**Abstract**

**Background:** We previously demonstrated that single nucleotide polymorphism (SNP) and haplotypes were associated with aspirin hypersensitivity in asthmatics. We investigated the genetic effects of the SNPs and haplotypes on the expression of the CysLTR2 gene.

**Methods:** We measured CysLTR2 protein and mRNA expression in EB virus-infected B cell lines from asthmatics having $ht^1+/+$ and $ht^2+/+$. A gel retardation assay was used to identify nuclear protein binding to the c.-819 promoter site. The function of promoter and 3'-UTR were assessed using pGL3 luciferase and pEGFP reporter system, respectively.

**Results:** We found that the expression of CysLTR2 protein was higher in B cell lines of asthmatics having $ht^2+/+$ than in those having $ht^1+/+$. PMA/ionomycin induced higher mRNA expression of CysLTR2 in B cell lines from $ht^2+/+$ asthmatics than those from $ht^1+/+$ asthmatics. A nuclear protein from the B cell lines showed stronger DNA binding affinity with a probe containing c.-819T than one containing c.-819G. The luciferase activity of the c.-819T type of CysLTR2 promoter was higher than that of the c.-819G type. EGFP expression was higher in the EGFP-c.2078T 3'-UTR fusion construct than in the c.2078C construct.

**Conclusion:** The sequence variants of CysLTR2 may affect its transcription and the stability of its mRNA, resulting in altered expression of CysLTR2 protein, which in turn causes some asthmatics to be susceptible to aspirin hypersensitivity.
Background

Aspirin-intolerant asthma (AIA) refers to the development of bronchoconstriction in asthmatics following the ingestion of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs). This syndrome is characterized by the ‘aspirin triad’ [1] syndrome of aspirin hypersensitivity, bronchial asthma, and chronic rhinosinusitis with nasal polyposis. A two-compartment model has been proposed in which both the augmentation of cysteinyl leukotriene (CysLT) production and the overexpression of the CysLTR1 receptor on inflammatory cells occur within the respiratory tract [2]. The overproduction of CysLTs has been demonstrated in the airways and circulation of asthmatics who are intolerant to aspirin [3-5]. CysLTs are important inflammatory mediators in the development of asthma, as they mediate bronchoconstriction while increasing mucus secretion, vascular permeability and cellular infiltration [6]. CysLTs exert their biological actions by binding two types of G-protein-coupled seven-transmembrane receptors: cysteinyl leukotriene receptor 1 (CysLTR1; MIM 300201), which is sensitive to the asthma drugs montelukast, zafirlukast and pranlukast [7,8] and CysLTR2 [9]. CysLTR2 has been documented to be expressed in lung interstitial macrophage [10], pulmonary vascular smooth muscle, endothelium [11-13], eosinophils [14], mast cells [15], B and T lymphocytes [16,17], raising the possibility that CysLTR2 may have an important role in allergic inflammation. Particularly, CysLTR2 may have an important role in remodeling and fibrosis pathways in B lymphocytes. Furthermore, CysLTR2 is located on chromosome 13q14.2-21.1, near a locus known to be susceptible to aspirin intolerance. These results suggest that sequence variants on the promoter (c.-819G>T) of CysLTR2 may affect the efficiency of its transcription with tight linkage to 3’-UTR variations or that the SNPs themselves on 3’-UTR may affect the stability of its mRNA [20]. The functional effects of these SNPs on transcription should be identified. Here, we investigated the genetic effect of the promoter and the 3’-UTR polymorphisms of CysLTR2 at the transcriptional and translational levels of the gene.

