

Original article

A haplotype in the inducible T-cell tyrosine kinase is a risk factor for seasonal allergic rhinitis

Background: Identification of disease-associated single nucleotide polymorphisms (SNPs) in seasonal allergic rhinitis (SAR) may be facilitated by focusing on genes in a disease-associated pathway.

Objective: To search for SNPs in genes that belong to the T-cell receptor (TCR) pathway and that change in expression in allergen-challenged CD4⁺ cells from patients with SAR.

Methods: CD4⁺ cells from patients with SAR were analysed with gene expression microarrays. Allele, genotype and haplotype frequencies were compared in 251 patients and 386 healthy controls.

Results: Gene expression microarray analysis of allergen-challenged CD4⁺ cells from patients with SAR showed that 25 of 38 TCR pathway genes were differentially expressed. A total of 62 SNPs were analysed in eight of the 25 genes; *ICOS*, *IL4*, *IL5*, *IL13*, *CSF2*, *CTLA4*, the inducible T-cell tyrosine kinase (*ITK*) and *CD3D*. Significant chi-squared values were identified for several markers in the *ITK* kinase gene region. A total of five SNPs were nominally significant at the 5% level. Haplotype analysis of the five significant SNPs showed increased frequency of a haplotype that covered most of the coding part of *ITK*. The functional relevance of *ITK* was supported by analysis of an independent material, which showed increased expression of *ITK* in allergen-challenged CD4⁺ cells from patients, but not from controls.

Conclusion: Analysis of SNPs in TCR pathway genes revealed that a haplotype that covers a major part of the coding sequence of *ITK* is a risk factor for SAR.

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Recent advances in high-throughput technologies allow genome-wide studies to find causal genetic variants in complex diseases like allergy. However, such association and gene expression studies have implicated a large number of genes and single nucleotide polymorphisms (SNP) in complex diseases (1). Each gene and SNP may only have a small effect in the disease. This complicates the identification of disease-associated SNPs and indicates the need for a hypothesis-based approach to find SNPs.

Recent studies of complex diseases, including allergic, neurological, haematological and oncological diseases (2–6) show that the chance of finding relevant SNPs increases by focusing on genes in disease-associated pathways. Genome-wide studies have shown that this likelihood may further increase by searching for polymorphisms in genes that change in expression in disease (7).

In this study, we hypothesized that disease-associated SNPs could be identified in seasonal allergic rhinitis (SAR) by combining these two approaches, i.e. to search for SNPs in genes that changed in expression in a disease-associated

pathway. Seasonal allergic rhinitis has a well-defined phenotype and pathogenesis (8) and is therefore particularly suited to examine this hypothesis. We focused on the T-cell receptor (TCR) signalling pathway and started by analysing expression of TCR genes in allergen-challenged CD4⁺ cells from patients with SAR. Differentially expressed genes were identified and SNPs were analysed in a subset of these genes.

Materials and methods

Subjects

For analysis of SNPs, peripheral blood samples were collected from 251 patients (116 female, 135 male) with symptomatic birch and/or grass pollen induced SAR and 386 healthy individuals (167 female, 219 male), serving as controls. The median (range) age of patients and controls was 32 (18–64) and 46 (27–69).

For analysis of gene expression in allergen-challenged CD4⁺ cells and proteins in cell supernatants, blood samples were obtained from 10 asymptomatic patients with SAR outside of the season and

five healthy controls. The median age of the patients and the controls was 19 (17–34) and 44 (20–48) years.

The diagnosis of birch and/or grass pollen induced allergic rhinitis was based on a positive history of SAR for at least 2 years and a positive skin prick test (SPT) or Phadiatop test (Pharmacia Upjohn, Uppsala, Sweden) to birch and/or grass. All patients were classified as having severe symptoms (itchy nose and eyes, sneezing, nasal secretion and nasal blockage) during pollen season, and they had all been treated with antihistamines and nasal steroids during pollen seasons in the previous years. All patients presented a wheal reaction diameter >3 mm towards birch or timothy (grass) in SPT (roughly corresponding to a 3+ or 4+ reaction when compared with histamine or a positive Phadiatop test), with at least class 2 in subsequent test with specific allergens. Approximately 44% of the patients were positive for birch and/or grass only, while c. 31% were positive (≥ 2) for one to two additional allergens, c. 20% for three to four and c. 4% for more than or equal to five additional allergens. The presence of additional allergens did not seem to affect the outcome of the analysis. According to ARIA-nomenclature, the patients are referred to as suffering from SAR, as the combination of birch and grass pollen allergy in some cases resulted in a relatively long period of symptoms. Controls had no history of allergic rhinitis and a negative SPT or Phadiatop test. Controls had no history of allergic rhinitis and a negative SPT or Phadiatop test. Both patients and controls were of European origin, with both parents born in Sweden. The Ethics Committee of the Medical Faculty, Lund University, approved the study and written informed consent was obtained from all subjects.

