Leukotriene B₄ receptor locus gene characterisation and association studies in asthma

Asif S Tulah¹,5, Bianca Beghé², Sheila J Barton³, John W Holloway⁴ and Ian Sayers¹*

Abstract

Background: Polymorphisms spanning genes involved in the production of leukotriene B₄ (LTB₄) e.g. ALOX5AP and LTA4H are associated with asthma susceptibility, suggesting a role for LTB₄ in disease. The contribution of LTB₄ receptor polymorphism is currently unknown. The aim of this study was to characterise the genes for the two pivotal LTB₄ receptors, LTB₄R1 and LTB₄R2 in lung tissue and determine if polymorphisms spanning these genes are associated with asthma and disease severity.

Methods: Rapid amplification of cDNA ends (RACE) was used to characterise the LTB₄R1 and LTB₄R2 gene structure in lung. The LTB₄R1/2 locus on chromosome 14q11.2 was screened for polymorphic variation. Six LTB₄R single nucleotide polymorphisms (SNPs) were genotyped in 370 Caucasian asthma families and 299 Adult Asthma Individuals (n=1877 total) and were evaluated for association with asthma and severity (BTS) outcome measures using Family Based Association Test, linear regression and chi square.

Results: LTB₄R1 has complex mRNA arrangement including multiple 5'-untranslated exons, suggesting additional levels of regulation. Three potential promoter regions across the LTB₄R1/2 locus were identified with some airway cell specificity. 22 SNPs (MAF>0.01) were validated across the LTB₄R locus in the Caucasian population. LTB₄R1 and LTB₄R2 SNPs were not associated with asthma susceptibility, FEV₁ or severity.

Conclusions: LTB₄R1 and LTB₄R2 show splice variation in the 5'-untranslated region and multiple promoter regions. The functional significance of this is yet to be determined. Both receptor genes were shown to be polymorphic. LTB₄R polymorphisms do not appear to be susceptibility markers for the development of asthma in Caucasian subjects.

Keywords: Association, Asthma, Family based association test, Leukotriene, Leukotriene B₄ receptor, RACE, Severity

Background

Asthma is a multifactorial respiratory disease with genetic and environmental contributing factors. Leukotrienes are lipid mediators known to be involved in allergic conditions such as asthma and are generated by a series of enzymes and proteins which form the 5-lipoxygenase (5-LO) pathway [1]. There are two types of leukotriene; cysteinyl leukotrienes (CysLTs), which are potent bronchoconstrictors, and the dihydroxy leukotriene, leukotriene B₄ (LTB₄) a chemoattractant and activator of leukocytes. CysLTs have long been reported as important mediators contributing to inflammatory diseases such as asthma [2]. Recent data supports a role for LTB₄ in asthma pathophysiology. LTB₄ is elevated in the airways of asthma subjects and its concentration correlates with asthma severity [3,4].

Polymorphisms within genes encoding constituents of the 5-LO pathway provide excellent candidates for markers of asthma susceptibility. Polymorphisms in two 5-LO pathway genes; 5-lipoxygenase activating protein (ALOX5AP) and leukotriene A₄ hydrolase (LTA4H) have shown an association with LTB₄ overproduction from ionomycin-stimulated neutrophils and with myocardial infarction (MI) susceptibility [5,6]. 5-lipoxygenase activating protein (FLAP) is an adapter protein for the rate-limiting enzyme 5-lipoxygenase and is involved in the production of all leukotrienes; however LTA₄H is specifically involved in LTB₄ production. We and others have recently provided preliminary evidence that SNPs...
spanning ALOX5AP and LTA4H are asthma susceptibility markers and determinants of lung function [7,8]. Polymorphisms spanning ALOX5, LTC4S, CYSLTR1 and CYSLTR2 have also shown association with asthma-related traits, reviewed in [9].

Two G protein-coupled receptors (GPCRs) for LTB4 encoded by LTB4R1 and LTB4R2 have been described and are located together on chromosome 14q11.2 [10,11]. LTB4 via its receptors is important in the recruitment and activation of leukocytes to sites of inflammation [12] and these receptors have been proposed as potential therapeutic targets in asthma. Increasing our understanding of the expression, regulation and potential function of these receptors may provide important information for the design of therapeutic agents. Currently, relatively little is known about LTB4R1 and LTB4R2 gene structure, splice variation and polymorphic variation and the contribution of polymorphic variation to asthma and disease severity.

