 0.035 for departure from HWE (data not shown). Therefore, genotyping errors can most probably be excluded. Genotype coverage was always >90% (Table 2).

A graphical overview of the 13 SNPs examined in relation to the exon/intron structure of the human STAT6 gene is given in Figure 1.

Association results for the SNPs

Table 2 represents a summary of all confirmed SNPs and their parental allele frequencies (prevalence) within the sib-pair study. There was no linkage/association of any SNP on the asthmatic disease (Table 2) and this was the same for peripheral eosinophil cell count (Table 3). Weakly positive effects were seen by in2SNP3 (prevalence 43.93%; Table 2) on total IgE (P = 0.0200; Table 3), in17SNP1 (prevalence 39.61%; Table 2) on total IgE (P = 0.0260; Table 3), 3′UTRSNP4 (prevalence 35%; Table 2) on total IgE (P = 0.0280; Table 3) and 3′UTRSNP3 (prevalence 7.92%; Table 2) on the variable SLOPE from the resulting dose-response curve after bronchial challenge (P = 0.0370; Table 3). However, the most prominent effect was seen by the SNP in intron 18, in18SNP1 (prevalence 10%; Table 2) on total IgE level (P = 0.0070; Table 3).

Due to a low number of informative genotypes (prevalence 1.92%) the SNP in intron 16, in16SNP1, has not been tested in the quantitative analysis.

Haplotypes

The 12 most frequent haplotypic combinations of the 13 SNPs found by the method of Rohde and Fuerst (35) are shown in Table 4. Although complicated by missing genotypes they make up 93.81% of all possible haplotypes. Of the remaining 6.19%, all haplotypes had a frequency <0.01% (data not shown). Interestingly, haplotype numbers 5 (1112112212121, frequency 6.19%, Table 4) and 10 (111112212121, frequency 2.12%; Table 4) contain all alleles of the different SNPs that have shown weak or significant effects on one of the quantitative traits examined (see also Table 3). Therefore, we tested these haplotypes, together with the three most frequent ones, for association with one of the quantitative traits using a quantitative TDT model. However, no significant associations were obtained (all P-values >0.06, data not shown).

Pairwise linkage disequilibrium (LD) tested by the maximum-likelihood method of Excoffier and Slatkin (37) showed most SNPs in significant linkage disequilibrium (P < 0.05) using 10,000 permutations (data not shown). LD departures were only seen in pairs 5′flankingSNP1/5′flankingSNP2 (exact P = 0.3606), 5′flankingSNP1/in18SNP1 (P = 0.0755), in2SNP2/in18SNP1 (P = 0.1246), in16SNP1/3′UTRSNP2 (P = 0.0981), in16SNP1/3′UTRSNP3 (P = 0.3106),
However, four of the self-found SNPs in the 3′UTR, 3′UTR SNP1-4, which were taken directly from the public SNP database (www.ncbi.nlm.nih.gov/SNP; column 7), all other SNPs were found by sequence analysis. The position of each variation is given in base pairs (column 2) according to the GenBank entry (column 4). With the exception of in2SNP1-3, in8SNP1-2 and in12SNP1, which were taken directly from the public SNP database (www.ncbi.nlm.nih.gov/SNP; column 7), all other SNPs were found by sequence analysis. However, four of the self-found SNPs in the 3′UTR, 3′UTR SNP1-4, have been entered into the public SNP database during the study (column 7). Primers used for PCR, (together with corresponding annealing temperatures in °C), sequencing and PROBE-extension reactions are also listed (column 8, 9 and 10).

Table 1. Overview of the 16 SNPs of the human STAT6 gene (column 1) that were genotyped in our sib-pair study using MALDI-TOF mass spectrometry or sequencing (column 6).

