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<th>Lack of association of TIM3 polymorphisms and allergic phenotypes</th>
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<td><strong>Author(s)</strong></td>
<td>Zhang, J; Daley, D; Akhabir, L; Stefanowicz, D; Chan-Yeung, M; Becker, AB; Laprise, C; Pare, PD; Sandford, AJ</td>
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Research article

Lack of association of TIM3 polymorphisms and allergic phenotypes

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Abstract

Background: T-cell immunoglobulin mucin-3 (TIM3) is a T H1-specific type I membrane protein that regulates T H1 proliferation and the development of immunological tolerance. TIM3 and its genetic variants have been suggested to play a role in regulating allergic diseases. Polymorphisms in the TIM3 promoter region have been reported to be associated with allergic phenotypes in several populations. The aims of this study were to examine whether genetic variation in the promoter region of TIM3 influenced transcription of the gene and risk for allergic phenotypes.

Methods: We performed 5’ rapid amplification of cDNA ends and reverse transcription-polymerase chain reaction. We screened for polymorphisms in the promoter region. Deletion analysis was used to localize the promoter region of TIM3. Genotyping was performed by TaqMan assays in three asthma/allergy population samples.

Results: We found two regions with promoter activity in TIM3. One region was from -214 bp to +58 bp and the other from -1.6 kb to -914 bp relative to the transcription start site. None of the single nucleotide polymorphisms (SNPs) or haplotypes affected the transcriptional activity in reporter gene assays. No association between the SNPs and any phenotype was observed in the study cohorts.

Conclusion: Our findings indicate that SNPs and haplotypes in the TIM3 promoter region do not have a functional effect in vitro and are not associated with allergic diseases. These data suggest that polymorphisms in the TIM3 promoter region are unlikely to play an important role in susceptibility to allergic diseases.

Background

Asthma is a chronic inflammatory disease of the airways that is a major cause of morbidity in developed countries and has been increasing in prevalence [1,2]. Asthma is a common disease caused by interactions between multiple genes of small to modest effect and equally important environmental factors. Asthma susceptibility has been linked to several loci e.g. chromosomes 5, 6, 11, 12 and 14.
expressed preferentially on T H1 cells after activation of naive CD4+ T-helper cells. T H1 cells mediate immune function and chromosomal location both TH2 cells mediate immune responses to extracellular pathogens and produce cytokines such as interferon-γ, IL2, tumour-necrosis factor-α and lymphotoxin. T H12 cells mediate immune responses to intracellular pathogens, delayed-type hypersensitivity reactions, and produce cytokines such as interferon-γ, IL2, tumour-necrosis factor-α and lymphotoxin.

Recent association studies suggested that polymorphisms in the TIM3 promoter region may be associated with asthma-related phenotypes in both Caucasian and Asian population samples [10-12]. Other studies have demonstrated associations of TIM1 polymorphisms with asthma and related traits [11,13,14]. In the present study, we performed an association study in three asthma/allergy population samples to investigate the role of polymorphisms in the TIM3 promoter region and determined whether these polymorphisms affected TIM3 transcriptional activity.

Methods

Study populations

We used three independent asthma/allergy population samples: the Canadian Asthma Primary Prevention Study (CAPPS) cohort, the Study of Asthma Genes and the Environment (SAGE) birth cohort and the Saguenay-Lac-St-Jean (SLS)/Québec City (QC) Familial Collection (Table 1). The study protocols were approved by ethical review boards at all participating institutions. Informed consent was obtained from each individual or his/her guardian.

