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COPD

Genetic regulation of expression of leukotriene A4 hydrolase

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ABSTRACT In chronic inflammatory lung disorders such as chronic obstructive pulmonary disease (COPD), the concurrent organ-specific and systemic inflammatory responses lead to airway remodelling and vascular dysfunction. Although a major common risk factor for COPD, cigarette smoke alone cannot explain the progression of this disease; there is increasing evidence that genetic predisposition also plays a role in COPD susceptibility and progression. A key enzyme in chronic lung inflammation is leukotriene A4 hydrolase (LTA4H). With its aminopeptidase activity, LTA4H degrades the neutrophil chemoattractant tripeptide PGP.

In this study, we used the luciferase reporter gene analysis system and quantitative trait locus analysis to explore the impact of single-nucleotide polymorphisms (SNPs) in the putative promoter region of *LTA4H* on LTA4H expression.

We show that not only is the putative promoter of *LTA4H* larger than previously reported but also that SNPs in the expanded promoter region regulate expression of LTA4H both in cell-based systems and in peripheral blood samples from human subjects.

These findings provide significant evidence for an active region upstream of the previously reported *LTA4H* promoter, which may have implications related to ongoing inflammatory processes in chronic lung disease.

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SNPs in the putative promoter region of *LTA4H* specifically affect the expression of leukotriene A4 hydrolase http://ow.ly/VCSNE

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Introduction

Chronic obstructive pulmonary disease (COPD) is an inflammatory lung disorder that is currently the third-leading cause of death in the USA and is noted to have an increasing incidence worldwide [1]. Mortality related to COPD is not solely associated with loss of lung function but is also associated with the development of associated cardiovascular disease.

Neutrophils play a critical role in the inflammatory response observed in COPD [2]. Although numerous mediators have been associated with acute neutrophilic inflammatory responses in disease (including interleukin (IL)-8 and leukotriene (LT)B4), there are few specific pathways that have been determined to regulate a chronic, neutrophilic inflammatory response. Recently, our laboratory has characterised a novel neutrophil chemoattractant, a tripeptide (PGP), which has a significant role in the chronic neutrophilic inflammatory response [3, 4]. Our research described a self-propagating inflammatory pathway that involves the activation of neutrophils, releasing matrix metalloprotease-9 and prolyl endopeptidase. This proteolytic cascade cleaves collagen to generate PGP [5–8]. PGP subsequently recruits additional neutrophils by mimicking a key sequence found in IL-8, which leads to further recruitment and activation of more neutrophils [4]. We further showed that acute inflammation can be terminated by LTA4 hydrolase (LTA4H) [5].

LTA4H is a major enzyme of the 5-lipoxygenase pathway, activation of which leads to the biosynthesis of proinflammatory leukotriene lipid mediators from arachidonic acid [9]. LTA4H catalyses the hydrolysis of LTA4 into LTB4, a potent proinflammatory lipid mediator that not only recruits but also activates neutrophils. More recently, our laboratory has demonstrated that PGP is regulated by the aminopeptidase activity of LTA4H [3]. LTA4H degrades PGP into PG and free proline, and decreases neutrophil influx. Thus, the regulation of the presence and activities of this enzyme has a crucial role in the regulation of the chronic neutrophilic inflammatory response in human disease. This finding demonstrated a central regulatory enzyme of inflammation that generates one chemoattractant (LTB4) and degrades another (PGP). Recently, our group has demonstrated that LTA4H aminopeptidase activity is reduced in COPD subjects, leading to increased PGP levels [10].

Genetic studies have reported associations of the promoter and coding region of *LTA4H* with vascular disease and COPD [11–17]. In this article, we explore the role of genetic variation in the expression of *LTA4H* in cell-based systems and confirm these findings at the population level in results from a previously published blood expression quantitative trait locus (eQTL) analysis. Our results identify specific genetic variants that regulate the expression of *LTA4H*.

Materials and methods

Cloning promoter fragment to reporter gene

To clone putative promoter fragments of *LTA4H*, we used human genomic DNA obtained from human cervical cancer cells (HeLa), purified with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). All primers were ordered from IDT Integrated DNA Technologies (Coralville, IA, USA). Different fragments of the putative promoter were amplified by PCR using the Phusion Hot Start High-Fidelity DNA Polymerase Kit (New England BioLabs, Ipswitch, MA, USA). Fragments were sequenced at the Howell and Elizabeth Heflin Center for Genomic Science at the University of Alabama at Birmingham.

Mutagenesis

In order to create all necessary single-nucleotide polymorphism (SNP) variants, DNA sequences were subject to mutagenesis utilising the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The obtained constructs were then verified through sequencing.

