

Localization and Up-Regulation of Cysteinyl Leukotriene-2 Receptor in Human Allergic Nasal Mucosa

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ABSTRACT

Background: Cysteinyl leukotrienes (CysLTs) are lipid mediators that have been implicated in the pathogenesis of allergic rhinitis. Pharmacological studies using CysLTs indicate that 2 classes of receptors exist, namely, CysLT₁ and CysLT₂ receptors. The former class of receptors is sensitive to the CysLT₁ antagonists currently used to treat asthma and allergic rhinitis, and its localization has been previously examined by our group using immunohistochemistry and in situ hybridization techniques. We investigated the expression and localization of the CysLT₂ receptor in human nasal mucosa by western blot and immunohistochemical analyses.

Methods: Human turbinates were obtained after turbinectomy from 16 patients with nasal obstruction refractory to medical therapy. To identify the cells expressing the CysLT₂ receptor, double immunostaining was performed by using anti-CysLT₂ receptor antibody and anti-CD31 (endothelial cell) antibody or anti-smooth muscle actin antibody.

Results: A 39 kDa band was detected on the western blots of human turbinates samples by using the anti-CysLT₂ receptor antibody. The expression level of the CysLT₂ receptor in patients with nasal allergy was higher than that in patients with non-allergic rhinitis. The immunohistochemical study also showed an intense immunoreactivity for CysLT₂ receptor in both vascular endothelial cells and vascular smooth muscles.

Conclusions: The results indicated that the CysLT₂ receptor plays a primary role in the vascular responses in the upper respiratory tract.

KEY WORDS

allergic rhinitis, CysLT₂ receptor, immunohistochemistry, Western blot

INTRODUCTION

The allergic response is a complex process involving the interaction of many mediators. Among these mediators, cysteinyl leukotrienes (CysLTs) are important for the pathogenesis of airway allergic diseases such as allergic rhinitis and asthma.¹ Pharmacological studies using CysLTs indicate that at least 2 classes of receptors exist, namely CysLT₁ and CysLT₂ receptors.^{2,3} Our previous immunohistochemical study showed that anti-CysLT₁ receptor antibody labeled eosinophils, mast cells, macrophages, neutrophils, and vascular endothelial cells in human nasal mucosa,⁴ suggesting that in allergic rhinitis, the

major targets of the CysLT₁ antagonists are vascular bed and infiltrated leukocytes.

While the CysLT₁ receptor has been studied in great detail in relation to allergic rhinitis and asthma,¹ our knowledge of the CysLT₂ receptor function is still rather limited, which is in part because of the lack of a specific receptor antagonist. To understand the role of the CysLT₂ receptor in nasal allergy, it is very important to gather information about the localization of the receptor. However, few studies have shown the CysLT₂ receptor expression in nasal specimens, specifically in epithelial cells,⁵ submucosal glands,⁶ and inflammatory leukocytes.⁵⁻⁷ In the present study, we performed immunohistochemical analysis on sur-

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Table 1 Demographic characteristics of allergic and non-allergic patients

	Allergic rhinitis N = 10	Non-allergic patients N = 6	p Value
Sex (male/female)	4/6	3/3	NS
Age	34 ± 16	38 ± 10	NS
Specific IgE to house dust mite (d1) (kU/L)	5.3 ± 4.6	<0.35	0.004
Total IgE (kU/L)	331 ± 297	100 ± 61	NS
Blood eosinophils (cells/ μ L)	441 ± 296	137 ± 57	0.04
Local eosinophils (cells/mm ²)	58.2 ± 25.6	9.2 ± 10.7	0.003
Total nasal symptom score (TNSS)	5.8 ± 2.8	2.3 ± 1.0	0.03
Nasal obstruction score	3.2 ± 0.8	1.7 ± 0.8	0.02
Sneezing score	1.7 ± 1.4	0.5 ± 0.5	NS
Rhinorrhea score	1.0 ± 1.3	0.2 ± 0.4	NS

Data expressed as mean ± SD or No. NS, not significant.

gical specimens to demonstrate the localization of CysLT₂ receptor in nasal structural cells. The western blot analysis also confirmed the up-regulation of the CysLT₂ receptor in perennial allergic nasal mucosa.