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Variation in bp according to the GenBank accession no.</th>
<th>Position in the gene</th>
<th>GenBank accession no.</th>
<th>Confirmed Genotyping MALDI (M)/sequencing (S)</th>
<th>GenBank ref. SNP ID (rs)/GenBank assay ID (rs)</th>
<th>PCR primer</th>
<th>Annealing temp. (°C)</th>
<th>PROBE-extension primer/sequencing primer</th>
</tr>
</thead>
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<td>5′flanking SNP1</td>
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<td>5′flanking region</td>
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<td>Yes</td>
<td>S</td>
<td>New</td>
<td>5′-GCCACATTTGACCTCTAAATGCTG-3′</td>
<td>5′-CTGAGGGCTGAGACATTGCC-3′</td>
</tr>
<tr>
<td>5′flanking SNP2</td>
<td>2438C→T</td>
<td>5′flanking region</td>
<td>AF067572</td>
<td>Yes</td>
<td>M</td>
<td>New</td>
<td>5′-CCAAGCATTTGCTCTTTT AAC-3′</td>
<td>5′-CTGAGGGCTGAGACATTGCC-3′</td>
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<tr>
<td>in2SNP1</td>
<td>5021C→T</td>
<td>Intron 2</td>
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<td>M</td>
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<td>Intron 2</td>
<td>AF067572</td>
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<td>M</td>
<td>232750</td>
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<td>5′-GAAACCTGCTATCCTAC ACTC-3′</td>
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<td>M</td>
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<td>5′-GAAACCTGCTATCCTAC ACTC-3′</td>
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<td>Intron 8</td>
<td>AF067572</td>
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<td>M</td>
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<td>5′-TCAAGGCGCTTGAGC AGAC-3′</td>
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<td>9234C→G</td>
<td>Intron 8</td>
<td>AF067572</td>
<td>No</td>
<td>M</td>
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<td>in12SNP1</td>
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<td>Intron 12</td>
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<td>M</td>
<td>703816</td>
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<td>5′-TCAAGGCGCTTGAGC AGAC-3′</td>
</tr>
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<td>Intron 17</td>
<td>AF067572</td>
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<td>M</td>
<td>730816</td>
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<td>M</td>
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<td>3′UTR</td>
<td>AF067572</td>
<td>Yes</td>
<td>M</td>
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<td>3′UTR</td>
<td>AF067572</td>
<td>Yes</td>
<td>M</td>
<td>703817</td>
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<td>5′-TCAAGGCGCTTGAGC AGAC-3′</td>
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<tr>
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<td>4610A→G</td>
<td>3′UTR</td>
<td>AF067572</td>
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<td>S</td>
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<td>4671A→G</td>
<td>3′UTR</td>
<td>AF067572</td>
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<td>4703A→G</td>
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<td>5′-GAGGATTTGGGTCTCTGACGCC-3′</td>
<td>5′-GAGGATTTGGGTCTCTGACGCC-3′</td>
</tr>
</tbody>
</table>


3 in eosinophil cell count could be observed (Table 1). However, a significant association of allele A4 with an increase in total IgE levels, eosinophil cell count, and variable SLOPE from the resulting dose-response curve after bronchial challenge, within 108 Caucasian sib-pair families. We could show that the SNP in intron 18, in18SNP1, is significantly associated with an increase in total IgE levels, whereas one allele of the dinucleotide polymorphism (GT repeat) in the first exon (exon 1) shows a significant association with a change in eosinophil cell count.

Of the 13 SNPs verified in our sib-pair study none was found in the coding region of the gene. Our results are consistent with previous data from Heinzen et al. (38) who screened the coding region (exon 3 to the third of exon 23) of the gene by SSCP analysis but did not find any common variation. These findings demonstrate the highly conserved character of the human STAT6 gene.

Despite this high conservation there are alternative splice products of the human STAT6 pre-mRNA, which are missing exon 2, exons 2 and 4 and parts of exon 16, respectively (20). As these variants are expressed to varying extents in vivo (20) there is the possibility of a reciprocal influence of these products resulting in an exact regulation of the STAT6 activity. Therefore, we also looked for variations in the corresponding exon/intron boundaries that could influence alternative splicing.