The CAPPS cohort was initiated in 1995 and recruited from two Canadian cities, Vancouver and Winnipeg [15,16]. Infants were recruited who were at high risk for the development of asthma, defined as those who had at least one first-degree relative with asthma or two first-degree relatives with other allergic diseases. In total, there were 545 families recruited into this study (549 infants, 4 sets of twins). At the 7-year time point loss to follow-up was minimal, with 86% of the families completing a questionnaire. Spirometry and methacholine challenge testing were performed at the 7-year time point. The diagnoses of asthma and other atopic disorders were made by a pediatric allergist based on a detailed history and physical examination. Atopy was defined as at least one positive skin prick test. Methacholine challenge testing was carried out according to Cockcroft et al. [17]. The provocative concentration of methacholine that induced a 20% decrease in FEV1 from post-saline value (PC20) was determined. AHR

Table 1: Sample sizes by study, phenotype and ethnic background

<table>
<thead>
<tr>
<th>Phenotype</th>
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<th>SAGE</th>
<th>SLS/QC</th>
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Caucasian Samples (complete trios)

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<td>AHR</td>
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<tr>
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Non Caucasian Samples (complete trios)

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Combined Analysis (complete trios)

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<th>SAGE</th>
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<tr>
<td>Atopic Asthma</td>
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<td>92</td>
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*Non Caucasian samples included individuals of Asian (Chinese, Korean and Japanese) and Canadian First Nations descent.

*Combined Analysis also contains trios with mixed and unknown ethnicity.

CAPPS – Canadian Asthma Primary Prevention Study Cohort; SAGE – Study of Asthma Genes and the Environment birth cohort; SLS/QC – Saguenay – Lac-St-Jean/Québec City Familial Collection; AHR – airway hyperresponsiveness.
for this cohort and the SAGE cohort was defined as a PC$_{20}$ of less than 3.2 mg/ml methacholine [18,19].

SAGE is a population-based sample of 16,320 children, born in the province of Manitoba, Canada in 1995 [20]. In 2002, the families were sent a questionnaire to determine their health and home environment exposure. Children were classified according to the presence of asthma (n = 392), hay fever/food allergy (n = 192) or neither (n = 3002). All the children in the asthma and allergy groups were invited to participate in the study, together with a random sample (n = 200) of children with neither condition. A pediatric allergist assessed the presence of asthma based on a detailed history and physical examination, a methacholine challenge test was administered and skin prick tests for common allergens were performed. In total, 725 families were recruited into the study, including 247 with an asthmatic child and 328 with an atopic child.

The SLSJ/QC Familial Collection is comprised of 306 families from the Saguenay-Lac-Saint-Jean (n = 227) and Quebec City (n = 79) regions of Quebec, Canada [21,22]. There is at least one adult asthmatic proband in each family. Asthma was assessed using a respiratory health questionnaire and pulmonary function tests. AHR was defined as a PC$_{20}$ < 8 mg/ml at the time of recruitment. If PC$_{20}$ was not measurable, a 15% increase in FEV$_1$ after inhalation of a bronchodilator or a variation in PEF of at least 12% within a 2-week period was also considered diagnostic of AHR. Participants were defined as having asthma if they had a reported history of asthma that was validated by a physician, or if they showed asthma-related symptoms and a positive PC$_{20}$ at the time of recruitment. Subjects were defined as atopic if they had at least one positive response to a skin prick test. Subjects with a PC$_{20}$ > 8 mg/ml were considered not to have AHR; non-asthmatics were those who had no history of physician-diagnosed asthma, no symptoms of asthma and a PC$_{20}$ greater than 8 mg/ml; non-atopics were those who had no positive response on skin prick test.

**Expression of TIM3 in tissues**

The Human Multiple Tissue, Human Immune System cDNA Panels and Human Blood Fraction Panel (BD Biosciences/Clontech, Palo Alto, CA, USA) were used to analyze expression of TIM3 in various tissues. The PCR primers for the gene expression study are listed in Table 2. Resting CD14+ (monocytes), CD4+ (T helper/inducer cells), CD8+ (T suppressor/cytotoxic cells) and CD19+ (B lymphocytes) cells were positively selected from mononuclear cells from healthy donors by immunomagnetic separation with Dynabeads M-450 (Dynal, Oslo, Norway). Cells were activated with pokeweed mitogen (Invitrogen, San Diego, CA, USA) and concanavalin A (ICN, Costa Rica).
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 glyceraldehyde 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 34 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 amplifying TIM3 and G3PDH, respectively.

