

Determination of structure and transcriptional regulation of *CYSLTR1* and an association study with asthma and rhinitis

Zhang J, Migita O, Koga M, Shibasaki M, Arinami T, Noguchi E.
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Pediatr Allergy Immunol 2006; 17: 242–249. © 2005 Blackwell Munksgaard

Pharmacologic studies have revealed that cysteinyl leukotrienes (CYS-LTs) act through two receptors, cysteinyl leukotriene receptor 1 (CYSLTR1) and CYSLTR2. CYSLTR1 antagonists are widely used to treat asthma and rhinitis. In this study, we characterized the genomic structure and transcriptional regulation of *CYSLTR1* and examined associations between *CYSLTR1* polymorphisms and asthma/rhinitis. The experiment of rapid amplification of cDNA end revealed that *CYSLTR1* contains three exons and that the entire open reading frame is located in exon 3. Reverse transcriptase-polymerase chain reaction showed that there were multiple splice variants of *CYSLTR1* and that the transcript expression patterns differed from tissues and cell types. The promoter region of *CYSLTR1* is from –665 to –30 bp relative to the transcription start site. We identified four polymorphisms (c.-618-434T/C, c.-618-275C/A, c.-618-136G/A, and 927C/T), and transmission disequilibrium tests revealed that none of these polymorphisms was associated with the development of asthma/rhinitis. However, the TCG and CAA haplotypes in the promoter region caused different transcriptional activity. Our findings indicate that *CYSLTR1* polymorphisms are not likely to be involved in the development of asthma/rhinitis, but it is possible that these polymorphisms could influence drug responses in individuals with atopic diseases.

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Key words: asthma; cysteinyl leukotriene receptor 1; 5' rapid amplification of cDNA end; splicing variance; polymorphism

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Accepted 4 October 2005

Asthma and allergic rhinitis are considered chronic inflammatory diseases characterized by infiltration of inflammatory cells such as T cells, eosinophils and mast cells (1, 2). Among chemical mediators known to be involved in the pathophysiology of bronchial asthma and rhinitis, cysteinyl leukotrienes (CYSLTs) are considered to be some of the most important mediators because they cause bronchoconstriction, vascular hyperpermeability, and mucous hypersecretion (3, 4). CYSLTs act through cell surface receptors. Pharmacologic studies have revealed that CYS-LTs activate at least two receptors, cysteinyl leukotriene receptor 1 (CYSLTR1) and CYSLTR2 (5–7). These receptors have different potencies and affinities for endogenous agonists and different selectivities for antagonists (8, 9). In 1999, the *CYSLTR1* was cloned by ligand fishing

for orphan 7-transmembrane (7TM) receptors (10, 11). The cDNA encodes a protein of 337 amino acids and maps to human chromosome Xq21.1. Northern blotting revealed that *CYSLTR1* is expressed in spleen, peripheral blood leukocytes, lung, pancreas, small intestine, and prostate (10, 11). *In situ* hybridization experiments showed that *CYSLTR1* is present in human lung smooth muscle and lung macrophages (12). In intracellular calcium mobilization assays, CYSLTs increased Ca^{2+} concentrations with a rank order of potency of leukotriene D₄ (LTD₄) > LTC₄ > LTE₄ (10, 11, 13). CYSLTR1 antagonists, such as montelukast (singulair), zafirlukast (accolate) and pranlukast (onon), have been widely used to treat asthma. CYSLTR1 antagonists have also proven effective for treatment of allergic rhinitis (14). Equilibrium

and kinetic binding studies yielded a rank order of potency for CYSLTR1 of zafirlukast = montelukast > pranlukast (15).