Allergen challenge of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) from 10 patients and five controls were prepared and stimulated with the relevant pollen extract or diluent as described by Benson et al. (9). For gene expression studies, CD4+ cells were enriched from the allergen-challenged PBMC using anti-CD4-coated paramagnetic microbeads and a MACS (magnetic cell sorter) system according to the instructions of the manufacturer (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). cRNA was extracted according to the procedure described (9).

Gene expression microarray analysis of allergen-challenged CD4+ cells from patients with SAR

Gene expression microarray analyses of CD4+ cells were performed using DNA microarrays measuring the expression of 22 283 genes and variants (HuGe U133A GeneChip; Affymetrix, Santa Clara, CA, USA). CD4+ cells from three patients with SAR were analysed after challenge with allergen or diluent as previously described (9).

Quantitative real-time PCR analysis of *ITK* expression in CD4+ cells

Double-stranded cDNA was prepared from CD4+ cells that had or had not been challenged with allergen from 10 patients with SAR and five healthy controls using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) and random hexamer primers supplied in the kit. Quantitative PCR was performed using the ABI7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Validated expression assays were used, Hs00178571_m1 for *ITK*, and for endogenous control human GUSB (beta-glucuronidase) a FAM/MGB Probe

(Non-Primer Limited; Applied Biosystems) was used. The assays and TaqMan PCR mastermix (Applied Biosystems) were pipetted in a 384-well plate using a liquid-handling Biomek FX robot (Beckman Coulter Inc., San Diego, CA, USA). A total reaction volume of 10 μ l was used and each sample was pipetted in triplicates for accuracy measurements. Reactions are characterized by comparing threshold cycle (C_T) values. The C_T is a unitless value defined as the fractional cycle number at which the sample fluorescence signal passes a fixed threshold. Samples with high starting copy number show an increase in fluorescence early in the PCR process, resulting in a low C_T number, whereas lower starting copy number results in higher C_T numbers. A C_T -value for each sample was set at the exponential phase of the PCR.

Statistical analysis of real-time PCR data and *ITK* expression

The relative standard curve method was used. Two standard curves with known relative amounts of total RNA were analysed for the gene of interest (*ITK*) and for the endogenous control gene (*GUSB*). For each unknown sample, the relative amount was calculated using linear regression analysis from their respective standard curves relative, and normalized *ITK* expression value were obtained by subtracting the *ITK* C_T -value from the *GUSB* C_T -value (ΔC_T). The significance of the different mean ΔC_T between cases and controls was assessed using a *t*-test for equality of means and a two-tailed *P*-value was obtained. The reference gene, *GUSB* was equally expressed in the two groups.

Analysis of IL-5 and IL-13 in supernatants from allergen-challenged PBMC

Analysis of IL-5 and IL-13 protein was performed using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems Ltd, Abingdon, UK).

Genotyping

Genes that showed a more than fivefold increase in expression in CD4+ cells in patients with SAR were selected for association analysis: *ICOS*, *IL5*, *IL13*, *CSF2*, *CTLA4* and *IL4*. In addition, two genes were selected based on reports in the literature: *CD3D* and *ITK*. To provide a comprehensive overview of the genetic variation in the investigated genes, we selected SNPs distributed over the genes: promoter SNPs that might affect the transcriptional activity, nonsynonymous SNPs and SNPs in intronic and 3'UTR regions, all with reported frequencies in HapMap or dbSNP. Genotypes were determined using the SEQUENOM-MASSARRAY MALDI-TOF system (Sequenom Inc, San Diego, CA, USA). The system analyses allele-specific primer extension products using mass-spectrometry. Assay design was made using the MASSARRAY ASSAY DESIGN, version 2.0 software. Primers were obtained from Metabion GmbH (Planegg, Germany) and all reactions were run under the same conditions except for the primer annealing temperature of the primary PCR. PCR reactions were performed in a total volume of 6 μ l containing 2.5 ng of template DNA, 1.25 $\times$ HotStar Taq PCR buffer, 0.15 units of HotStar Taq polymerase, 3.5 mM MgCl₂, 0.5 mM dNTPs and 100 nM of each primer. Amplifications were performed using GeneAmp 9700 (Applied Biosystems, Carlsbad, CA, USA) machines with dual-384 heads as follows: 95°C for 15 min, 45 cycles of 95°C for 20 s, 56, 60 or 64°C for 30 s, 72°C for 60 s and finally 72°C for 3 min. Dephosphorylation of unincorporated dNTPs was achieved using shrimp alkaline phosphatase.