METHODS

TISSUE PREPARATION

Human inferior turbinates were obtained after turbinectomy from 16 patients with nasal obstruction refractory to medical therapy. Informed consent was obtained from all patients, and this study was approved by the ethics committee of Sapporo Medical University (approval number: 24-14). All patients were nonsmokers, of which 10 had perennial allergy to mites as diagnosed by questionnaires and the CAP test (Pharmacia, Uppsala, Sweden). The remaining 6 patients who had concomitant septoplasty because of symptomatic nasal septal deviation, with a negative CAP test for major inhaled allergens such as mites, birch, grass, mugwort and Japanese cedar were included in non-allergic rhinitis group as control. Severity of nasal symptoms (nasal obstruction, sneezing and rhinorrhea) was assessed on 0-4 category scale and Total Nasal Symptom Score (TNSS) was calculated. All medications, including antibiotics, were prohibited for at least 3 weeks prior to the study. Demographic and clinical characteristics of the patients are summarized in Table 1. The nasal mucosal specimens dissected from the cartilage, were either frozen immediately in liquid nitrogen and stored at -70°C for protein extraction or fixed in 10% formalin for immunohistochemistry.

WESTERN BLOT ANALYSIS

The nasal samples were homogenised in the Tissue Protein Extraction Reagent (T-PER, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with the Protease Inhibitor Cocktail (Sigma, St. Louis, MO, USA). Protein extracts were then purified using the PAGEprep™ Advance Kit (Thermo Fisher Scientific), and protein concentrations in the homogenates

were determined by the BCA Protein Assay Kit (Thermo Fisher Scientific). Protein extracts (20 μ g of protein each sample) were separated in a 4-12% sodium dodecyl sulfate (SDS)-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA, USA). Membranes were subsequently blocked and probed with a rabbit polyclonal anti-human CysLT₂ antibody (1 : 500; ab75160, Abcam, Cambridge, UK). A rabbit polyclonal anti-human antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was also used as an internal control. The bands were visualized using WesternBreeze® Chromogenic Immunodetection Kit (Invitrogen). The ratio of the protein levels of the CysLT₂ receptor to GAPDH was determined by densitometric analysis.

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Deparaffinized sections were initially incubated with 3% H₂O₂ in methanol for 30 min to quench endogenous peroxidase activity. After microwave treatment (10 min at 500 W in citrate buffer), the sections were incubated in blocking solution (10% normal goat serum in PBS) for 30 min before overnight incubation with the primary antibody at 4°C. A rabbit polyclonal anti-human CysLT₂ antibody raised against a peptide derived from the first extracellular domain of the human CysLT₂ receptor (ab115453, Abcam) was used as the primary antibody at a concentration of 10 μ g/ml. The sections were washed, and incubated for 30 min with EnVision⁺, Peroxidase (Dako, Carpinteria, CA, USA). A further wash in PBS was followed by developing in DAB (Dako) as a chromogen for signal visualization. The slides were counterstained with Mayer's haematoxylin and mounted on a cover slip using a mounting medium.

To identify the subsets of cells expressing CysLT₂ receptor, the following monoclonal antibodies were