Luciferase reporter gene assay

To study the effect of SNPs on expression, we subcloned the DNA fragment with or without particular SNPs into a promoter-less vector containing a luciferase reporter gene. For luciferase expression, we used the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). HeLa cells were co-transfected with a pGL-Basic vector containing a firefly luciferase (which included our putative promoter fragments with or without SNPs) and an internal control pRL-TK vector expressing *Renilla* luciferase, as a control for transfection normalisation. The internal control provided the basal response, thus minimising experimental variability caused by transfection with different lengths of DNA.

Cell culture and treatment

HeLa cells were grown in Eagle's MEM plus 10% fetal calf serum, 2 mM L-glutamine, 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 1% nonessential amino acids (Sigma, St Louis, MI, USA), and

incubated in humidified air containing 5% CO_2 at 37°C. At 80% confluence, cells were transfected with different DNA constructs using Oligofectamine 2000 (ThermoFisher, Waltham, MA, USA) as a transfection reagent and incubated for 24 h before harvesting.

LTA4H *eQTL data*

To examine the impact of genetic variation near the *LTA4H* promoter *in vivo*, we queried two disease-associated SNPs within 6 kb of the ATG start site (rs17025122 and rs7971150) for evidence of association with *LTA4H* expression in publically available data from a previously published eQTL meta-analysis in whole blood samples from 5311 subjects (http://genenetwork.nl/bloodeqtlbrowser/) [18]. Local association data and linkage disequilibrium patterns based on 1000 Genomes EUR data were visualised using Locuszoom (https://statgen.sph.umich.edu/locuszoom/) [19].

Statistical analysis

Descriptive statistical analysis, including calculation of means and standard deviations, were conducted for all quantitative measures. The two-tailed Student t-test was used for comparisons between two groups and one-sided ANOVA was used for comparisons between three or more groups for *in vitro* studies. The results were considered significant at the 95% confidence level, or p-values of 0.05 or less ($p<0.05$).

Results

SNPs in promoter region of LTA4H

The *LTA4H* gene was first described by Mancini and Evans [20], and their report served as a basis for further insight into its transcriptional regulation. Further investigation led to the description of a 2021-bp long promoter containing a positive regulatory region and two specific protein–DNA complexes in this element [21].

In the present study, we analysed two SNPs upstream of the reported promoter for *LTA4H*. These SNPs, rs17025122 (−4.1 kb from ATG) and rs7971150 (−5.4 kb from ATG), have previously been associated with vascular disease and showed a possible association with COPD in a European case–control cohort [22].

To explore the effect of these SNPs on the expression of *LTA4H*, we subcloned them into a promoter-less vector with a luciferase reporter gene (figure 1). As a result, we generated a 2173-bp (~2.2-kb) control promoter that matched the previously reported promoter for *LTA4H* and did not contain any studied SNPs. Various extended promoter constructs were generated containing rs17025122 (termed the 4.1-kb fragment) or rs7971150 (termed the 5.4-kb fragment) (table 1).

The 4.1-kb promoter fragment was generated in two versions, one containing the ancestral allele (4.1 kb G) and the other containing minor allele (4.1 kb A). The four versions of the 5.1-kb long putative promoter fragment contained either or both of rs17025122 (minor allele A or ancestral allele G) and rs7971150 (minor allele A or ancestral allele G), in all possible combinations of the biallelic SNPs.

24 h after transfection of these constructs, HeLa cells were ruptured and luciferase expression was recorded as the luminescent signal generated in the enzymatic reaction. These results provided a robust readout of the luciferase expression level and imply a similar outcome for actual expression of *LTA4H in vivo*. Expression of luciferase in this system showed DNA length-specific activity, as evidenced by higher

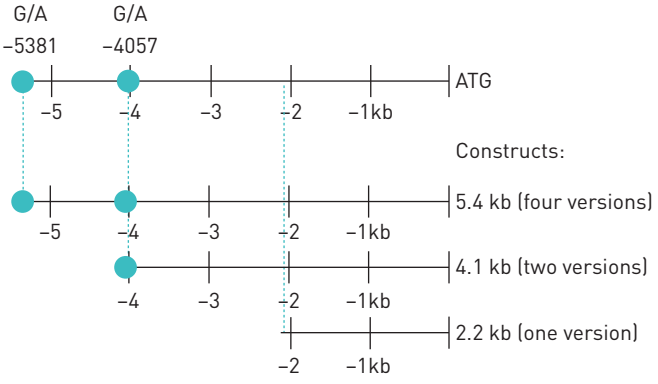

FIGURE 1 Putative promoter of the leukotriene A4 hydrolase gene (*LTA4H*). Visualisation of the putative promoter constructs includes two single-nucleotide polymorphism (SNPs) that overlap in association with cardiovascular disease and chronic obstructive pulmonary disease. Circles represent the ancestral/SNP version of the nucleotide.