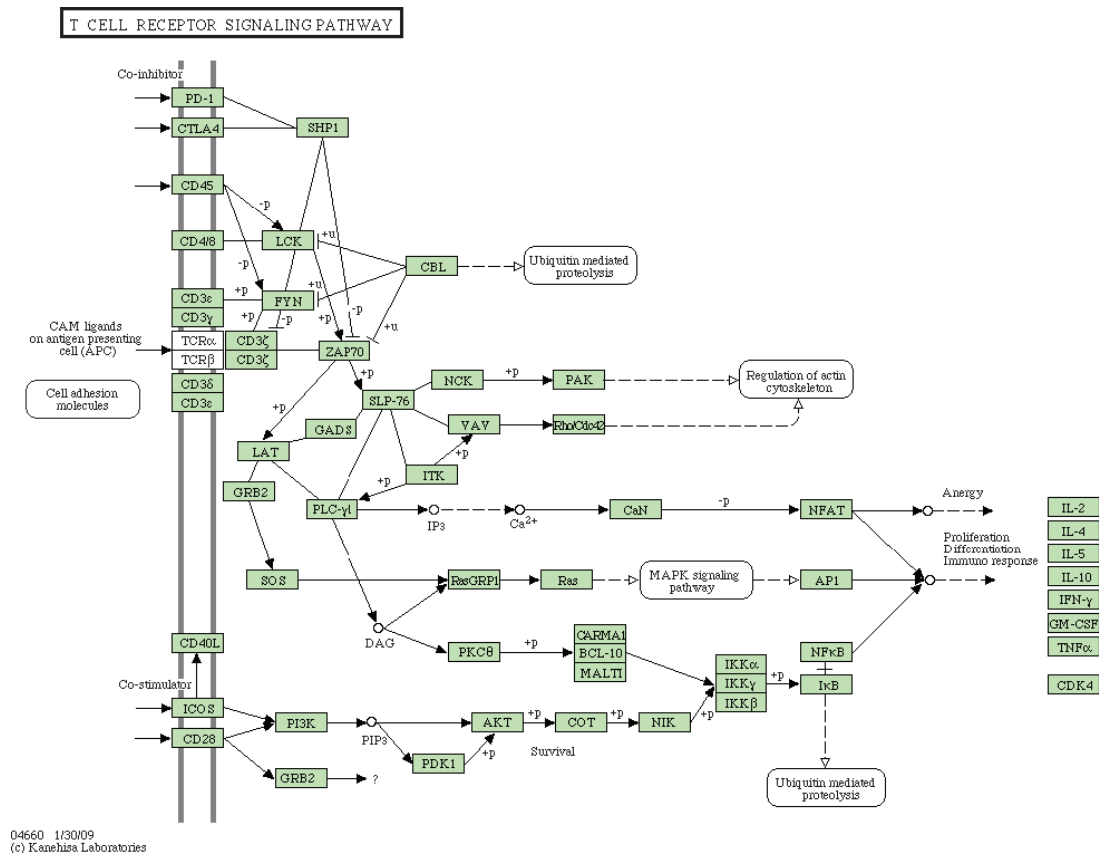


Figure 1. Genes in the T-cell receptor signalling pathway (from the KEGG pathway database, <http://www.genome.jp/kegg/pathway/hsa/hsa04660.html>). The expression levels of these genes were analysed in allergen-challenged CD4⁺ cells from patients with seasonal allergic rhinitis.

Concentrations of individual hME primer pairs were adjusted to even out peak heights in the mass spectrum. The extension reactions were then made by mixing the adjusted MassEXTEND primer mix (containing approximately 1 μ M of each primer) with hME EXTEND mix containing buffer and 50 μ M of each d/ddNTP mix and 1.25 units of Thermo Sequenase. PCR amplification of hME reactions was performed as follows: 94°C for 2 min and 99 cycles of 94°C for 5 s, 52°C for 5 s and 72°C for 5 s. The samples were then manually desalted by using 6 mg of Clean Resin and a dimple plate and subsequently transferred to a 384-well SpectroCHIP (Sequenom Inc., San Diego, CA, USA) using a nanodispenser.