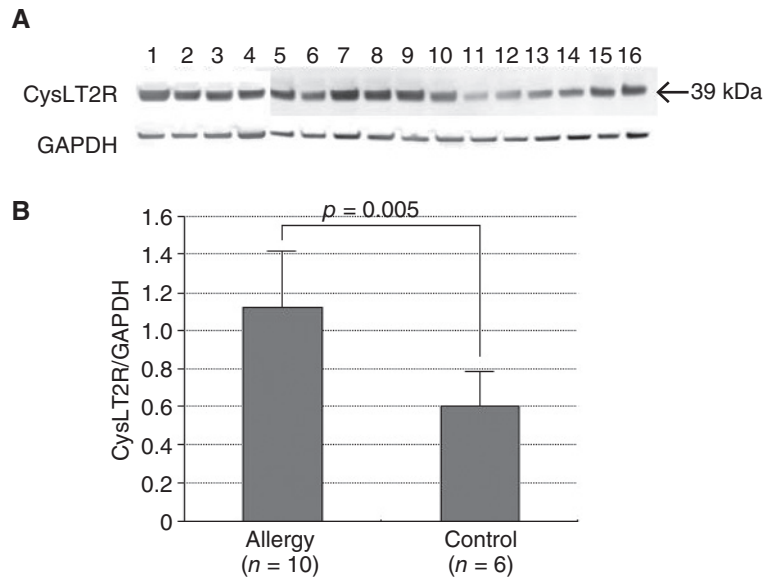


Fig. 1 **A:** Western blots showing CysLT₂ receptor protein level in allergic nasal mucosa (lane 1-10) and nonallergic nasal mucosa (lane 11-16). The antibody recognized a single, 39-kDa protein -the CysLT₂ receptor. **B:** Densitometric analysis of the relative protein level of CysLT₂ receptor as shown. The data were expressed as mean + SD and normalized to GAPDH.

used: anti-CD31 (JC70A clone, Dako) for vascular endothelial cells, anti-smooth muscle actin (1A4 clone, Dako) for vascular smooth muscle, and anti-human CD45 (2B11 + PD7/26 clone, Dako) for leukocyte common antigen. For double staining, deparaffinized sections were incubated overnight at 4°C with a combination of rabbit polyclonal anti-human CysLT₂ antibody and one of mouse monoclonal anti-human phenotypical marker antibody.

The sections were washed in PBS and incubated for 30 min with both Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1 : 50; Molecular Probes, OR, USA). The sections were mounted using Vectashield® mounting medium with 4',6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and examined under an Olympus BX51 microscope fitted with a DP70 CCD camera (Olympus Optical, Tokyo, Japan). All image analysis was performed with DP Controller and DP Manager Software (Olympus Optical). Using this method, the CysLT₂ receptor-expressing cells were visualized as red, vascular endothelial cells were green, and the combined signal was visualized as yellow.

STATISTICAL ANALYSIS

The protein levels of the CysLT₂ receptor and GAPDH detected in the duplicate experiment of western blot analysis were determined, and data were presented as mean ± standard deviation. The non-

parametric Mann-Whitney U test was used to evaluate differences between perennial allergic rhinitis and control subjects. Values of $P \leq 0.05$ were considered to be statistically significant.

RESULTS

WESTERN BLOTTING

To determine the protein size of the CysLT₂ receptor in human nasal mucosa, its expression was examined by using western blot analysis. As shown in Figure 1 A, a single band of the predicted molecular weight (approximately 39 kDa) was detected. Densitometric quantification of the bands was performed to determine the expression levels of the CysLT₂ receptor and GAPDH in allergic and non-allergic nasal mucosa (Fig. 1B). The ratio of CysLT₂ receptor/GAPDH in nasal mucosa from patients with nasal allergy was significantly higher than that from non-allergic patients (allergic rhinitis: 1.12 ± 0.29 , control: 0.60 ± 0.16 , $p = 0.005$). No significant correlation was found between the level of CysLT₂ receptor and nasal symptom score, total and specific IgE, local and blood eosinophil number (data not shown).

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The distribution of the CysLT₂ receptor in nasal mucosa was examined by means of immunohistochemistry. As shown in Figure 2A, a significant immunoreactivity for the CysLT₂ receptor was detected in blood vessels. In contrast, epithelial cells and submucosal