5' Rapid Amplification of cDNA ends (5'RACE)
We performed 5' RACE experiments using commercially available RACE-ready human leukocyte and spleen cDNAs (Marathon Ready cDNA, BD Biosciences/Clontech) according to the manufacturer's instructions. Primers used for amplification for the first round PCR and for the nested PCR are shown in Table 2. The amplified RACE product was cloned into pCR2.1 TOPO-TA cloning vector (Invitrogen). Plasmids were purified by column chromatography (Invisorb Spin Plasmid Mini Kit,Invitek GmbH, Berlin) and subjected to direct sequencing with M13 primers.

Single nucleotide polymorphism (SNP) screening and genotyping
Approximately 2500 bp of the 5' flanking region upstream of the transcription initiation site of TIM3 was amplified by PCR from genomic DNA of 19 unrelated healthy Caucasians. Subsequently, the products were subjected to direct sequencing with a Big-Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA). Genotyping of the two tag SNPs was done by TaqMan Assay-on-Demand™ SNP typing (Applied Biosystems).

Plasmid construction, transfection and luciferase assay
Genomic fragments of the 5' flanking region of exon 1 of TIM3 were amplified. PCR products were digested with XhoI and KpnI overnight at 37°C and then subcloned into the pGL3-Basic vector (Promega, Madison, WI, USA) digested with XhoI and KpnI. The clones were sequenced to confirm that the inserts were correct. The YT human T NK cell line provided by Dr. Zacharie Brahmi as a gift was resuspended in RPMI 1640 (Sigma-Aldrich Co, St. Louis, MO, USA) with 20% FBS. Approximately 1 × 10⁷ YT cells were cotransfected with 30 μg of test construct and 150 ng of pPL-TK (Promega) by electroporation with a Gene PulSar II (Bio-Rad, Hercules, CA) set at 300 V and 975 μF. Transfected cells were harvested 24 h after transfection. Cells were lysed by the addition of 200 μl of lysis buffer (Promega). Twenty μl of each lysate was used for 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. The signal was read using a POLARstar OPTIMA (BMG, Alexandria, VA, USA) fluorimeter. Reporter activity is presented as the mean of at least five independent measurements.

Statistical analysis
Differences in transcriptional activity in the reporter gene assays were analyzed by ANOVA and unpaired t-tests. We tested for association with asthma, atopy, atopic asthma and airway hyper-responsiveness phenotypes using the Family based Association Test (FBAT) software [23].

Results
Tissue expression of TIM3
Expression of TIM3 was analyzed by PCR-based methods (Figure 1). TIM3 was strongly expressed in placenta, lung, kidney, spleen, and leukocytes. In the Human Blood Fraction Panel TIM3 was more highly expressed in active CD4+ cells than resting CD4+ cells. However it was more highly expressed in resting CD8+ cells than in active CD8+ cells. TIM3 was also strongly expressed in resting CD14+ cells. No splicing variants were found.

Isolation of 5'-full-length TIM3 transcripts and structure of the human TIM3 gene
Current information at the time of the experiment (January 2007) in the NCBI database http://www.ncbi.nlm.nih.gov indicated that TIM3 was composed of seven exons and the translational start site was contained within exon 1. TIM3 was highly expressed in leukocytes and spleen and therefore 5’ RACE experiments were performed with cDNAs derived from these cell types. We were able to identify an additional 25 bp of sequence on the 5’ side of the known cDNA sequence (Figure 2). No additional novel exons were detected.

Polymorphism screen
We screened for polymorphisms in the TIM3 promoter region using DNA from 19 unrelated normal subjects and found six polymorphisms -574 G/T, -882 C/T, -1516 G/T, -1571delC, -1766G/T (rs10061463) and -1922 G/A (rs12186731) in TIM3 (Figure 2). Among the six polymorphisms four (-574 G/T, -882 C/T, -1571delC and -1766G/T) were in perfect linkage disequilibrium (r² = 1). There were only three haplotypes formed by the six polymorphisms.

