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An IL-13 promoter polymorphism associated with increased risk of allergic asthma

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IL-13 plays a crucial role in the development of allergic asthma by several mechanisms, including induction of IgE antibodies, airway eosinophilia and hyper-reactivity. We previously established a deregulated production of IL-13 by T cells from allergic asthma patients. In this report we describe the identification of a novel IL-13 promoter polymorphism (C to T exchange) at position –1055. The IL-13 –1055 TT genotype is associated with allergic asthma ($P = 0.002$), altered regulation of IL-13 production ($P < 0.002$), and increased binding of nuclear proteins to this region. We postulate that the presence of this polymorphism predisposes to the development of allergic asthma.

Keywords: *human; cytokines; allergy and immunology; genetics; Th2 cells*

Introduction

Allergic asthma is controlled by environmental and genetic factors. A number of genetic markers located on chromosome 5q31–33, 12q15–24 and 11q13, are linked to characteristics of allergic asthma, such as enhanced serum IgE levels and bronchial hyper-responsiveness (BHR).[1–6] The 5q31–33 region contains a number of genes which could be involved in the aetiology of allergic asthma. These include: Th2 cytokines IL-4, IL-13, IL-5, the p40 chain of the Th1 cytokine-inducer IL-12, IL-3, IL-9, CD14, the β_2-adrenergic receptor, the corticosteroid receptor and the transcription factors, Interferon regulatory factor 1 (IRF1) and transcription factor-7 (TCF7). In mice the control of Th2 responses is also associated with a region on murine chromosome 11 that is syntenic with human chromosome 5q31.[7] In humans, the severity of *Schistosoma mansoni* infection is linked to a marker on chromosome 5q31.[8] Resistance to this disease is regulated by Th2 cytokines, in particular IL-13.[9] These findings all point towards a strong genetic influence of the chromosome 5q31 region on the development of Th2-mediated diseases, such as allergic asthma. Because of their biological effects, IL-4 and IL-13, located at close proximity on 5q31, are very likely candidates to be involved in the inheritance of allergic asthma. Firstly, IgE synthesis is dependent on the presence of either IL-4 or IL-13,[10,11] and secondly these cytokines induce VCAM-1 expression on endothelial cells which may cause the accumulation of eosinophils at the site of the allergic reaction.[12,13]

Recently, three groups have independently stressed the specific role of IL-13 in the effector phase of asthma in mice. In immunised mice, the neutralisation of IL-13 leads to reduction of airway hyper-responsiveness (AHR) and pulmonary mucus formation,[14,15] while neutralisation of IL-4 does not. In addition, administration of IL-13 to non-immunised mice, or selective expression of IL-13 in the lungs of transgenic mice leads to the entire asthmatic phenotype.[14,15,17] Thus, IL-4 may be the most important cytokine for the induction of Th2 responses, including IL-13 production,[18,19] whereas IL-13 dominates the effector phase of asthma. The important role of Th2 cytokines in human asthma is underscored by the fact that T cells, acquired from bronchial biopsies, display an increased capacity to produce IL-4, IL-5 and IL-13.[20–23] For the IL-4 promoter and IL-4 receptor α chain, functional polymorphisms have been reported.[24–27] There is no published information on IL-13 polymorphisms.

Previously we have established that the production of IL-13 by T cells is inhibited by a calcium-inducing signal.[28] The magnitude of this negative signal can be analysed when T cells are stimulated with anti-CD28 and PMA (ie in the absence of a calcium signal), to obtain maximal IL-13 production. Additional stimulation with anti-CD2, anti-CD3 or a calcium ionophore, results in inhibition of IL-13 production on the protein as well as the mRNA level, which is reversed by CsA[28] (TCTM van der Pouw Kraan, personal observations). IL-13 production by T cells from allergic asthma patients is less sensitive for this inhibitory signal.[29] This led us to the hypothesis that an IL-13 promoter polymorphism is responsible for the reduced sensitivity of IL-13 production for the inhibitory signal. Here we describe the identification of such a polymorphism and its relation to allergic asthma.