TABLE 1 Generation of putative promoter constructs containing single-nucleotide polymorphisms (SNPs)

Distance from first methionine kb	Ancestral	SNP	Construct version			
			All ancestral	All SNPs	SNP fragment	
					5.4 kb	4.1 kb
2.2						
4.1	G	**A**	G	**A**		
5.4	G	**A**	GG	**AA**	**A**G	G**A**

luciferase activity of the ancestral version of the 5.4-kb fragment (4.1 kb G and 5.4 kb GG) compared to the 2.2-kb promoter region (figure 2a), suggesting a longer than anticipated promoter region. Of note, key constructs were tested in 16HBE14o- cells with similar results.

We next utilised an online software tool from Genomatix (http://www.genomatix.de/en/index.html) to determine whether the studied SNPs may impact transcription factor binding sites (TFBSs) in this extended enhancer region. This software utilises a large library of matrix descriptions for TFBSs to locate matches in DNA sequences. For the 4.1-kb SNP site, the software identified one TFBS which was unchanged between the ancestral and derived allele. For the 5.4-kb SNP site, the software predicted six TFBSs for the ancestral version (5.4 kb G) and 12 TFBSs for the derived version (5.4 kb A) (table 2). This *in silico* analysis showed how significant a difference in one base might be in gene expression.

To explore the effect of SNP on the expression of the reporter gene *in vitro*, we used the luciferase assay. For the 4.1-kb promoter fragment, there was no statistical difference in luciferase expression between the minor

TABLE 2 Software prediction of transcription factors binding to putative promoter of the leukotriene A4 hydrolase gene (*LTA4H*) containing particular single-nucleotide polymorphisms

Marker ID	rs17025122	rs7971150
Distances from ATG bp	−4057	−5381
Ancestral	G	G
Transcription factor binding sites	1) Human acute myelogenous leukaemia factors	1) cAMP-responsive element binding proteins 2) PAR/bZIP family 3) Cart-1 4) LIM homeodomain factors 5) PAX3 binding sites 6) GC-box factors SP1/GC
SNP	A	A
Transcription factor binding sites	1) Human acute myelogenous leukaemia factors	1) CLOX and CLOX homology (CDP) factors 2) cAMP-responsive element binding proteins 3) PAR/bZIP family 4) Cart-1 5) LIM homeodomain factors 6) Fork head domain factors 7) HOX transcription factors 8) Paralogue HOX genes 1–8 from the four HOX clusters A–D 9) Cart-1 10) Paralogue HOX genes 1–8 from the four HOX clusters A–D 11) CLOX and CLOX homology (CDP) factors 12) GC-box factors SP1/GC

PAR/bZIP: proline- and acidic amino acid-rich basic leucine zipper; Cart-1: cartilage homeoprotein 1; PAX3: paired-box transcription factor 3; SP1: specificity protein 1; HOX: homeobox.