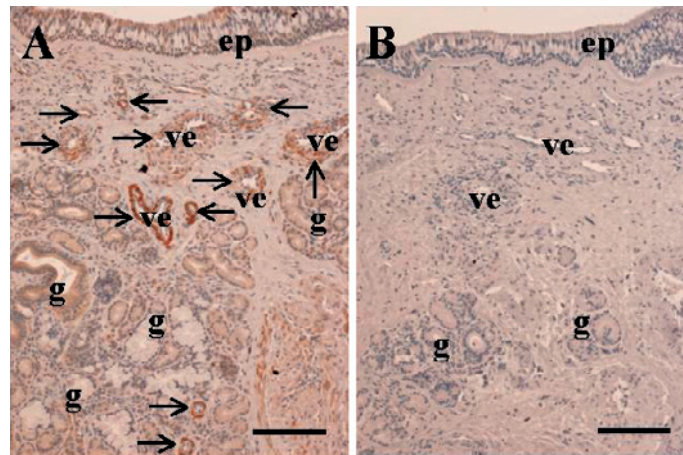


Fig. 2 Immunohistochemical staining for CysLT₂ receptor in human nasal mucosa. Inferior turbinates were stained with anti-human CysLT₂ receptor antibody (**A**) or normal rabbit immunoglobulin (**B**). CysLT₂ receptor immunoreactivity was observed mainly on blood vessels (arrow). ep, epithelial cells; g, submucosal glands; v, blood vessels. Scale bar = 200 μ m.

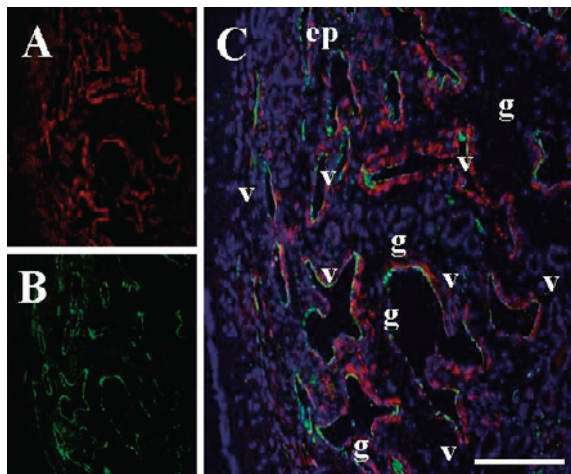


Fig. 3 Identification of CysLT₂ receptor on blood vessels of the nasal mucosa. (**A**): CysLT₂ receptor positive cells. (**B**): Anti-CD31 stain as an identification marker for vascular endothelial cells. (**C**): Overlay image. CysLT₂ protein (red) shows co-localization with anti-CD31 antibody (green) and the combined signal is visualized as yellow. Not only vascular endothelial cells, but also vascular smooth muscles express CysLT₂ receptor. ep, epithelial cells; g, submucosal glands; v, blood vessels. Scale bar = 200 μ m.

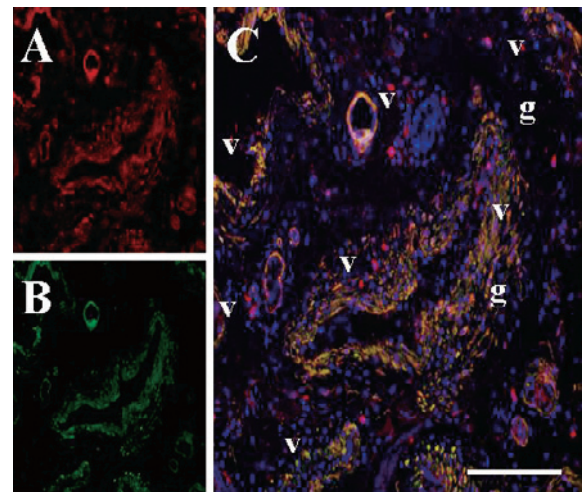


Fig. 4 Identification of CysLT₂ receptor on vascular smooth muscle of the nasal mucosa. (**A**): CysLT₂ receptor positive cells. (**B**): Anti-smooth muscle actin stain as an identification marker for vascular smooth muscle. (**C**): Overlay image. CysLT₂ protein (red) shows co-localization with anti-smooth muscle actin antibody (green) and the combined signal is visualized as yellow. Vascular smooth muscles express CysLT₂ receptor. g, submucosal glands; v, blood vessels. Scale bar = 100 μ m.