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Results

Identification of a C to T substitution at position –1055 of the IL-13 promoter that is associated with allergic asthma

The IL-13 promoter –1360 to ± –108 region was analysed by direct PCR sequencing, which allows determination of both alleles simultaneously. After identification of a nucleotide exchange (C to T) immediately adjacent to the consensus NF-AT site at position –1055, we quickly screened the remainder of the samples for the presence of this polymorphism by an oligonucleotide ligase assay (OLA), that specifically detects the genotypes at position –1055. Analysis of 101 allergic asthma patients and 107 non-atopic controls, for the presence of the IL-13 –1055 C to T substitution (Table 1), revealed a higher frequency of homozygous –1055 T carriers in the allergic asthma group (13/101). This was in contrast to 2/107 in the control group, $P = 0.002$, odds ratio = 7.8, RR = 6.9). Within the patient group, the genotypes were not related to total or specific serum IgE levels or BHR.

The –1055 TT genotype is associated with altered regulation of IL-13 production

In our earlier work, it was established that T cell IL-13 production is inhibited by a calcium-inducing signal, which can be reversed by CsA.[28] For part of our currently analysed samples, it was certain through previous work that this negative signal is not presented as effectively in T cells from allergic asthma patients.[29] We used stimulation conditions that allowed us to specifically measure the strength of the inhibitory calcium-dependent signal for IL-13 production. T cells were stimulated with anti-CD28 and PMA to obtain optimal IL-13 production, the additional inhibitory signal was delivered by anti-CD2. In this report we compared these data to the –1055 genotypes in the corresponding subjects. In Figure 1 the distribution of the –1055 genotypes is indicated for both patients and non-atopic controls. Subjects homozygous for –1055 T displayed a significant decreased relative inhibition of IL-13 production upon additional stimulation with anti-CD2, compared to the –1055 CC or CT genotypes ($P = 0.0016$ and $P = 0.0002$, respectively). Within the patient group the –1055 TT genotypes were also significantly less inhibited by additional CD2-stimulation ($P = 0.003$), compared to the remainder of these patients.

The –1055 C to T change results in increased binding of nuclear proteins

To provide an explanation for the functional relevance of the –1055 C to T change we analysed the binding of nuclear proteins to this region using electromobility shift assays (EMSA). As probes we used the NF-AT site containing either a C, or a T at position –1055 (–1055-C or –1055-T). Nuclear proteins of T cells either unstimulated or stimulated with anti-CD3, anti-CD28 and PMA, formed more intense bands with the –1055-T sequence, than with the –1055-C sequence (Figure 2). With extracts of stimulated cells in particular, a high molecular weight complex was clearly visible with the –1055-T-, but not with the –1055-C sequence, while a complex with increased mobility (marked with an asterix) was of equal intensity. These differences in band intensity were consistently observed in three independent experiments and could not be attributed to a difference in specific labelling of the probes. These differences were confirmed with cross-competition experiments using unlabelled –1055-T, –1055-C and an NF-AT sequence present in the IL-4 promoter, in combination with –1055-T as a probe and nuclear proteins from activated T cells (Figure 3). Unlabelled –1055-T effectively competed for the high molecular weight complex, whereas –1055-C hardly affected binding of nuclear proteins to –1055-T. The IL-4 NF-AT sequence, which contains a T at the same position as the –1055-T sequence, also effectively competed, but not the upper band. This indicates that the C to T change at position –1055 of the NF-AT sequence results in increased binding of nuclear proteins.

Table 1 The –1055 TT genotype is more frequent in allergic asthma patients

–1055 Genotype	*Allergic asthma patients n = 101*	*Non-atopic controls n = 107*
CC	57 (56%)	77 (72%) $P = 0.02$
CT	31 (31%)	28 (26%)
TT	13 (13%)	2 (2%) $P = 0.002$

Patients and non-atopic controls were genotyped by sequencing and OLA.

