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ORIGINAL INVESTIGATION

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TGFβ1 allele association with asthma severity

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Abstract Transforming growth factor β1 (TGFβ1) is a multifunctional cytokine involved in pro- and anti-inflammatory pathways and is expressed in several cell types. Subepithelial fibrosis is one of the principle features of airway remodelling in asthma and is increased in severe patients. TGFβ1 is implicated in fibrosis, including the deposition of extracellular matrix proteins. TGFβ1 mRNA levels in eosinophils are increased in severe asthmatics relative to mild asthmatics. Therefore, TGFβ1 is a promising candidate gene for contributing to asthma severity. Four polymorphisms located in the promoter region and signal peptide (C–509T, 72insC, T869C and G915C) were genotyped in groups of severe asthmatic, mild asthmatic or control individuals defined by steroid usage and pulmonary function. Significant differences (P=0.016) were found between the groups for the genotype frequencies at C–509T, attributable mainly to a greater relative frequency of homozygosity for the –509T allele in the severe group compared to the mild and control groups. Individuals homozygous for –509T were also homozygous at the other variant sites for the 72C, 869C and 915G alleles (haplotype 1). The T allele creates a putative YY1 transcription factor binding site, but binding between YY1 and the DNA sequence of the T allele was not detected in vitro. In this study, we show that the –509T variant on haplotype 1 is the most informative marker of the TGFβ1 contribution to asthma severity.

Introduction

Asthma is a complex polygenic disorder, resulting from a combination of genetic and environmental factors. The severity of the disease phenotype varies between individuals and it is likely that the inheritance of certain combinations of polymorphisms in a number of genes will comprise the genetic component of asthma severity.

Candidate genes for asthma severity include those involved in chronic airway inflammation, treatment response (particularly to corticosteroids) and airway remodelling. This is in contrast to genes thought to be associated with the 'initiation' of asthma, such as those involved in the control of IgE levels and bronchial hyperreactivity, for which genome screens have been performed (Daniels et al. 1996; Collaborative Study on the Genetics of Asthma 1997; Hizawa et al. 1998; Ober et al. 1998; Wjst et al. 1999). Polymorphisms associated with severity (based on FEV_1) have been identified in the pro-inflammatory genes IL-4 (Chouchane et al. 1999; Sandford et al. 2000; Burchard et al. 1999) and TNF-α (Chagani et al. 1999), the IL-4 receptor (Rosa-Rosa et al. 1999) and the anti-inflammatory gene IL-10 (where severity was based on steroid usage) (Lim et al. 1998). The Gly16 variant of the β-adrenergic receptor has been shown to be associated with severe, steroid-dependent asthma (Reihsaus et al. 1993), but other studies have found no association between the receptor and asthma severity (Weir et al. 1998; Mak et al. 1999). No genetic associations with airway remodelling in asthma severity have previously been reported.

One of the principle features of airway remodelling is subepithelial fibrosis, which is increased in severe asthmatics (Minshall et al. 1997). Transforming growth factor β1 (TGFβ1) is implicated in several aspects of fibrosis, including the deposition of extracellular matrix proteins such as collagens and fibronectin (Roche et al. 1989; Sime et al. 1997; Ignotz et al. 1986). TGFβ1 mRNA levels in eosinophils are significantly increased in patients with severe asthma compared to mild asthma (Minshall et al. 1997; Ohno et al. 1996).

TGFβ1 is, therefore, a promising candidate gene for asthma severity. Previous studies have reported a single polymorphism in the TGFβ1 promoter (C/T at position –509) that has been linked to elevated TGFβ1 plasma levels (Grainger et al. 1999) and IgE levels (Hobbs et al.

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1998), and induces a YY1 consensus binding site within a negative regulatory region of TGFβ1 transcription.

Four polymorphisms located in the promoter region and signal peptide (Derynck et al. 1985; Kim et al. 1989) were genotyped in groups of individuals classified as severe or mild asthmatic based on steroid usage and pulmonary function, and also control samples from a blood bank population. Three of the variants were single nucleotide polymorphisms (SNPs), C–509T, T869C and G915C, and the remaining variant was an insertion of an additional C nucleotide into a run of seven consecutive Cs at position 72 (72insC). If there was an association between a variant and asthma severity, a difference in genotype frequency would be expected between the phenotypic groups.

