

5' Flanking region polymorphism of the gene encoding leukotriene C₄ synthase does not correlate with the aspirin-intolerant asthma phenotype in the United States

Rachel Van Sambeek, BA,^a Donald D. Stevenson, MD,^c Mathew Baldasaro, MS,^a Bing K. Lam, PhD,^a JiLiang Zhao, PhD,^a Satoshi Yoshida, MD, PhD,^b Chandri Yandora, PhD,^b Jeffrey M. Drazen, MD,^b and John F. Penrose, MD^a *Boston, Mass, and La Jolla, Calif*

Background: Approximately 10% of patients with asthma have a distinct clinical entity in which their symptoms are exacerbated by aspirin and most other nonsteroidal anti-inflammatory agents. These individuals typically have significant basal overproduction of cysteinyl leukotrienes, and within their biosynthetic pathway, the terminal enzyme, leukotriene C₄ synthase (LTC₄S), is significantly overexpressed. A single nucleotide polymorphism consisting of an adenine (A) to cytosine (C) transversion –444 nucleotides upstream of the ATG translation start site in the LTC₄S gene has been associated with a relative risk of 3.89 for the aspirin-intolerant phenotype in Polish patients.

Objective: These studies were undertaken to further investigate the functional effect of this allele in LTC₄S gene expression and subsequently to determine whether an association between the presence of this polymorphism and aspirin-intolerant asthma existed within patients of the United States.

Methods: Functionality of the C-444 allele was assessed by using promoter-reporter constructs and transient transfection assays in the THP-1 monocytic cell line. Genotyping was performed on 137 unaffected control subjects, 33 patients with aspirin-tolerant asthma, and 61 patients with aspirin-intolerant asthma from the United States.

Results: Promoter-reporter constructs containing the C-444 allele revealed no significant upregulatory or downregulatory effects in the transcription of the LTC₄S gene. The LTC₄S genotype distribution was consistent with the Hardy-Weinberg equilibrium in patients with aspirin-tolerant asthma and unaffected control subjects but not in patients with aspirin-intolerant asthma; however, the distributions were not significantly different among the phenotype groups.

Conclusions: Our data demonstrate that the C-444 allele in the LTC₄S gene is not statistically different among patients with the aspirin-intolerant asthmatic phenotype, patients with the aspirin-tolerant asthmatic phenotype, and unaffected control subjects in the United States. This finding, along with the lack of functionality of this polymorphism, suggest that it is not related to a specific asthma phenotype and may represent a population-stratified polymorphism within patients of eastern European descent. (*J Allergy Clin Immunol* 2000;106:72-6.)

Key words: Aspirin-intolerant asthma, cysteinyl leukotrienes, leukotriene C₄ synthase, polymorphism

Bronchial asthma is a disease characterized by airway hyperresponsiveness with superimposed inflammation, resulting in intermittent airflow obstruction. It has been estimated that 10% of patients with moderate-to-severe persistent asthma have a distinct clinical entity in which exacerbation of their symptoms is precipitated by oral administration of aspirin and most other nonsteroidal anti-inflammatory agents (NSAIDs).¹ Although the cysteinyl leukotrienes (LTs) appear to be mediators of asthma in general, individuals with the phenotype of aspirin-intolerant asthma (AIA) typically have significant constitutive overproduction of cysteinyl LTs further upregulated by aspirin administration.²⁻⁴ Additionally, treatment of patients with AIA with LT-modifying drugs markedly attenuates their pathophysiologic responses to aspirin.⁵⁻⁷

The generation of the proinflammatory cysteinyl LTs occurs by means of the sequential action of 4 proteinases. The first 3 of these proteins, cytosolic phospholipase A₂, 5-lipoxygenase (ALOX5) activating protein (formerly referred to as 5-lipoxygenase activating protein), and ALOX5 work in concert to form LTA₄, a pivotal but unstable intermediate.⁸⁻¹⁰ LTA₄ is then converted to LTC₄ by the terminal enzyme of the pathway, LTC₄ synthase (LTC₄S),¹¹ which is expressed in only a limited number of hematopoietic cells involved in allergic inflammation, such as eosinophils, mast cells, basophils, and monocytes-macrophages.¹² A carrier-mediated process¹³ then allows transport of LTC₄ to the extracellular environment for enzymatic conversion to its bioactive metabolites LTD₄ and LTE₄.^{14,15}

An immunohistochemical study of the enzymes of this pathway performed in a functional biopsy study of

From ^athe Department of Medicine, Harvard Medical School and the Division of Rheumatology, Immunology, and Allergy, ^bDivision of Pulmonary and Critical Care, Brigham and Women's Hospital, Boston; and ^cthe Division of Allergy, Asthma and Immunology, Scripps Clinic and the Scripps Research Institute, La Jolla.

