

Characterization of two polymorphisms in the leukotriene C₄ synthase gene in an Australian population of subjects with mild, moderate, and severe asthma

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Background: The cysteinyl-leukotrienes (cys-LTs) are proinflammatory mediators that are important in the pathophysiology of asthma. LTC₄ synthase is a key enzyme in the cys-LT biosynthetic pathway, and studies in small populations have suggested that a promoter polymorphism (A₋₄₄₄C) in the gene might be associated with asthma severity and aspirin intolerance.

Objective: We sought to screen the LTC₄ synthase gene for polymorphisms and to determine whether there is an association between these polymorphisms and asthma severity or aspirin sensitivity in a large, well-phenotyped population and to determine whether this polymorphism is functionally relevant.

Methods: The coding regions of the LTC₄ synthase gene were screened for polymorphisms and the A₋₄₄₄C polymorphism was analyzed in a large Australian white adult population of mild (n = 282), moderate (n = 236), and severe asthmatic subjects (n = 86) and nonasthmatic subjects (n = 458), as well as in aspirin-intolerant asthmatic subjects (n = 67). The functional activity of the promoter polymorphism was investigated by transient transfection of HL-60 cells with a promoter construct.

Results: A new polymorphism was identified in intron 1 of the gene (IVS1-10C>A) but was not associated with asthma. Association studies showed that the A₋₄₄₄C polymorphism was weakly associated with asthma per se, but there was no association between the C₋₄₄₄ allele and chronic asthma severity or aspirin intolerance. A meta-analysis of all the genetic studies conducted to date found significant between-study heterogeneity in C₋₄₄₄ allele frequencies within

different clinical subgroups. In vitro functional studies showed no significant differences in transcription efficiency between constructs containing the A₋₄₄₄ allele or the C₋₄₄₄ allele. **Conclusions:** Our data confirm that, independent of transcriptional activity, the C₋₄₄₄ allele in the LTC₄ synthase gene is weakly associated with the asthma phenotype, but it is not related to disease severity or aspirin intolerance. (J Allergy Clin Immunol 2004;113:889-95.)

Key words: LTC₄ synthase, asthma, disease severity, aspirin-intolerant asthma, promoter polymorphism, Australian population

Asthma is a chronic inflammatory disorder of the airways and is characterized by variable airflow obstruction and bronchial hyperresponsiveness. It varies considerably in its severity; some patients have extremely severe disease, whereas others have minimal symptoms. The primary cause of asthma is unknown, although atopy and allergy are the strongest risk factors. Aspirin-intolerant asthma (AIA) is a specific subtype of asthma,¹⁻⁴ in which individuals with AIA usually develop symptoms after the third decade of life in response to ingestion of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs) and often present with the so-called aspirin triad (asthma, chronic rhinosinusitis, and nasal polyps).⁵ Estimates of the prevalence of AIA in adult asthmatic subjects vary from 3% to 20%.^{4,6-9}

Many mediators have been implicated in the pathophysiology of asthma, including the cysteinyl leukotrienes (cys-LTs). The cys-LTs are potent proinflammatory mediators that are known to promote smooth muscle contraction, mucus hypersecretion, airway edema, and eosinophil recruitment to the airways.¹⁰ The cys-LT synthetic pathway has been well characterized, and its 3 primary enzymes are 5-lipoxygenase (ALOX5), 5-lipoxygenase activating protein (ALOX5AP), and LTC₄ synthase.