Genetic analysis

Single nucleotide polymorphisms that produced good quality data were used to analyse patients and controls for associations between genetic variation in the genes and allergic rhinitis. First, the genotype frequencies were calculated and tested for Hardy–Weinberg equilibrium (HWE) using a chi-squared test. Next, allele and genotype frequencies were investigated for associations with allergic rhinitis using chi-squared homogeneity tests. Genotype data from selected SNPs were also used to estimate linkage disequilibrium and to investigate the haplotype frequencies for the indicated region of *ITK*. Haplotypes were estimated using the program PHASE.

Results

Gene expression microarray analysis of genes involved in T-cell receptor signalling

Genes involved in TCR signalling were identified using the KEGG pathway database (Fig. 1). The expression levels of these genes were analysed in CD4⁺ cells from patients with SAR that were challenged *in vitro* with either allergen or diluent. The TCR pathway represented in KEGG contains 38 genes, 25 of which were differentially expressed (Table 1). Six genes that showed a more than fivefold increase in expression in CD4⁺ cells in patients with SAR were selected for association analysis: *ICOS*, *IL4*, *IL5*, *IL13*, *CSF2* and *CTLA4*. Two additional TCR pathway genes were selected based on data in the literature: *CD3D* and *ITK* (10).

Association study of genes involved in TCR signalling

A total of 53 SNPs distributed over the genes *ICOS*, *IL4*, *IL5*, *IL13*, *CSF2*, *CTLA4*, *CD3D* and the inducible T-cell tyrosine kinase (*ITK*) were genotyped. All inves-

Table 1. T-cell receptor signalling genes that were differentially expressed in allergen-challenged CD4+ cells from patients with seasonal allergic rhinitis

Gene name	Gene ID	Fold change*
Inducible T-cell co-stimulator	<i>ICOS</i>	30
Interleukin-5	<i>IL5</i>	30
Interleukin-13	<i>IL13</i>	16
Colony stimulating factor 2	<i>CSF2</i>	16
Cytotoxic T-lymphocyte-associated protein 4	<i>CTLA4</i>	12.6
Interleukin-4	<i>IL4</i>	6.5
Mitogen-activated protein kinase	<i>MAP3K8</i>	4.9
Lymphocyte-specific protein tyrosine kinase	<i>LCK</i>	3.9
Protein kinase C, θ	<i>PRKCQ</i>	3.8
CD3G antigen	<i>CD3G</i>	3.7
Linker for activation of T cells	<i>LAT</i>	3.5
IL-2-inducible T-cell kinase	<i>ITK</i>	3.3
Tumour necrosis factor	<i>TNF</i>	3.1
CD3Z antigen	<i>CD3Z</i>	2.9
CD3E antigen	<i>CD3E</i>	2.8
Pyruvate dehydrogenase kinase 1	<i>PDK1</i>	2.8
RAS guanyl releasing protein 1	<i>RASGRP1</i>	2.7
FYN oncogene	<i>FYN</i>	2.7
Interferon- γ	<i>IFNG</i>	2.6
Lymphoma translocation gene	<i>MALT1</i>	2.3
Zeta-chain (TCR) associated protein kinase	<i>ZAP70</i>	2.2
Calcium binding protein P22	<i>CHP</i>	0.4
Interleukin-2	<i>IL2</i>	0.4
Nuclear factor of activated T cells 5	<i>NFAT5</i>	0.1
Mucosa associated lymphoid tissue		

*Ratio between median signal value of allergen-challenged/diluent challenged CD4+ cells from patients with SAR (data derived from gene expression microarray analysis).

tigated SNPs were polymorphic and produced good quality data according to quality scores obtained from the SEQUENOM software. Genotype frequencies were calculated and subsequently tested for HWE in nonallergic individuals. No significant deviation from HWE was observed. Furthermore, assuming an odds-ratio of 1.5 and additive inheritance, our material comprising 251 patients and 381 controls yields a power of approximately 0.4 for a range of intermediate allele frequencies.