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Reprint requests: John F. Penrose, MD, Brigham and Women's Hospital, Smith Building, Room 626C, 1 Jimmy Fund Way, Boston, MA 02115.

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Abbreviations used

AIA: Aspirin-intolerant asthma
ALOX5: 5-Lipoxygenase
AP: Activating protein
ATA: Aspirin-tolerant asthma
LT: Leukotriene
LTC₄S: Leukotriene C₄ synthase
NSAID: Nonsteroidal anti-inflammatory drug

patients with AIA and aspirin-tolerant asthma (ATA) revealed that the patients with AIA had increased cysteinyl LTs in their bronchoalveolar lavage fluid.¹⁶ This finding, as well as a reduction in airflow from lysyl-aspirin administration and a reduction in the threshold for aspirin tolerance, correlated to the overexpression of LTC₄S in eosinophils of the bronchial mucosa. The presence of other eicosanoid biosynthetic enzymes, including ALOX5, ALOX5 activating protein, and prostaglandin G-H synthase 1, were not significantly different between the groups nor did they correlate with the clinical features noted above.¹⁶ Subsequently, a single-nucleotide polymorphism, an adenine (A) to cytosine (C) transversion in the 5' untranslated region of the LTC₄S gene located 444 nucleotides upstream from the ATG translation start site, was reported in 47 patients with AIA from Poland (presumably of eastern European descent) with LTC₄S overexpression.¹⁷

In this study we sought to examine the functional effect of this polymorphism on LTC₄S gene expression and then to determine its relationship to patients with the AIA phenotype in the United States. As an *in vitro* means to study gene expression, we used promoter-reporter constructs containing this sequence variant and compared the values with those of the wild-type sequence. This was followed by genotyping of patients with AIA in the United States to relate the naturally occurring presence of this sequence variant to the AIA phenotype.

METHODS

Assessment of the functional effect of the C allele in the -444 position of the 5' flanking region of the LTC₄S gene

To assess the relative functional contribution of the C allele, a 550-bp DNA fragment of the proximal 5' flanking region of the LTC₄S gene was produced by means of restriction enzyme digest of a P1 plasmid containing the human LTC₄S gene. This DNA fragment encompasses the 5' flanking region of the gene extending 3' to the ATG translation start site and was used as a template to further create a site-directed mutagenic fragment that incorporated the polymorphic cytosine transversion at nucleotide -444. These fragments of DNA were individually ligated upstream of the luciferase gene in the promoterless and enhancerless pGL3 basic vector and purified in a cesium chloride gradient. Nucleotide sequences were determined to ensure no mutations had occurred during PCR and subcloning before they were electrophoretically transfected into the LTC₄S-expressing THP-1 monocytic cells. The experiments also included, as a negative control, the promoterless and enhancerless pGL3 basic vector. Cells (5×10^6) were transfected with various

constructs by using diethylaminoethyl-dextran grown for 18 hours after transfection, washed in HBSS without calcium and magnesium, and lysed in 500 μ L of lysis buffer. Samples were assayed for luciferase activity by the addition of luciferin, and luminescence was quantitated with a Monolight luminometer.

Patients

Sixty-one individuals with AIA (59 white patients, which included 2 Polish patients and 2 Hispanic patients), 33 white patients with ATA, and 137 white individuals without asthma were studied. Patients with AIA all exhibited chronic rhinosinusitis with abnormal sinus x-ray findings. Patients with AIA were initially identified by a history of asthma attacks after ingestion of NSAIDs and subsequently underwent inpatient oral challenges with aspirin at the General Clinical Research Center of the Scripps Research Institute. Positive responses were defined by the development of respiratory reactions at a mean provoking dose of 60 mg of aspirin.¹⁸ Patients with ATA underwent oral aspirin challenges in the Scripps Research Center or gave a history of ingesting aspirin or NSAIDs in the past month without adverse effect. Normal control subjects did not have asthma, as determined by history, and could ingest aspirin without adverse effects.