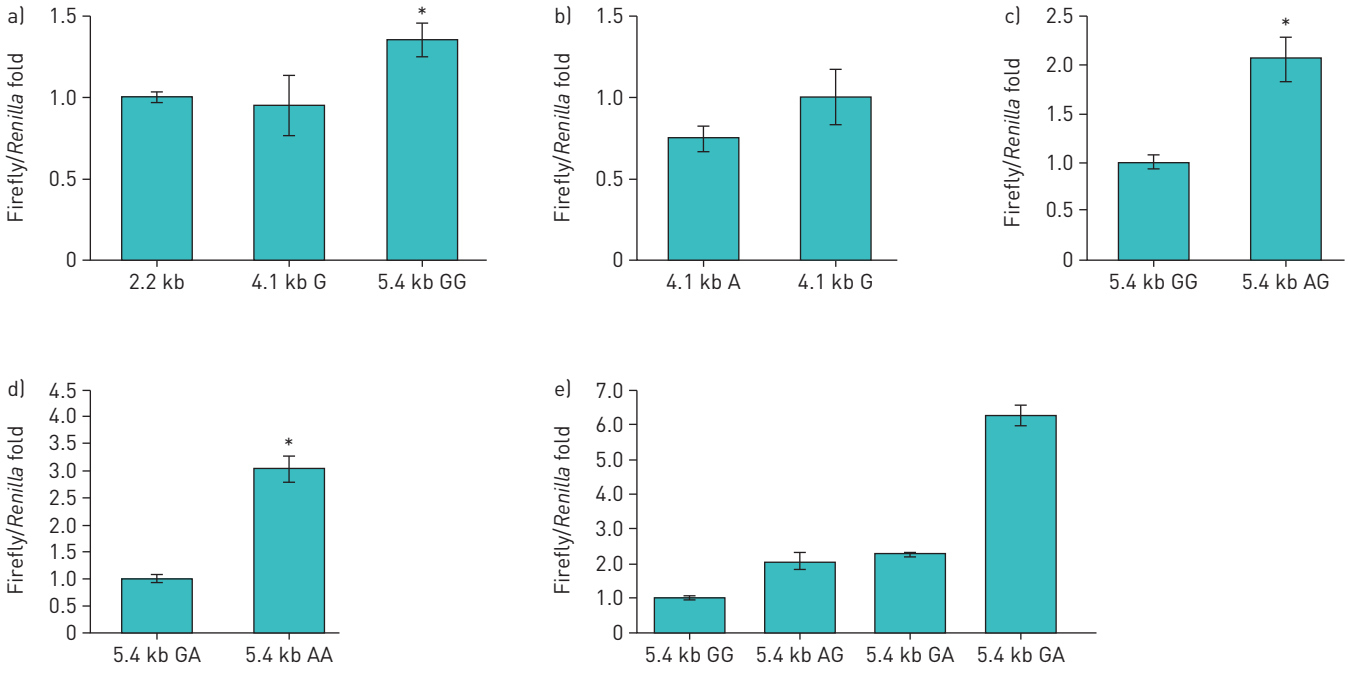

FIGURE 2 Expression of luciferase reporter gene in various promoter sequences. HeLa cells were grown to ~80% confluence, and co-transfected with plasmids carrying a particular version of the putative promoter (with or without a single-nucleotide polymorphism (SNP)) expressing a firefly luciferase as a reporter gene and expressing *Renilla* luciferase as an internal control that served as the baseline response. a) Putative leukotriene A4 hydrolase gene (*LTA4H*) promoter fragments (ancestral forms of SNPs for the 4.1- and 5.4-kb fragments) expressed the luciferase reporter gene at higher levels than the control promoter (n=10). b) The 4.1-kb putative *LTA4H* promoter (ancestral: G; SNP: A) expressed luciferase reporter gene at similar levels (n=10). c) The 5.4-kb putative *LTA4H* promoter demonstrated increased luciferase gene expression with SNP at the 5.4-kb position compared to the ancestral allele (n=10). d) The 5.4-kb putative *LTA4H* promoter demonstrated increased luciferase gene expression with the derived allele at both the 4.1- and 5.4-kb sites relative to the derived allele at the 4.1-kb site and ancestral allele at the 5.4-kb site (n=10). e) The 5.4-kb putative *LTA4H* promoter with derived alleles at both the 4.1- and 5.4-kb sites expressed the luciferase gene six-fold more than the 5.4-kb putative promoter fragment containing ancestral alleles at both the 4.1- and 5.4-kb sites. *: p<0.05.

allele (4.1 kb A) and the ancestral allele (4.1 kb G) (figure 2b). This is in agreement with the transcription factors binding software prediction. Interestingly, when maintaining the 4.1-kb allele as the ancestral allele, the 5.4-kb derived allele (5.4 kb AG) demonstrated a statistically significant increase in luciferase expression

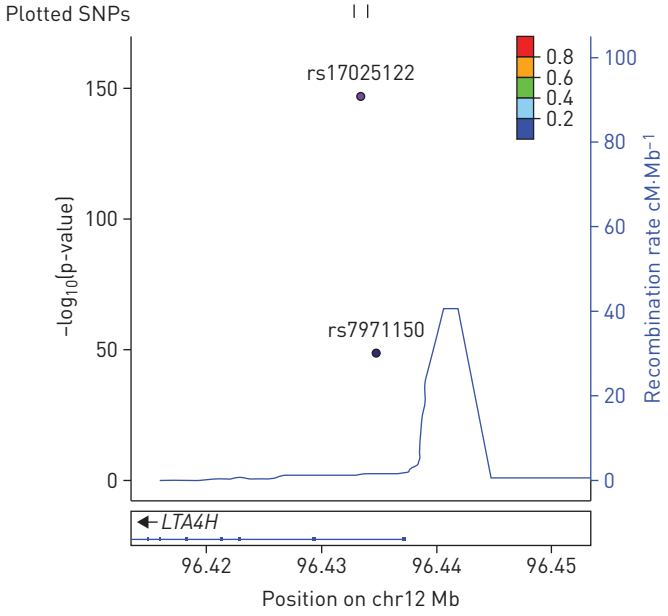

FIGURE 3 Expression quantitative trait locus (eQTL) expression of leukotriene A4 hydrolase (LTA4H) mRNA in peripheral blood. Locus plot showing eQTL association p-values for tested single-nucleotide polymorphisms (SNPs) near LTA4H in blood eQTL data previously published by Westra *et al.* [18]. Chr: chromosome.