Results

Flow cytometric analysis of CysLTR2 expression between haplotypes 1 and 2

To investigate the effect of the SNPs on the production of CysLTR2 protein in the B cell lines, we measured CysLTR2 protein levels on the surface of B cell lines for ht1 homozygotes (ht1+/+, N = 8) and ht2 homozygotes (ht2+/+, N = 9) using immunofluorescence staining and flow cytometric analysis (Figure 1). Approximately 50% of EBV-infected B cells expressed CysLTR2 on their surface (Figure 1A). The positive rate of CysLTR2 on the B cell lines was not significantly different between the two haplotypes (ht1+/+, 46.96 ± 5.10% vs. ht2+/+, 48.26 ± 7.36%, P > 0.05; Figure 1B). The amount of CysLTR2 protein among CysLTR2 positive B cells, however, was significantly different (Figure 1C). The mean fluorescence intensity was significantly higher in B cells of the subjects having ht2+/+ than in those having ht1+/+ (5.07 ± 0.21 vs. 3.90 ± 0.45, P = 0.04). These results indicate that single nucleotide polymorphisms on the promoter or 3’-UTR region may affect protein expression of the CysLTR2 gene.

Comparison of mRNA levels of the CysLTR2 gene between haplotypes 1 and 2

To investigate the genetic effect of these polymorphisms on the mRNA expression of CysLTR2 in B cell lines, we measured the CysLTR2 mRNA levels between ht1+/+ and ht2+/+ by RT-PCR. Semi-quantitation of CysLTR2 mRNA
induced mRNA expression of the CysLTR2 gene in EBV-infected B cells of $ht2^{+/+}$ ($N = 6$) relative to $ht1^{+/+}$ ($N = 6$, $P = 0.016$; Figure 2B). These results indicate that the protein expression of the CysLTR2 gene may be regulated at transcriptional levels.

**Comparison of promoter activity between T and G type of CysLTR2 promoter c.-819 T>G**

Because the SNPs of CysLTR2 haplotype 1 and 2 are located on the promoter region and on the 3’-UTR region, we investigated the individual effect of these two SNPs on CysLTR2 mRNA expression. First, we compared the activities of promoters possessing c.819T or G by luciferase reporter assay as described in the Methods.

Due to 293 T cells expressing CysLTR2 mRNA by PMA/ionomycin stimulation (Figure 3A), the CysLTR2 promoter-luciferase constructs were transfected into the cells and the differences in luciferase activities between c.819T and T were analyzed. Luciferase activity was normalized by β-galactosidase activity co-transfected as an internal control. In the absence of stimulation, no difference was observed in luciferase activities between genotypes (Figure 3B, c.819G; 41.83 ± 6.01 vs. c.819T; 38.12 ± 2.28, $P$<0.01 calculated using a Mann–Whitney U-test).

**Comparison of promoter activity between T and G type of CysLTR2 promoter c.-819 T>G**

Because the SNPs of CysLTR2 haplotype 1 and 2 are located on the promoter region and on the 3’-UTR region, we investigated the individual effect of these two SNPs on CysLTR2 mRNA expression. First, we compared the activities of promoters possessing c.819T or G by luciferase reporter assay as described in the Methods.

Due to 293 T cells expressing CysLTR2 mRNA by PMA/ionomycin stimulation (Figure 3A), the CysLTR2 promoter-luciferase constructs were transfected into the cells and the differences in luciferase activities between c.819T and T were analyzed. Luciferase activity was normalized by β-galactosidase activity co-transfected as an internal control. In the absence of stimulation, no difference was observed in luciferase activities between genotypes (Figure 3B, c.819G; 41.83 ± 6.01 vs. c.819T; 38.12 ± 2.28, $P$<0.01 calculated using a Mann–Whitney U-test).

**Comparison of promoter activity between T and G type of CysLTR2 promoter c.-819 T>G**

Because the SNPs of CysLTR2 haplotype 1 and 2 are located on the promoter region and on the 3’-UTR region, we investigated the individual effect of these two SNPs on CysLTR2 mRNA expression. First, we compared the activities of promoters possessing c.819T or G by luciferase reporter assay as described in the Methods.