Determination of the C allele in the -444 position of the 5' flanking region of the LTC₄S gene

Genomic DNA was isolated from either buccal scrapings or peripheral blood leukocytes of these individuals, and a 447-bp fragment in the 5' flanking region of the LTC₄S gene that encompassed the C-444 allele was amplified by means of PCR. The oligonucleotide primers 5'-TGCATGGAACCCCTACAACGACTAAG-3' and 5'-GCCAAGCCAGCAGGGAGGAAACCAAA-3' were used. PCR conditions were as follows: denaturing at 96°C for 1 minute, followed by 25 cycles of denaturing at 96°C for 20 seconds, annealing at 60°C for 30 seconds, and chain elongation at 72°C for 70 seconds. This was followed by a chain elongation at 72°C for 5 minutes.

The presence of this mutation creates a restriction enzyme recognition site for the MspI enzyme 182 bp from the 5' end of this PCR product, allowing the DNA in affected individuals to be genotyped by means of RFLP analysis. An inherent MspI site is located 233 bp from the 3' end of this PCR amplicon, and digestion of this DNA product with MspI from the individuals that are homozygous for the wild-type AA genotype yields 214-bp and 233-bp fragments. For DNA with the heterozygous AC genotype, 182-bp, 32-bp, 214-bp, and 233-bp fragments were expected; in the individuals with the homozygous CC genotype, only 182-bp, 32-bp, and 233-bp fragments were expected. When the DNA was resolved in 2% agarose gels, the 32-bp DNA fragment was not visualized (Fig 1).

Statistical analyses

Analyses were performed to compare genotype frequencies among the phenotype groups by using exact statistical techniques. Fisher exact tests were used to compare the proportions of individuals homozygous for the C allele and, separately, the proportions homozygous for the A allele. Exact Jonckheere-Terpstra tests were used to compare the number of C alleles carried by each individual as a dose-response comparison. The Hardy-Weinberg equilibrium was addressed by testing whether the data were consistent with a disequilibrium coefficient of zero.¹⁹ The Fisher exact test was also used to calculate the power of the study. The study achieved 80% power to detect the smallest clinically meaningful difference in proportions of individuals homozygous for the C allele of 8% in the unaffected population and 25% in the AIA population.

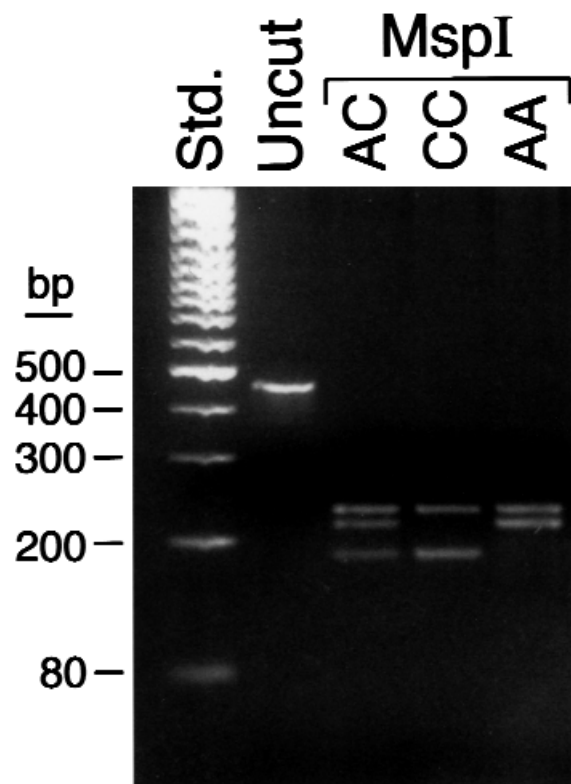


FIG 1. RFLP analysis. A representative example of the MspI-digested DNA fragments in a 2% ethidium-stained agarose gel from an AC heterozygote, CC homozygote, and a wild-type AA homozygote are shown.

RESULTS

Functional effect of the C allele in the -444 position of the 5' flanking region of the *LTC₄S* gene

The C allele at the -444 position within the context of the 550-bp construct of the 5' flanking region of the *LTC₄S* gene in THP-1 cells provided luciferase activity that was equal to the activity of the wild-type construct ($n = 7$, Fig 2). With the negative control value serving as 1, the fold-increase of luciferase activity for the wild-type A allele at the -444 position is 81.7, whereas for the C allele at the -444 position, the fold-increase is 83.6.