Materials and methods

Patients

Patients with mild asthma (Table 1) suffered intermittent symptoms treated with infrequent (<2 times per week) short-acting inhaled β_2-agonists, did not use maintenance inhaled corticosteroids and had normal lung function. Patients with severe asthma (Table 1) had daily symptoms requiring regular inhaled β_2-agonist therapy and high-dose inhaled (≥800 μg/day beclomethasone dipropionate or equivalent) or oral corticosteriods, and had impaired lung function. A group of anonymous control samples were obtained from a blood donor clinic to reflect the random population. All subjects were white Caucasians. Mild asthmatics were recruited for clinical trials and patients with severe asthma from outpatient clinics at the Royal Brompton Hospital, London, UK. The study was approved by the Research Ethics Committee of the Royal Brompton Hospital and each subject gave written informed consent.

Genotyping

Ten millilitres of venous blood was taken from each patient and stored at –70 °C. Genomic DNA was extracted from whole blood using DNeasy tissue kit (Qiagen, Crawley, UK).

PCR reactions

Primers pairs were designed to amplify regions containing the polymorphisms from GenBank (J04431 and J05114), (C–509T: 5′-GGGGACAGTAAATGTATGG-3′, 5′-TGAGACACAGGGGAGCC-3′; 72insC: 5′-ACCTCCCTCCGCGGAGC-3′, 5′-TCTCCCGACCAGCTCGTCC-3′T869C: 5′-GTGCCGGGGGGCGCCGCCTCCCCCATGCCTCCCTC-3′, 5′-CAGCACCAGTAGCCACAGCAGCGGTAGCCGCAGC-3′; G915C: 5′-CGCTGCTGTGGCTACTGGT-3′, 5′-CTCCGGTTCTGCACTCTCC-3′). The T869C primers have one altered nucleotide in each (underlined) from the genomic sequence in order to create a *Bgl*I site to detect the variant, and to destroy an additional site that would have resulted in a digest product too small to detect on an agarose gel. The C–509T variant was amplified using the BIOTAQ DNA polymerase kit (Bioline, London, UK). Each 10 μl PCR reaction contained 50 ng DNA, NH_4 reaction buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer and 0.5 U *Taq* polymerase. Cycling conditions were as follows: 94 °C for 10 min, 35 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, followed by 72 °C for 10 min. All other variants were amplified using the GC-rich PCR system (Roche, Lewes, UK). The 10 μl reactions contained 50 ng DNA, Reaction Buffer (including 1.5 mM $MgCl_2$), 1–2 M Resolution Solution, 200 μM dNTP (except for the 72+C PCR, which used 200 μM dATP, dTTP, dGTP and 2 μM of dCTP), 5 pmol of each primer and 0.4 U Enzyme Mix. For radioactive analysis of the 72insC variant, 0.01 μl of α-^{32}P dCTP was added to the reaction. Cycling conditions were as above, but using an annealing temperature of 52 °C.

Table 1 Clinical details of subjects. Mean values for the phenotypic groups are given

	Severe asthmatics	Mild asthmatics
Mean age (years)	44.8	27.9
Number of females	79	30
Number of males	43	61
Mean FEV_1 (forced expiratory volume in 1 s) % predicted	63.5	90.9

Restriction digests

C–509T, T869C and G915C were detected by the creation or loss of a restriction specifications [*Dde*I (Promega), *Bgl*I and *Fse*I (New England Biolabs, Hitchin, UK)]. Products were electrophoresed on 4% agarose gels and samples genotyped from the digested band sizes.

Size variation detection

The 72insC variant was detected due to size variation by electrophoresing denatured PCR product on a 6% polyacrylamide gel at 65 W.