glands showed little or no expression. Specificity of the staining was confirmed by the absence of labeling with normal rabbit immunoglobulin (Fig. 2B). To further clarify the vascular cell types expressing the CysLT₂ receptor, double immunofluorescence staining was performed for CysLT₂ receptor (Fig. 3A, 4A)

and for CD31 and smooth muscle actin, which are as a vascular endothelial cell marker (Fig. 3B) and a vascular smooth muscle marker (Fig. 4B), respectively. As shown in Figure 3C, 4C, not only vascular endothelial cells but also vascular smooth muscle showed intense immunoreactivity for the CysLT₂ receptor. In

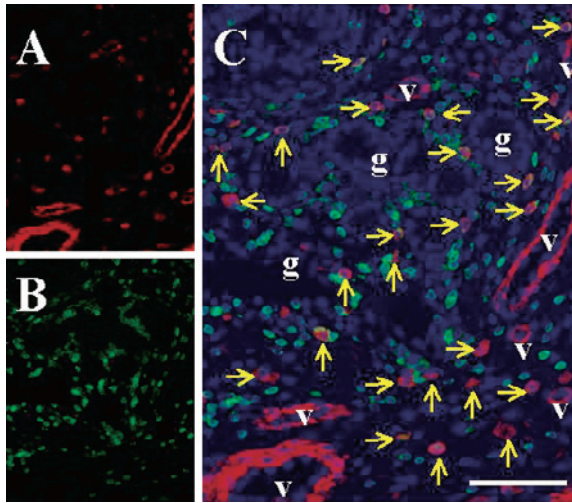


Fig. 5 Identification of CysLT₂ receptor on inflammatory leukocytes in the nasal mucosa. (A): CysLT₂ receptor positive cells. (B): Anti-CD45 stain as an identification for leukocyte common antigen. (C): Overlay image. CysLT₂ protein (red) shows co-localization with anti-CD45 antibody (green) and the combined signal is visualized as yellow. Not only blood vessels, but also some inflammatory leukocytes (arrow) express CysLT₂ receptor. g, submucosal glands; v, blood vessels. Scale bar = 100 μ m.

order to clarify the CysLT₂ receptor on inflammatory leukocytes, we performed double immunofluorescence staining for CysLT₂ receptor (Fig. 5A) and leukocyte common antigen (Fig. 5B). As shown in Figure 5C, some inflammatory leukocytes express CysLT₂ receptor. The further double immunohistochemical staining revealed that the majority of these CysLT₂ receptor positive cells were mast cells and macrophages (data not shown). The pattern of the immunohistochemical findings for all 16 patients were remarkably similar.

DISCUSSION

The results from this study, obtained nasal surgical specimens, indicate that the nasal CysLT₂ receptor localizes exclusively in blood vessels. In the past, only few studies have been designed to explore the expression of the CysLT₂ receptor in nasal mucosa biopsies from patients with allergic rhinitis.⁵⁻⁷ Corrigan *et al.* showed the presence of the CysLT₂ receptor in inflammatory leukocytes, mucus glands and epithelial cells in nasal biopsies from patients with chronic rhinosinusitis.⁵ On the other hand, the CysLT₂ receptor immunoreactivity in sinus mucosa obtained from endoscopic sinus surgery was mainly detected in inflammatory leukocytes.^{6,7} These previous reports did not examine the expression of the CysLT₂ receptor in blood vessels.⁵⁻⁷ To the best of our knowledge, our study shows for the first time, the presence of CysLT₂ in human nasal blood vessels.

The studies using mice with deletions⁸ or overexpression⁹ of the CysLT₂ receptor in the lungs have suggested a prominent role for the receptor in mediating vascular permeability. In vitro, human umbilical vein endothelial cells (HUVECs) express the CysLT₂ receptor mRNA in vast excess (>4000-fold) of the CysLT₁ receptor mRNA, and Ca²⁺ fluxes caused by CysLTs in HUVECs may result from perturbation of the CysLT₂ receptor rather than the expected CysLT₁ receptor.¹⁰ Therefore, the CysLT₂ receptor-mediated nasal vascular response may affect nasal vascular permeability and consequent plasma leakage may be cause local tissue edema and watery rhinorrhea. Moreover, CysLTs have been found to elicit several specific functional responses in endothelial cells, eg, the synthesis of platelet activating factor,¹¹ secretion of von Willebrand factor, and surface expression of P-selectin.¹² These effects may be in part due to activation of the CysLT₂ receptor.