The aims of this study were to 1) investigate the gene structure of human LTB4R1 and LTB4R2 in cells and tissues relevant to asthma; 2) determine the extent and nature of polymorphic variation across the receptor locus and 3) determine if LTB4R polymorphisms were associated with asthma, lung function and disease severity in asthma families and adult asthma subjects. Our data suggest that LTB4R1 and LTB4R2 have complicated gene structure and that polymorphisms spanning the LTB4R locus are not determinants of asthma susceptibility.

**Methods**

**Cell culture and RNA/cDNA preparation**

Human airway smooth muscle (HASM) cells were isolated and cultured as previously described [13]. Primary human bronchial epithelial cells (HBEC) were obtained from Lonza (Wokingham, UK) and cultured in bronchial epithelial growth medium (BEGM, Lonza, UK), using bronchial epithelial differentiation medium (BDEM, Lonza, UK) cells were differentiated at air-liquid interface [14,15]. The bronchial epithelial cell line BEAS-2B and a leukemic monocyte cell line, THP-1, were also cultured as described previously [14,16]. Commercial RNA for the lung, brain and placenta was obtained from Ambion (Huntingdon, UK) and peripheral blood mononuclear cells (PBMC), polymorphonuclear cells (PMN) was obtained from 3H Biomedical (Uppsala, Sweden). Cells were lysed and RNA extracted (from at least two different donors) using the RNeasy mini kit (Qiagen, Crawley, UK), as described by the manufacturer. cDNA was prepared using the Superscript first strand cDNA synthesis kit (Invitrogen, Paisley, UK) using random hexamers and 0.5-1.0μg total RNA per reaction, as directed by manufacturer.

**Rapid amplification of cDNA ends (RACE)**

RACE was performed using the GeneRacer kit (Invitrogen) and Superscript II as directed [14]. RACE-ready lung cDNA was synthesised from 1μg total lung RNA obtained from Ambion (Huntingdon, UK) as described. Gene-specific primers were designed for 5’ and 3’ RACE in the coding region in an overlapping fashion. Plasmid DNA from RACE PCR clones was prepared using the DNA Miniprep kit (Qiagen) and sequenced with M13F and M13R vector primers using Big Dye v3.1 (Applied Biosystems) and an ABI 310 DNA sequencer. Sequence data was aligned to the human database using the Basic Local Alignment Search Tool (BLAST) 2 sequence alignment program.

**Polymorphism screening**

The extended LTB4R1 and LTB4R2 genomic region identified by RACE (~11.5kb, NCBI build 37: +14:24776125–24787584) was amplified by multiple PCR reactions and screened for polymorphisms by direct sequencing using DNA extracted from whole blood of 35 individuals from the Nottingham Adult Asthma Cohort recruited on the basis of physician diagnosed asthma and no other respiratory illness with <10 pack-years smoking history. These subjects had severe asthma as defined by British Thoracic Society (BTS) step ≥ 3. Single nucleotide polymorphisms (SNPs) were identified by examining chromatograms and BLAST analysis of sequencing traces. Any potential SNP identified was validated by sequencing on the reverse strand of DNA. Ethical approval was obtained from the Nottingham University Hospitals local ethics committee.

**Subjects for association analyses**

341 Caucasian families (n=1508) with at least two biological siblings with physician diagnosed asthma were recruited from the Southampton area. This cohort has been described in detail previously [17]. Baseline FEV1 (forced expiratory volume in one second) was measured as best of three values within 5% performed using Vitalograph dry-wedge bellows spirometer (Vitalograph Ltd, Buckingham, UK) and determined 14 days after respiratory tract infection or use of bronchodilator or anti-allergic medication. 46 Caucasian families (n=184) with at least two biological siblings with physician diagnosed asthma from the Nottingham area [17] were also recruited. Baseline lung function tests were performed, FEV1 defined as the best of three values. The Nottingham and Southampton cohorts were combined to generate a UK family cohort (n=370). A cohort comprising 299 unrelated adult European Caucasian individuals recruited from Nottingham and Padova was used [18]. Individuals were 16–60 years, had asthma for >1 year with no other respiratory illness and <10 pack-years
Smoking history. Baseline FEV$_1$ was measured. All subjects were classified according to British Thoracic Society Step Guidelines (BTS steps, ranging from step 1 to step 5) based on physician prescribed medication [19]. Ethical approval was obtained from the Nottingham University Medical School, the Padova Local Ethics Committees and the Southampton and South West Hampshire and the Portsmouth and South East Hampshire Local Research Ethics Committees. Informed consent was provided by the adult (or parent/guardian for child subjects).