Electrophoretic mobility shift assay (EMSA)

The YY1-GST expression plasmid was kindly given by V. Janssens, Leuven, Belgium. Expression in BL21 cells and purification of the fusion protein was performed as described for the pGEX system (Pharmacia, Little Chalfont, UK). One pair of complementary oligonucleotides was designed for each variant (–509C: 5′-ACCCTTCCATCCCTCAGGTGTCCT-3′, 5′-AGGACACCTGAGGGATGGAAGGGT-3′; and –509T: 5′-ACCCTTCCATCCTTCAGGTGTCCT-3′, 5′-AGGACACCTGAAGGATGGAAGGGT-3′; the variant nucleotides are underlined). The oligonucleotides were annealed as described by Newton and Adcock (2000). Annealed –509C and –509T oligonucleotides, plus a consensus YY1 double stranded oligonucleotide (Santa Cruz Biotechnology, Santa Cruz, USA), were radiolabelled (γ-^{32}P ATP) using T4 PNK (Bioline, London, UK). Radiolabelling, binding reactions, nondenaturing PAGE and autoradiography were all carried out as described by Newton and Adcock (2000). Ten microgrammes of YY1-GST protein was used per binding reaction, and competition experiments were performed by adding unlabelled probe at a concentration 100 times that of the labelled probe to be used in the non-competition reactions, followed by addition of the radiolabelled probe 15 min later. To prepare A549 nuclear lysate, confluent cells were stimulated with IL-1β (1 ng/ml) or TNF-α (10 ng/ml) and the nuclear lysate was then extracted as described by Newton and Adcock (2000). Ten microgrammes of lysate was used per binding reaction as above.

Statistical analysis

Genotype frequencies were analysed using the chi-squared test and *P*-values calculated using the Instat programme.

Transcription factor binding site databases

Sequences were searched for potential transcription factor binding sites at the following website: http://transfac.gbf.de/TRANSFAC.

Results

One hundred and twenty-two severe asthmatic, 91 mild asthmatic and 122 control subjects were genotyped at the four polymorphic sites: C–509T, 72insC, T869C and G915C. Genotype frequencies within the phenotypic groups are detailed in Table 2. Using the chi-squared (χ^2) test, a significant difference (P=0.016) was found between the groups for the genotype frequencies of just one of the polymorphisms, the C–509T variant. This result was largely attributable to a greater proportion of individuals with the T,T genotype in the severe group relative to the other two groups. A significant difference was also detected for this variant when the mild asthma group alone was compared to the severe group (P=0.034). The difference was even more significant however when the genotypes of just the severe and control groups were compared (P=0.004). The difference between the mild asthmatic and the control group frequencies was only just significant (P=0.040).

It has been predicted that the T allele creates a YY1 transcription factor binding site (Hobbs et al. 1998), and so an EMSA reaction was carried out to compare the binding of YY1 to regions of the TGFβ1 promoter sequence containing either the C or T nucleotide. Binding was not observed for either variant. To investigate whether other proteins show a binding preference for either variant, a second EMSA reaction was performed using nuclear lysate from an A549 cell line. Again binding was not detected to either variant. In addition, a transcription factor binding site database was screened using each allele sequence, but no binding site matches were found.

It was noted that individuals homozygous for –509T were also homozygous at the other variant sites for the 72C, 869C and 915G alleles. Hence each individual carried two copies of the same haplotype, which was termed haplotype 1 (Table 3). The three haplotypes were deduced from individuals homozygous at all four polymorphic sites (Table 3). These three haplotypes can account for each individual's pair of haplotypes in all the three groups, although it has not been proven that additional less common haplotypes do not occur in the heterozygous individuals.

Table 2 The frequencies of genotypes at the four polymorphic sites within each phenotype group

Variant	Genotype	Frequency within group (%)		
		Severe asthmatic	Mild asthmatic	Control
–509	C,C	48.4	58.2	54.1
	C,T	37.7	34.1	43.4
	T,T	13.9	7.7	2.5
72insC	7,7	87.7	81.3	77.9
	7,8	12.3	18.7	21.3
	8,8	0	0	0.8
869	T,T	39.3	39.6	39.3
	T,C	41.8	46.1	50.0
	C,C	18.9	14.3	10.7
915	G,G	87.7	81.3	77.9
	G,C	12.3	18.7	21.3
	C,C	0	0	0.8

Discussion

A major characteristic of asthma severity is an increase in subepithelial airway fibrosis, which may be promoted by TGFβ1. One or more polymorphisms in the TGFβ1 gene acting to increase the level or the function of the protein may be associated with asthma severity. To investigate this, four polymorphic sites, C–509T, 72insC, T869C and G915C, in the promoter and signal peptide regions of TGFβ1 were genotyped in three phenotypic groups of severe or mild asthmatics and control individuals. Three haplotypes were deduced (Table 3), which were all present in each cohort.