Although the third SNP in intron 2, in2SNP3 showed a weak positive effect on total IgE levels (P = 0.0200; Table 3), none of the examined SNPs in intron 2 is located in a region that seems to be directly involved in the splicing mechanism. Also for the most interesting SNP in intron 18, in18SNP1, which is significantly associated with an increase in total IgE levels (P = 0.0070; Table 3) a direct effect on the splicing of the neighbouring exons is unlikely. The only SNP examined in our study that could have such a direct influence on the splicing mechanism is in16SNP1, which is located only 9 bp downstream of the 3′ end of exon 16. However, no association of this SNP on the asthmatic disease could be observed. Because of its low frequency in this study (prevalence 1.92%; Table 2) no significant association with any atopy or asthma was found in the Japanese population (39). Another study, testing for association of this SNP on the asthmatic disease could be observed. Because of its low frequency in this study (prevalence 1.92%; Table 2) no significant association with any atopy or asthma was found in the Japanese population (39).

The parental allele distributions are given as total numbers and relative frequencies (prevalence). Analysis was performed with the RC-TDT (33) considering all children of each family. Exact P-values are given (52).

### 5′flankingSNP2/3′UTRSPN5 (P = 0.3531), in16SNP1/3′UTRSPN5 (P = 0.8554), 3′UTRSPN1/3′UTRSPN5 (P = 0.0559).

### GT repeat in exon 1

A recently described dinucleotide polymorphism (GT repeat) in the first exon (exon 1) (32) has been tested within 108 families of our sib-pair study. We could identify five alleles, alleles A1–A5, which are listed, together with their parental allele frequencies, in Table 5. Interestingly, allele A1 (prevalence 40.14%; Table 5) and allele A3 (prevalence 43.51%, Table 5) were the most frequent ones and this was also the case for the parental number of A1 and A3 heterozygotes (data not shown).

To determine the number of GT repeats of the different alleles, we sequenced PCR products of persons homozygous for one of the five alleles. Thus, allele A1 contains 13 repeats of GT and allele A5 17 repeats (Table 5).

There was no linkage/association of any of the five alleles on the asthmatic phenotype, total IgE level and bronchial responsiveness to methacholine challenge (SLOPE) (Tables 5 and 6). However, a significant association of allele A4 with an increase in eosinophil cell count could be observed (P = 0.0010; Table 6).

### DISCUSSION

As the human STAT6 gene represents one of the most promising candidate genes for asthma and other allergic diseases on the chromosomal region 12q13–q24, we systematically screened all 23 exons and the promoter region of the gene for common polymorphisms (especially SNPs). We identified six novel SNPs which were tested, together with 10 additional SNPs from the public database and a dinucleotide polymorphism (GT repeat) within exon 1, for association with asthma and related phenotypes, total serum IgE levels, eosinophil cell count, and variable SLOPE from the resulting dose-response curve after bronchial challenge, within 108 Caucasian sib-pair families. We could show that the SNP in intron 18, in18SNP1, is significantly associated with an increase in total IgE levels, whereas one allele of the dinucleotide polymorphism (GT repeat) in the first exon (exon 1) shows a significant association with a change in eosinophil cell count.

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In accordance with the latter findings, no association of this variant with asthma and the quantitative traits was found in our study (Tables 2 and 3). These findings show that there are marked differences not only between different ethnic groups but also within one ethnic group underlining the genetic heterogeneity of complex diseases like asthma.

The function of the 3′UTR of eukaryotic genes is not fully understood at the moment. There is rising evidence for its essential role in the appropriate expression of many genes by affecting translation, coding capacity, mRNA stability and localization of the mRNA in the cytoplasm (40). Some human diseases, e.g. myotonic dystrophy (DM), can be put down to mutations in the 3′UTR of genes (40). Only two of five SNPs within the 3′UTR examined in our study showed weak positive effects: 3′UTRSNP4 on total IgE levels (\(P = 0.0280\); Table 3) and 3′UTRSNP3 on SLOPE (\(P = 0.0370\); Table 3). Therefore, a direct influence of these SNPs on the regulation of STAT6 expression can most probably be excluded.

Interestingly, only two haplotypes, haplotype number 5 and haplotype number 10, contain all alleles of the different SNPs that showed weak or significant effects on one of the quantitative traits (Table 4). Therefore, an association of these haplotypes

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**Table 3.** Association results for the quantitative traits logarithmized total serum IgE levels, eosinophil cell counts and bronchial responsiveness to methacholine (SLOPE) tested by the QTDT by Abecasis et al. (34) using genotypes from all family members and incorporating age and gender as covariates.