Due to 293 T cells expressing CysLTR2 mRNA by PMA/ionomycin stimulation (Figure 3A), the CysLTR2 promoter-luciferase constructs were transfected into the cells and the differences in luciferase activities between c.819T and T were analyzed. Luciferase activity was normalized by β-galactosidase activity co-transfected as an internal control. In the absence of stimulation, no difference was observed in luciferase activities between genotypes (Figure 3B, c.819G; 41.83 ± 6.01 vs. c.819T; 38.12 ± 2.28, $P$<0.01 calculated using a Mann–Whitney U-test).
> 0.05). However, PMA/ionomycin stimulation significantly induced luciferase activity in c.-819T but not in the G type. As a result, the luciferase activity of the c.-819T type of the CysLTR2 promoter was significantly higher than that of the c.-819G type (60.79 ± 4.21 vs. 35.30 ± 2.70, respectively, \( P = 0.002 \)). These data suggest that transcription of the CysLTR2 gene may be differently regulated when stimulated according to the nucleotide polymorphism at the -819 locus on the promoter.

**Nuclear factor binding site on the region of the promoter containing CysLTR2 c.-819T>G**

To confirm whether the locus on the promoter of CysLTR2 (c.-819G>T) provided the binding sites for a transcription factor, gel shift assays were performed using the nuclear extract from EBV-infected B cells. DNA-protein complexes were detected with the probe for CysLTR2 c.-819T (Figure 4C). The intensity of this complex was higher when hybridized with the probe for c.-819T than with the probe for the c.-819G and decreased through competition with the unlabeled probe of each type. Nuclear extract from the PMA/ionomycin-stimulated B cell lines showed stronger DNA-protein binding affinity in the T type of probe than the unstimulated B cell lines. This indicates that the CysLTR2 promoter region around c.-819T may be the binding site for a transcription factor. Since a TFSEARCH search revealed putative binding sites for several candidate transcription factors around the c.-819T>G allele, such as HFH-2 and SRY (Figure 4A and 4B), we performed a supershift assay for SRY and Foxd3, which is the human homolog of HFH-2. However, neither HFH-2 nor SRY showed the supershift in EMSA (data not shown).

**Comparison of 3’-UTR activity between C and T types of the CysLTR2 3’- UTR c.2078C>T**

To evaluate the effect due to sequence variance of 3’-UTR c.2078C>T on CysLTR2 protein expression, the fluorescence intensity of 293 T cell lines transfected with each type (c.2078C or T) of EGFP-CysLTR2 3’-UTR fusion construct was analyzed using flow cytometry (Figure 5A). The EGFP expression levels of the EGFP-CysLTR2 3’-UTR fusion constructs were analyzed as the mean fluorescence intensity of EGFP of all cells analyzed. On flow cytometry analysis, side scatter and forward scatter were not different between the two cell types. The relative EGFP expression level of the 3’-UTR (c.2078T) fusion construct was significantly higher than that of the c.2078C construct (Figure 5B; 0.96 ± 0.07 vs. 0.48 ± 0.06, respectively, \( P = 0.002 \)), indicating that the mRNA stability of the c.2078T type of CysLTR2 3’-UTR was higher than that of c.2078C.
Discussion

We demonstrated that the sequence variant on the promoter (c.-819G>T) of the CysLTR2 gene affects the efficiency of its transcription and the sequence variant on the 3'-UTR (c.2078C>T) modulates mRNA degradation, resulting in different expressions of CysLTR2 protein according to the gene genotypes. We previously identified three novel SNPs in the promoter and 3' untranslated region of CysLTR2. Three SNPs (c.-819G>T, c.2078C>T, and c.2534A>G) and ht1 [G-C-A-A, ht2 [T-T-G-A] were strongly associated with the drop in FEV1 induced by aspirin challenge in asthmatics [20]. To identify the functional effects of these SNPs on transcription and translation of the CysLTR2 gene, we first measured the protein expression of the CysLTR2 gene. CysLTR2 is known to be expressed by lung interstitial macrophages [10], pulmonary vascular smooth muscle, endothelium [11-13], eosinophils [14] and mast cells [15]. These cells are difficult to obtain and maintain from our asthmatics subjects, so we used immortalized B cells induced by EB virus from subjects whose genotypes had been analyzed in a previous study [20]. We demonstrated that the B cell lines having ht2/-/+ had a higher translational and transcriptional expression levels of CysLTR2 than those having ht1/-/+ especially when stimulated (Figure 1 and 2). These results indicate that the genetic differences affect the amount of protein and mRNA expression in the CysLTR2 gene. In our previous studies, ht2 homozygote asthmatics showed the highest responses to aspirin challenge, while ht1 homozygotes showed the lowest responses [20]. Taken together, these data suggest that the greater response to aspirin challenge in asthmatics may be due to higher expression of CysLTR2 protein on the B cells and presumably on the other CysLTR2 gene-expressing cells, such as lung interstitial macrophages [10], eosinophils [14] and mast cells [15].

