

A polymorphism in the promoter region of the human interleukin-16 gene is not associated with asthma or atopy in an Australian population

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Summary

Background IL-16 is an immunomodulatory cytokine whose expression is increased in the bronchial mucosa, bronchoalveolar lavage fluid and induced sputum of asthmatic patients. It has been suggested that IL-16 has a regulatory role in the pathophysiology of asthma. A single-nucleotide polymorphism (T₋₂₉₅C) has been described in the promoter region of the gene and it has been hypothesized that this polymorphism may be associated with altered levels of IL-16 expression, and account for the increased levels of IL-16 seen in the asthmatic airway.

Objective To determine the association between the T₋₂₉₅C promoter polymorphism and asthma, disease severity and atopy in a large Australian Caucasian population.

Methods We used PCR and restriction fragment length polymorphism analysis to establish the allele frequency of the T₋₂₉₅C promoter polymorphism in a random Australian Caucasian population ($n = 176$) and to characterize the polymorphism in a large Australian Caucasian population of mild ($n = 273$), moderate ($n = 230$) and severe ($n = 77$) asthmatic patients, and non-asthmatic controls ($n = 455$). Genotype association analyses were performed using χ^2 tests.

Results The polymorphic C allele was present in 19% of the asthmatic population and 21% of the non-asthmatic population. There was no association between the polymorphism and asthma ($P = 0.153$) nor with asthma severity ($P = 0.417$) or atopy ($P = 0.157$) in this population.

Conclusion Although it has been hypothesized that the T₋₂₉₅C promoter polymorphism may be associated with increased IL-16 gene expression, it is not associated with asthma, disease severity or atopy in this Australian population.

Keywords asthma, atopy, Australian population, IL-16, single-nucleotide polymorphism

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Introduction

Asthma is a chronic respiratory disease characterized by reversible bronchoconstriction, airway hyper-responsiveness and inflammation. Infiltration of the airways by a variety of inflammatory cells including eosinophils, mast cells and T lymphocytes, is a characteristic feature [1]. T-helper lymphocytes (Th cells) are present in increased numbers [2] and have a central role in promoting airway inflammation through the synthesis and release of inflammatory cytokines and mediators [3, 4].

IL-16 is an immunomodulatory cytokine that acts as a chemoattractant for CD4⁺ cells and contributes to their recruitment and activation at sites of inflammation associated with asthma [5]. Notably, in 1993, asthma was the first disease shown to be directly associated with increased IL-16 expression [5, 6]. Increased levels of IL-16 have been

demonstrated in the bronchial mucosa of the asthmatic lung [2], and the level of IL-16 expression within the bronchial epithelium correlates with CD4⁺ cell infiltration, including Th cells [7]. Allergen challenge leads to a further increase in the levels of IL-16 in the airways of atopic asthmatic subjects, suggesting that a phenotypic change in atopic asthma patients, such that they are primed for increased IL-16 production [8]. IL-16 exerts its biological activities through direct stimulation of the surface molecule CD4, which is present on a number of inflammatory cells including eosinophils and Th cells [8], although recent findings suggest that other receptors independent of CD4 may also be involved [9]. IL-16 is involved in promoting Th cell migration, proliferation and synthesis of inflammatory cytokines [5], responses that are involved in the development of airway inflammation in asthma [1].

It appears that IL-16 expression is complex and controlled at both the transcriptional and post-translational levels [10]. The gene encoding IL-16 is located on chromosome 15q26.3 [11] and consists of seven exons and six introns [8], with a well-characterized promoter region [10]. A DNA fragment spanning nucleotides -408 to $+88$ (relative to the

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transcription start site) has been found to exert constitutive promoter activity [10]. Recently, a single-nucleotide polymorphism (SNP) was described in the IL-16 gene (T₋₂₉₅C) in this region of the promoter, and it has been hypothesized that this polymorphism may be associated with altered levels of gene expression [12], and may partially account for the increased levels of IL-16 seen in the asthmatic airways. This polymorphism has not been previously studied in asthma, however, recent studies have suggested that this polymorphism may be involved in the development of other inflammatory diseases including inflammatory bowel disease [13] and allergic contact dermatitis [14].