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<thead>
<tr>
<th>SNP</th>
<th>Allele 1/allele 2</th>
<th>Allele associated with higher IgE level</th>
<th>(P)-value total IgE</th>
<th>Allele associated with higher eosinophil cell count</th>
<th>(P)-value eosinophil cell count</th>
<th>Allele associated with higher SLOPE</th>
<th>(P)-value SLOPE</th>
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<td>2 (A) &gt;0.1000</td>
<td></td>
<td>1 (G) &gt;0.1000</td>
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<td>2 (G) &gt;0.1000</td>
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<tr>
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</table>

Twelve SNPs of the human STAT6 gene were genotyped in 108 sib-pair families. Due to a low number of informative genotypes the SNP in intron 16, in16SNP1 (prevalence 1.92%), has not been tested. In column 2 the allele with the higher frequency (prevalence) is (1), the minor one is (2).
Recent studies have shown that dinucleotide repeats can influence the transcription or translation of genes. Gabellini (41) demonstrated that a GT repeat in intron 2 of the human cardiac Na+Ca2+ exchanger gene (NCX1) functions as a strong intronic splicing enhancer that could be involved in the regulation of NCX1 expression. Furthermore, a significant correlation between the CA repeat polymorphism in the first intron of the human IFNG gene, another asthma candidate gene on the chromosomal region 12q13–q24, and the in vitro production of IFNG could be observed (42).

Exon 1 of the human STAT6 gene is non-coding and part of the 5′UTR (20). There is strong evidence that certain features within the 5′UTR of eukaryotic genes are involved in the regulation of translation of the mRNA and that mutations, affecting the 5′UTR, can contribute to the pathogenesis of several human diseases (43). If the GT repeat polymorphism, directly or indirectly, influences such regulatory elements, the consequence would be an abnormal translation of the STAT6 mRNA.

Th2-cells (Th2 lymphocytes) and the cytokines they secrete play a central role in the development of eosinophilia, a key feature of asthma and allergic diseases (22). As STAT6 is involved in the IL-4 and IL-13 signalling pathway (22), two of the main cytokines released by Th2-cells, a higher translation rate of the STAT6 mRNA due to the dinucleotide polymorphism in the 5′UTR would lead to a higher amount of STAT6 protein resulting in a higher eosinophil chemottractance and activity. This would explain why this repeat is associated with an increase in eosinophil cell count in our study (Table 6). Even if the mechanism of how this dinucleotide polymorphism contributes to the development of eosinophilia remains still unknown it represents a useful marker mutation for association and linkage analysis of asthma and allergic diseases.

Nevertheless, we conclude that a major contribution of the variants in the STAT6 gene to the pathology of asthma in our study is unlikely from these results. However, an involvement of STAT6 in the development of eosinophilia and changes of total IgE levels can be assumed. As asthma and allergy are multifactorial diseases, characterized by high genetic heterogeneity between different ethnic groups, it is possible that the influence of STAT6 on asthma and related traits differs in different populations. Additional genetic and epidemiological studies as well as functional analysis of human STAT6 are required to fully elucidate the role of this interesting gene in the development of asthma and allergic diseases.
MATERIALS AND METHODS

Probands
108 Caucasian asthma sib-pair families (474 probands, 216 parents, 258 children) were collected since the beginning of 1994, mainly in paediatric university clinical centres in Germany and Sweden (44). Only families with at least two children with confirmed clinical asthma were included. Prematurity, low birth weight, as well as any other severe pulmonary disease, were reasons for exclusion. Ninety-seven of these families participated in our previous genome-wide searches for linkage to asthma and related phenotypes (13,19). Each study participant, including all children, signed a consent form. All study methods have been approved by the ethics commission of ‘Nordrhein-Westfalen’, Germany.