TABLE 3 Cumulative data of two single-nucleotide polymorphism (SNP) sites in expression quantitative trait locus analysis: the effect of SNP on the leukotriene A4 hydrolase (*LTA4H*) expression

SNP	Chromosomal position bp	p-value
rs17025122 (−4.1 kb)	94 957 553	1.46×10^{-147}
rs7971150 (−5.4 kb)	94 958 877	2.06×10^{-49}

relative to the 5.4- and 4.1-kb ancestral alleles (5.4 kb GG) (figure 2c). Surprisingly, the presence of the derived allele at 5.4 kb in the derived allele background at 4.1 kb (5.4 kb AA) had a synergistic effect and expressed luciferase over three-fold stronger than the ancestral allele at 5.4 kb with the derived allele at 4.1 kb (5.4 GA) (figure 2d). Overall, when the 4.1- and 5.4-kb sites contained the minor allele (5.4 kb AA), the putative promoter fragment expressed over six-fold higher reporter gene levels compared to the double ancestral form (5.4 kb GG) (figure 2e). These results highlight that, although the 4.1-kb derived allele has little impact on luciferase expression, there is an additive effect of the derived allele at 4.1 kb and 5.4 kb, which has notable impact on luciferase expression. These data suggest that changes at the 5.4-kb site may have upstream effects on transcription factor binding at the 4.1-kb site and highlight the importance of downstream elements in regulation of the expression of *LTA4H*.

Genetic regulation and transcription factor binding at LTA4H *promoter-region SNPs* in vivo

Figure 3 shows that two candidate SNPs, rs17025122 (4.1 kb) and rs7971150 (5.4 kb), are strongly associated with *LTA4H* expression *in vivo* in publically available eQTL data from an analysis of blood expression from over 5000 subjects [18]. These SNPs demonstrated notable p-values, with rs17025122 (4.1 kb) with p-value of 1.46×10^{-147} and rs7971150 (5.4 kb) with p-value of 2.06×10^{-49} (table 3).

To determine whether there is evidence of transcriptional activity in these SNP sites in ENCODE (Encyclopedia of DNA Elements) epigenomic data, we queried the Haploreg web interface (www.broadinstitute.org/mammals/haploreg/haploreg.php) for evidence of enhancer activity or experimental evidence of transcriptional factor binding in cell lines or primary cells at rs17025122 and rs7971150 or SNPs in perfect linkage disequilibrium. Both SNPs lie within enhancer regions (K562 cells for rs7971150 and multiple cell types for rs17025122) and both lie within regions of experimentally observed transcription factor binding [23].

Discussion

Inflammation is a critical feature of COPD and recent data have suggested that PGP peptide may serve as a critical component of the inflammatory response in COPD [4–6]. In fact, a recent report showed that PGP levels in COPD sputum improved during treatment with chronic azithromycin in concert with reduced exacerbation frequency. PGP was the only inflammatory biomarker that demonstrated marked responsiveness with azithromycin treatment [24]. The regulation of this peptide, therefore, is likely to be of critical importance in the progression of chronic lung inflammation. The results reported herein suggest that the promoter region for *LTA4H* is longer than previously thought and demonstrate allele-specific expression of *LTA4H* in an *in vitro* system, which may have significant implications for COPD biology. Therefore, it is possible that genetic variability might portend increased presence and/or activity of this enzyme, which in turn might create a more potent pro-inflammatory environment for people who smoke and/or have COPD with particular SNPs.

Importantly, population-level eQTL analyses confirm that SNPs near the *LTA4H* promoter impact *LTA4H* mRNA expression *in vivo*. Ongoing studies may suggest specific patient genotypes that confer high risk of development and progression of COPD. Indeed, our data strongly suggest that cigarette smoke exposure induces LTA4H expression in airway epithelial cells [3]. Future studies will focus on the expression of SNPs with cigarette smoke exposure to determine critical gene-by-environment interaction.

The combination of an *in vitro* expression system coupled with large COPD cohorts with genetic data, such as COPDGene, will also establish a paradigm for future studies of other enzymes critical to the unrelenting inflammatory response observed in COPD and may suggest new therapeutic disease targets in this disorder.

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