

ARTICLE

# Physical map of an asthma susceptibility locus in 7p15-p14 and an association study of *TCRG*

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Chromosome 7p15-p14 showed genome-wide significant linkage to asthma related traits among the Finnish and French-Canadian families. As an essential step toward cloning the susceptibility gene, a detailed physical map of the region is needed. In this study we report a dense set of carefully tested, new microsatellite markers for fine mapping embedded in a continuous, easy-to-read, physical map of the region that includes the known genes and putative transcripts. Even though susceptibility genes for asthma are difficult to predict from a multitude of unknown genes mapped to the region, *TCRG* encoding the  $\gamma$ -chain of the heterodimeric  $\gamma/\delta$  T cell receptor is a potential candidate. We present linkage and association results for *TCRG* in two independent Finnish family sets by using four highly polymorphic microsatellites spanning 169 kb across the locus. Linkage results confirmed our previous findings, but our study did not provide any evidence on behalf of a strong association of *TCRG* with either high serum total Immunoglobulin (IgE) level or asthma. Our results suggest that some other known or yet unidentified gene in the linkage region is the true asthma susceptibility gene.

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## Introduction

Recent genome wide scans have suggested several regions potentially linked to asthma and related traits. One of the loci of interest is located on 7p15-p14. Among Finnish asthma families, this locus was the only locus that reached genome wide significance level.<sup>1</sup> The finding was further confirmed among French-Canadian families and in another independent data set from Finland.<sup>1</sup> Daniels *et al.*<sup>2</sup> have reported six regions of possible linkage, including 7p14-p15, and established by simulations that at least some of them are likely to be true positives. German,<sup>3</sup> French,<sup>4</sup> and Italian families<sup>5</sup> have shown some evidence of linkage, but

there are also genome scans with inconclusive results,<sup>6–8</sup> some of which have been done in ethnic populations other than Caucasian.<sup>9,10</sup> Furthermore, clinically distinct autoimmune diseases may be controlled by a common set of susceptibility genes.<sup>11</sup> 7p15-p14 has also been linked to diseases such as multiple sclerosis<sup>12</sup> and inflammatory bowel disease,<sup>13</sup> and genomic regions homologous to human 7p15-p14 have been linked to insulin dependent diabetes<sup>14</sup> and inflammatory arthritis<sup>15</sup> in rat models.

Implicating a susceptibility gene for such a complex disorder as asthma is difficult. As an essential step toward identifying a susceptibility gene for asthma, we need a detailed physical map of the linkage region that includes the known genes, putative transcripts, and genetic markers. In this study we report a continuous, easy-to-read, physical map of the region with structural and functional information of known genes. We also present our linkage and

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association results for *TCRG* among Finnish asthma families by using four highly polymorphic microsatellite markers within the *TCRG* locus. Our results suggest that *TCRG* is not significantly associated with asthma or high serum IgE level, and thus, *TCRG* might not be the major susceptibility gene for asthma in 7p15-p14.

## Materials and methods

### Construction of the physical map

We started the construction of a chromosome 7 physical map with the microsatellite markers found to be linked with high serum IgE level and asthma.<sup>1</sup> First, we used these markers to determine the corresponding region in the Radiation hybrid (RH) map (<http://www.ncbi.nlm.nih.gov/genemap99>), and to identify STSs, genes, ESTs, clones and contigs located in the region. Second, we used RH distances of the STS, genes, and ESTs mapped into clones/contigs, and the order of clones/contigs in NCBI Human Chromosome 7 contig map (<http://www.ncbi.nlm.nih.gov/genome/seq/chr.cgi?CHR=7>) to roughly organise the clones and contigs. Third, to determine the exact order and orientation of clones and contigs, we explored public databases with different contig and clone end sequences, cDNA and EST sequences by BLAST. To fill in the gaps in public sequence, we also used the Celera database (<http://public.celera.com/index.cfm>). Fourth, we compared the organisation of our map to different genomic databases to make sure that no major discrepancies were present. The comparisons were done using the Human Genome Browser (<http://genome.ucsc.edu/goldenPath/octTracks.html>), the NCBI Entrez Genomes Database (<http://www.ncbi.nlm.nih.gov/genome/seq/>), the Sanger Center Ensemble Database (<http://www.ensembl.org/>), and the Celera database. During the process additional information was also collected from the NCBI databases (<http://www.ncbi.nlm.nih.gov/index.html>) UniSTS, UniGene, OMIM, LocusLink, and from the Marshfield Genetic Map (<http://research.marshfieldclinic.org/genetics>). Finally, cDNA sequences were used to map the exons of known genes in genomic sequence. The physical map was drawn based on collected data of clones, genes and markers using the PhysiMap program (<http://www.ee.oulu.fi/~polvi/physimap.htm>) developed specifically for this purpose.