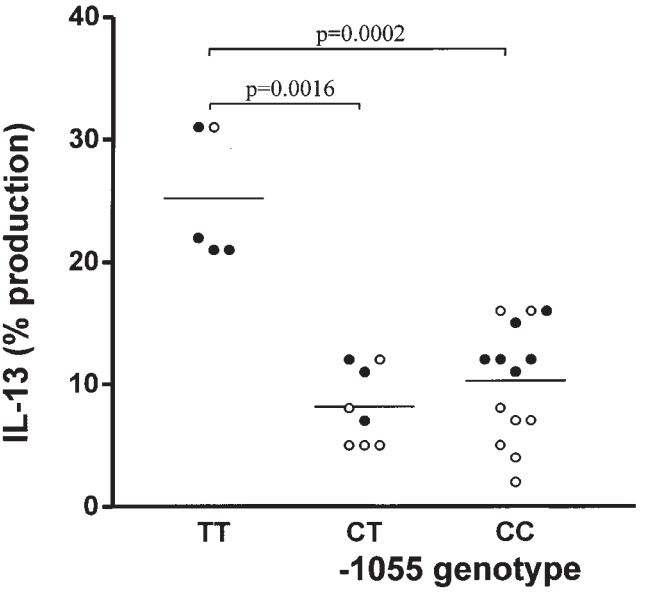

Figure 1 The –1055 TT genotype is associated with reduced inhibition of IL-13 production by anti-CD2. T cells from allergic asthma patients (●) and controls (○) were stimulated for 3 days with anti-CD28 and PMA, in the absence or presence of anti-CD2. The ratio of IL-13 production in the presence and absence of anti-CD2 is shown.

Discussion

The –1055 C to T change in conjunction with the most distal NF-AT consensus sequence (GGAAAA) at position –1061 to –1056 of the IL-13 promoter, is the first polymorphism that is described for IL-13. This polymorphism is particularly interesting due to the observation that several groups have identified the IL-13/IL-4 gene cluster on chromosome 5q31–33 as a region that is associated with

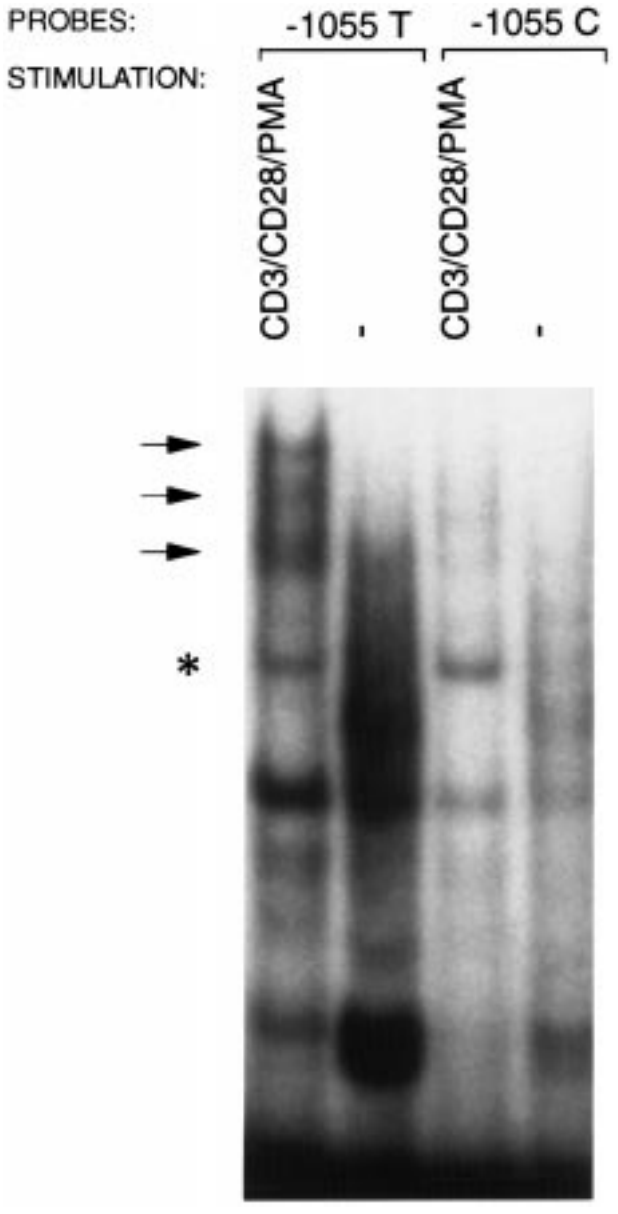

Figure 2 Increased binding of nuclear proteins to the –1055 T allele. ^{32}P-labelled probes of the –1055 T or –C allele were incubated with nuclear extracts from unstimulated (–) or anti-CD3, anti-CD28 plus PMA-stimulated cells. DNA-protein complexes were separated by polyacrylamide gel electrophoresis. One representative experiment out of three is shown. The arrows indicate a high molecular weight complex selectively binding to the –1055-T sequence; the asterix indicates bands of equal intensity formed with nuclear extracts from activated T cells and the –1055-C and –1055-T sequence.