LTC₄ synthase is a membrane-bound glutathione transferase expressed only by cells of hematopoietic origin and is a key enzyme in the synthesis of cys-LTs, converting LTA₄ to LTC₄.¹¹ The gene encoding LTC₄ synthase is located on the long arm of chromosome 5 (5q35)^{12,13} in a region implicated in asthma by linkage analysis and adjacent to a region (5q31-33) that includes a number of candidate asthma genes.¹⁴ The LTC₄ synthase gene spans about 2.5 kilobases, containing 5 small exons

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Abbreviations used

AIA: Aspirin-intolerant asthma
 ALOX5: 5-lipoxygenase
 ALOX5AP: 5-lipoxygenase activating protein
 ATA: Aspirin-tolerant asthma
 bp: Base pair
 cys-LT: Cysteinyl leukotriene
 NSAIDs: Nonsteroidal anti-inflammatory drugs
 LTC₄: Leukotriene C₄
 SSCP: Single stranded conformational
 polymorphism

and a number of putative DNA binding motifs or regulatory sites in the 5' untranslated region.¹²

Sanak et al^{15,16} described an adenine (A) to cytosine (C) transversion 444 base pairs (bp) upstream [-444] of the translation start site of the LTC₄ synthase gene and reported that the polymorphic C₋₄₄₄ allele occurred more commonly in patients with AIA (relative risk, 2.62-3.89). A 5-fold greater expression of LTC₄ synthase has been demonstrated in individuals with AIA when compared with patients with aspirin-tolerant asthma (ATA)¹⁷; furthermore, the expression of LTC₄ synthase mRNA has also been shown to be higher in blood eosinophils from asthmatic subjects compared with control subjects and was particularly increased in eosinophils from patients with AIA.¹⁶ Preliminary studies by Sampson et al¹⁸ have also shown an association between severe asthma and the polymorphic C₋₄₄₄ allele, suggesting a genetic propensity for increased cys-LT production might be a predisposing factor for severe asthma irrespective of aspirin sensitivity.

These findings suggest an important role for LTC₄ synthase in asthma severity and AIA. However, these initial studies were carried out in European populations, with relatively small numbers of individuals and with limited definition of their asthma severity. We hypothesized that, in an Australian population, polymorphisms in the LTC₄ synthase gene would be associated with asthma severity and with AIA. We screened all 5 exons of the LTC₄ synthase gene for polymorphisms, and we conducted an association study in a large, white, adult population of nonasthmatic control subjects and patients with asthma who were carefully characterized with respect to asthma severity and aspirin intolerance.

METHODS

Subjects

Patients with asthma (n = 604) and nonasthmatic control subjects (n = 458) participated in the association study. All participants were interviewed by one of the investigators. Asthma was defined as doctor-diagnosed asthma, and the nonasthmatic control subjects had no history of asthma. All participants were unrelated white subjects between 18 and 89 years of age, and they were recruited by random mailings and through physician referrals from the Sir Charles Gairdner Hospital, Western Australia (n = 1019), the Prince Charles Hospital, Queensland (n = 16), and the Alfred Hospital, Victoria (n = 27), Australia. All subjects gave written informed consent and

completed a comprehensive questionnaire that was used in the assessment of phenotype. Approximately 15 mL of blood was obtained from each participant, and lung function was assessed by spirometry. Assessment of atopic status was based on a positive skin prick reaction (wheal diameter, >3 mm) to at least 1 of 5 common aeroallergens: cat, dog, house dust mite, mold mix (*Alternaria tenuis*, *Aspergillus* mix, *Cladosporium*, *Penicillium* mix), and grass pollen mix (Kentucky Blue, Orchard, Red Top, Timothy, Sweet Vernal, Meadow Fescue, Perennial Rye). To establish allele frequencies in an Australian white population, blood samples were collected from 170 random white blood donors from the Red Cross Blood Services in Western Australia. The study protocol was approved by the Human Research Ethics Committee of the Sir Charles Gairdner Hospital and the Research Ethics Committees at the Prince Charles and Alfred Hospitals.

Disease severity

To assist in allocating patients to different asthma severity groups, we used criteria slightly modified from those specified by the Australian National Asthma Council, Australia¹⁹ as well as the National Asthma Education and Prevention Program (NAEPP) Expert Panel Report.²⁰ The following criteria were used to assess asthma severity: (1) lung function (% predicted FEV₁); (2) use of oral corticosteroids in the last 12 months; (3) daily inhaled corticosteroid (beclomethasone equivalent) dose; (4) weekly frequency of use of rescue medication; (5) weekly frequency of daytime symptoms; (6) weekly frequency of nighttime waking due to asthma; (7) unplanned visits to a general practitioner in the last 12 months; and (8) 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 overall category in which they were classified for the majority of criteria.