### Design of new microsatellite markers

To create a dense map of polymorphic markers spread evenly across the linkage region, we screened the publicly available genomic sequence for potentially polymorphic short sequence repeats. The repeats were initially tested by PCR amplification in 10 unrelated individuals. If allelic polymorphism was detected, the Mendelian inheritance was confirmed in 10 nuclear families. Detailed information on primers, PCR conditions and allele frequencies are available in the GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/>) and at our www site (<http://www.genome.helsinki.fi/english/research/projects.xml>).

## Two independent family collections for linkage and association analysis

First family recruitment was done in the Kainuu province in central eastern Finland in 1994 and 1996. The methods for recruitment, control for population stratification, and clinical evaluation have been described previously in detail.<sup>16,17</sup>

The second family collection was recruited from the province of North Karelia in southern eastern Finland in 1999. Like Kainuu, it represents the late settlement region of Finland, but with a distinct population history than that of the first data set, ie the settlers came from a different donor population. The study subjects were initially identified by a population survey.<sup>1,18</sup> All participants were invited into a clinical trial to evaluate their history of atopic disorders (FinnAllergy study). Proband ( $n=791$ ) were carefully studied clinically. Among the participants, there were 180 individuals with IgE level >100 kU/l. They were contacted by a letter and asked to join a genetic study with their nuclear family members. Altogether 85 families and 517 individuals were recruited; 26 of the families were trios. In 69 families the proband reported atopic symptoms in multiple family members and other siblings. All participants filled out a questionnaire including questions of respiratory symptoms, smoking habits, medication and physician-made diagnosis of asthma or other atopic disorders.

Total serum IgE level was determined by Diagnostics CAP FEIA (Kabi Pharmacia, Sweden) in both the Kainuu and North Karelia study groups.

### Linkage and association analysis for *TCRG*

*TCRG* was mapped to the genomic clones AC006033 and AC07245 using exon sequences for V1, V2, V3, V4, V5, V5P, V6, V7, V8, VA, V9, V10, VB, V11, JP1, JP, J1, C1, JP2, J2, and C2 retrieved from the international ImMunoGeneTics database (IMGT) (<http://imgt.cines.fr:8104>).<sup>19</sup> All polymorphic sites (*TCR4*, *TCR6*, and *TCR7*) and two flanking markers D7S2497 (heterozygosity 0.87) and MN35 were genotyped in both sample sets. For genotyping, DNA was extracted from blood leukocytes by a standard non-enzymatic method. The primer pairs were fluorescently labelled (Genosys, UK). PCR amplification was done separately for each marker and run pooled on an ABI377 sequencer. Linkage and association analyses were performed using GENEHUNTER 2.0<sup>20</sup> and Haplotype Pattern Mining (HPM),<sup>21</sup> respectively.

For haplotyping, large pedigrees were divided into trios using an in-house computer programme. The programme identifies the maximal number of trios that are not overlapping and in which one or two members were affected (not both parents). Trios that included members who had not been genotyped or members with unknown phenotype were excluded. Haplotyping was done within each trio and four independent chromosomes were obtained from each trio. In case of ambiguities (missing genotype data, identical heterozygous genotypes in all of the family members, or Mendel errors), data for that marker were discarded. If the child was

affected, the transmitted chromosomes were considered disease associated and the non-transmitted chromosomes as controls. If one of the parents was affected, his/her chromosomes were considered disease associated and the spouse's chromosomes as controls. If both the parent and the child were affected, only the non-transmitted chromosome of an unaffected parent was considered as the control and the other three as disease associated. These haplotypes, labelled as disease-associated or control, were then used as input for HPM.