Allele and genotype frequencies for the C allele in the -444 position of the 5' flanking region of the *LTC₄S* gene

In the present study 18 (30%) of the patients with AIA, 14 (42%) patients from the ATA group, and 53 (39%) individuals from the unaffected group were heterozygous for the C allele. Additionally, 8 (13%) of the patients with AIA, 4 (12%) patients from the ATA group, and 11 (8%) individuals from the unaffected group were homozygous for the C allele (Table I). The distribution of genotypes within the unaffected population and within the ATA population were consistent with the Hardy-Weinberg equilibrium ($P > .75$). Although the distribution of geno-

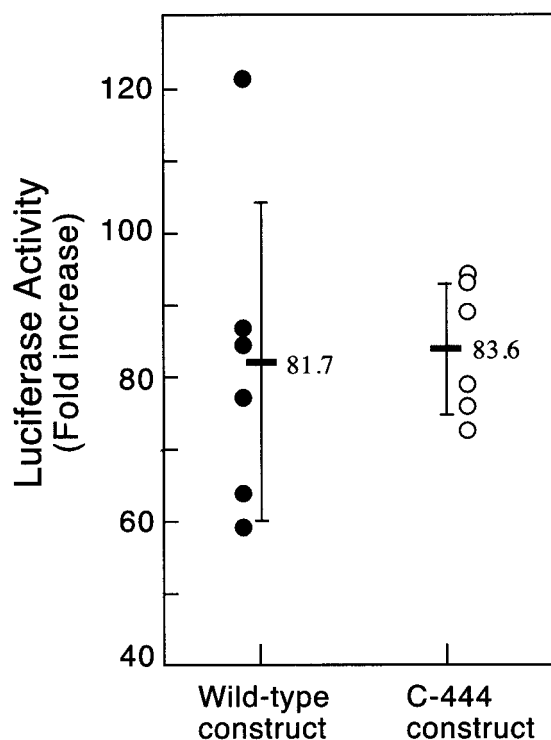


FIG 2. Effect of the C-444 single-nucleotide polymorphism in *LTC₄S* gene expression. Luciferase activity of THP-1 cells after transient transfection with a promoterless and enhancerless luciferase control is shown (as fold increase) in comparison with constructs that contain an insert with 550 bp of the promoter region of the *LTC₄S* gene with the naturally occurring A allele (wild-type construct) or with the C single-nucleotide polymorphism inserted by means of site-directed mutagenesis (C-444 construct).

types within the AIA population trended toward a significant level, the P value of .038 did not achieve the statistically significant level of $\geq .05$ used in tests of Hardy-Weinberg equilibrium.

Although the C allele in the AIA phenotype trended toward significance, when the C allele frequencies for the unaffected patients, patients with ATA, and patients with AIA were calculated, the P values of .274, .333, and .279, respectively, demonstrated no statistically significant differences among the 3 groups as either AC or CC genotype.

DISCUSSION

Typically, individuals with AIA have significantly increased levels of cysteinyl LTs in their urine, and immunohistochemical analysis has revealed that the level of *LTC₄S* overexpression in the aspirin-intolerant asthmatic phenotype correlates with overproduction of cysteinyl LTs and bronchial hyperreactivity to lysine aspirin. Furthermore, of the proteins in LT and prostanoid biosynthetic pathways, only *LTC₄S* is significantly overexpressed, which suggests that a defect in the regulation of this gene seems reasonable. On the basis of these data, *LTC₄S* appears to be a prime candidate gene for AIA.

TABLE I. Genotype frequency

	AA	AC	CC	χ^2 Value	P value*
Unaffected (n = 137)	0.532 (73)	0.386 (53)	0.080 (11)	0.099	.752
ATA (n = 33)	0.454 (15)	0.424 (14)	0.121 (4)	0.0682	.794
AIA (n = 61)	0.574 (35)	0.295 (18)	0.131 (8)	4.31	.038

*P values test the null hypothesis of the Hardy-Weinberg equilibrium calculated by using one degree of freedom.