Phenotyping
Asthma in the children was initially defined by clinical history and validated later by interview questions (13). All clinical data were entered twice into the database. Asthma was the primary trait examined; however, all participants were also examined for associated phenotypes such as positive skin prick tests for common allergens, serological IgE measurement and eosinophil cell count (13). Pulmonary function tests were performed by forced expiration in a sitting position using a nose clip. Forced flow volume tests were performed until three reproducible loops were achieved. Of these, the trial with the maximum sum of the forced vital capacity (FVC) and forced expiratory volume during 1 s (FEV1.0) was used for the analysis (45). Bronchial challenge was performed using methacholine with increasing doses of 0, 0.156, 0.312, 0.625, 1.25, 2.5, 5, 10 and 25 mg/ml administered during five consecutive breaths with 14 µg delivered from the Vilbiss 646 nebulizer chambers using a ZAN 200 breath-triggered pump (ZAN, Oberthulpa, Germany) (46). The provocation was stopped at the occurrence of symptoms or a decrease of 20% from the baseline FEV1.0. The variable SLOPE from the resulting dose-response curve was calculated as described earlier (47). The dose necessary for a 20% fall of FEV1.0 is given as PD20. Probands with a PD20 <8 mg/ml were considered to be bronchial hyper-reactive.
During patient visits at the clinic, interviews were completed for each participant. Blood was taken from a cubital vein with a closed EDTA-coated system (Sarstedt, Nuembrecht, Germany) for either DNA-isolation or serological analysis. Total IgE was determined using the ELISA technique (Pharmacia, Freiburg, Germany). Total cell counts were measured in a Coulter counter (Beckman Coulter, Inc., Fullerton, CA) (10³/µl) and eosinophil cells were counted manually as a percentage of all nucleated cells (range 0.00-0.15) and multiplied with the total cell count.

DNA preparation
DNA was isolated from peripheral white blood cells using the salt method (48) and in a few cases also with the Qiamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol.

SNP screening
To find common SNPs in the human STAT6 gene we performed sequence-analysis of defined PCR products of all 23 exons and the promoter region of the human STAT6 gene. The screening panel included 32 independent adult probands of a population-based study enriched with 16 asthmatic patients (36). The entire region was covered by PCR fragments each containing one or two entire exons plus 20–100 bp of the neighbouring introns. Because of its large size exon 23 was split into five single fragments. All PCR- and sequencing-primers were designed using the Vector NTI suite 6.0 (www.informaxinc.com).

PCR was then carried out in a volume of 50 µL containing 80 ng of DNA template, 25 pmol of each PCR-primer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 U Taq polymerase and the buffer recommended by the supplier (all from MBI Fermentas, Vilnius, Lithuania). Thermocycling started with a single denaturation step for 5 min at 95°C, following 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min and extension at 72°C for 1 min. One final extension step was added for 10 min at 72°C. Purification of the PCR-products were performed on the BIOMEK 2000 robotic system (Beckman Coulter Inc.) using the Nucleo-Spin Robot 96-Extract-Kit (Macherey-Nagel GmbH & Co. KG, Dueren, Germany). Samples were eluted with 50 µl elution buffer (Macherey-Nagel GmbH & Co. KG).

Cycle sequencing of the purified PCR products followed a standardized protocol (Applied Biosystems, Foster City, CA). Sequencing of the products were performed on the ABI prism 3100 Genetic Analyser (Applied Biosystems). The sequences were analysed using the alignment program of the Vector NTI database (Vector NTI suite 6.0; www.informaxinc.com).