The HPM algorithm finds all the haplotype patterns up to a given length whose association to the trait, measured by a  $2 \times 2$   $\chi^2$ -test, exceeds a given threshold value. To make the algorithm more robust with respect to marker mutations, genotyping errors, and missing data, a user-specified number of gaps is allowed in the patterns. Based on the found patterns, each marker is given a score based on the number of patterns spanning across the marker. HPM estimates the significances of the obtained scores against the null hypothesis that there is no association. The null distributions are estimated by permuting the trait values and recalculating the scores several times. Comparing the observed scores to these distributions yields marker-wise  $P$  values that are comparable with each other.

To estimate the overall significance of detected association, accounting simultaneous testing of multiple markers, we performed nested permutation tests. The lowest marker-wise  $P$  value is compared to the distribution of the lowest marker-wise  $P$  values obtained when the HPM analysis is repeated several times with permuted data, yielding a corrected  $P$  value. The procedure has been described in detail (see [www.acm.org/sigkdd/kdd2001](http://www.acm.org/sigkdd/kdd2001), and [www.cs.helsinki.fi/TR/C.html](http://www.cs.helsinki.fi/TR/C.html)).<sup>22,23</sup>

## Results

### Physical map

The clones, contigs, genes and markers that are located on 7p15-p14 and linked to asthma are shown in Figure 1. Altogether 184 clones were placed into the map. Nine gaps were detected between the following nine clones:

AC006331.2/AC005105.2, AC006027.1/AC005154.1, AC073068.8/AC006033.2, AC073345.10/AC005483.1, AC004987.2/AC072061.8, AC026866.8/AC004988.2, AC011296.2/AC015515.3, AC011738.4/AC005189.1, and AC006454.3/AC004453.1.

The orientation of the following five pairs was determined by using cDNA sequences of genes that had exons in both flanking clones:

AC006331.2/AC005105.2, AC006027.1/AC005154.1, AC073345.10/AC005483.1, AC026866.8/AC004988.2, and AC011738.4/AC005189.1.

The genes were *Cre-Bpa*, *NOD1* (OMIM#605980), *RPF-1*, *FLJ11808*, and *STK17A* (OMIM#604726), respectively. The

orientation of the remaining four pairs AC073068.8/AC006033.2, AC004987.2/AC072061.8, AC011296.2/AC015515.3 and AC006454.3/AC004453.1, and the length of gaps, was determined using Genomic Scaffold sequences of the public Celera database.

After the assembly of the clones, we compared their order and orientation to the most complete draft assembly of the human genome (<http://genome.cse.ucsc.edu/goldenPath/octTracks.html>). Draft sequence showed high concordance with our map. However, two major discrepancies were observed. First, NT\_000369 showed reverse orientation in the draft sequence, but our assembly results could be confirmed by the Celera and Sanger Institute Ensemble databases. Second, seven clones in our map were absent in the corresponding region of the draft sequence. Five of the clones (AC087072.2, AC087069.2, AC078843.2, AC026866.8, and AC017116.5) could be found in the NCBI Entrez Genome Database placed between the same clones as in our map. In addition, AC015515.3 was shown to partially overlapping with AC027269.5, as in our map. The position of the clone AC087070.2 was confirmed from the Sanger Center Ensemble Database.

When we compared the comprehensive genetic map to our physical map (Figure 2), the order for six markers (D7S1806, D7S1514, D7S668, D7S2469, D7S2548, and D7S678) differed. In 11 cases, two or more markers were mapped into the same genetic position, on our physical map the marker order could be now verified.

Figures 1 and 2 are available in more detail at our web site (<http://www.genome.helsinki.fi/eng/research/asthma/pubs.html>).

### New microsatellite markers

We have carefully tested 53 new microsatellites for the region; 31 of them that were robust to type, polymorphic, and followed Mendelian inheritance in 10 families were chosen for an association study (<http://www.genome.helsinki.fi/eng/research/asthma/pubs.html>). The intermarker distance for markers shown in the asthma linkage region (Figure 1) is on average 210 kb (range from 17 to 656 kb).

### Genes in the region

Altogether 87 known and putative genes were identified in the region (please see <http://www.genome.helsinki.fi/eng/research/asthma/pubs.html> for specific data). Most genes encode proteins with known structure or/and function, but also genes with only predicted protein product are included. The region includes one cluster of genes (*HOXA1-13*) belonging to a HOX-gene family while the remaining genes encode proteins that fall functionally and structurally into multiple categories.