Studies have indicated that polymorphisms in genes encoding proteins of the leukotriene synthetic pathway contribute to intra-patient variations in clinical responses to CYSLTR1 antagonists. CYSLTs are synthesized through the 5-lipoxygenase (5-LO, *ALOX5*) pathway of arachidonic acid metabolism. *ALOX5* is the first committed enzyme in the biosynthetic pathway leading to production of the leukotrienes (16). *ALOX5* gene promoter polymorphism has been shown to be associated with altered responses to treatment with antileukotriene drugs (17, 18) and airway hyper-responsiveness to methacholine (19). Another member of the pathway, LTC4 synthase (*LTC4S*), is a terminal enzyme that catalyzes the conjugation of unstable LTA4 with reduced glutathione to form LTC4. The -444A/C polymorphism in *LTC4S* is predictive of the clinical response to pranlukast in a Japanese population (20).

These findings suggest that *CYSLTR1* may be a good candidate gene for asthma and allergic rhinitis. A recent screen of the NCBI database (<http://www.ncbi.nlm.nih.gov>) revealed that *CYSLTR1* contains three exons, but the genomic structure and regulatory region of *CYSLTR1* have not been fully characterized. To date, there has been only one study to examine possible associations of allergic diseases with *CYSLTR1* polymorphisms. 927C/T polymorphism in *CYSLTR1* was examined with asthma in 585 Japanese asthmatic patients and 323 controls, but no association was observed (21). In the present study, we characterized the genomic structure of human *CYSLTR1* and performed association studies of asthma/allergic rhinitis with *CYSLTR1* polymorphisms in a Japanese population.

Methods

Subjects

Probands of asthmatic families were mite-sensitive asthmatic children who visited the Pediatric Allergy Clinic of the University Hospital of Tsukuba. A full verbal and written explanation of the study was given to all family members interviewed, and 137 families (466 members), including 45 families used for our previous genome-wide screening (22), gave informed consent and participated in this study. Criteria used for the diagnosis of asthma were as described previously (22).

Probands of the allergic rhinitis families were children with allergic rhinitis who were recruited from three elementary schools and a junior high school in Matsukawa, a farming community in central Japan where orchard grass pollens are thought to be a major cause of seasonal allergic rhinitis. Children with allergic rhinitis were identified through a questionnaire, and diagnosis was made by physicians. Other family members were contacted, and a total of 48 families (188 individuals) who gave informed consent participated in this study. Criteria used for the diagnosis of seasonal allergic rhinitis were as described previously (23). This study was approved by the Committee of Ethics of the University of Tsukuba.

Mutation screening and genotyping

All exons and exon-intron junctions and the 5' flanking region of *CYSLTR1* were amplified by polymerase chain reaction (PCR) from genomic DNAs of 24 unrelated Japanese females with seasonal allergic rhinitis. Genotyping was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3100 autosampler (Applied Biosystems).

5' rapid amplification of cDNA end

We carried out 5' rapid amplification of cDNA end (5' RACE) experiments using commercially available RACE-ready human leukocyte and spleen cDNAs (Marathon Ready cDNA; BD Biosciences/Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Primers used for the amplification were: 5'-GCCATTGCCAAAGAAGCCTACAACAG-3' and 5'-CCATCCTAATACGACTCACTATAGGGC-3' (AP1) for the first round PCR, and 5'-TGCTCTTTCTGGCACTGGAGTACCTT-3' and 5'-ACTCACTATAGGGCTCGAGCGGC-3' (AP2) for the nested PCR. The amplified RACE product was cloned in pCR2.1 TOPO-TA cloning vector (Invitrogen, San Diego, CA, USA). Each white (positive) clone was isolated and grown in Luria-Bertani medium with ampicillin overnight. Plasmids were purified by column chromatography (Invisorb Spin Plasmid Mini Kit; Invitex GmbH, Berlin, Germany) and subjected to direct sequencing with M13 primers.