Next, we assessed the regulating mechanisms of the three SNPs (c.-819G>T, c.2078C>T and c.2534A>G) on CysLTR2 protein expression using promoter activity assays and 3'-UTR function analysis because the sequence variants on the promoter (c.-819G>T) of the CysLTR2 gene may affect the efficiency of its transcription and the SNPs on the 3'-UTR may affect the stability of its mRNA. Luciferase activity of the CysLTR2 promoter was higher in the T type transfected 293T cells than in the G type, which indicates that the sequence variants on the promoter are regulators for CysLTR2 gene expression (Figure 3). The genetic role of the promoter (c.-819G>T) was demonstrated by the nuclear protein binding assay (Figure 4). The binding affinity of this DNA-binding factor was higher for c.-819T than for c.-819G with or without PMA/ionophore stimulation (Figure 4). In the steady state, this DNA-binding factor may bind constitutively to the promoter close to the c.-819G>T locus and induce CysLTR2 gene expression. Moreover, PMA/ionophore-stimulated signaling may cause an increase in this binding factor on the promoter, which would induce greater CysLTR2 gene expression. With c.-819T, PMA/ionophore-induced signaling may cause higher-level binding of the DNA-binding factor than it does with c.-819G, which would increase CysLTR2 gene expression. We performed a supershift assay for the candidate factors: SRY and HFH-2. Neither SRY nor FoxD3 (human homolog of HFF2) antibodies showed supershifts in EMSA. The nature of the putative DNA-binding factor therefore remains to be clarified.

In the evaluation of the sequence variance of the 3'-UTR (c.2078G>T and c.2534A>G) on CysLTR2 gene expression, the former was chosen since the c.2534A>G is located 124 bp downstream from the polyadenylation site and would not be present in mature mRNA. The expression level of fluorescence containing the c.2078T type was significantly higher than that of c.2078C (Figure 5). This indicates that mRNA degradation of the T type of CysLTR2 3'-UTR was much slower than that of the C type. Based on the results of our promoter and 3'-UTR functional study, the sequence variance of the two loci may influence CysLTR2 gene expression cooperatively. To validate the results of our promoter and 3'-UTR functional study, we performed the additional experiment using the vectors containing both promoter and 3'-UTR regions of CysLTR2. Ht1 (c.-819G and c.2078G) and Ht2 (c.-819T and c.2078T) were inserted into pGL3-basic luciferase reporter system, respectively. The expression level of luciferase containing the ht2 was significantly higher than that of ht1, especially when stimulated with PMA plus ionomycin (data not shown). These results indicate that the combined genetic differences affect the amount of protein and mRNA expression in CysLTR2 gene. In contrast to the extensive studies on the functions of the CysLT1 receptor [21,22], the functional characterization of the CysLT2 receptor has been limited because of a lack of specific competitive antagonists. Recent experimental studies have demonstrated unanticipated functions for the cysLTs, acting non-redundantly through CysLTR2. CysLTR2, but not CysLT1, is the receptor responsible for mediating the contribution of the CysLTs to experimental bleomycin-induced chronic pulmonary inflammation and fibrosis [23], and for the production of IL-8 by IL-4-primed cultured human mast cells [24]. However, the impact of the receptor has not been revealed in chronic inflammatory airway diseases such as asthma. In particular, any in vivo role for the CysLTR2 receptor remains unknown. To the best of our knowledge, our study is the first to clearly demonstrate the genetic relationship between the CysLTR2 receptor nucleotide polymorphism and aspirin hypersensitivity in asthmatics at the level of gene function.
**Conclusion**

The sequence variants on the promoter and on the 3’-UTR of CysLTR2 affect the efficiency of its transcription and the stability of its mRNA, resulting in the alteration of protein expression of CysLTR2. This change causes a certain number of asthmatics to be susceptible to aspirin hypersensitivity. This functional evidence may have genetic value as a diagnostic biomarker and could provide fundamental information for the development of therapeutic strategies.