### TCRG linkage and association analysis

To study the role of *TCRG* as a potential candidate locus in asthma, we first screened altogether eight tandem repeats



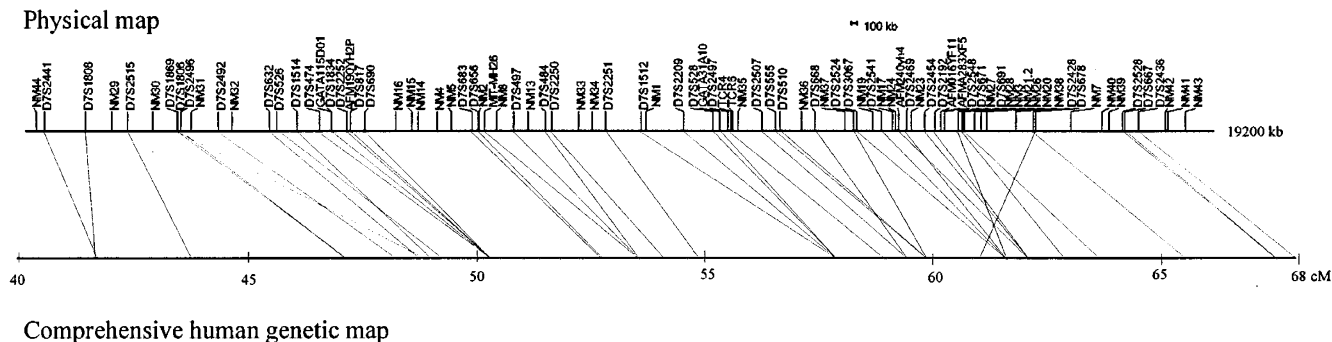


Figure 2 Refined localization of microsatellite markers on the physical map in comparison to their localisation on the genetic map.

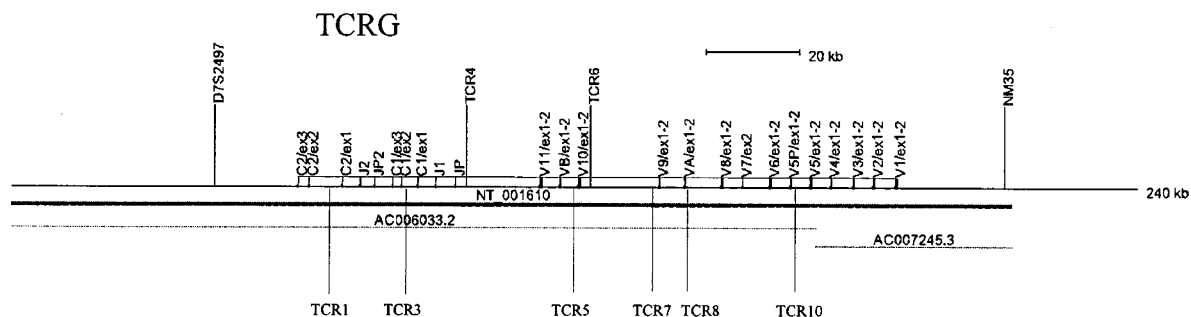


Figure 3 Organization of constant (C), joint (J), and variable (V) genes in the *TCRG* locus including microsatellites screened for polymorphisms (below) and four markers found polymorphic and genotyped in two Finnish data sets (above).

within the locus (Figure 3). Three of them (TCR4, TCR6, and TCR7) were confirmed to be highly polymorphic in 20 chromosomes tested and showed Mendelian inheritance in 10 nuclear families. These three markers and two flanking markers D7S2497 and NM35, spanning a 169 kb haplotype, were then genotyped in two Finnish family sets (1150 individuals). Later, however, TCR7 was excluded from the analysis, since sequencing of some individuals with ambiguous genotypes showed that even alleles of same size were actually different in sequence because of several SNPs within the repeat and single nucleotide insertions in a string of adenines upstream of the repeat. The remaining markers showed strong linkage disequilibrium across the region when analysed in consecutive pairs by  $2 \times 2$  contingency tables ( $P < 10^{-28}$ ).