Expression of *CYSLTR1* in tissues

Human multiple tissue, human immune system, and human blood fraction cDNA panels

Table 1. Sequence of primers used in reverse transcriptase-polymerase chain (RT-PCR) reaction and plasmid constructs

<i>RT-PCR</i>	
Primer pair I–III	
Forward	5'-AGAGAACAACCTCGTCCCTGCT-3'
Reverse	5'-CTTGATTGCGGAAGTCATCA-3'
Primer pair II–III	
Forward	5'-AGCGTGGGCTTCCTCTAATA-3'
Reverse	5'-CTTGATTGCGGAAGTCATCA-3'
<i>Plasmid constructs</i>	
24 bp	
Forward	5'-ATTATAAGCTTAAGCAGGACGAGTGTCTCTCTT-3'
Reverse	5'-ATTATAGATCTAAGAGAGAACTCGTCCCTGCTT-3'
–30 bp	
Forward	5'-ATTATAAGCTTAAGCAGGACGAGTGTCTCTCTT CAGCTTAGTCTC-3'
Reverse	5'-ATTATAGATCTCTACAGTCACAGAAATCACAGAG AACTAAGCTGAAG-3'
–108 bp	
Forward	5'-ATTATAAGCTTAAGCAGGACGAGTGTCTCTC-3'
Reverse	5'-AATTATAGATCTTCAAGTTTCACATAATGCCAGTT-3'
–345 bp	
Reverse	5'-AATTATAGATCTTTCAACACAGGACCATGA-3'
–665 bp	
Reverse	5'-AATTATAGATCTGGCTACTGCTGGGCTCATATT-3'
–1253 bp	
Reverse	5'-AATTATAGATCTTGTTGTGCTGGGGTATGGTA-3'
–1604 bp	
Reverse	5'-AATTATAGATCTAGGGGAGTGTAGCAGAGCAA-3'

The forward primer for the constructs of –345 bp, –665 bp, –1253 bp and –1604 bp is the same as that of –108 bp.

(Clontech) were used to analyze expression of *CYSLTR1* in various tissues. PCR primers are listed in Table 1. Resting CD14+ (monocytes), CD4+ (T helper/inducer cells), CD8+ (T suppressor/cytotoxic cells) and CD19+ (B lymphocytes) cells were positively selected from mononuclear cells produced by healthy donors by immunomagnetic separation with Dynabeads M-450 (Dyna, Oslo, Norway). Cells were activated with pokeweed mitogen (PWM; Invitrogen) and concanavalin A (ConA; ICN, Costa Mesa, CA, USA) by standard methods, and the degree of activation of lymphocytes was estimated on the basis of morphological criteria (blast morphology and mitoses) and expression of two activation markers, CD25 (interleukin-2 receptor) and CD71 (transferrin receptor). We used glycerol-3-phosphate dehydrogenase (G3PDH) as an internal control for PCR. Amplification conditions were an initial denaturation step at 94°C for 10 min followed by 40, 34 and 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s for primer pairs I–III, II–III and G3PDH, respectively.

RNA preparation and reverse transcriptase-PCR analysis

Lymphocytes from peripheral blood were isolated by Ficoll–Paque gradient centrifugation

(Amersham Biosciences, Piscataway, NJ, USA). Total RNA was isolated from lymphocytes with RNeasy® MiNi Kit (Qiagen, San Diego, MD, USA) and cDNA was prepared with Taqman reverse transcription (RT) reagents (Applied Biosystems) at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. Amplification condition consisted of an initial denaturing step of 95°C for 10 min, followed by 38 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, with a final incubation at 72°C for 3 min. PCR products were separated by electrophoresis on 15% polyacrylamide gel at 4°C overnight. Bands were excised from the gels and purified with QIA® II Gel Extraction Kit (Qiagen). PCR products were then cloned in pCR2.1 TOPO-TA cloning vector (Invitrogen). Direct sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI 3100 autosequencer (Applied Biosystems).

Plasmid construction and transfection, and luciferase assay

Genomic fragments of the 5' flanking region of exon 1 of *CYSLTR1* were amplified with the primers listed in Table 1. PCR products were digested with *Bgl*II and *Hind*III overnight at 37°C and then subcloned in pGL3-Basic vector (Promega, Madison, WI, USA) digested with *Bgl*II and *Hind*III. U937 cells (Cell Resource Center for Biomedical Research, Tohoku University, Miyagi, Japan) were resuspended in RPMI 1640 (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal calf serum (Sigma-Aldrich). Approximately 1×10^7 cells were cotransfected with 30 µg of test construct and 150 ng of pRL-TK (Promega) by electroporation with a Gene Pulsar II (Bio-Rad, Hercules, CA, USA) set at 350 V and 950 µF for U937. Transfected cells were harvested 5 h after transfection. Cells were lysed by the addition of 200 µl of lysis buffer (Promega). A volume of 20 µl of each lysate was used for the luciferase assay with the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase values were normalized to the Renilla luciferase values of pRL-TK, which were determined at the same time. Reporter activity is presented as the mean of at least three independent measurements.