We hypothesized that individuals with the IL-16 T₋₂₉₅C promoter polymorphism may be at increased risk of developing airway inflammation and asthma, and may be more likely to have increased disease severity, or an increased risk of developing atopy. We investigated these associations in a large well-characterized Australian Caucasian population of asthmatic subjects and non-asthmatic controls.

Methods

Subjects

In order to determine the frequency of the T₋₂₉₅C polymorphism in the general population, we analysed this polymorphism in DNA from a random Australian Caucasian population ($n = 176$), collected from random blood donors at the Red Cross Blood Bank in Perth, Western Australia. A further 455 control individuals with no evidence of asthma and 580 randomly recruited subjects with doctor-diagnosed asthma, including 273 individuals with mild asthma, 230 individuals with moderate asthma and 77 individuals with severe asthma participated in the association study. All subjects were unrelated and completed a detailed questionnaire and spirometry. Blood samples were obtained for DNA extraction. All individuals were tested for atopic status by skin prick test, with a positive reaction resulting in a weal of 3 mm or greater diameter than the saline control to at least one of the five aeroallergens tested (including cat, dog, house dust mite, mould mix and grass pollen mix). Patients with either (a) a smoking history prior to their diagnosis of asthma of more than 10 pack-years, or (b) a smoking history of 5–10 pack-years with other evidence of chronic obstructive pulmonary disease, were excluded from the study. The study protocol was approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee and all subjects provided informed, written consent to participate in this study.

Classification of asthma severity

To assist in allocating patients to groups with varying degrees of severity, criteria specified by the Australian National Asthma Council [15] and the National Asthma Education and Prevention Program [16] were used. The following criteria were used to assess asthma severity: (i) lung function (% predicted FEV₁); (ii) use of oral corticosteroids in the last 12 months; (iii) daily inhaled corticosteroid (beclomethasone equivalent) dose; (iv) weekly frequency of use of rescue medication; (v) weekly frequency of daytime symptoms; (vi)

weekly frequency of night-time waking because of asthma; (vii) unplanned visits to a general practitioner in the last 12 months; (viii) hospital admissions for asthma in the last 12 months. Patients were classified as having mild, moderate or severe asthma with respect to their score for each criterion and were placed in the category in which they were classified for the majority of criteria.

Molecular methods

DNA was extracted from buffy coats using a commercially available DNA extraction kit (Qiagen, Hilden, Germany), following the manufacturers' instructions.

PCR and restriction fragment length polymorphism (RFLP) analysis was optimized following a previously described protocol [12]. The PCR reaction uses a mutagenized primer pair and the underlined base shows the modification introduced in the forward primer to create a restriction site recognized by restriction endonuclease *AhdI*: 5'-CTC CAC ACT CAA AGC CTT TTG TTC CTA TGA-3' and 5'-CCA TGT CAA AAC GGT AGC CTC AAG C-3'. The PCR reaction contained 1 × PCR buffer (Qiagen), 1 × Q solution (Qiagen), 2 mM MgCl₂, 200 μM each of dATP, dTTP, dCTP and dGTP (Promega Corporation, Madison, WI, USA), 20 pmol of each primer and 1 U of *Taq* DNA polymerase (Qiagen). PCR amplification conditions were optimized with an initial denaturation step at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min; and a final extension step at 72 °C for 10 min in a thermal cycler (Eppendorf, Hamburg, Germany).

Following PCR, RFLP analysis was used to detect the presence of the T₋₂₉₅C polymorphism on the basis that the restriction endonuclease *AhdI* (New England Biolabs, Beverly, MA, USA) recognizes the restriction sequence GACNNN^{*}NNGTC, present only in amplified DNA fragments containing the C₋₂₉₅ allele. The restriction mix contained 1 × restriction buffer 4 (New England Biolabs), 1 × bovine serum albumin (New England Biolabs) and 2 U *AhdI* (New England Biolabs). Restricted products were electrophoresed on 3% agarose gels (Amresco, Cleveland, OH, USA). Expected fragment sizes were 280 bp for the T₋₂₉₅ allele, and 246 bp for the C₋₂₉₅ allele. Figure 1 shows an example of PCR-RFLP analysis for the IL-16 T₋₂₉₅C polymorphism.

Statistical methods

Genotype and allele frequencies were calculated for the random Australian Caucasian group and each patient group.