**SNP selection and genotyping**

**LTB4R** SNPs were chosen for their ability to tag linkage disequilibrium (LD) blocks using Tagger software [20] or for inferred function. Sequencing data from the severe asthma subjects and available HapMap data (Build 36) were used to select the six SNPs for analysis. We acknowledge a limitation of the current study is the use of this available HapMap build that has now post SNP selection been superseded by 1000 genomes data. Six SNPs which captured the information for eight SNPs (data not shown). 5

**Association analyses**

The family based association test (FBAT) software (version 1.5.1) [23] was used for association analyses in the family cohorts between **LTB4R1** 1.5.1) [23] was used for association analyses in the family cohorts between **LTB4R2** (ver-

**Results**

**Identification of LTB4R1 and LTB4R2 splice variants and promoter regions**

There was ubiquitous expression of **LTB4R1** and **LTB4R2** mRNA in the airway and periphery cells, including lung, HASM, HBEC, PBMC, PMN, BEAS-2B and THP-1 (data not shown). 5

**LTB4R1** 5

**LTB4R2** showed a more conserved structure based on RACE data. Only one 5

**LTB4R1** 5

**LTB4R2** sequencing

Direct sequencing in 35 individuals revealed 22 SNPs validated across the region (Table 1 and Figure 1). Four were novel SNPs and identified at low frequency (MAF <0.01) at position −7686, −5918, −5866, −5737 (in the **LTB4R2** 5'-untranslated region), all positions are relative to the **LTB4R1** ATG. 18 of these SNPs have now been reported (1000genomes and NCBI Build 37). Of note, the non-synonymous SNPs identified in Asian populations in **LTB4R1** (rs34645221, Ala79Ser and rs17849864, Leu346Phe) and **LTB4R2** (rs1950504, Asp196Gly) were
not validated in the UK population analysed. Two synonymous coding region SNPs were identified, both in LTB4R1. rs3742511 (Ser18Ser) located in the extracellular N-terminus and rs1046584 (Gly309Gly) located in the cytosolic C-terminus.

**LTB4R polymorphisms are not associated with asthma, lung function or disease severity**

The clinical characteristics for all study cohorts are shown in Table 2. The family cohort contains children with asthma (mean age sibling 1 was 13.3±4.4 years and sibling 2 was 10.3±4.6 years) and the second asthma cohort is comprised of asthma adults (mean age 39.2±12.3 years, n=299). Genotyping data for the six SNPs did not show deviation from the Hardy-Weinberg Equilibrium (p>0.05). Minor allele frequencies (MAF) were similar across the different study cohorts. The haplotype structure of the LTB4R region generated using the genotyping data in the cohorts is shown in Figure 3. Analysis of LD across these SNPs indicated some redundancy in the genotyping with high LD between several LTB4R SNPs e.g. rs11158635, rs2516564 and rs2224122.
Our data indicate no LTB4R SNPs tested were associated with asthma diagnosis, FEV1 or severity (BTS) in the families (Table 3). In the Adult Asthma Cohort we completed baseline percent predicted FEV1 analyses (Table 4), and again did not observe any significant associations. We retrospectively evaluated SNPs spanning ALOX5AP (8 SNPs) and LTA4H (6 SNPs) that encode for proteins involved in LTB4 production and had previously been associated with asthma susceptibility [7] with BTS defined severity (step 1–5). Interestingly, while no association survived correction for multiple testing, modest associations in the family cohort were observed with multiple ALOX5AP SNPs, e.g. SG13S41G (intron 4) (p=0.005, z=+2.778) and SG13S114A (intron 1) (p=0.017, z=+2.397). LTA4H SNP also showed modest association e.g., rs2540482C (5’UTR) (p=0.014, z=−2.468) (Table 5). Our previous study [7] analysed the effect of these same SNPs in determining asthma severity, but used an in house generated asthma severity score, which showed the same SG13S41G SNP with a p=0.021 and the same direction of effect as the present study. In the Adult Asthma Cohort dichotomised analysis of BTS 1 versus BTS ≥4 showed no significant association with any SNP tested (data not shown).