Both linkage and haplotype association analyses were done for two phenotypes: asthma and high total serum IgE level, with new markers added. For comparison, exactly the same pedigrees were included into the non-parametric multipoint linkage analysis as in our genome scan.<sup>17</sup> For the *TCRG* markers, the best linkage was found to high IgE level when both data sets were pooled together (NPL score 3.3,  $P=0.0009$ , information content 0.86 at D7S2497) (Table 1). For asthma, NPL scores was 2.2 across *TCRG* ( $P=0.003$ , information content 0.80) in the Kainuu data set, among

the North Karelia families asthma status was unknown. As expected, the overall linkage for 7p14-p15 was at the same level as before.<sup>1</sup> The best linkage hits were outside the *TCRG* locus; for IgE at D7S691 (NPL score 4.0,  $P=0.0001$ , information content 0.80) in the combined data set and for asthma in the Kainuu data set, at GATA31A10 (NPL score 2.7,  $P=0.004$ , information content 0.66).

By using the HPM algorithm,<sup>21</sup> the best allele and haplotype associations for high IgE were found again in the combined data set. Altogether 596 chromosomes were retrieved from multiplex and nuclear families. Table 2 illustrates the best haplotype associations for high IgE level. All found associations remained rather weak ( $\chi^2 \leq 7.5$ ). For asthma, the number of associated haplotypes and the strength of found associations remained even lower (Table 2).

To study if the overall haplotype distribution in *TCRG* differs among the disease associated compared to control chromosomes, HPM analysis was done using the following parameters: maximum pattern length four markers, one gap allowed for missing data and possible errors, and  $\chi^2$  threshold for the association 3.0. The observed scores (range from 6 to 22) (=number of qualified haplotype patterns spanning across the marker) for associated haplotypes remained low for both phenotypes even with this low threshold for association. Permutation test showed no

**Table 1** NPL-scores, nominal *P* values, and information content for the phenotypes 'high serum total IgE level' in two independent data sets and 'asthma' in one data set. Non-parametric multipoint linkage analysis is done together with the previously published fine mapping markers on the chromosome 7<sup>1</sup>

Markers	Sample sets and phenotypes											
	High serum total immunoglobulin E									Asthma		
	Kainuu n=38 pedigrees			North Karelia n=29 pedigrees			All n=67 pedigrees			Kainuu n=72 pedigrees		
	NPL score	P value	Information content	NPL score	P value	Information content	NPL score	P value	Information content	NPL score	P value	Information content
D7S2497	2.9	0.003	0.80	1.7	0.05	0.94	3.3	0.0009	0.86	2.2	0.01	0.73
TCR4	2.9	0.003	0.80	1.8	0.04	0.94	3.3	0.0008	0.86	2.2	0.01	0.74
TCR6	2.9	0.003	0.80	1.8	0.04	0.94	3.3	0.0008	0.87	2.2	0.01	0.74
NM35	2.9	0.003	0.80	1.7	0.04	0.94	3.2	0.001	0.86	2.2	0.01	0.74

**Table 2** Best haplotype associations of TCRG for high serum IgE level in the combined data set (343 affected and 253 control chromosomes) and for asthma in the Kainuu data set (362 affected and 298 control chromosomes) when 30% missing data, but no errors were allowed for the haplotype patterns

Total	All	High IgE	Confidence	$\chi^2$	D7S2497	TCRG markers		
						TCR4	TCR6	NM35
497	18	16	89	7.5	–	3	5	3
544	45	34	76	6.3	–	–	5	3
497	12	11	92	5.9	–	1	5	3
518	15	13	87	5.4	–	1	5	–
544	7	7	100	5.2	–	–	1	5
518	7	7	100	5.2	–	3	1	–
497	7	7	100	5.2	–	3	1	5
573	7	7	100	5.1	5	3	1	5
497	17	14	82	4.4	–	1	3	5
493	6	6	100	4.4	5	3	1	–
573	17	14	82	4.3	11	3	5	3
493	13	11	85	3.9	4	1	5	–
573	5	5	100	3.7	3	3	5	3
497	5	5	100	3.7	–	2	2	1
518	27	20	74	3.2	–	3	5	–

Total	All	Asthma	Confidence	$\chi^2$	D7S2497	TCRG markers		
						TCR4	TCR6	NM35
562	49	36	73	6.9	2	2	–	–
640	35	26	74	5.5	2	2	2	7
545	28	21	75	4.5	2	2	2	–
579	32	23	72	3.8	–	2	2	–
627	168	103	61	3.7	–	–	2	–
558	31	22	71	3.2	–	2	2	7

Total, the number of chromosomes in which the number of missing alleles within the pattern does not exceed 30%; All, the number of haplotypes with a specific pattern; Asthma/high IgE, the number of disease associated haplotypes with the specific pattern; Confidence, percentage of haplotypes with the specific pattern that are associated with the disease,  $\chi^2$ , value for disease association of the specific haplotype pattern.

statistically biased haplotype distribution for asthma (marker-wise *P* values 0.47–0.76) and borderline significance for high IgE level (lowest marker-wise *P* value 0.038 at TCR6).