SNP genotyping
With the exception of 5′flankingSNP1, 3′UTRSNP3, 3′UTRSNP4 and 3′UTRSNP5 (Table 1), which were genotyped by sequencing, all other SNPs of the STAT6 gene were genotyped performing a primer oligo base extension (PROBE) reaction (49,50) following analysis with MALDI-TOF mass spectrometry (Sequenom Inc., San Diego, CA) (49–51). Therefore PCRs were performed under standard conditions using one biotinylated PCR primer (PCR primer with biotinylation at the 5′ end). The reaction volume was 50 µl including 60–80 ng of DNA-template, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 25 pmol of the non-biotinylated PCR-primer, 10 pmol of the biotinylated PCR-primer, 0.5 U Taq polymerase and the buffer recommended by the supplier (all from MBI Fermentas). The biotinylated PCR primer produced DNA strands complementary to the PROBE-extension primer (49,50). PCR cycling started with a single denaturation step for 10 min at 95°C, following 45 cycles of denaturation for 45 s at 95°C, annealing for 45 s (annealing temperatures are listed in Table 1) and extension at 72°C for 45 s. One final extension step was added for 10 min at 72°C. PCR products were then bound via biotin-streptavidin coupling to paramagnetic beads according to standardized protocols (Sequenom Inc.). The following PROBE reactions, as well as sample preparation for the analysis with the MALDI-TOF mass spectrometry, were performed with the MULTIMEK 96 automated 96-channel pipettor (Beckman Coulter Inc.) using the MassEXTEND Reagents Kit (Sequenom Inc.). The resulting primer extension products were then spotted on a 96-element silicon chip (SpectroChip, Sequenom Inc.) with the SpectroJet NP nanoliter pipetting system.
(Sequenom Inc.) and analysed in fully automated mode by a MassARRAY mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany; Sequenom Inc.) according to the manufacturer’s adjustments. The resulting mass spectra were further processed and analysed by analysis software (SpectroTYPER; Sequenom Inc.) (51). All assays for the PROBE reactions and the equivalent PCR were created using the SpectroDESIGNER software (Sequenom Inc.). The PCR-, sequencing- and PROBE-extension primers used for genotyping of the SNPs as well as the corresponding annealing temperatures for the PCRs are listed in Table 1.

Genotyping of the STAT6 exon 1 GT repeat

PCR was carried out in a total volume of 25 µl containing 40–60 ng of genomic DNA, 10 pmol of a 6-FAM-labelled forward primer (5′-GAGGGGACCTGGGTAGAAGGA-3′), 6-FAM-labelling at the 5′ end, 10 pmol of a non-labelled reverse primer (5′-CACCCCCATGCCTCATCAG-3′), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.25 U of Taq polymerase and the buffer recommended by the supplier (all from RBI Fermentas). Thermocycling started with a single denaturation step of 95°C for 5 min, following 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C and extension at 72°C for 1 min. One final extension step was added for 10 min at 72°C. After PCR, 1 µl of PCR products plus 0.3 µl of GENESCAN 400HD (ROX) molecular weight standard (Applied Biosystems) were denatured in 15 µl of formamide (Applied Biosystems) for 2 min at 90°C and immediately chilled on ice. Fragment analysis of the PCR-products was performed under standard conditions on the ABI prism 3100 Genetic Analyser (Applied Biosystems) with POP4 polymer (Applied Biosystems). Fragment lengths were determined using the GeneScan Analysis Software v3.7 (Applied Biosystems).

The number of GT repeats for each allele was determined by sequencing PCR fragments of probands being homozygous for one allele. Cycle sequencing was carried out using the unlabelled forward primer mentioned above and followed a standardized protocol (Applied Biosystems). Sequencing of the products were performed on the ABI prism 3100 Genetic Analyser (Applied Biosystems).

Statistical analysis

For the qualitative trait asthma the RC-TDT (33) was used to test for linkage in the presence of association (LD) within families. For the quantitative trait, logarithmized total serum IgE levels, the methacholine challenge were analysed by the QTDT based on the extended orthogonal decomposition model (34) using genotypes of all family members and incorporating age and gender as covariates. The likelihood ratio test was based on 1000 permutations.

Haplotype frequency estimation of the 13 SNPs was carried out by a standard EM algorithm (37) including nuclear family information (35). The method uses children’s genotype to exclude those haplotype pairs from the calculation that are compatible with the genotype of the parents but contradictory for the children, leading to an improved and more accurate haplotype frequency estimation (35).

Statistical analysis was done with SAS release 8.1 (data handling, basic descriptions, RC-TDT), QTDT package (34) for the analysis of the quantitative traits, Arlequin release 2.0 (EM) algorithm for the analysis of haplotypes and pair-wise LD and SIB-PAIR release 0.996 (Mendel check).

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