atopy and asthma.[1–6] The observation that the frequency of the –1055 TT genotype is higher in allergic asthma patients compared to non-atopic controls implies that this genotype could be associated with atopy, asthma or both. CsA is known to prevent the nuclear translocation of NF-AT through inhibition of the calcium-dependent phosphatase calcineurin. In the absence of CsA, calcineurin activates NF-ATp/c by dephosphorylation,[30] after which NF-AT translocates to the nucleus where it can bind to specific DNA sequences that control gene transcription. Because CsA up-regulates IL-13 production, we speculated that the binding of NF-AT to the IL-13 promoter inhibits IL-13 production. Interestingly, NF-AT1 (= NF-ATp) or NF-AT4-deficient mice, show an enhanced expression of IL-4 and IL-13.[31,32] In particular the NF-AT1 and NF-AT4 double deficient mice show a dramatic and specific upregulation of Th2 cytokine expression. In these mice NF-ATc is present in large amounts in the nucleus and may be responsible for the observed effects, indicating that NF-AT1 and 4 act as repressors of Th2 cytokine production, while NF-ATc could be a positive regulator. This may also apply to the human IL-13 promoter, which contains three potential NF-AT binding sites. Therefore, alteration of the –1055 sequence at the most distal NF-AT site may influence binding of proteins of the NF-AT family members. The C to T change in the IL-13 promoter closely resembles an *in vitro* nucleotide exchange that has been studied in the human IL-2 promoter NF-AT site. When the GGAAAAAC sequence was changed to GGAAAATT, an increased binding of human nuclear proteins was observed in this region, comparable to our results.[33]

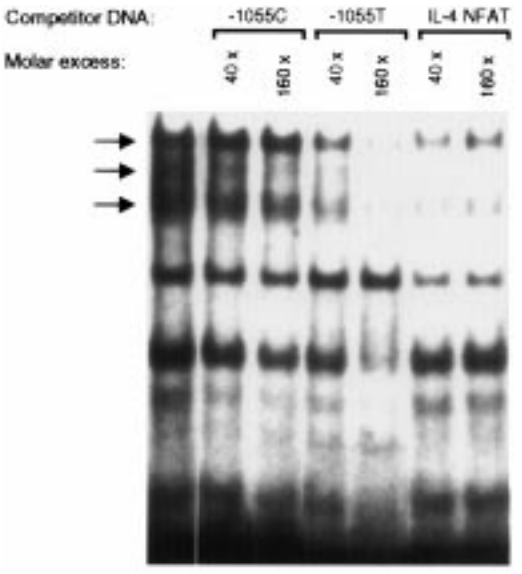

Figure 3 Cross competition of nuclear protein/–1055 T allele interactions. Nuclear extracts from activated T cells (anti-CD3, anti-CD28 and PMA) were incubated with ^{32}P-labelled –1055 T. Protein DNA interactions were competed for by unlabelled –1055 T, –1055 C, or the NF-AT sequence from the IL-4 promoter.

It is also interesting to note that only the –1055 TT genotype is associated with both allergic asthma and altered regulation of IL-13 production. The –1055 CT genotype was equally distributed over patients and controls and also did not differ from the –1055 CC genotype in terms of IL-13 production. This implies that the C allele is dominant. The mechanism of this dominance is at present unclear. An explanation could be provided by the possibility of mono-allelic expression. Expression of only one allele has recently been described for the IL-2 and IL-4 genes.[34–36] In most cells a single allele is expressed, without preference for either allele. This process could be less random when different promoter sequences are present in the two alleles. If the –1055 C allele is preferentially expressed, then only in the absence of a C allele, the T allele would result in a phenotype.

A polymorphism in the IL-4 promoter has been ident-

ified (–590 C to T change) in 15 out of 44 asthma patients, which is associated with higher IgE levels within this group and increased binding of nuclear proteins to this region.[26] Two other functional polymorphisms are described in the IL-4 Receptor α chain, located on chromosome 16. The alleles leading to an increased sensitivity to IL-4 are strongly associated with atopy (defined by enhanced serum IgE levels or specific IgE), and with atopic asthma.[24,25] We cannot exclude the possibility that the association of the IL-13 polymorphism with allergic asthma, is a reflection of a linkage disequilibrium with another polymorphism in the 5q31 region. However the observations that this polymorphism leads to altered IL-13 production and increased binding of nuclear proteins to this region, suggest that the polymorphism itself is involved in the genetic susceptibility to allergic asthma. The ultimate goal of genetic studies will be the identification of combinations of several polymorphic genes that may cooperate, either independently or in synergy, to influence the allergic asthma phenotype.