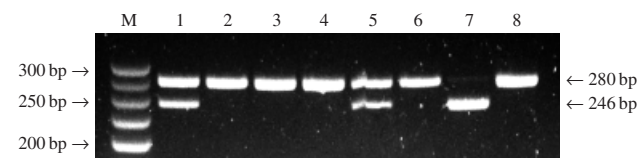


Fig. 1. PCR and restriction fragment length polymorphism (RFLP) analysis for IL-16 T₋₂₉₅C. PCR-RFLP analysis of IL-16 promoter polymorphism in eight unrelated subjects: lanes 2, 3, 4, 6 and 8, homozygous for the T₋₂₉₅ allele (TT); lanes 1 and 5, heterozygous for the T₋₂₉₅ allele and the C₋₂₉₅ allele (TC); lane 7, homozygous for the C₋₂₉₅ allele (CC); M, 25 bp DNA Step Ladder (Promega Corporation).

Table 1. Demographics of the study population

Phenotype	n	Females (%)	Atopy (%)	Mean age (years $\pm$ SD)	% predicted FEV ₁ (mean $\pm$ SEM)	Oral corticosteroids* (%)	Frequency of oral corticosteroid use (mean times/year)	Inhaled corticosteroids† (%)	Mean inhaled corticosteroid dose‡ (mcg/day $\pm$ SEM)	β ₂ -adrenoceptor agonists§ (%)
Non-asthma	455	55.6	49	50.6 $\pm$ 14.2	101.7 $\pm$ 2.5	Nil	Nil	Nil	Nil	Nil
All asthma	580	63.3	83.1	49.1 $\pm$ 15.5	—	—	—	—	—	—
Mild asthma	273	61.5	85.0	47.2 $\pm$ 15.1	92.2 $\pm$ 1.0	5	1.3	59.0	890 $\pm$ 49	82.0
Moderate asthma	230	64.8	81.3	50.8 $\pm$ 15.9	74.2 $\pm$ 1.4	38	1.5	95.6	1475 $\pm$ 65	94.3
Severe asthma	77	64.9	81.8	50.6 $\pm$ 14.6	61.3 $\pm$ 3.2	64	2.1	100	2530 $\pm$ 174	100

*Percentage of patients who had used oral corticosteroids in the last 12 months. †Percentage of patients using inhaled corticosteroids. ‡Percentage of patients using β₂-adrenoceptor agonists.

Comparisons of allele and genotype distribution were performed using χ^2 tests, using the R Statistics Program [17]. The power of the completed experiment was determined using GraphPad StatMate version 2 for Windows (GraphPad Software, San Diego, CA, USA), where $\alpha = 0.05$ (two-tailed) and $\delta = 0.80$. This study had 80% power to detect a change in odds ratio (OR) of 1.28, with a significance level of 0.05.

Results

The allelic frequency of the IL-16 T₋₂₉₅C polymorphism in random Australian Caucasian subjects was 0.216 and was therefore considered likely to be informative in this population.

Association analyses

A population of 1035 unrelated Australian Caucasian subjects were recruited. All subgroups had similar age and gender distributions, although the asthma group as a whole had a significantly higher percentage of atopic subjects (83%) when compared with the non-asthmatic control group (49%) ($P < 0.0001$). Daytime symptoms of asthma occurred, on average, between 3.9 times per week in individuals with mild asthma, 6.8 times per week in individuals with moderate asthma and nine times per week in individuals with severe asthma. Only half the patients with mild asthma ever woke from sleep because of their asthma, while all patients with severe asthma woke from sleep every night because of their asthma. Unplanned visits to the GP over the last 12 months varied between 0.51 per year in patients with mild asthma, 1.4 per year in patients with moderate asthma and 4.2 per year in patients with severe asthma. Hospital admissions over the last 12 months had occurred in less than 5% of patients with mild asthma, in 30% of patients with moderate asthma and in greater than 70% of patients with severe asthma. Further characteristics of these groups are presented in Table 1.