Discussion
Both LTB4R1 and LTB4R2 receptors are potentially important drug targets for conditions driven by inflammation involving LTB4. LTB4 production and activity is thought to be particularly important in severe asthma where a neutrophilic inflammation is more commonly observed [24]. The aims of this study were to characterise the LTB4R1/2 locus at the molecular level to identify key regulatory regions (TSS, promoter regions), splice variation and polymorphic variation in lung tissue and to investigate the potential contribution of polymorphic variation to asthma susceptibility and severity. Our data show that LTB4R1 and LTB4R2 mRNA is ubiquitously expressed in multiple lung and peripheral cell types and that these genes are complex and have variation in 5’-untranslated regions and predicted promoter regions which may be functional in terms of cell-specific regulation. We also show that the LTB4R1/2 locus is polymorphic (22 SNPs spanning ~11.5kb, MAF>0.01), with most variation in the untranslated regions. This study does not provide evidence supporting a role for LTB4R SNPs in susceptibility to develop asthma or severity phenotypes using asthma enriched families and adult asthma subjects. However, retrospective analyses of SNPs spanning ALOX5AP and LTA4H provided some evidence for association with BTS defined severity although this did not survive correction for multiple testing. This study represents the first characterisation of the LTB4R locus with respect to gene structure in the lung and the first evaluation of LTB4R SNPs for association with asthma susceptibility and severity.
| SNP          | Alleles (major/minor) | Location | Gene location (amino acid) | Individuals sequenced (n) | Minor allele frequency |
|--------------|-----------------------|----------|----------------------------|---------------------------|------------------------|
| rs2332320    | T/C                   | −8639    | LTB4R2 5'UTR               | 43                        | C=0.128                |
| rs1053648    | C/T                   | −8513    | LTB4R2 5'UTR               | 43                        | T=0.035                |
| rs1053649    | A/C                   | −8456    | LTB4R2 5'UTR               | 43                        | C=0.035                |
| rs11158634   | C/G                   | −8401    | LTB4R2 5'UTR               | 43                        | G=0.244                |
| rs11158635   | G/T                   | −8088    | LTB4R2 5'UTR               | 43                        | T=0.244                |
| Novel 1      | C/G                   | −7686    | LTB4R2 5'UTR               | 35                        | G=0.014                |
| rs2144492    | C/A                   | −7446    | LTB4R2 5'UTR               | 35                        | A=0.043                |
| rs2180197    | G/C                   | −7266    | LTB4R2 5'UTR               | 35                        | C=0.043                |
| rs45512098   | C/G                   | −6743    | LTB4R2 5'UTR               | 41                        | G=0.024                |
| rs2516564    | C/T                   | −6215    | LTB4R2 5'UTR               | 41                        | T=0.22                 |
| rs2748543    | C/A                   | −6184    | LTB4R2 5'UTR               | 42                        | A=0.27                 |
| Novel 2      | G/A                   | −5918    | LTB4R2 5'UTR               | 42                        | A=0.012                |
| Novel 3      | C/G                   | −5866    | LTB4R2 5'UTR               | 42                        | G=0.012                |
| Novel 4      | G/C                   | −5737    | LTB4R2 5'UTR               | 42                        | C=0.012                |
| rs2224122    | G/C                   | −1444    | LTB4R1 5'UTR               | 43                        | C=0.163                |
| rs374510     | G/C                   | −1177    | LTB4R1 5'UTR               | 44                        | C=0.011                |
| rs3742511    | T/C                   | +53      | LTB4R1 ORF (S/S)           | 39                        | C=0.026                |
| rs1046584    | C/T                   | +926     | LTB4R1 ORF (G/G)           | 39                        | T=0.28                 |
| rs1046587    | G/A                   | +1202    | LTB4R1 3'UTR               | 41                        | A=0.048                |
| rs4981503    | G/T                   | +143S    | LTB4R1 3'UTR               | 41                        | T=0.11                 |
| rs3181384    | C/T                   | +2118    | LTB4R1 3'UTR               | 42                        | T=0.286                |
| rs111415008  | A/C                   | +2555    | LTB4R1 3'UTR               | 41                        | C=0.012                |

The LTB4R locus was sequenced in 35 asthmatic subjects. 22 SNPs were validated, of which four were novel. Those SNPs with rs numbers correspond to those reported in the 1000 genomes project and dbSNP (NCBI build 37).