AIA

AIA is a complex disorder to define, because different individuals often have a specific subset of symptoms, and a single asthmatic episode might be coincidentally associated with taking aspirin or NSAIDs. Hence, for population studies of patients with ATA or AIA, it is usual to have either a study group of patients who have undergone an aspirin challenge and/or a well-characterized group of asthmatic and nonasthmatic individuals who have taken aspirin or other NSAIDs and have had no adverse reactions or a strong history of multiple asthma reactions to these drugs. All patients recruited for this study completed a questionnaire that included a section in which adverse reactions to aspirin or NSAIDs were fully documented and contained questions on history of nasal polyps and age of onset, indication of the specific drug(s) known to result in the adverse reaction (from a list of named drugs including aspirin, aspirin derivatives, and NSAIDs), the number of occasions on which adverse reactions were experienced, the time to onset of symptoms, incidence of hospitalization after ingestion of these drugs, and specific symptoms (from a comprehensive list of possible symptoms). Individuals reporting any adverse reaction to these drugs were further categorized into (1) individuals reporting minimal respiratory response or poorly defined responses, (2) individuals who had experienced more than 1 episode with characteristic symptoms of AIA, or (3) individuals who had undergone a formal aspirin challenge or who reported multiple characteristic episodes usually resulting in hospitalization. The final analysis excluded all individuals in the first category and only included individuals in the second and third categories as having AIA, whereas all other patients with asthma were defined as having ATA. All nonasthmatic individuals reporting any adverse skin or respiratory reactions to aspirin or other NSAIDs were also excluded from the final analysis. Sixteen patients with AIA were recruited through the Prince Charles Hospital, and another 8

TABLE I. Demographics of the total study population

Phenotype	N	Females (%)	Atopy (%)	Mean age (y ± SD)	% Predicted FEV ₁ (mean ± SEM)	Oral corticosteroids* (%)	Frequency of oral corticosteroid use (mean times/y)	Inhaled corticosteroids† (%)	Mean inhaled steroid dose‡ (µg/day ± SEM)	β ₂ -Adrenoceptor agonists§ (%)
Non-asthma	458	56	51	50.6 ± 14.2	101.7 ± 2.5	Nil	Nil	Nil	Nil	Nil
All asthma	604	63	83	49.0 ± 15.5	—	—	—	—	—	—
Mild asthma	282	61	84	47.3 ± 15.1	92.2 ± 1.0	5	1.3	59	890 ± 49	82
Moderate asthma	236	64	81	50.8 ± 15.9	74.2 ± 1.4	38	1.5	95.6	1474.5 ± 65	94.3
Severe asthma	86	65	78	50.1 ± 13.6	59.7 ± 2.6	79	2.9	98.2	2729.2 ± 165	97.5
All AIA	110	72	65	49 ± 15.0	—	—	—	—	—	—
Category 1 ¹ AIA	43	79	72	47 ± 16.5	—	—	—	—	—	—
Category 2 ² AIA	41	78	54	48 ± 16.4	80.2 ± 4.7	41	2.5	93	1889 ± 211	87.8
Category 3 ³ AIA	26	50	73	52 ± 10.2	74.1 ± 4.4	48	2.0	88	890 ± 134	88.4
ATA	496	62	85	49 ± 15.4	80.8 ± 0.9	27	1.97	68.9	1449.2 ± 54	88.5

*Percentage of patients who had used oral corticosteroids in last 12 months.

†Percentage of patients using inhaled corticosteroids.

‡Beclomethasone equivalent.

§Percentage of patients using β₂-adrenoceptor agonists.¹Category 1: Individuals reporting minimal or no respiratory response to aspirin.²Category 2: Individuals who had experienced more than one episode with characteristic symptoms of AIA.³Category 3: Individuals who had undergone a formal aspirin challenge or reported multiple episodes of AIA.

individuals who reacted positively to a formal aspirin challenge were recruited from the Alfred Hospital, Melbourne.

DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes by using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, or by using the salt extraction method described by Miller et al.²¹

PCR-RFLP and PCR—single stranded conformational polymorphism analysis

Detailed methodologies for the PCR-RFLP and PCR—single stranded conformational polymorphism (SSCP) analyses can be viewed (see e-text in the Journal's Online Repository www.mosby.com/jaci). PCR products were amplified in standard reactions of 20 or 30 µL by using specific primers as shown in Table E1 (in the Journal's Online Repository at www.mosby.com/jaci). Forty-two DNA samples (14 mild asthma, 14 severe asthma, and 14 nonasthma) were screened for polymorphisms in all 5 exons by using PCR-SSCP analysis. The amplified products, spanning each exon, were electrophoresed on MDE (BioWhittaker, Rockland, Maine) SSCP-polyacrylamide gels with 3 gel conditions: room temperature, 4°C, and room temperature with 10% (wt/vol) added glycerol. The A₋₄₄₄C promoter polymorphism and a new polymorphism in intron I (IVS1-10c > a) were detected by using PCR-RFLP analysis with the restriction endonucleases *Msp*I and *Bse*RI, respectively.

Sequence analysis

To characterize and confirm polymorphisms, the PCR products were sequenced commercially by using the primers previously used for PCR amplification (see Table E1 in the Online Repository).

Functional assays

To test the functional activity of the promoter polymorphism (A₋₄₄₄C), a portion of the LTC₄ synthase promoter including the ATG

translation start site and extending 5' of the polymorphic site at position -444 (−528 to +35), containing either A₋₄₄₄ or C₋₄₄₄, was amplified from genomic DNA and inserted into a pGL3-basic vector containing a luciferase reporter gene (Promega, Madison, Wis). The sequence of the insert was confirmed by sequence analysis. HL-60 promyelocytic leukemia cells that were confirmed as expressing LTC₄ synthase by RT-PCR (see Fig E1 in the Journal's Online Repository at www.mosby.com/jaci) were transfected with the constructs by electroporation, and cell lysates were assayed for luciferase activity by using a dual-reporter assay system (Promega).

Statistical analysis

Genotype and allele frequencies were calculated for the random white group and each of the patient groups. Univariate comparisons of allele and genotype distribution were performed by using χ² tests and logistic regression analysis, with the R Statistics Program.²² The influence of the LTC₄ synthase promoter polymorphism on specific asthma phenotypes was also examined by using multivariate logistic regression analysis and, where indicated, proportional odds logistic regression analysis. Hardy-Weinberg equilibrium analysis for each group was evaluated by the exact test, implemented in R Statistics Program.

RESULTS

Subjects

In total, 604 patients with asthma and 458 nonasthmatic control subjects participated in this study. All patients were white, and the country of birth of all 4 grandparents was known for 515 subjects (49%). Ninety-six percent of grandparents came from Northern Europe or Australia. Patients with asthma were subdivided by disease severity, and Table I illustrates the demographics of the population

TABLE II. Genotype and allele frequencies of the LTC₄ synthase A₄₄₄C promoter polymorphism

Phenotype	N	Genotype			Allele		HWE P value
		AA	AC	CC	A	C	
Random white population	170	0.53	0.38	0.09	0.57	0.43	
Non-asthma controls	458	0.56	0.38	0.07	0.74	0.26	.903
All asthma	604	0.48	0.44	0.08	0.70	0.30	1.000
Mild asthma	282	0.47	0.43	0.10	0.69	0.31	1.000
Moderate asthma	236	0.50	0.43	0.07	0.71	0.29	.637
Severe asthma	86	0.51	0.43	0.07	0.73	0.28	.611
AIA, Category 2	41	0.50	0.32	0.18	0.56	0.34	.074
AIA, Category 3	26	0.60	0.32	0.08	0.76	0.24	.586
ATA	496	0.47	0.45	0.08	0.69	0.31	.206

HWE, Hardy-Weinberg equilibrium.