If there was an association between a polymorphism and asthma severity, a difference in genotype frequency would be expected between the mild and severe groups. A significant difference was found between the genotype frequencies of the C–509T variant when the severe and mild asthma groups and controls were compared. This was mainly attributable to a higher level of homozygosity for –509T in the severe group. Almost twice as many (1.8 times) severe asthmatics carried the T,T genotype compared to the mild asthmatics, and this difference was increased to 5.6 times relative to the controls. A significant difference also existed between the severe and mild groups alone and a lower level of significance was observed for the difference between the mild asthmatics and controls. These results suggest that the allele is associated with the severity rather than the initiation of asthma or atopy, in contrast to studies where polymorphisms have

Table 3 Three haplotypes identified within the asthma phenotype groups. Note that the haplotype frequencies are estimated assuming that only the three known haplotypes exist in heterozygote individuals

Haplotype number	Haplotype				Frequency		
	–509	72	869	915	Severe	Mild	Control
1	T	7	C (Pro)	G (Arg)	0.34	0.25	0.24
2	C	7	T (Leu)	G (Arg)	0.60	0.66	0.64
3	C	8	C (Pro)	C (Pro)	0.06	0.09	0.11

been compared between asthmatics and controls, such as in IL-13 (Heinzmann et al. 2000).

There are three possible explanations for the –509T association with asthma severity. Firstly, –509T may be the functional variant in the TGFβ1 association with asthma severity. Since the variant is within the promoter region of TGFβ1 (Kim et al. 1989), it may act to create or destroy a transcription factor binding site. However, although the T allele creates a putative YY1 binding site (Hobbs et al. 1998), binding between YY1 and the DNA sequence was not detected in vitro. It is possible that the site for another binding protein could be affected, but the absence of binding between proteins expressed by lung epithelial A549 cells, in which TGFβ1 is expressed (Vodovotz et al. 1999), and the T and C alleles, does not favour this hypothesis. In addition, no transcription factor binding sites in two databases matched to either allele. This is consistent with the non-significant result obtained when the –509 alleles were compared for TGFβ1 activity using a luciferase reporter gene assay (Luedecking et al. 2000), although a slight increase was observed for the T allele. However, it is possible that the conformation of the native promoter allows distinct co-activators or transcription activator factors (TAFs) to bind to upstream regions altering the subsequent expression of TGFβ1, which may be affected by the sequence variant.

A second possible explanation for the –509T association is that the variant is in linkage disequilibrium with the true functional polymorphism. The –509T allele is the only marker for haplotype 1, and so in studies where associations have been found between –509T and an increase in TGFβ1 plasma levels (Grainger et al. 1999) and IgE levels (Hobbs et al. 1998), the allele may in fact be in linkage disequilibrium with the true causative variant linked elsewhere on haplotype 1. None of the other three tested variants were significantly associated with severity when analysed alone, implying that it must be another variant linked elsewhere on the chromosome. SNPs are estimated to occur approximately one in every 350 bp of DNA (Cargill et al. 1999), so several additional variants in the gene would be expected.

Finally, the TGFβ1 contribution to asthma severity may comprise the interaction of two or more polymorphisms linked to haplotype 1. This phenomenon has been identified in the β_2-adrenergic receptor gene, where associations were found between bronchodilator response to β-agonist in asthmatics and haplotype pairs, but not individual SNPs (Drysdale et al. 2000).

In conclusion, this study suggests that although there is no functional evidence for the associated individual polymorphism, the TGFβ1 –509T variant on haplotype 1 is the most informative marker of the TGFβ1 contribution to asthma severity. However, in order to substantiate this positive finding further, confirmation of the same association in other studies and population groups is necessary.

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