Our present immunohistochemical studies have clearly shown the expression of the CysLT₂ receptor not only in vascular endothelial cells, but also in vascular smooth muscle cells. With regards to the expression of the CysLT₂ receptor in human vascular smooth muscle cells, only the presence of its mRNA in coronary artery has been reported using RT-PCR and in situ hybridization.¹³ When human coronary smooth muscle cells are stimulated with LTC₄, the intracellular calcium concentration increases, which suggests that the contraction induced by LTC₄ may be mediated by the CysLT₂ receptor.¹³ In contrast, activation of the CysLT₂ receptor in endothelial cells leads the release of a relaxant factor, nitric oxide (NO).¹⁴ Because no data on the experimental model of allergic rhinitis using CysLT₂ receptor-deficient mice or CysLT₂ receptor antagonists, the biological significance of the CysLT₂ receptor in nasal vasculature remains unclear.

The expression of CysLT₂ receptor was found not only on nasal vasculature, but also on some infiltrated leukocytes such as mast cells and macrophages in the nasal mucosa. It has been reported that mast cells, macrophages and eosinophils in nasal lavage from patients with active seasonal allergic rhinitis expressed CysLT₂ receptor.¹⁵ The roles of CysLT₂ receptor in these inflammatory leukocytes still remain unclear.

Our present western blot analysis has showed that the expression of the CysLT₂ receptor increased significantly in allergic nasal mucosa compared to non-allergic samples. Previous studies on paranasal sinus mucosa have also shown that the CysLT₂ receptor mRNA expression in chronic rhinosinusitis was significantly higher than that in controls.^{6,7} In addition, mucosal hyper-responsiveness, a pivotal pathophysiological characteristic of allergic rhinitis has been observed in patients with perennial allergic rhinitis.¹⁵ It is therefore, logical to hypothesize that hyperrespon-

siveness might stem from the up-regulation of the leukotriene receptors. The CysLT₂ receptor expression is subjected to regulation in vitro. Our previous study showed that CysLTs and Th2 cytokines such as IL-13 enhanced the expression of CysLT₂ receptor in human monocytes in vitro.¹⁶ Some studies have also demonstrated increased local levels of IL-4 and IL-13 following allergen provocation in allergic rhinitis.¹⁷ Therefore, it is possible that the CysLT₂ receptor in allergic airways may be up-regulated by IL-13 released from leukocytes.

To further clarify the role of CysLTs in the allergic airways, a dual CysLT₁/CysLT₂ receptor antagonist might be more appreciate. However, the pharmacology of the CysLT₂ receptor is less defined than that of CysLT₁ receptor, mainly because of the lack of a selective antagonist. The dual CysLT₁/CysLT₂ receptor antagonist BAY u9773 may act as a CysLT₁ receptor agonist.¹⁸ Thus, the clinical advantages of dual CysLT₁ and CysLT₂ receptor antagonists for the treatment of allergic rhinitis are still unknown.

In conclusion, our results indicate that the CysLT₂ receptor plays a primary role in the vascular responses in the upper respiratory tract. The present findings should be of considerable interest for elucidating the potential roles of the CysLT₂ receptor in upper airway diseases such as allergic rhinitis.