Statistical analysis

Transmission disequilibrium test (TDT)-chromosome X was analyzed with the UNPHASED package (<http://www.litbio.org/access.htm>) (24). Differences in transcriptional activity in reporter assays were analyzed with the Wilcoxon test.

Results

Isolation of 5' full-length *CYSLTR1* transcripts and structure of the human *CYSLTR1* gene

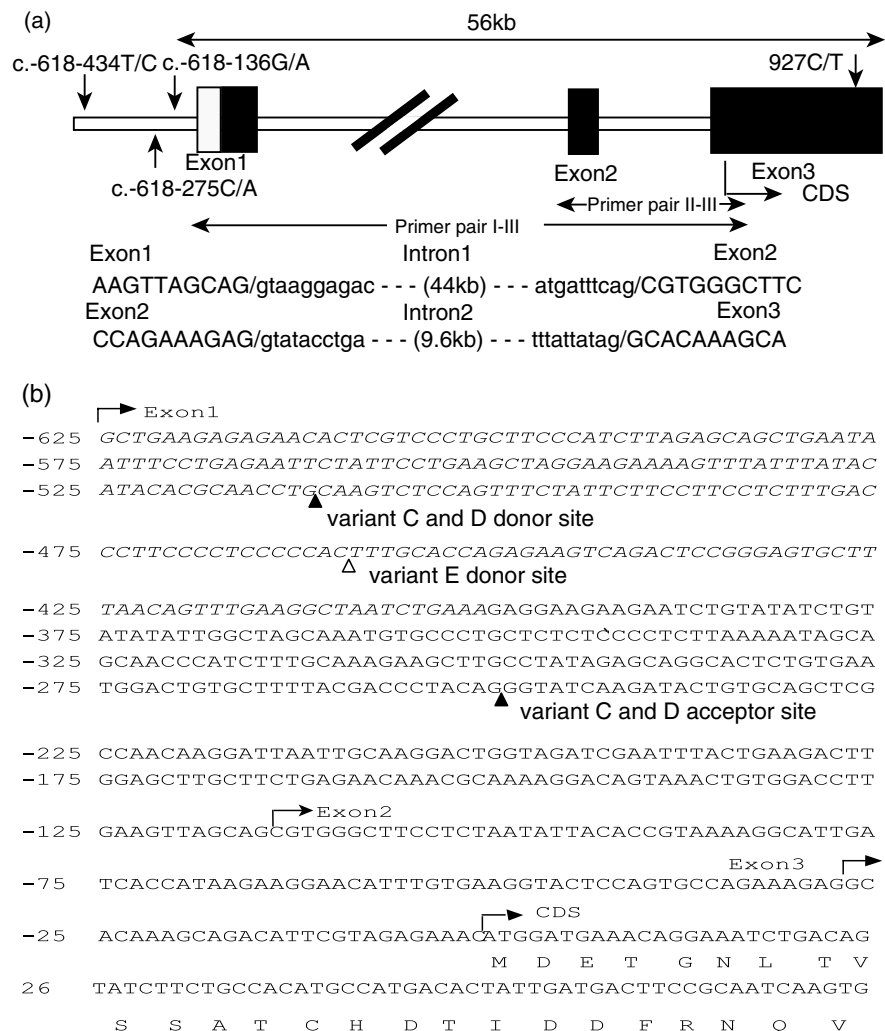
Current information in the NCBI database (<http://www.ncbi.nlm.nih.gov>) indicates that *CYSLTR1* is composed of three exons and two introns, and the cDNA is 1537 bp long (NM_006639). Our present 5' RACE experiments with cDNAs derived from human leukocytes and spleen were successful and added an additional 225 bp of sequence to the 5' side of the available sequence (Fig. 1a, b). No additional novel exons were detected.