studied. All groups 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 nonasthmatic control group (51%) ($P < .0001$). Daytime symptoms of asthma occurred, on average, 3.9 times per week in individuals with mild asthma, 6.8 times per week in individuals with moderate asthma, and 9 times per week in individuals with severe asthma. Less than half the patients with mild asthma ever woke from sleep because of their asthma, whereas all patients with severe asthma woke from sleep every night because of their asthma. Unplanned visits to the family doctor for asthma during the previous 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 during the previous 12 months occurred in less than 5% of patients with mild asthma, in 30% of patients with moderate asthma, and in 70% of patients with severe asthma.

AIA and ATA

In total, 496 individuals were classified as having ATA, and, of the 1062 individuals participating in the study, 110 individuals reported adverse reactions to aspirin or other NSAIDs. Of these, 67 were classified as category 2 or 3; 24 were classified as mild, 25 as moderate, and 18 as severe asthmatic subjects.

The mean ages of the individuals included in this analysis did not differ significantly between categories. Women outnumbered men 3.6:1 in Category 2, whereas the gender distribution in Category 3 (formal challenge or multiple episodes) was 1:1. Although 61% of the patients in AIA Categories 2 and 3 were atopic, this did not differ significantly from the asthma population as a whole ($\chi^2 = 1.87$; $P = .17$). The majority of Category 3 patients had a history of nasal polyps (73%), whereas only 24% of Category 2 patients reported a history of nasal polyps ($\chi^2 = 4.78$; $P = .03$).

PCR-SSCP analysis

All 5 exons of the LTC₄ synthase gene were screened for polymorphisms by using PCR-SSCP analysis. No new polymorphisms were found in the coding region of the LTC₄ synthase gene. However, with primers spanning exon 2, a previously undescribed polymorphism in intron 1 was identified. Sequence analysis showed a single nucleotide C to A transversion 10 bp upstream of exon 2 (IVS1-10c > a) as shown in Fig E2 (in the Journal's Online Repository at www.mosby.com/jaci).

PCR-RFLP analysis

IVS1-10c > a. The IVS1-10c > a polymorphism altered a BseRI restriction site as shown in Fig E3 (in the Journal's Online Repository at www.mosby.com/jaci). DNA from individuals homozygous for the wild-type C allele yielded fragments of 60, 70, and 236 bp, whereas DNA from individuals homozygous for the polymorphic A allele showed fragments of 60 and 306 bp. DNA from heterozygous individuals had all 4 fragments. Genotypes were analyzed for a total of 243 individuals (24 with severe asthma, 43 nonasthmatic control subjects, and 176 random white subjects). Three individuals were homozygous AA, 3 were heterozygous AC, and 237 were homozygous CC, giving an allele frequency of 0.98 for the wild-type C allele and 0.02 for the polymorphic A allele.

A₄₄₄C promoter polymorphism. We investigated the A₄₄₄C polymorphism in 604 asthmatic subjects, including 67 individuals with AIA, and 458 nonasthmatic subjects. Table II summarizes the genotype and allele frequencies for each group studied. In all population groups, the distribution of genotypes was consistent with Hardy-Weinberg equilibrium.

There was no association between the polymorphic C₄₄₄ allele and disease severity in this population ($\chi^2 = 1.47$; $P = .23$). However, comparing asthma with nonasthma, there was a trend toward an association between asthma and the polymorphic C₄₄₄ allele (allelic association: $\chi^2 = 4.79$, $P = .03$; genotypic association: $\chi^2 = 5.11$, $P = .08$). This effect was not influenced by age, smoking status, or ethnicity (crude allelic odds ratio, 1.24; 95% CI, 1.02-1.51; age-gender-smoking adjusted odds ratio, 1.26; 95% CI, 1.03-1.54; age-gender-smoking—British ancestry odds ratio, 1.38; 95% CI, 1.01-1.89). In further analysis that separated asthma and atopic status, the association was shown to be with asthma ($\chi^2_1 = 5.62$; $P = .018$) rather than with atopy or atopic asthma ($\chi^2_2 = 2.73$; $P = .25$).