All SNPs were subsequently analysed for association with SAR using a chi-squared homogeneity test, both at the allele and genotype levels. In *ITK*, no significant differences were found between the allergic and nonallergic groups when analysing the genotype frequencies. However, when analysing the allele frequencies, four SNPs showed nominally significant values ($P < 0.05$). With one exception, none of the SNPs in the other seven genes; *ICOS*, *IL5*, *IL13*, *CSF2*, *CTLA4*, *IL4* and *CD3D* was found to be associated with SAR, neither at the allele nor at the genotype level (data not shown). The exception is a single SNP (rs2276424) in *CD3D*. This SNP is located in a haplotype region together with two additional SNPs. None of these shows an increased chi-squared value in spite of very strong linkage

Table 2. Differentially expressed genes HWE test and association test results for all investigated SNPs in *ITK*

SNP	Location	Minor allele frequency	HWE, controls (χ^2)	Association test, genotype freq. (χ^2)	Association test, allele freq. (χ^2)
rs445940	Intron-1	0.13	0.44	2.21	1.96
rs2431105	Intron-1	0.11	0.00	1.45	1.44
rs31225	Intron-1	0.48	0.42	0.29	0.00
rs246856	Intron-1	0.30	1.30	4.66	3.98
rs11742812	Intron-1	0.24	0.26	1.12	0.40
rs30140	Intron-4	0.29	1.11	0.20	0.01
rs152114	Intron-4	0.30	0.84	4.93	4.02
rs3846684	Intron-5	0.36	0.01	5.12	2.57
rs25778	Intron-6	0.30	0.93	4.93	4.12
rs30114	Intron-6	0.30	3.22	3.00	3.10
rs339749	Intron-7	0.30	0.82	4.82	4.35
rs4122648	Intron-7	0.24	0.43	1.01	0.37
rs248662	Intron-8	0.29	0.00	3.30	3.02
rs13355489	Intron-8	0.13	1.26	4.12	1.82
rs10866648	Intron-9	0.42	0.12	5.02	2.50
rs30118	Intron-9	0.45	0.72	0.84	0.84
rs30119	Intron-10	0.30	0.64	5.23	4.45
rs3734024	Intron-10	0.41	1.04	2.15	1.80
rs6876898	Intron-11	0.23	1.66	2.20	1.17
rs13190673	Intron-13	0.19	2.58	1.60	0.18
rs25775	Intron-13	0.30	0.78	5.14	3.61
rs27341	Intron-16	0.42	2.50	2.84	1.43
rs27988	Exon-17	0.09	3.40	1.77	0.44
rs451494	5'UTR	0.13	0.43	2.24	1.99
rs31208	5'UTR	0.13	0.82	3.01	2.98

SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; *ITK*, inducible T-cell tyrosine kinase.

HWE tests and association tests for allele frequencies were performed with 1 d.f., whereas the association tests for genotype frequencies were performed with 2 d.f.. Significant values ($P < 0.05$) are given in bold.

disequilibrium (LD). Therefore, this marker was not further investigated. The results for the *ITK*-markers prompted us to analyse more SNPs in *ITK* to further strengthen the results. In a second round of genotyping, nine additional SNPs were analysed. These were tested for HWE in patients and controls, as well as for the association at the genotype and allele level. Out of the 25 SNPs analysed in *ITK*, all were in HWE and five showed nominally significant associations with allergic rhinitis at the allele level ($P < 0.05$). Table 2 summarizes the results of the HWE tests and chi-squared homogeneity tests for allele and genotype frequencies for all investigated SNPs in *ITK*. As all five significant markers (marked in bold in Table 1) are located within a region of strong LD according to HapMap data, the haplotypes for only these markers were determined. The results show two major haplotypes accounting for 99% of all patient chromosomes and 97% of all control chromosomes. The most common haplotype (GGGAA) had a frequency of 75% in patients and 68% in controls, whereas the less common haplotype (AAACG) had a frequency of 24% in patients and 29% in controls. As expected, all five

markers belong to the same haploblock, with the more common haplotype overrepresented in patients. Thus, the difference between patients and controls is a difference at the haplotype level rather than a number of single SNP differences.

Analysis of *ITK* in allergen-induced proliferation of Th2 cells

To analyse the role of *ITK* in allergen-induced proliferation of T helper 2 (Th2) cells, PBMC from patients with SAR were challenged with allergen *in vitro*. Inducible T-cell tyrosine kinase expression was subsequently analysed in Th cells, extracted from the allergen-challenged PBMC, using real-time PCR and normalized to the reference gene *GUSB*. The mean ΔC_T value for allergen-challenged CD4⁺ T cells was 3.0 ± 1.5 compared with 1.4 ± 0.6 in diluent-challenged Th cells ($P < 0.01$).