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**Table 2 Clinical characteristics of the study cohorts**

|                   | Pedigrees | Asthma Families | Sibling 1 | Sibling 2 | Adult Asthma Cohort |
|-------------------|-----------|-----------------|-----------|-----------|---------------------|
| **Age (years, mean±SD)** | 26.2±1.6  | 13.3±4.4        | 10.3±4.6  | 39.2±12.3 | 65.6                |
| **Gender (% Female)**     | 50.8      | 54.6            | 53.7      | 100       | 100                 |
| **Asthma (% Doctor diagnosed)** | 61.6      | 100             | 100       | 100       | 100                 |
| **FEV1 (% Predicted) (mean±SD)** | ND        | 95±15.6         | 96.2±14.9 | 92.3±20.57 | 64.3                |
| **Positive skin prick test (%)** | 62.5      | 73.9            | 65.1      | 47.4      | 46.4                |
| **Eczema (% questionnaire)**                  | 41.1      | 53.5            | 55.1      | 31.8      | 31.8                |
| **Hay fever (% questionnaire)**               | 50.2      | 64.9            | 47.4      | 64.5      | 64.5                |
| **Log total serum IgE (mean)**                | 2.04      | 2.33            | 2.33      | 2.03      | 2.03                |

**Step on BTS guidelines (%)**:
- **Step 1**: 19.1, 24.9, 23.8, 13.7
- **Step 2**: 32.5, 52.7, 55.7, 17.7
- **Step 3**: 6.1, 11.6, 10.2, 50.9
- **Step 24**: 4.7, 9.2, 6.7, 17.7

**n**: 1578, 370, 361, 299
Table 3 LTB4R SNP association analysis with asthma, FEV₁ and BTS score 1 to 5 in 370 families

| SNP no.* | SNP     | Gene Location | Alleles | MAF  | Asthma Z-score | P-value | Fam | FEV₁ Z-score | P-value | Fam | BTS (1–5) Z-score | P-value |
|----------|---------|---------------|---------|------|----------------|---------|-----|---------------|---------|-----|------------------|---------|
| LT B4 R  |         |               |         |      |                |         |     |               |         |     |                  |         |
| 1        | rs2332320 | 5'UTR         | T/C     | 0.096| −0.173         | 0.863   | 121 | −0.350        | 0.726   | 118 | −1.000           | 0.317   |
| 2        | rs11158635 | 5'UTR         | G/T     | 0.211| −0.648         | 0.517   | 211 | −0.813        | 0.416   | 204 | +0.386           | 0.699   |
| 3        | rs2516564  | 5'UTR         | C/T     | 0.212| −1.109         | 0.268   | 221 | −1.288        | 0.198   | 218 | −0.060           | 0.952   |
| LT B4 R  |         |               |         |      |                |         |     |               |         |     |                  |         |
| 4        | rs2224122  | 5'UTR         | C/G     | 0.214| −0.907         | 0.365   | 217 | −1.193        | 0.233   | 212 | +0.103           | 0.918   |
| 5        | rs1046587  | 3'UTR         | G/A     | 0.494| −1.204         | 0.229   | 287 | −1.391        | 0.164   | 287 | −1.006           | 0.314   |
| 6        | rs3181384  | 3'UTR         | C/T     | 0.215| −0.895         | 0.371   | 215 | −1.190        | 0.234   | 211 | +0.157           | 0.875   |