**Methods**

**EV virus-infected B cell culture**

Venous whole blood was obtained from asthmatics having the haplotype 1 (common allele homozygotes (GCAA: c.-819G>cT, c.2078C>T, c.2534A>G and c.2545+297A>G) and haplotype 2 (rare allele homozygotes (TTGG)) and peripheral blood mononuclear cells (PBMC) were separated using discontinuous Histopaque gradients solution (Sigma, St. Louis, MO). PBMC were infected with B95-8 supernatant and incubated with 0.5 μg/ml of cyclosporine A (Sigma) for 3 weeks. Cells were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with L-glutamine (2 mM), HEPES (25 mM), and 10% FBS (Invitrogen Life Technologies). All cell lines were maintained in culture at 37°C in an atmosphere containing 5% CO₂. Cells were reseeded at a density of 5 × 10⁴ cells/ml every 3-4 days. This study was performed with the approval of the Ethics Committee of the Soonchunhyang University Hospital and informed written consent was obtained from all the study subjects.

**Flow cytometry analysis of CysLTR2 protein expression**

The expression of CysLTR2 in B cell lines was assessed with the use of a polyclonal anti-CysLTR2 antibody (Cayman Chemicals, Ann Arbor, MI) directed against amino acids 1-18 of the N-terminal portion of the receptor. For flow cytometry studies, B cell lines were washed twice with PBS and labeled for 30 min at 4°C with anti-CysLTR2 antibody (or irrelevant antibody for control). Cells were then washed twice with PBS and incubated for 30 min at 4°C with FITC-conjugated goat anti-rabbit IgG (BD Pharmingen™, BD Biosciences, San Diego, CA). As an isotype-matched control, FITC-conjugated mouse IgG1κ (BD Biosciences) was similarly treated. Finally, cells were washed again and resuspended in PBS. An analysis of fluorescence staining was performed with a FACSCalibur flow cytometer and Cell Quest Software (Counter Corporation, Miami, FL). The levels of CysLTR2 expression were determined as the percentage of positive staining and the ratio of mean FL1 channel values of CysLTR2 stain. The percentage of positive staining was measured as the cells showing fluorescence above that of the control antibody. The ratio of mean FL1 channel values was determined as the FL1 level of CysLTR2 stain to that of the isotype-matched control stain.

**Semi-quantitation of mRNA expression using reverse transcriptase polymerase chain reaction**

Total RNA was extracted using TRI Reagent (guanidium thiocyanate-phenol mixture; Molecular Research Center, Inc., Cincinnati, OH) and chloroform. Possible DNA contamination was eliminated by treatment with 1 μl of RNase-free DNase I (10,000 U/ml; Stratagene, La Jolla, CA) for 15 min at 37°C. The reaction was stopped with 0.5 μl of 2 mM EDTA. DNase I-treated RNA was heated at 65°C for 5 min with 1 μl of oligo deoxynucleotide-15 primer (500 μg/ml) and 10 mM dNTP mix, then quickly chilled on ice. After centrifugation, DNase I-treated RNA were incubated at 42°C for 2 min with 0.1 mM DTT and 1 μl of SuperScript II™ RT (200 U/μl; Invitrogen Life Technologies) at 42°C for 50 min, and then heat inactivated at 70°C for 15 min. Following reverse transcription (RT), cDNA was aliquoted into tubes containing specific primer pairs (Table 1) for human CysLTR2 and GAPDH genes to