Although detailed studies of LTC_4S regulation have not been reported, induction does occur in response to phorbol 12-myristate 13-acetate, presumably acting through the nuclear transcription factors activating protein 1 and activating protein 2, for which consensus sequences are present in the 5' flanking region of the gene.^{20,21} Because the C allele at position -444 in the 5' flanking region of this gene creates a third binding motif for binding of activating protein 2, it has been postulated that the presence of this additional site could lead to overexpression of the LTC_4S protein. By using in vitro promoter-reporter constructs, site-directed mutants of the 5' flanking region for the LTC_4S gene were made, incorporating the C allele into a 550-bp fragment. These promoter constructs were cloned into luciferase reporter constructs, and transfection into LTC_4S -expressing THP-1 cells did not reveal a significant functional effect as measured by luciferase activity. This data suggests that under the transfection conditions described, the C allele has no effect on the transcription of the LTC_4S gene, leading us to reconsider the possibility that the polymorphism might not be associated with increased expression of the LTC_4S gene product. This was addressed by a genotypic analysis of patients with AIA in the United States, comparing the allele frequency to patients with ATA and normal individuals.

Our genotype analysis of AIA demonstrated that the frequency of the C allele is not comparatively different from the values in the aspirin-tolerant asthmatic subjects and nonasthmatic control subjects, respectively. This held true for both AC and CC genotypes, supporting that no gene dose-response effect was present. Thus neither allele frequency nor genotype correlates with the aspirin-intolerant phenotype in patients from the United States.

The basis for the disparity of these results may lie at several levels. One consideration of the variance in allele frequency between aspirin-intolerant asthmatic individuals in eastern Europe and the United States is the method of analysis. In both studies the technique of RFLP analysis was used as a means for genotyping, taking advantage of a new *MspI* restriction site introduced by the single-nucleotide polymorphism. Although the size of the PCR amplicon differed between the two studies, it seems unlikely that the efficiency of the restriction digest might account for the significant differences noted in the Polish patients compared with the US patients. An additional consideration for explaining the differences between these populations exists in the selection of patients who may exhibit variable levels of cysteinyl LT generation, aspirin sensitivity, or both. Aspirin challenge techniques in the United States and Poland are similar and accurate

enough such that detection of truly aspirin-sensitive asthmatic subjects occurred in both studies. Despite this, direct comparisons of the level of aspirin intolerance are essentially impossible to compare, and although cysteinyl LT generation was not measured in all 61 of our patients, elevated urine levels are present in many of them.²²

Each of the patients reported here had significant decrements in pulmonary function after challenge with aspirin. Furthermore, although similar clinical characterization was reported in the patients of eastern European descent, to our knowledge neither the basal nor aspirin-triggered cysteinyl LT levels were examined. Although differences in age or sex did not exist, ethnic background of the patients might be an important difference between the two studies. Only two patients of Polish extraction were found in our sample of 61 patients with AIA; however, 5 were of European descent. It seems likely that there is a population bias for the C allele that exists in the European population compared with the patients in the United States. Reports of allelic variants associating with disease are increasing, and the phenomenon of ethnic restriction has been described for mutations in other candidate genes for allergic inflammation, such as the β -chain of the high-affinity IgE receptor and the platelet-activating factor acetyl hydrolase gene.^{23,24} In the case of the C allele at position -444 in the LTC_4S gene, however, there has not been adequate demonstration that the presence of this allele in the eastern European population is dose dependent or that affected individuals overproduce cysteinyl LTs either basally or after aspirin administration. Furthermore, comparison of the allele frequencies of the C allele in unaffected individuals and patients with ATA did not differ between European and US populations; however, the allele frequency of 0.436 observed in Polish patients with AIA is substantially higher than the value of 0.274 found in the US patients with the same phenotype.¹⁷

Thus although the gene for LTC_4S appears to be a likely candidate gene for the AIA phenotype, the C allele at position -444 in the 5' flanking region of this gene does not appear to be causative in either occurrence or severity of the AIA phenotype in patients in the United States. This point is further substantiated by the lack of upregulation caused by the C allele, as measured by in vitro promoter-reporter assays. Although it is possible that the association of this allele with AIA in Polish patients was a spurious finding, the most likely explanation for the discrepancy between the two sets of results is the confounding effect of population stratification. The intriguing finding of overexpression of LTC_4S in patients with AIA, however, suggests that screening for an additional polymorphism of the LTC_4S gene may be productive. One

final consideration is that re-examination of the patients with AIA may reveal subsets of patients with specific clinical or biochemical patterns, such as overproduction of cysteinyl LTs, that correlate to this or other alleles.

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