To evaluate the overall significance of this smallest marker-wise *P* value observed, it should be further corrected for multiple testing. Since most of the variables (tested markers and phenotypes) are highly correlated, a simple Bonferroni adjustment is obviously too conservative. To

estimate the effect of simultaneous testing of multiple markers, we used a second level permutation test.<sup>22,23</sup> The *P* value of 0.038 was compared to the distribution of the lowest marker-wise *P* values obtained when the HPM analysis was repeated several times with permuted data, yielding a corrected *P* value of 0.059. Previously we have estimated by simulations that a correction of 1.3 should be used for the two correlated phenotypes,<sup>1</sup> yielding a final *P* value of 0.08.

## Discussion

Chromosome 7p14-p15 has been linked to asthma related traits in Finnish, French-Canadian, and Australian populations<sup>1,2</sup> and to other immune mediated disorders.<sup>11</sup> For positional cloning projects, an accurate physical map is crucial. Although several maps of the region are publicly available, none of them includes all information and discrepancies exist. Based on our calculations in the Finnish data, the appropriate confidence region for positional cloning of the susceptibility gene for asthma is from the marker D7S526 to D7S1830 (24 cM).<sup>24</sup> Our ~20 Mb physical map covers most of the region with 184 genomic clones and 87 genes. Only information that could be verified from several sources is indicated on the map. Fifty-three new markers were added to 50 previously published markers. They form a highly informative marker map across the region with an average marker density of 210 kb. With a couple of exceptions, physical distances between the markers matched well with the genetic distances (1 cM~1 Mb), but the more precise map allowed us to order the markers correctly (Figure 2).

As a potentially interesting candidate, *TCRG* has been mapped to this region.<sup>25</sup> *TCRG* (~130 kb) consists of a cluster of 14 highly polymorphic variable (V1-V11, V5P, VA and VB, including five pseudogenes), five joining (JP1, JP, J1, JP2, and J2), and two constant genes (C1 and C2) that are rearranged during  $\gamma/\delta$  T cell differentiation to encode  $\gamma$ -chain of the heterodimeric T cell receptor<sup>19,26</sup> (Figure 3).  $\gamma/\delta$  T cells are believed to maintain homeostasis in the airway mucosa during immuno-inflammatory stress and form the first line defence on epithelial surfaces before a  $\alpha/\beta$  T cell response is fully developed.<sup>27</sup> Several studies on  $\gamma/\delta$  deficient-mice have suggested that  $\gamma/\delta$  T cells have an essential role in the development of the IgE response, eosinophilic bronchitis, and bronchial hyperreactivity when mice are sensitised with oval albumine.<sup>25,28-30</sup> Among asthma patients increased numbers of  $\gamma/\delta$  T cells in bronchoalveolar lavage fluid compared to controls have been reported.<sup>31,32</sup>  $\gamma/\delta$  T cells are potentially also involved in the development of other immune diseases.<sup>33-37</sup>

Because the germline sequence of the *TCRG* locus consists of highly homologous elements, genotyping of single nucleotide polymorphisms described in different T cell clones can be extremely difficult.<sup>20</sup> We chose to apply in our linkage analysis four highly polymorphic and stable microsatellite markers. Our analysis showed that *TCRG* is definitively located within the linkage peak that we have reported previously, but the observed NPL scores did not exceed that of the neighbouring markers. Our study did not provide any evidence for a strong association of *TCRG* with either high serum total IgE level or asthma. All found associations were weak and distributed in multiple, rare *TCRG* haplotypes. Since asthma is a complex disorder with interactions of many cell types and tissues, it is very difficult to exclude any of the genes of the region. As a new

working hypothesis, we must consider the possibility that some other known or yet unidentified genes in the linkage region will show a stronger association to asthma.

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