**Table 1: Primer sequences used in this study**

| Approach          | Orientation | Location | Primer Sequence                  |
|-------------------|-------------|----------|----------------------------------|
| RT-PCR            |             |          |                                  |
| CysLTR2           | Forward     | +29/+46  | 5'-CATCCATCTCCGTATCAG-3'          |
|                   | Reverse     | +734/+717| 5'-GCTTTTCGTGAGAAAACC-3'         |
| GAPDH             | Forward     |          |                                  |
|                   | Reverse     |          |                                  |
| Promoter construct| Forward     | -1342/-1323| 5'-TTTTCTGCGTTGGTGTTGG-3'              |
|                   | Reverse     | +178/+159| 5'-TGGAACACCAATTTCCCAAG-3'         |
|                   | Nested, Forward | -1008/-987| 5'-ATTTAGCTAGCCAAAAACATTTAATGAACTTAG-3' |
|                   | Nested, Reverse | -4/-30   | 5'-ATTATCTCGAGGGGGTAAAGAAAAACAGACACAAAAAG-3' |
| UTR construct     | Forward     | +501/+517| 5'-GGCTTCTCATAACTGC-3'            |
|                   | Reverse     | +2787/+2770| 5'-GGTTAGCAAAATGTCTTG-3'          |
|                   | Nested, Forward | +1042/+1062| 5'-AAATAGCCGCCGCCGAGCTCTTAGTAGAGACCTG-3' |
|                   | Nested, Reverse | +2410/+2385| 5'-TTATCGCAGCGGCTGTTAGGAAGAACAGCTTTATTC-3' |

Underlined nucleotide sequences are added to the 5' end of oligonucleotide primer for cloning. These cloning sites are Nhe I (GCTAGC), Xho I (CTCGAG), and Not I (GGGGCCGC), and they are preceded by A and T residues at random.
amplify into 706-bp and 300-bp PCR products, respectively. Amplification was performed on a thermocycler for 30 cycles (one cycle: 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C) with the initial denaturation at 95°C for 5 min and a final extension at 72°C for 7 min. Amplified PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining.

Preparation of the promoter (c.-819T>G)-luciferase and the EGFP-3′-UTR (c.2078C>T) reporter constructs

The promoter and UTR region of CysLTR2 were amplified by PCR. Primary PCR products were amplified using the genomic DNA of allele-matched B cell lines as templates from the CysLTR2 promoter (1520-bp fragment, from -1342 to +178) and UTR (2287 bp fragment, from +501 to +2787) sequence. The primary PCR reaction mixture was diluted and used as a template for a nested PCR using the nested primer. The secondary PCR primer for the promoter (1027-bp fragments, from -1008 to -4) and UTR (1395-bp fragments, from +1042 to +2410) region contained a Nhel-XhoI and NotI-NotI restriction site. The primers used are listed in Table 1. The amplified PCR product was cloned into a pGEM-T easy vector (Promega, Madison, WI) and digested with Nhel and XhoI (New England Biolabs, Beverly, MA). The fragment was subcloned into the Nhel and XhoI sites of a promoterless luciferase reporter pGL3-Basic vector (Promega). The UTR fragment was cloned into the NotI site of an enhanced green fluorescent protein (EGFP) expression plasmid, pEGFP-N1 vector (BD Biosciences). Plasmids were isolated using the Qiagen plasmid purification kit (Qiagen, Santa Clarita, CA).

Transient transfection and assays of luciferase and fluorescence intensity

The 293 T cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Rockville, MD) with L-glutamine (2 mM), HEPES (25 mM), 10% heat-inactivated FBS (Invitrogen Life Technologies), 100 U/ml penicillin, and 100 ng/ml streptomycin (Life Technologies-BRL, Gaithersburg, MD) at 37°C in humidified air containing 5% CO2. One day before transfection, 5 × 105 cells were seeded per well in 2 ml without antibiotics to obtain 90-95% confluence at the time of transfection. The 293 T cells were then transiently transfected with pGL3-promoter or pEGFP-UTR of the CysLTR2 gene using LF2000 (Invitrogen Life Technologies; recommended μg DNA: μl lipofectamine = 1:2) in OptiMEM (Life Technologies-BRL) without serum. One microgram of pSV-β-galactosidase control vector (Promega) was co-transfected to normalize transfection efficiencies. For promoter construct transfection, PMA (10 ng/ml) and ionomycin (2 μM) was added for 24 h after transfection. After 48 h of transfection, cultured cells were washed twice with PBS and solubilized by scraping with 400 μl of reporter lysis buffer (Promega).