Transcriptional activity of the 5’ flanking region of TIM3
To examine the transcriptional activity in the 5’ flanking region of TIM3, we constructed plasmids that contained sequences from -2220, -1702, -914, -452, -241 and +63 relative to the transcription initiation site. The primers used for plasmid construction are listed in Table 2. The expression of TIM3 in YT cells was confirmed by RT-PCR (data not shown). The constructs were then transiently transfected into YT cells. Deletion analysis revealed that promoter
**Figure 1**
Expression of TIM3 in multiple human tissues. Results of PCR amplification of cDNA from different organs (A), the immune system (B), and blood fractions (C) are shown. G3PDH was included as an internal control. MC, mononuclear cells; R, resting; A, activated; NC, non-template control.

**Figure 2**
Genomic structure of the human TIM3 gene. The open boxes represent the positions of exons 1–7. The shaded box is the region we extended in our 5’RACE experiment. TIM3 contains seven exons and the coding sequence (CDS) starts in exon 1. The downward arrows indicate the SNPs in the promoter region. The four red arrows indicate SNPs that are in perfect LD.
activity of TIM3 in YT cells required at least 241 bp of upstream sequence and the maximal reporter gene expression was observed with the -1702 bp construct (Figure 3). There were five polymorphisms, -574 G/T, -882 C/T, -1516 G/T, -1571delC and -1766G/T in this region. To determine whether the five polymorphisms and their haplotypes were functional, we generated luciferase reporter gene constructs that contained the 5′ flanking region of TIM3 from exon 1 to -1702 bp with three different haplotypes, i.e., haplotype CCGGC, haplotype GCTCG and haplotype TTG-T. The results showed there was no difference in expression level between each haplotype (Figure 3).

FBAT analysis
To determine whether the previously reported TIM3 associations [10-12] were present in the CAPPS, SAGE and SLSJ/QC populations, we chose -882 C/T and -1922 G/A as tag SNPs. However -1922 G/A was in a region of repetitive sequence. Therefore, rs13170556, which was in perfect linkage disequilibrium (LD) with -1922 G/A, was genotyped in our samples. Both polymorphisms (rs13170556 and rs10061463) were in Hardy-Weinberg equilibrium (p > 0.1) in all cohorts. We performed FBAT analysis to test for association with asthma, atopy, atopic asthma and AHR. The results were corrected by the number of SNPs tested within TIM3 (n = 2) and the effective number of independent phenotypes (n = 3). We found that rs13170556 was associated with asthma in the CAPPS cohort in both the Caucasians only analysis and in the combined analysis of the Caucasian families with the non Caucasian families (p = 0.0138 and 0.0085, respectively) (Table 3). However, after correction for multiple testing we found no evidence for association in any of the three cohorts individually or in joint analysis of all the cohorts (Table 3). Similarly, there was no association found for -882C/T with any phenotype in any of the analyses (Table 4).

Discussion
In the present study, we determined the expression pattern of TIM3 in human cells. We investigated the genomic structure and transcriptional activity of TIM3 and investigated polymorphisms in the promoter region of TIM3 in multiple cohorts. We isolated the full-length genomic consistent with previous reports. In the mouse, CD4+ cells as well as resting CD8+ cells and CD14+ cells, consistent with previous reports. In the mouse, TIM3 was expressed in both CD4+ and CD8+ cells [24,25] and in human peripheral blood mononuclear cells TIM3 was expressed at a higher level on CD14+ cells and CD8+ cells than on CD4+ cells [26]. TIM3 was also reported to be expressed in NK and NTK (NK-like T) cells [26,27]. In our results, TIM3 was expressed at a higher level in activated CD4+ cells than in resting CD4+ cells but conversely expression was higher in resting CD8+ than in activated CD8+ cells. Our results demonstrate that the expression level of TIM3 is not only differentially regulated in subsets of T cells but is also determined by the activation state of the cell.