Tissue expression of *CYSLTR1*

Splice variants of *CYSLTR1* were examined with cDNA derived from lymphocytes. Five splice variants were detected, and the structure of each variant is shown in Fig. 2. Variants A and C were the dominant transcripts in lymphocytes. Variant

A was the longest transcript, whereas variant C lacked a part of exon 1. Variant B lacked all of exon 2, and both variant D and variant E lacked a part of exon 1 and all of exon 2. Expression of *CYSLTR1* in various human tissues was analyzed with a PCR-based method (Fig. 3a–c). We designed primers to amplify the regions between exons 1 and 3 (expected product size 691 bp, primer pair I–III) and between exons 2 and 3 (expected product size 189 bp, primer pair II–III) (Fig. 1a). Transcripts including exons 2 and 3 were observed in most tissues analyzed. We observed multiple splice variants in the transcripts including exons 1, 2 and 3. Variant A was the major product in lung, and several splice variants were observed in brain, placenta, liver, kidney and pancreas (Fig. 3a). Variants A and C were observed in tonsil, leukocytes, spleen and lymph node. Variant C was expressed weakly in thymus and bone marrow (Fig. 3b). Variant A was present in resting CD4⁺, CD14⁺, CD8⁺ and CD19⁺ cells, and in activated CD4⁺ cells

Fig. 1. Genomic structure and cDNA sequence of human cysteinyl leukotriene receptor 1 (*CYSLTR1*). (a) Top, genomic structure of human *CYSLTR1* gene. Boxes mark the positions of exons 1–3. The open box is the region we extended in our 5' rapid amplification of cDNA end (5' RACE) experiment. *CYSLTR1* contains three exons, and the coding sequence (CDS) starts with exon 3. Arrows indicate the locations of the polymorphisms. The positions of primer pairs I–III and II–III are shown. Bottom, genomic sequences of exon–intron junctions in *CYSLTR1*. (b) Sequence of the human *CYSLTR1* cDNA. Numbers are relative to the translation initiation site. Boundaries between exons are indicated by arrows. The 5' RACE extended sequence is italicized. Splice sites for variants C and D are indicated with closed triangles, and the splice site for variant E is shown with an open triangle.



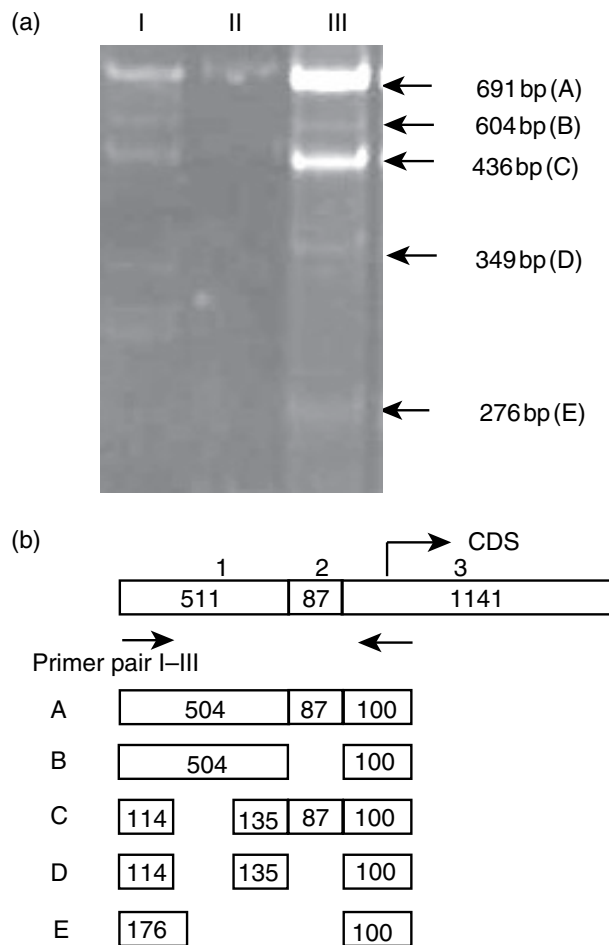


Fig. 2. Splice variants of cysteinyl leukotriene receptor 1 (*CYSLTR1*). There are five *CYSLTR1* variants in lymphocytes (a) and the structure of each variant is shown in (b). The size in bp of each exon is indicated in the box. CDS and arrows indicate the start site of the coding sequence.