There was no significant difference in allele frequencies between Category 2 and 3 AIA (Irwin-Fisher exact $P = .12$), although these groups were relatively small. When comparing the allele and genotype frequencies between the nonasthmatic subjects, ATA subjects, and all AIA subjects, there was a moderately significant difference ($\chi^2 = 15.12$; $P = .04$), probably because of the differences in genotype frequencies between nonasthmatic subjects and Category 2 AIA group ($P = .05$).

and between nonasthmatic subjects and ATA group ($P = .03$). Importantly, there were no significant differences found when comparing any AIA (Categories 2 and 3 together) to nonasthma ($\chi^2 = 3.45$; $P = .12$) or any AIA with ATA ($\chi^2 = 3.82$; $P = .07$). Overall, the C allele was increased slightly in the ATA group and Category 2 AIA group, and proportional odds logistic regression analysis (regressing on the number of C alleles) showed similar results to the χ^2 analysis ($P = .03$).

There was no association between C_{444} genotypes and nasal polyps ($\chi^2 = 1.53$; $P = .47$). The age of onset of asthma was available for 581 subjects, and the Kruskal-Wallis test showed no significant association between age of onset of asthma and LTC_4 synthase genotype ($P = .08$).

We performed a meta-analysis of all the genetic studies conducted to date and found significant between-study heterogeneity in C_{444} allele frequencies within different clinical subgroups. The nonasthma control genotype frequencies did not differ significantly from one another, despite the different ethnic backgrounds from which the studies were derived (Polish: $q = 0.27$, $n = 75^{16}$; American: $q = 0.278$, $n = 137^{23}$; Spanish: $q = 0.293$, $n = 61^{24}$; Dutch: $q = 0.28$, $n = 92^{25}$; two Japanese populations: $q = 0.22$, $n = 171^{26}$ and $q = 0.20$, $n = 110^{27}$; British: $q = 0.31$, $n = 180^{28}$; and this Australian population: $q = 0.26$, $n = 456$). However, in the case of ATA, the C_{444} allele frequency was significantly decreased in the Japanese population, and the other studies can be pooled ($P = .36$). In the case of AIA, the C_{444} allele was significantly increased only in the Polish sample (the other studies can be pooled [$P = .51$]). Pooling 4 studies^{16,23,26-28} and this Australian population, the allelic odds ratio for “all” asthma for the C_{444} allele under a random effects model was 1.11 (95% CI, 0.91-1.36).

Luciferase assay

To determine the functional promoter activity of the C_{444} allele, HL-60 cells were shown to express LTC_4 synthase as shown in Fig E1 and then transfected with a pGL3 construct containing promoter sequence with either the A_{444} or the C_{444} allele. Several separate experiments ($n = 8$) revealed that there was no significant difference in luciferase activity between the A_{444} and C_{444} constructs (Fig 1).

DISCUSSION

This is the most significant and thorough association analysis of the $A_{444}C$ promoter polymorphism conducted to date. It involved a large population of subjects with mild, moderate, and severe asthma and nonasthmatic control subjects. With PCR-SSCP analysis, we have screened all 5 exons of the LTC_4 synthase gene for polymorphisms and have not found any new polymorphisms in the coding region. However, we have identified a new polymorphism (c > a) in intron 1 of the gene, 10 bp upstream of exon 2 (IVS1-10c > a). This polymorphism is rare, occurring in only 2% (6/242) of the