TIM3 is expressed in human NK cells both at the mRNA and protein levels [26,27]. We found that TIM3 was also expressed in one type of NK cell line, the YT cell line, which was used in the reporter gene assays. We identified TIM3 promoter activity in the -241 bp and -1702 kb regions relative to the transcription initiation site. Conserved non-coding sequences may contain transcriptional regulatory elements participating in the temporal and tissue-specific expression patterns of genes [28,29]. In the UCSC website http://genome.ucsc.edu/ there are three conserved regions in the TIM3 promoter (Figure 3B) and the first conserved region contributes to the -241 bp promoter region and the last two regions contribute to the -1702 bp promoter region. There are five SNPs in the -1.7 kb region and the -1516 G/T, -1571delC and -1766G/T SNPs flank the conserved sequence. However, the haplotype formed by these SNPs did not affect the promoter activity (Figure 3C). We also stimulated the YT cell line with IL-2 at different concentrations but we found no difference in promoter activity among the different haplotypes after the stimulation (data not shown).
Figure 3
Promoter activity assay of the human TIM3 gene promoter constructs. (A) Luciferase activity is presented relative to the PGL3 basic vector after each construct was transfected into YT cells. All values are the mean ± SD of at least five independent experiments. (B) Comparison of the promoter activity and the conserved regions in the human genome. (C) Comparison of the promoter activity between the haplotypes.
sons was performed to avoid false positive results. Although a nominal association of rs13170556 was found in the CAPPS cohort it was not significant after correction for multiple comparisons. Moreover, the association was not replicated in the other two cohorts and in the combined analysis of all three cohorts. Therefore, the association was likely a statistical artifact rather than a true positive result.

We did not analyze other phenotypes such as total or specific serum IgE in this study. We did not analyze haplotypes in the patient cohorts as we believe that this would have been inappropriate since we used tag SNPs from HapMap and it has been suggested that in this scenario there is little benefit of exhaustive haplotype testing [30]. In addition, we used the most powerful approach given our study design, there is high LD in the region, the marker coverage was not dense and our single SNP main effects were negative. All these factors made it unlikely that we would have benefited from haplotype tests.

The power to detect an association in this study varied with the phenotype, allele frequency and cohort considered. Power was calculated using the TDT Power Calculator [31]. For a major allele ‘A’ and minor allele ‘a’, we assumed the penetrance of the three genotypes was AA = 0.1, Aa = 0.2 and aa = 0.5. For an allele frequency of 0.13 and the phenotype of allergic asthma in the CAPPS cohort
(i.e. 37 trios) the power to detect an association was only 0.41. However, for a sample size of 96 trios (e.g. AHR in the SAGE cohort) the power was 0.80 and was >0.80 for all other phenotypes in all cohorts in the Caucasians.

**Conclusion**
Our findings indicate that SNPs and haplotypes in the TIM3 promoter region do not have a functional effect in vitro and are not associated with allergic diseases. These data suggest that polymorphisms in the TIM3 promoter region are unlikely to play an important role in susceptibility to allergic diseases.

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

**Authors’ contributions**
JZ participated in the genotyping, performed the remainder of the molecular analysis and produced the first draft of the manuscript. DD performed the analysis of the genetic epidemiological data and helped to draft the manuscript, LA participated in the genotyping. DS participated in the genotyping, MC-Y participated in the recruitment of the patient cohorts and helped to draft the manuscript, AB participated in the recruitment of the patient cohorts and helped to draft the manuscript, PDP participated in the recruitment of the patient cohorts, the design of the study and helped to draft the manuscript, AJS participated in the design of the study.

**Table 4: Allele frequencies of the rs10061463 polymorphism in the three study cohorts for each phenotype**

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<td></td>
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<td>Frequency</td>
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<sup>a</sup>Combined analysis of the Caucasian families with the non Caucasian families
<sup>b</sup>Combined analysis of the three cohorts (CAPPS, SAGE and SLSJ/QC)
<sup>c</sup>corrected p value

CAPPS – Canadian Asthma Primary Prevention Study Cohort; SAGE – Study of Asthma Genes and the Environment birth cohort; SLSJ/QC – Saguenay – Lac-St-Jean (SLSJ)/Québec City (QC) Familial Collection; AHR – airway hyperresponsiveness
study and helped to draft the manuscript. All authors read and approved the final manuscript.

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References


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