and monocytes, but was not expressed in activated CD8+ cells (Fig. 3c). We could not find a human sequence corresponding to band X, which was observed in kidney, fetal liver, tonsil and leukocytes (Fig. 3a, b), by BLAST search with human genome sequence database (<http://www.ncbi.nlm.nih.gov/BLAST/>), therefore, we considered band X as PCR artifact.

Polymorphism search and TDT analysis

We screened for polymorphisms in DNAs from 32 unrelated female subjects and found four polymorphisms c.-618-334T/C (rs321029), c.-618-275C/A (rs2637204), c.-618-136G/A (rs2806489) and 927C/T (rs20995), in *CYSLTR1* (Fig. 1a). We numbered these according to the recommendation of nomenclature system published previously (25). These four polymorphisms are in

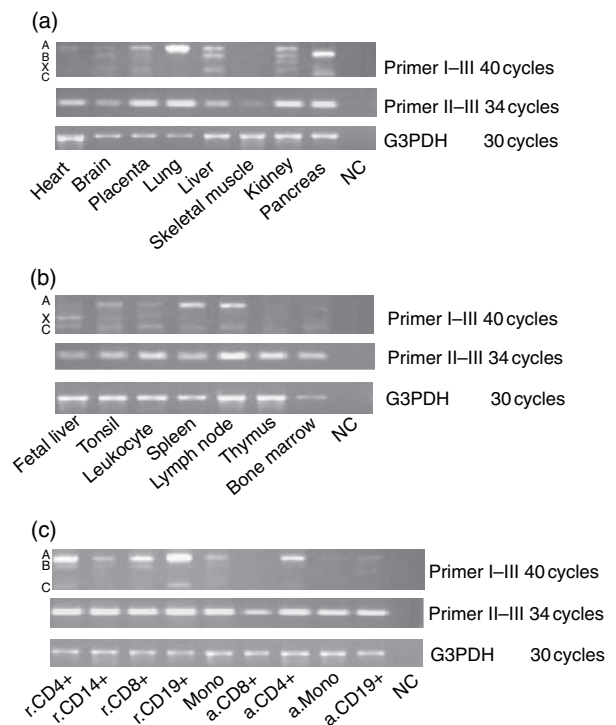


Fig. 3. Expression of cysteinyl leukotriene receptor 1 (*CYSLTR1*) in human multiple tissue panels. Results of polymerase chain reaction amplification of body organs (a), immune system (b), and blood fractions (c) are shown. Glycerol-3-phosphate dehydrogenase is included as an internal control. Mono, monocytes; r, resting; a, activated; NC, no-template control. Four bands (A, B, X and C) were observed, and the splice structures of bands A, B and C are depicted in Fig. 2b.

complete linkage disequilibrium with each other ($r^2 = 1$). We performed TDT analysis of chromosome X, and none of the polymorphisms was associated with asthma or allergic rhinitis (CAAT haplotype: transmitted 26, not transmitted 26, $p = 1.0$ for asthma; and transmitted 12, not transmitted 10, $p = 0.67$ for rhinitis). The frequency of CAAT haplotype in parents was 56% and that of the TCGC haplotype was 44%.

Transcriptional activities of 5' flanking region of *CYSLTR1*

To examine the transcriptional activity in the 5' flanking region of *CYSLTR1*, we constructed plasmids that contained sequences from -1604, -1235, -665, -345, -108, and -30 to +24 relative to the transcription initiation site. The primers used for plasmid construction are listed in Table 1. The expression of *CYSLTR1* in U937 cells was confirmed by RT-PCR (data not shown). These constructs were then transiently transfected into U937 cells. Deletion analysis revealed that the region driving maximal reporter

gene expression in U937 cells was located –665 bp proximal to the transcription initiation site (Fig. 4). Two larger constructs, one from –1604 and the other from –1235, have lower luciferase activities than construct –665, suggesting that negative regulatory sites may be present in this region.