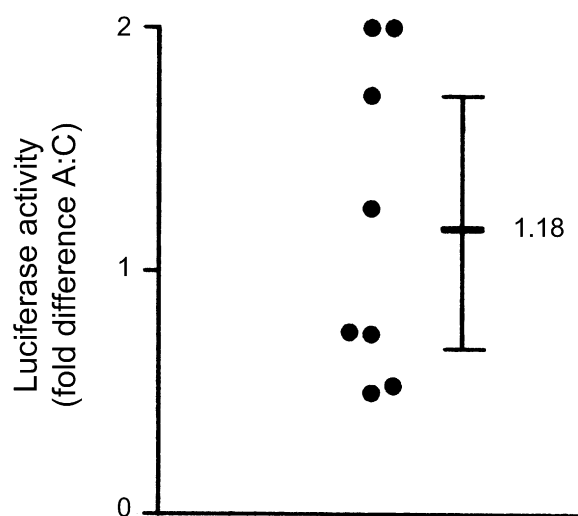


FIG 1. Luciferase activity of HL-60 cells after transient transfection with constructs containing a 563-bp fragment containing the promoter region of the LTC_4 synthase gene, with either the wild type A_{444} allele (A) or the polymorphic C_{444} allele (C). Luciferase assays were normalized against a *Renilla* luciferase control. The results ($n = 8$) are expressed as a ratio of luciferase activity for the A_{444} construct replicates to the C_{444} construct replicates in each experiment.

population studied, and was not associated with asthma or atopy. Although it is relatively close to the intron/exon splice site, it does not appear to alter splicing of the gene on computer modeling (<http://genes.mit.edu/GENSCAN.html>).

In addition to the newly described intron 1 polymorphism in the LTC_4 synthase gene, we have also characterized the promoter polymorphism ($A_{444}C$) initially described by Sanak et al,¹⁵ and we found a weak association between asthma per se and the C_{444} allele. We did not find any statistical evidence for an association of the C_{444} allele with asthma severity in contrast with the early data from Sampson et al.¹⁸ However, our study supports the more recent findings of this group in a British population²⁸ and those of Brauer et al,²⁵ which showed no relationship between LTC_4 synthase genotype and markers of severity in a Dutch population. Kawagishi et al²⁷ were also unable to show an association between LTC_4 synthase genotype and respiratory function or dose of inhaled corticosteroid in a Japanese population.

Although there was no significant association between the $A_{444}C$ promoter polymorphism and AIA, we did find a moderately significant difference when comparing allele and genotype frequencies among the AIA, ATA, and nonasthma groups, and this is likely to be attributable to frequency differences between nonasthmatic subjects and subjects with ATA. Thus, in contrast to the findings of Sanak et al,^{15,16} we did not find evidence of an association between the polymorphic C_{444} allele and AIA in this Australian white population. Other investigators have also reported a lack of association between the C_{444} allele and AIA in white populations from the Netherlands and the

United States.^{23,25} Our study showed a slight overrepresentation of the C₄₄₄ allele in the ATA group, a finding similar to that of Van Sambeek et al.²³ However, in a Japanese population, the C₄₄₄ allele was significantly underrepresented in the ATA group, whereas C₄₄₄ allele frequencies were similar in the AIA and nonasthma populations.²⁷ It has been suggested that the unique association between the C₄₄₄ allele and AIA in the Polish population ($q = 0.44$) represents a population-stratified polymorphism within patients of eastern European descent.^{23,27} This is further supported by our meta-analysis that demonstrated that our study was in agreement with previous replication studies of the C₄₄₄, in finding only a small association with asthma in general, rather than specifically with aspirin intolerance.

Previous studies in European and Turkish populations have shown a biased female:male ratio of 2.3:1 in AIA, and only one third of patients with AIA were atopic.²⁹⁻³¹ The demographics of our patient group differed significantly, particularly with respect to gender ratios and atopy status. Our population had a female:male ratio of 3.6:1 in patients reporting aspirin intolerance but approximately 1:1 in patients who had a positive aspirin challenge or repeated severe episodes of asthma after ingesting aspirin/NSAIDs, and 61% of our AIA patients were atopic. Previous studies in Australian populations, reported by Vally et al,⁹ reported a female:male ratio of between 1.4 and 5.5:1, with the prevalence of atopy ranging between 37% and 49% in those populations. In large population studies it is impractical to challenge all patients, but there are several characteristics favoring a diagnosis of AIA.³² We used a comprehensive questionnaire and documented in detail adverse reactions to aspirin and other NSAIDs. This enabled us to characterize patients with a degree of certainty. Despite this, it is important to acknowledge that a small number of individuals included in our ATA group might have occult sensitivity to aspirin and would react positively to a formal aspirin challenge. However, these small numbers would be unlikely to change the overall findings of this population-based association study.