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REFERENCES

- Shirasaki H. Cysteinyl leukotriene receptor CysLT₁ as a novel therapeutic target for allergic rhinitis. *Expert Opin Ther Targets* 2008;**12**:415-23.
- Lynch KR, O'Neill GP, Liu Q *et al*. Characterization of the human cysteinyl leukotriene CysLT₁ receptor. *Nature* 1999;**399**:789-93.
- Heise CE, O'Dowd BF, Figueroa DJ *et al*. Characterization of the human cysteinyl leukotriene 2 receptor. *J Biol Chem* 2000;**275**:30531-6.
- Shirasaki H, Kanaizumi E, Watanabe K *et al*. Expression and localization of the cysteinyl leukotriene 1 receptor in human nasal mucosa. *Clin Exp Allergy* 2002;**32**:1007-12.
- Corrigan C, Mallett K, Ying S *et al*. Expression of the cysteinyl leukotriene receptors cysLT₁ and LT₂ in aspirin-sensitive and aspirin-tolerant chronic rhinosinusitis. *J Allergy Clin Immunol* 2005;**115**:316-22.
- Perez-Novoa CA, Claeys C, Van Cauwenberg P, Bachert C. Expression of eicosanoid receptors subtypes and eosinophilic inflammation: implication on chronic rhinosinusitis. *Respir Res* 2006;**7**:75.
- Ouyang Y, Kamijo A, Murata S *et al*. Expression of cysLT₁ and cysLT₂ receptor in chronic hyperplastic eosinophilic sinusitis. *Acta Histochem Cytochem* 2009;**42**:191-6.
- Beller TC, Maekawa A, Friend DS, Austen KF, Kanaoka Y. Targeted gene disruption reveals the role of the cysteinyl leukotriene 2 receptor in increased vascular permeability and in bleomycin-induced pulmonary fibrosis in mice. *J Biol Chem* 2004;**279**:46129-34.
- Hui Y, Cheng Y, Smalera I *et al*. Directed vascular expression of human cysteinyl leukotriene 2 receptor modulates endothelial permeability and systemic blood pressure. *Circulation* 2004;**110**:3360-6.
- Stostrom M, Johansson A, Schroder O, Qiu H, Palmblad J, Haeggstrom JZ. Dominant expression of the CysLT₂ receptor accounts for calcium signaling by cysteinyl leukotrienes in human umbilical vein endothelial cells. *Arterioscler Thromb Vasc Biol* 2003;**23**:e37-41.
- McIntyre TM, Zimmerman GA, Prescott SM. Leukotriene C₄ and D₄ stimulate human endothelial cells to synthesize platelet-activating factor and bind neutrophils. *Proc Natl Acad Sci U S A* 1986;**83**:2204-8.
- Datta YH, Romano M, Jacobson BC, Golan DE, Serhan CN, Ewenstein BM. Peptideleukotrienes are potent agonists of von Willebrand factor secretion and P-selectin surface expression in human umbilical vein endothelial cells. *Circulation* 1995;**92**:3304-11.
- Kamohara M, Takasaki J, Matsumoto M *et al*. Functional characterization of cysteinyl leukotriene CysLT₂ receptor on human coronary artery smooth muscle cells. *Biochem Biophys Res Commun* 2001;**287**:1088-92.
- Walch L, Norel X, Gascard JP, Brink C. Functional studies of leukotriene receptors in vascular tissues. *Am J Respir Crit Care Med* 2000;**161**:S107-11.
- Figueroa DJ, Borish L, Baramki D, Philip G, Austin CP, Evans JF. Expression of cysteinyl leukotriene synthetic and signaling protein in inflammatory cells in active seasonal allergic rhinitis. *Clin Exp Allergy* 2003;**33**:1380-8.
- Shirasaki H, Seki N, Fujita M *et al*. Agonist-and T(H)2 cytokine-induced up-regulation of cysteinyl leukotriene receptor messenger RNA in human monocytes. *Ann Allergy Asthma Immunol* 2007;**99**:340-7.
- Ghaffar O, Laberge S, Jacobson MR *et al*. IL-13 mRNA and immunoreactivity in allergen-induced rhinitis: comparison with IL-4 expression and modulation by topical glucocorticoid therapy. *Am J Respir Cell Mol Biol* 1997;**17**:17-24.
- Labat C, Ortiz JL, Norel X *et al*. A second cysteinyl leukotriene receptor human lung. *J Pharmacol Exp Ther* 1992;**263**:800-5.