Discussion

Identification of disease-associated SNPs in SAR is complicated by the large number of genes involved in this disease. Although both genes and SNPs may have a large effect on the disease in combination, on their own, each of them may have only small effects. Recent studies of allergy and other complex diseases have indicated that the likelihood of finding disease associated SNPs increases if genes in disease-associated pathways or related genes are examined (2–6). The simple rationale was that disease associated genes are more likely to contain relevant SNPs. This idea was further supported by a genome-wide study which showed that such SNPs could be identified by analysing genes that changed in expression in disease (7).

In this study, we hypothesized that disease-associated SNPs could be identified in SAR by combining these two approaches, i.e. to search for SNPs in genes that changed in expression in a disease-associated pathway. We focused on the TCR signalling pathway and started by analysing expression of TCR genes in allergen-challenged CD4⁺ cells from patients with SAR. CD4⁺ cells are known to have a key regulatory role in allergic disease and genes involved in TCR signalling were therefore likely candidates for SNP analysis (8). Indeed, out of 38 analysed TCR genes, 25 changed in expression. Single nucleotide polymorphisms were analysed in a subset of these genes, namely five genes whose expression levels changed more than five-fold (*ICOS*, *IL4*, *IL5*, *IL13*, *CSF2*, *CTLA4*). While large gene expression changes can be seen as a promising and unbiased criterion for prioritization of candidate genes for SNP studies, several gene expression microarray studies indicate that relevant genes may show lower changes. We therefore choose to also include two other genes with expression changes of a lower magnitude, CD3D and *ITK*, because of their roles in allergic

inflammation, based on the literature (10, discussed in detail below).

A total of 53 SNPs distributed over the genes were initially studied for association with allergic rhinitis. Significant chi-squared values were identified for several markers in the *ITK* gene region. Further study revealed a total of five SNPs significant at the 5% level and an additional four SNPs showing increased chi-squared values. Haplotype analysis of the five significant SNPs identified two common haplotypes, which showed different frequencies in cases and controls. The more common haplotype shows an increased frequency in cases, implicating this haplotype as a risk factor for allergic rhinitis. This haplotype region covers a major part of the coding sequence of the *ITK* gene, but does not include the promoter region and exon 1. To our knowledge, SNPs in *ITK* have previously only been analysed in one study of asthma and atopy, which showed that SNPs in the 5' genomic region in *ITK* were associated with atopy (10). Of the other genes investigated in this study, previous associations studies in allergic diseases have demonstrated association to SNPs in *CD3D*, *ICOS*, *IL5* and *IL13* (5, 11–14). It should, however, be noted that the material investigated in this study is of limited size. Moreover, the results could be affected by sensitization characteristics; it is conceivable that mono-sensitized patients could have different genetic backgrounds than poly-sensitized patients.

The identification of disease-associated SNPs in *ITK* is consistent with its functions; *ITK* has an important role in Th2 cell proliferation and a recent murine model of allergic asthma showed significant decreases of airway inflammation in *ITK* $-/-$ mice. Airway hyperresponsiveness, tracheal responses, lung inflammation, eosinophil infiltration, mucous production and Th2 cytokine production all decreased (15, 16). Inducible T-cell tyrosine kinase is also involved in the migration of T cells to sites of inflammation. By contrast, the functional role of *ITK* in human allergic inflammation is not well characterized. Using gene expression microarrays, we found increased expression of *ITK* in allergen-challenged CD4⁺ cells compared to diluent-challenged cells from patients with SAR. This finding was validated by real-time PCR in an independent material of patients with SAR and healthy controls. Taken together these findings support that *ITK* also could have an important role for proliferation of Th2 cells in SAR. Interestingly, a recent study indicated that signals required for Th2 responses and migration are differentially sensitive to *ITK* activity (17). The authors suggested that this may have clinical implications for the treatment of allergic disease, in that inhibitors to target *ITK* activity have been developed. These could be modulated to alter T-cell migration without systemic effects on T-cell functions. The possibility that different structural variants affecting the expression or function of *ITK* could be involved in human allergic inflammation is important for proper directions for future research.

In summary, gene expression analysis of genes belonging to relevant pathways may help to identify candidate genes for SNP studies in allergic diseases. This approach led to the identification of a haplotype that covers a major part of the coding sequence of *ITK* as a risk factor for SAR. This renders *ITK* and its protein as good candidates for further studies in SAR.

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