Genotypic distributions (Table 2) conformed to Hardy–Weinberg equilibrium in all groups studied ($P \geq 0.126$). Atopic status was significantly associated with asthma in our population ($P < 0.0001$). There was no significant difference in allele or genotype frequencies between phenotypic groups, and no association between the C₋₂₉₅ polymorphism and asthma (OR = 1.00, 95% confidence intervals 0.95–1.06; $P = 0.153$), disease severity ($P = 0.417$) or atopy ($P = 0.157$). There was also no association between

Table 2. Genotype and allele association analyses for IL-16 T₋₂₉₅C polymorphism

Subject group	n	Genotype (%)			Frequency of C allele
		TT	TC	CC	
Random Australian Caucasian	176	60	36	3	0.216
Non-asthmatic controls	455	67	28	5	0.188
All asthma	580	62	34	4	0.211
Mild asthma	273	60	37	4	0.220
Moderate asthma	230	62	33	5	0.217
Severe asthma	77	70	27	3	0.162
Non-atopic	329	64	31	5	0.208
Atopic	705	64	32	4	0.198

T₋₂₉₅C genotypes and atopic status within the asthmatic population ($P = 0.626$). The tight confidence intervals for asthma, with a half-width of 0.05, strongly exclude most effects.

Discussion

We hypothesized that individuals carrying the IL-16 T₋₂₉₅C promoter polymorphism would be at increased risk of developing asthma or atopy, or developing more severe disease. However, in a comprehensive study of this polymorphism in a large, well-defined asthma population, we have shown no association between the promoter polymorphism T₋₂₉₅C in the IL-16 gene and asthma, disease severity, or atopy. To our knowledge, this is the first study that has investigated the association between the IL-16 T₋₂₉₅C polymorphism and these phenotypes.

Asthma is a chronic respiratory disorder that affects one in four children [18] and one in 10 adults [19] in Australia. At present, inhaled glucocorticosteroids have a major role in the management of asthma in Australia, however, a significant number of patients have severe disease despite regular therapy with both inhaled and systemic glucocorticosteroids [20]. As such there is an ongoing need to investigate the mechanisms of asthma and to determine the relevant genes and genetic polymorphisms so as to optimize current therapies and to develop new treatments for asthma.

IL-16 was initially termed Lymphocyte Chemoattract Factor after its discovery in 1982 and was designated IL-16 in 1995 [21]. T lymphocytes, eosinophils, mast cells, epithelial cells, dendritic cells, fibroblasts and neuronal cells are all important cell types in asthma and are known to be capable of synthesizing IL-16 [8]. Asthma is associated with increased expression of IL-16 [5]. IL-16 has been identified in the bronchoalveolar lavage fluid (BALF) obtained 4 h after allergen challenge in subjects with asthma, representing approximately 80% of total chemoattractant activity, while it was not detected in BAL of non-asthmatic individuals, irrespective of atopic status [8]. It has been postulated that a phenotypic change occurs in the airway epithelium of asthmatic subjects such that they are primed for increased IL-16 production [8].

A T₋₂₉₅C polymorphism is present within a region shown to be necessary for promoter activity of the gene [10] and while the functional significance of this polymorphism is unknown, it is reasonable to hypothesize that it may alter levels of gene expression, resulting in increased levels of IL-16 and the recruitment, proliferation and activation of Th cells. This in turn may result in increased airway inflammation characteristic of asthma.

Although it has been shown that the IL-16 T₋₂₉₅C promoter polymorphism is significantly associated with allergic contact dermatitis, suggesting that genetic variation in IL-16 may play a role in allergic sensitization and the development of contact dermatitis [14], we have not been able to show a similar association with asthma.

We have shown that this polymorphism is present in a random Australian Caucasian population at a frequency of 0.216. This is consistent with previously published results that indicate approximate allele frequencies of 0.22 and 0.18 in

individuals from Japanese and Thai populations, respectively [12], and 0.22 in individuals from a German population [13]. Moreover, the results of this study suggest that Caucasian individuals with this promoter polymorphism are not at increased risk of developing asthma or atopy, nor are they at increased risk of having more severe asthma, as there is no significant difference in the polymorphism frequency between individuals with asthma and individuals without asthma, regardless of their disease severity.

In conclusion, we have shown that a previously described SNP in the promoter region of the IL-16 gene, T₋₂₉₅C, is not associated with asthma, asthma severity or atopy in an Australian Caucasian population, irrespective of its possible effects on gene expression and the development of other inflammatory diseases.

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