Cells were centrifuged and the supernatant was stored at -70°C. β-galactosidase activities were assessed by ortho-nitrophenyl-D-galactopyranoside (ONPG) hydrolysis using a β-Galactosidase Enzyme Assay System Kit (Promega) and total protein was measured using a BCA protein assay kit (Pierce, Rockford, IL). Luciferase activity was measured using the Luciferase Assay System (Promega) by a Turner 20/20 n luminometer (Turner Biosystems, Inc., Sunnyvale, CA) and expressed as relative luciferase activity to that of empty pGL3-basic vector. The expression levels of EGFP with the 3′-UTR of CysLTR2 were assessed by measuring the mean channel values of FL1 in flow cytometry (BD Biosciences) at 48 h after transfections. The relative mean value of FL1 was calculated as described above. The ratio of mean FL1 channel values was determined as the FL1 level of the transfectant stain to that of the isotype-matched control stain.

Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared by the modified method of Schreiber et al. [25]. The PMA and ionomycin stimulated B cell lines and unstimulated B cell lines were harvested after washing twice with HBSS. Cells were washed with buffer A (10 mM HEPES, 15 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin; Calbiochem, La Jolla, CA). The cell pellet was resuspended in buffer B (buffer A containing 0.2% Nonidet P-40) and incubated for 10 min. Nuclei were pelleted by centrifugation and resuspended in buffer C (buffer A containing 0.25 M sucrose). Nuclei were again pelleted, then resuspended in buffer D (50 mM HEPES, 400 mM KCl, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin; Calbiochem) and incubated with shaking for 30 min. All procedures were performed on ice. The mixture was centrifuged, and the supernatant was stored at -70°C. DNA-protein binding assays were performed using the Gel Shift Assay System (Promega) following the manufacturer’s instructions. Ten-microgram aliquots of nuclear extracts were incubated in 10 μl of total reaction volume containing binding buffer (Promega) for 10 min with or without unlabeled oligonucleotide probe. The 32P-labeled oligonucleotide probe was added to the reaction mixture and then incubated for 20 min. The reaction products were analyzed by electrophoresis in a 4% polyacrylamide gel with 0.5× TBE buffer. Gels were dried and analyzed by autoradiography. The sequences of the oligonucleotide probes used were forward: 5′-TTTTG TTTTG TTGTT [G/T]TTT TTTT TTTT G-3′, reverse: 5′-CAAAA AAAA AAAA [C/A]AAACA ACAAA ACAAA A-3. To determine the specificities of the DNA-protein complexes, 1.75 pmol of unlabeled probe was incubated with the nuclear extract for 10 min before the addition of the labeled probe. For the supershift assay, 200 μg of goat anti-human SRY Ab
(Santa Cruz Biotechnology, Santa Cruz, CA) or goat anti-human FoxD3 Ab (Santa Cruz Biotechnology) was incubated with the nuclear extract for 30 min before the addition of the labeled probe.

**Statistical analysis**

Data are expressed as the mean ± standard error of the mean (SEM). The program SPSS (version 10.0; SPSS Inc., Chicago, IL) was used for all analyses. Differences between independent groups and samples were compared using the nonparametric Kruskal-Wallis H-test for continuous data. If significant differences were found, a Mann-Whitney U-test was applied to compare differences between two samples. Wilcoxon’s signed-ranks test was used for two related samples.

**Abbreviations**

AAIA: Aspirin-intolerant asthma; NSAID: non-steroidal anti-inflammatory drugs; CysLT: cysteiny1 leukotriene; CysLT1R: cysteiny1 leukotriene receptor; FEV1: Forced expiratory volume in one second; SNP: Single nucleotide polymorphism; LD: Linkage disequilibrium; PBMC: peripheral blood mononuclear cells; EBV: Epstein-Barr virus; EGFP: Enhanced green fluorescent protein; EMSA: Electrophoretic mobility shift assays; SEM: Standard error of the mean.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

JAS performed all the experimental steps, HSC provided experimental assistance, SMP drafted the manuscript, CSP provided analytic support and supervised the project; ASJ, SWP, JSP, STJ, GLIL, TR, MKK, ISC and IYC provided analytic and data assistance. BLP and HDS supervised the SNP analysis. All authors have read and approved this final manuscript.