To determine whether the c.-618-434T/C, c.-618-275C/A, and c.-618-136G/A polymorphisms are functional, we generated luciferase reporter gene constructs that contained the 5' flanking region of *CYSLTR1* from exon 1 to –665 bp, and differed from wildtype only with c.-618-434C, c.-618-275A, and c.-618-136A. The three polymorphisms in the promoter (c.-618-434T/C, c.-618-275C/A, and c.-618-136G/A) and 927C/T are in complete linkage disequilibrium ($r^2 = 1$) with each other, so only two promoter haplotypes (TCG and CAA) exist in the population. The construct containing the TCG haplotype had higher transcriptional activity than that containing the CAA haplotype ($p = 0.049$), suggesting that the TCG and CAA haplotypes have different transcriptional activities and may contribute to altered expression of *CYSLTR1* (Fig. 5).

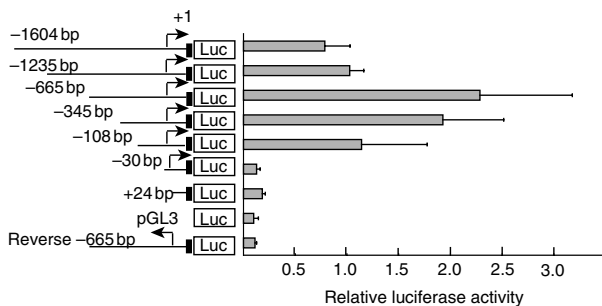


Fig. 4. Reporter activity in U937 cells. Each construct was transfected into U937 cell as described in *Methods*. Luciferase activity is presented relative to Renilla activity, and all values are the mean $\pm$ s.d. of at least three independent experiments.

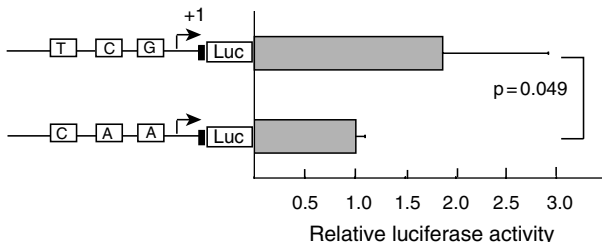


Fig. 5. Effect of the three polymorphisms on cysteinyl leukotriene receptor 1 (*CYSLTR1*) promoter activity. Luciferase activity is presented relative to Renilla activity, and values are the mean $\pm$ s.d. of at least three independent experiments.

Discussion

In the present study, we investigated the genomic structure and transcriptional activity of *CYSLTR1* and examined possible associations of *CYSLTR1* polymorphisms with asthma/rhinitis. We isolated the full-length genomic region of *CYSLTR1* and characterized the promoter region of *CYSLTR1*. We found four polymorphisms in *CYSLTR1*, but none was associated with asthma or allergic rhinitis by TDT.

Our 5' RACE analysis revealed that *CYSLTR1* contains three exons and extended the previous sequence data by 225 bp on the 5' side. Exons 1 and 2 are non-coding, and the entire open reading frame is located in exon 3. We found that there are five different *CYSLTR1* transcripts in lymphocytes. These transcripts do not change the putative open reading frame; all variants are predicted to encode identical proteins. However, it is possible that the differences in the transcripts influence the stability or secondary structure of the mRNA, which alters translation of the transcript to protein.

Genome-wide analyses of alternative splicing indicate that 40–60% of human genes have alternative splice forms, suggesting that alternative splicing is one of the most significant components of the functional complexity of the human genome (26). Recent studies showed that a majority of alternative splices change the protein product (26, 27). However, in the present study, the alternative splicing sites in the variants occurred outside of the coding region. This suggests that a strict conformation of the *CYSLTR1* protein is essential for its function in leukotriene signaling.