The z score indicates the direction of the association (+ means the allele was overtransmitted = risk and − means undertransmitted = protection with respect to asthma affection; for continuous traits + indicates the allele confers higher trait values and − indicates the allele confers lower trait values). The z score is a measure of transmission equilibrium i.e. the deviation from the number of times an allele was transmitted to affected offspring and the number of times it should be transmitted under the null hypothesis of no association, no linkage. Number of families in analysis (≥10 families). *SNP location shown on Figure 1.
LTB₄ production is increased in asthma [3] with levels highest in severe asthmatics when compared to moderate asthmatics and control subjects [24]. The LTB₄-LTB₄R interaction is responsible for the influx of inflammatory cells into the lung. A significantly reduced recruitment of eosinophils and neutrophils into the airways has been demonstrated in mice deficient in LTB₄ [26]. Murine studies also show LTB₄ is responsible for CD8⁺ T-cell mediated airway hyperresponsiveness through a mechanism involving mast cells [26]. These studies suggest LTB₄ contributes to asthma pathogenesis through the recruitment and activation of neutrophils and eosinophils. Blocking the LTB₄-LTB₄R interaction with the inhibitor LY293111 led to a reduction in BAL neutrophils [27] and with the inhibitor U75302 reduced the migration and proliferation of airway smooth muscle cells which contributes to airway remodelling [28]. These data suggested a potential role in both the inflammatory and structural changes observed in the asthmatic airway.

Polymorphisms spanning ALOX5AP and LTA4H show association with LTB₄ production from ionomycin stimulated neutrophils [5,6]. We have previously reported evidence that these same polymorphisms are associated with asthma susceptibility [7]. To date, little is known about the molecular structure of the two receptors for LTB₄ (LTB₄R1 and LTB₄R2) in lung and peripheral cells/tissues and regarding the effect of polymorphism contributing to asthma and severity phenotypes. Knowledge of the LTB₄R1 and LTB₄R2 isoforms and their expression pattern in effector cells will be useful when designing receptor antagonists and the effect of polymorphic variation across the receptors may show pharmacogenetic effects. We hypothesised that in addition to genes involved with LTB₄ synthesis, alterations in genes encoding LTB₄ target receptors may alter cellular responses to LTB₄, such as inflammatory cell influx and contribute to asthma susceptibility and severity.

LB₄R1 shows varied structure at the mRNA level with different 5' untranslated structures and transcription start sites (TSS), whereas LB₄R2 is more conserved. For LB₄R1, the open reading frame (ORF) being contained in a single exon which showed concordance with other GPCRs showing intronless 5' exons [29,30]. The homogeneity of

Table 4 Baseline lung function (FEV₁) and LTB₄R SNPs in the adult asthma cohort

| SNP     | MAF | p-value (R²) | Group (n) | Value, % (Mean ± SEM) |
|---------|-----|--------------|-----------|-----------------------|
| LTB₄R2  |     |              |           |                       |
| *rs2332320 0.10 | 0.703 | 0 (210) | 92.568 ± 1.424 |
| (5'UTR) | (0.001) | 1 (50) | 91.331 ± 2.918 |
| rs11158635 0.19 | 0.421 | 0 (168) | 93.592 ± 1.599 |
| (5'UTR) | (0.007) | 1 (79) | 90.047 ± 2.331 |
| rs2516564 0.19 | 0.422 | 0 (170) | 93.529 ± 1.592 |
| (5'UTR) | (0.007) | 1 (77) | 89.871 ± 2.365 |
|          |      | 2 (10)   | 90.412 ± 6.564 |

Table 5 ALOX5AP and LTA4H SNP association analysis with BTS score 1 to 5 in 370 families

| SNP     | Gene Location | Alleles | MAF | Fam | Z-score | P-value |
|---------|---------------|---------|-----|-----|---------|---------|
| ALOX5AP |               |         |     |     |         |         |
| rs115325 | 5'UTR         | G/A     | 0.109 | 118 | +0.977  | 0.329   |
| rs115314 | Intron1       | T/A     | 0.323 | 245 | +2.977  | 0.017   |
| rs3803277 | Intron2   | C/A     | 0.445 | 283 | +1.818  | 0.069   |
| rs13589  | Intron3       | G/A     | 0.042 | 54  | +1.886  | 0.059   |
| rs4668448 | Intron4   | C/T     | 0.242 | 239 | +1.183  | 0.237   |
| rs135322 | Intron4       | C/A     | 0.474 | 272 | +1.617  | 0.106   |
| rs13541  | Intron4       | A/G     | 0.067 | 80  | +2.778  | 0.005   |
| rs13535  | 3'UTR         | G/A     | 0.078 | 97  | +1.057  | 0.291   |