**Acknowledgements**

This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health, Welfare and Family Affairs, Republic of Korea (A010249). The biospecimens for this study were provided by the Soonchunhyang University Hospital, a member of the National Biobank of Korea.

**References**

1. Samter M, Beers RF Jr: Concerning the nature of intolerance to aspirin. J Allergy 1967, 40(5):281-293.
2. Sousa AR, Parikh A, Scadding G, Corrigan CJ, Lee TH: Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. N Engl J Med 2002, 347(19):1493-1499.
3. Picado C: Aspirin-intolerant asthma: role of cyclo-oxygenase enzymes. Allergy 2002, 57(Supp1 72):58-60.
4. Szczeklik A, Gryglewski RJ, Cerniawaska-Myslik G: Relationship of inhibition of prostaglandin biosynthesis by analogues to asthma attacks in aspirin-sensitive patients. Br Med J 1975, 1(593):67-69.
5. Szczeklik A: The cyclooxygenase theory of aspirin-induced asthma. Eur Respir J 1990, 3(5):588-593.
6. Henderson WR Jr: The role of leukotrienes in inflammation. Ann Intern Med 1994, 121(9):684-697.
7. Drazen JM, Israel E, O’Byrne PM: Treatment of asthma with drugs modifying the leukotriene pathway. N Engl J Med 1999, 340(3):197-206.
8. Leff AR: Regulation of leukotrienes in the management of asthma: biology and clinical therapy. Annu Rev Med 2001, 52(1):1-14.
9. Hui Y, Funk CD: Cysteinyl leukotriene receptors. Biochem Pharmacol 2002, 64(11):1549-1557.
10. Heise CE, O’Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, Stocca R, Bellefeuille JN, Abramowitz M, Cheng R, et al.: Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 2000, 275(39):30331-30336.
11. Coleman RA, Eglen RM, Jones RL, Narumiya S, Shimizu T, Smith WL, Dahlen SE, Drazen JM, Gardiner PJ, Jackson WT, et al.: Prostanoid and leukotriene receptors: a progress report from the IUPHAR working parties on classification and nomenclature. Adv Prostaglandin Thromboxane Leukot Res 1995, 23:283-285.
12. Labat C, Ortiz JL, Noirel X, Gorenne I, Verley J, Abram TS, Cuthbert NJ, Tudohope SR, Norman P, Gardiner P, et al.: A second cysteinyl leukotriene receptor in human lung. J Pharmacol Exp Ther 1992, 263(2):800-805.
13. Early SB, Barekzi E, Negri J, Hise K, Borish L, Steinke JW: Concordant modulation of cysteinyl leukotriene receptor expression by IL-4 and IFN-gamma on peripheral immune cells. Am J Respir Cell Mol Biol 2007, 36(6):715-720.
14. Duroudier NP, Tulah AS, Sayers J: Leukotriene pathway genetics and pharmacogenetics in allergy. Allergy 2009, 64(5):822-839.
15. Daniels SE, Bhattacharrya S, James A, Leaves NI, Young A, Hill MR, Corrigan C, Mallett K, Ying S, Roberts D, Parikh A, Scadding G, Lee PY, Faux JA: Expression of the type 2 receptor for cysteinyl leukotrienes (CysLT2R) by human mast cells: Functional distinction from CysLT1R. Proc Natl Acad Sci USA 2003, 100(20):11589-11593.
16. Early SB, Barekzi E, Negri J, Hise K, Borish L, Steinke JW: Concordant modulation of cysteinyl leukotriene receptor expression by IL-4 and IFN-gamma on peripheral immune cells. Am J Respir Cell Mol Biol 2007, 36(6):715-720.
Pre-publication history
The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2350/10/106/prepub