Materials and methods

Patients and control subjects

One hundred and one allergic asthma patients were recruited from the Outpatient Department of Pulmonology. Asthma was defined according to the criteria of the American Thoracic Society.[37] Atopy was defined by a positive skin test and a positive RAST for at least one inhalant allergen established during the previous 3 years. One patient was only positive in the skin prick test. Most patients displayed enhanced serum IgE levels, geometric mean of serum total IgE was 237 IU/ml. The geometric mean provocative concentration causing a 20% fall in FEVI (PC20) was 1.4 mg histamine or methacholine/ml. The study was approved by the local medical ethics committee and written consent was given by all patients. Healthy subjects without specific IgE for common inhalant allergens (RAST negative) and serum IgE levels below 100 IU/ml were selected as controls.

Sequence analysis of the IL-13 promoter

Genomic DNA was used as a template for amplification of the IL-13 promoter by PCR. The following primersets were used: (all containing a 5′ M13 sequence in the sense primer) region –1340 to –726: 5′TGTAAAAC GACGGCCAGTAGAGGGTGGGAATGACGTTC3′ and 5′ACTCCCAGACTCAAGCGATC3′, region –934 to –277: 5′TGTAAAACGACGGCCAGTAGACTGGTGAGCAAA GGGGATCAC3′ and 5′TCCAGCAGTTTTGCCTGTGA CAAC3′, region –566 to +97: 5′TGTAAAAC GACGGCCAGTACAGTGGGGCCAAG GAGGAATTAG3′ and 5′AAGGCAAGTGAGAGCAATGACCG3′ (GibcoBRL, Life Technologies, Gaithersburg, MD, USA). Thereafter PCR products were sequenced using the DYEnamic Direct cycle sequencing kit (Amersham, Bucks, UK), DYEnamic –21 M13 forward ET primers (Amersham) and the Applied Biosystems Model 373A DNA Sequencing System.

Oligonucleotide ligase assay (OLA)

PCR products (of the IL-13 promoter region –1340 to –726) were subjected to the OLA.[38] Two separate ligation reactions were carried out with either 5′ biotinylated –1055 C primer; 5′TGGAGGACTTCTAGTAAAAC3′ or 5′ biotinylated –1055 T primer; 5′TGGAGGACTTCTAG GAAAAT3′, both together with 3′ digoxygenine-labelled primer; 5′GAGGGAAGAGCAGGAAAAGG3′. The –1055 C primer contains a mismatch at position –1060 to increase specificity. Ligation of biotinylated primers with the digoxygenine-labelled primer was detected in streptavidin-coated microtiter plates (Pierce, Rockford, IL, USA) after incubation with alkaline phosphatase-labelled anti-digoxygenin antibodies (Boehringer) and substrate (BRL ELISA amplification system). Plates were read at 492 nm.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Peripheral blood mononuclear cells were stimulated with anti-CD3 (CLBT3/4.E) at 1 μg/ml, anti-CD28 (CLB-CD28) at 5 μg/ml and PMA at 10 ng/ml or left unstimulated at a density of 5×10^6/ml for 24 h. Nuclear extracts were prepared by the method described by Ohlsson.[39] Binding reactions were carried out as previously described[40] with minor modifications; binding reactions contained 5–15 μg nuclear protein, 5 mm Tris-HCl pH 7.5, 100 mm NaCl, 1 mm EDTA, 1 mm DTT, 1 × Complete protease inhibitor (Complete, Boehringer Mannheim, Germany), 5% glycerol, and 1 μg poly(dI-dC):(dI-dC) in a total volume of 25 μl. As probes we used: –1055-C (5′gatcGGACTTCTAGGAAAACGAGG GAAGAG3′), –1055-T (5′gatcGGACTTCTAGGAAAA TGAGGGAAGAG3′) and IL-4 NF-AT (5′gatc TTTACATTGGAAAATTTTATG3′) oligonucleotides.[41] Complexes were separated on 4% polyacrylamide gels.

Statistics

Frequencies of different genotypes were compared in the Fisher's exact test. For comparison of serum IgE, PC20 and IL-13 production in different genotype groups we used the Mann-Whitney U test. Two-tailed P values <0.05 were considered significant.

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