LTA4H

rs1978331 | Intron11 | T/C | 0.417 | 261 | -1.865 | 0.062 |
rs17677715 | Intron6  | T/C | 0.193 | 187 | -1.974 | 0.048 |
rs2540482 | 5'UTR    | T/C | 0.222 | 209 | -2.468 | 0.014 |
rs2606845 | 5'UTR    | A/G | 0.261 | 225 | -0.856 | 0.392 |
rs2540475 | 5'UTR    | C/T | 0.215 | 212 | -1.103 | 0.270 |

The z score indicates the direction of the association (for continuous traits + indicates the allele confers higher trait values and – indicates the allele confers lower trait values). The z score is a measure of transmission equilibrium i.e. the deviation from the number of times an allele was transmitted to affected offspring and the number of times it should be transmitted under the null hypothesis of no association, no linkage. Number of families in analysis (≥10 families).

Regression analysis was used to investigate the association between LTB₄R SNPs and baseline percent predicted FEV₁, in the Adult Asthma Cohort (n=299) using the additive model. 0, 1, 2 represent number of genotypes for major, heterozygotes and minor genotypes respectively. *Dominant model analysis was completed for rs2332320 due to low number of minor allele homozygotes in the additive model.
these results between templates provides support for these variants being authentic. Previous studies have suggested LTB4R1 contains one 5'-untranslated exon which is differentially spliced [10].

These different 5'-terminal exons give at least three different regions for transcriptional control across the LTB4R locus. Two different transcription start sites in LTB4R1 at positions −4150 to −4148 and −748 to −758 and in LTB4R2 at position −5512 (relative to LTB4R1 ATG) were identified. −4150 has been reported in THP-1 cells and found to be active [29], supporting our finding of this TSS in the lung. The sequence for these transcription start sites were also identified in other lung and peripheral cells based on our PCR screen. Further characterisation of the promoter regions identified is needed to determine whether they are cell-specific. Our bioinformatic screen for transcription factor binding sites has shown multiple Sp-1 and AP-1 motifs in the promoter regions defined by TSS −5512 and −4150 to −4148, but not in the −748 to −758 region. Sp-1 is responsible for basal transcription and AP-1 is involved in inflammation, suggesting these promoter regions may be utilised under these conditions. The promoter region defined by −748 to −758 does not contain these motifs, but contains a GR (glucocorticoid receptor) site. GRs are transcription factors that are activated when bound to steroids. Activated GR can interact with other transcription factors, which can be positive (anti-inflammatory) or negative. The latter is observed in patients with steroid resistant asthma where transcription factors inactive GR; a study has shown AP-1 may interfere with the binding of GR to DNA in steroid resistant patients [31]. Due to the close arrangement of LTB4R1 and LTB4R2 on chromosome 14q11.2 and the overlapping promoter region of LTB4R1 being in the ORF of LTB4R2 [11] raises the possibility that expression of these genes is regulated by the use of different promoters and may have cell-specific expression patterns. The complex 5'-untranslated region suggests transcriptional regulation may be important for tissue-specific regulation of the LTB4R1 gene. This complexity can also lead to decreased efficiency of translation [32], which may be an important consideration when developing antagonists to target these receptors.

We screened the LTB4R locus to determine the level of polymorphic variation across the receptors in the Caucasian population. 22 SNPs were validated in our UK population. Previously suggested non-synonymous polymorphisms (which were identified in the Japanese population) in LTB4R1 (rs34645221, Ala79Ser and rs17849864, Leu346Phe) and LTB4R2 (rs1950504, Asp196Gly) [33] were not validated. 14 validated SNPs were at the 5'-end of the LTB4R locus, in the predicted LTB4R2 promoter location. Of these 4 novel SNPs were identified. These had a low MAF (<0.05) and to date have not been reported in genetic databases or the 1000genomes browser. These could potentially affect transcriptional efficiency (of either LTB4R2 or both LTB4R1 and LTB4R2 due to the close location of these genes). Our data also suggest strong linkage disequilibrium between the SNPs and a conserved nature of the locus which gives support for the two genes being formed by duplication during evolution [11].