It has been suggested that the presence of the C₄₄₄ allele in the promoter region of the LTC₄ synthase gene results in an increase in transcriptional activity of this gene, thus supporting the functional significance of the A₄₄₄C polymorphism in individuals with AIA.¹⁶ However, our investigation showed that in vitro there was no significant difference in promoter activity between the A₄₄₄ and C₄₄₄ constructs. Although these results are in conflict with one report,¹⁶ they do support the findings of Van Sambeek et al²³ and Sayers et al,²⁸ which also showed that the C₄₄₄ allele had no effect on transcription of the LTC₄ synthase gene. It is likely that the differences in transcriptional activity between the first study and the more recent studies are the result of differences in the cell types and promoter fragment used. Sanak et al¹⁶ used 297 bp PCR-amplified fragments containing either allele, cloned into a β -galactosidase expression vector that was transfected into COS-7 cells and showed consistently raised levels of β -

galactosidase activity after transfection with the C₄₄₄ allele. Van Sambeek et al²³ used a 550-bp fragment of the proximal 5' flanking region of the LTC₄S gene (including the 5' flanking region of the gene and extending 3' to the ATG translational start site) with the C₄₄₄ transversion incorporated by site-directed mutagenesis. Sayers et al²⁸ generated a 1.3-kb fragment, corresponding to -1359 to -55, from genomic DNA. Both groups ligated the LTC₄ synthase promoter regions upstream of the luciferase gene in a pGL3 basic vector and transiently transfected these vectors into LTC₄ synthase-expressing THP-1 monocytic cells or HeLa and KU812F cells.^{23,28} We cloned a 563-bp fragment, generated from previously genotyped genomic DNA by PCR amplification, into a luciferase vector and transiently transfected this vector into promyelocytic HL-60 cells that also express LTC₄ synthase. Our findings that the activity of the wild-type A₄₄₄ construct was no different to that of the C₄₄₄ construct suggests that, although it is associated with asthma per se, the C₄₄₄ allele is not likely to be functionally associated with increased LTC₄ synthase transcription. It is possible that the larger fragments used in our study and the studies by Van Sambeek et al²³ and Sayers et al²⁸ included other modifying sequences that were not present in the smaller fragment cloned by Sanak et al,¹⁶ hence altering transcription of the promoter-reporter gene construct.

Candidate gene and association studies in asthma are often insufficient to confirm or refute the importance of specific polymorphisms in specific genes for several reasons,³³ including difficulty in phenotype definition, sample size, and population stratification.^{34,35} The strength of our association study lies in the attention paid to characterizing the phenotype, the large size of the population studied, and the use of a well-defined Australian white population.

Although previous studies suggested that a promoter polymorphism (A₄₄₄C) in the LTC₄ synthase gene is associated with severe asthma,^{15,18} we have not been able to confirm such an association in a large, carefully phenotyped population. LTC₄ synthase has also been identified as a likely candidate gene for the AIA phenotype; however, the polymorphic C₄₄₄ allele has been found to be associated with AIA only in a Polish population and not in American, Dutch, Japanese, and Australian populations. Previously reported in vitro functional assays were contradictory, showing either that the C₄₄₄ allele is associated with increased transcription of the gene,¹⁶ or that this allele does not alter gene transcription.^{23,28} Our functional assays confirm that there is no significant difference in activity between the wild-type A₄₄₄ and polymorphic C₄₄₄ alleles in vitro. Taken together with our observation that the C₄₄₄ allele was associated with asthma per se, these findings suggest that although polymorphisms in the LTC₄ synthase gene might be related to the pathogenesis of asthma, the A₄₄₄C promoter polymorphism is not associated with disease severity in an Australian population and/or aspirin intolerance in populations of non-Eastern European descent.

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