Tissue distribution of *CYSLTR1* has been examined extensively. Northern blot analysis revealed the highest level of expression in peripheral blood leukocytes, followed by spleen, lung, pancreas, small intestine and other tissues (10, 11). RT-PCR and *in situ* hybridization showed expression in eosinophils, neutrophils, B cells, T cells, mast cells, and CD14+, CD19+ and CD34+ cells (12, 28, 29). In the present study, we examined expression of the different transcripts in many tissues. Transcripts including exons 2 and 3 were observed in most tissues tested. However, expression of transcripts originating from exon 1 varied significantly in different tissues. Our data suggest that transcripts originating from exon 2 are the dominant transcripts in some tissues, such as thymus and bone marrow. Interestingly, transcripts from exon 1 were observed in resting CD8+ cells but not in activated CD8+ cells, suggesting that expression

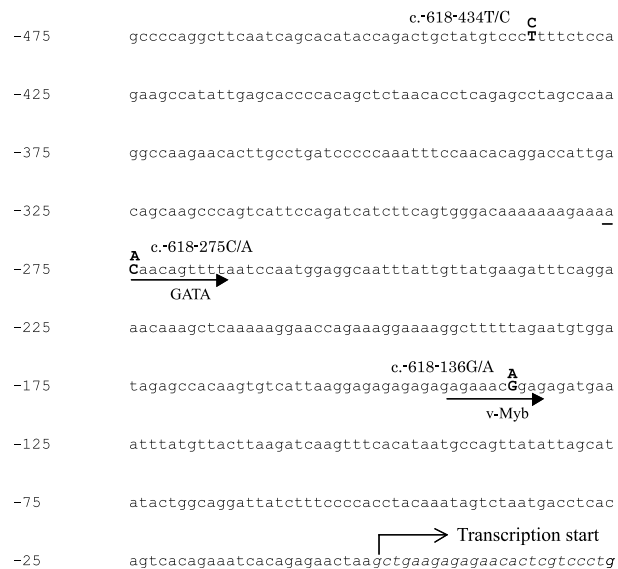


Fig. 6. *In silico* analysis of putative transcription factor binding to the polymorphic sites. The numbers are relative to the transcription initiation site. The polymorphisms are shown as bold, upper case. The transcription start site is indicated as an arrow, and transcribed sequences are italicized.

of transcripts from exon 1 is influenced by activation status. We found that *CYSLTR1* is expressed in CD14+ and CD19+ cells, which is consistent with previous results (12).

Our present TDT results were consistent with those of the previous study (21), suggesting that polymorphisms in *CYSLTR1* are unlikely to be involved in asthma. Case-control studies are the commonly used methods for genetic association studies. However, case-control studies are prone to a special form of confounding called population stratification, whenever the existence of one or more subpopulations may lead to a false association, be it positive or negative. TDT is one strategy for overcoming the problem of population stratification. However, our present sample size is not large (137 asthmatic families and 48 rhinitis families), and the possibility of a type II error cannot be excluded.

In the present study, we first identified the *CYSLTR1* promoter region, which is located -665 bp relative to the transcription initiation site, and showed that the TCG and CAA promoter haplotypes influence transcriptional activity. *In silico* analysis (<http://www.cbrc.jp/research/db/TFSEARCH.html>) revealed that several transcription factors could bind to the polymorphic sites in the *CYSLTR1* promoter. C to A substitution in the c.-618-275C/A site would affect the binding affinity of GATA-binding protein to this site (Fig. 6). The G to A change

in the c.-618-136G/A could decrease the affinity of binding of the v-Myb transcription factor to this site (Fig. 6). Therefore, it is possible that changes in expression of *CYSLTR1* due to these haplotypes may affect individual responsiveness to *CYSLTR1* antagonists. Further, studies of different populations are needed to clarify the roles of *CYSLTR1* polymorphisms in the development of atopic diseases.

Acknowledgments

We thank Mrs Shiota who helped to collect samples, and all family members who participated in this study.

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