Our studies did not observe any significant association with asthma, FEV1 or severity (BTS defined) in the asthma families or adult asthmatics for any LTB4R SNP analysed. These analyses suggest that these traits may not be genetically determined with respect to LTB4R polymorphism. Although there was no significant association, our data does show a constant direction of effect, suggesting this study may be underpowered to detect a subtle effect. Interestingly, there was modest evidence for a role of ALOX5AP and LTA4H SNPs associated with BTS defined asthma severity. These data therefore suggest genetically determined leukotriene production may be important in determining disease severity and not alterations in the downstream LTB4R receptor expression/activity. While no other study has assessed the role of LTB4R SNPs in asthma-related traits, research has involved SNPs spanning these genes in the cardiovascular field, where leukotrienes have also been shown to contribute to early atherosclerosis. rs1046587 and rs3181384 (both LTB4R1, 3'UTR) and three other SNPs spanning LTB4R were tested for association with carotid intima-media thickness in one study, but no association was observed after correction for multiple testing [34]. Also no association was observed with rs1046587 (LTB4R1, 3'UTR) and risk of ischemic stroke phenotypes in UK and German stroke cohorts [21]. This study did, however, identify significant or borderline association for the four other LTB4R polymorphisms tested: rs2748543 and rs3181384 (both in strong LD) with cardioembolic stroke in a UK cohort and rs1950505 and rs3742510 (also both in strong LD) with cardioembolic stroke in a German cohort [21]. Only rs2748543 was shown to be in LD with rs11158635 genotyped as part of this study.

Our study represents the first characterisation of the LTB4R locus with respect to gene structure in the lung and the first study to investigate association of LTB4R SNPs with asthma and importantly asthma severity where LTB4 has been suggested to have a more prominent role. We acknowledge the limitations of this study. One limitation was that RACE was conducted in the lung tissue which hampered our ability to detect cell-specific patterns of expression. Therefore it is likely that novel transcripts which may occur in other cells of the lung and periphery were not identified. To address some of these issues we profiled the variants identified by
5’RACE using PCR. Data suggest LTB4R1 and LTB4R2 show a ubiquitous expression profile in lung and peripheral cells and suggest that any antagonist targeting these receptors is unlikely to be cell-specific. We acknowledge that our sequencing cohort did not have the power to detect very rare SNPs (0.1%), however we did search the 1000genomes project resource to see if any of our identified rare variant SNPs (those with MAF<0.05) were validated by this project. Also there were modest numbers of families/individuals in our asthma cohorts used for association analyses. For this reason replication in larger additional cohorts is needed to validate our findings. Similarly, our asthma subjects had relatively preserved lung function which may have impeded our ability to detect association with FEV1 (% Predicted).

Conclusions
In conclusion, this study has shown that LTB4R1 and LTB4R2 have complicated structure and are highly polymorphic. We also report the first evidence that SNPs spanning these genes are not associated with asthma, lung function or asthma severity.

Abbreviations
S-LO: 5-lipoxygenase; BEDM: Bronchial epithelial differentiation medium; BEGM: Bronchial epithelial growth medium; BHR: Bronchial hyperresponsiveness; BLAST: Basic Local Alignment Search Tool; CYSLTs: Cysteinyl leukotriene; FBAT: Family based association test; FEV1: Forced expiratory volume in one second; FLAP: 5-lipoxygenase activating protein; GPCR: G protein-coupled receptor; HASM: Human airway smooth muscle; HBEC: Human bronchial epithelial cell; LD: Linkage disequilibrium; LTB4: Leukotriene B4; LTB4R: Leukotriene B4 receptor; RACE: Rapid amplification of cDNA ends; SNP: Single nucleotide polymorphism; TSS: Transcription start site; UTR: Untranslated region.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
IS and AST designed the study and drafted the manuscript. AST completed the laboratory experiments and statistical analyses. BB, JWH and SJB were involved with cohort organisation. All authors contributed to the final version of the manuscript.

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Author details
1Division of Therapeutics and Molecular Medicine, University of Nottingham, Queen’s Medical Centre, Nottingham, United Kingdom. 2Department of Oncology, Haematology and Respiratory Diseases, University of Modena & Reggio Emilia, Modena, Italy. 3MRC Lifecourse Epidemiology Unit, Faculty of Medicine, University of Southampton, Southampton, United Kingdom. 4Human Genetics and Medical Genomics, Human Development and Health, Faculty of Medicine, University of Southampton, Southampton, United Kingdom. 5Present address: Institute of Cellular Medicine, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom.

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