Significant linkage to chromosome 12q24.32–q24.33 and identification of SFRS8 as a possible asthma susceptibility gene

C Brasch-Andersen, Q Tan, A D Børglum, A Haagerup, T R Larsen, J Vestbo, T A Kruse

Background: Asthma is a complex genetic disorder. Many studies have suggested that chromosome 12q harbours a susceptibility gene for asthma and atopy. Linkage on chromosome 12q24.21–q24.33 was investigated in 167 Danish families with asthma.

Methods: A two step procedure was used: (1) a genome-wide scan in one set of families followed by (2) fine scale mapping in an independent set of families in candidate regions with a maximum likelihood score (MLS) of ≥1.5 in the genome-wide scan. Polymorphisms in a candidate gene in the region on 12q24.33 were tested for association with asthma in a family based transmission disequilibrium test.

Results: An MLS of 3.27 was obtained at 12q24.33. The significance of this result was tested by simulation, resulting in a significant empirical genome-wide p value of 0.018. To our Knowledge, this is the first significant evidence for linkage on chromosome 12q, and suggests a candidate region distal to most previously reported regions. Three single nucleotide polymorphisms in splicing factor, arginine/serine-rich 8 (SFRS8) had an association with asthma (p < 0.0020–0.050) in a sample of 136 asthmatic sib pairs. SFRS8 regulates the splicing of CD45, a protein which, through alternative splice variants, has an essential role in activating T cells. T cells are involved in the pathogenesis of atopic diseases such as asthma, so SFRS8 is a very interesting candidate gene in the region.

Conclusions: Linkage and simulation studies show that the very distal part of chromosome 12q contains a gene that increases the susceptibility to asthma. SFRS8 could act as a weak predisposing gene for asthma in our sample.

Bronchial asthma [MIM 600807] is the most common chronic disease affecting children and young adults. The high prevalence in the population and assumed heterogeneity make it difficult to characterise the mode of inheritance and the genes involved. Furthermore, environmental factors contribute to the manifestation of asthma, and the disease is most likely caused by interactions between multiple genes and various environmental exposures.

Several association and linkage studies of asthma and atopy have suggested a number of candidate genes and chromosomal regions. To date, positional cloning has one of the most reproduced regions showing evidence for linkage to asthma. Even though none of these reports has reached genome-wide significance, accumulating evidence suggests that chromosome 12q may harbour one or more susceptibility genes for asthma and atopy.

There seems to be more than one region on 12q with evidence of linkage to asthma and atopy. Most groups have focused on regions around the interferon-γ (IFNG) gene 12q13–q15 or distal to that region around 12q21–q24,12–14 where a number of potential candidate genes for asthma and atopy map. These include the nitric oxide synthase gene (NOS1) and the signal transducer and activator of transcription 6 gene (STAT-6).21 Results from the various studies have been conflicting with regard to both significance and phenotypes.

Three studies have identified linkage to asthma even more distal on chromosome 12q (12q24.31–q24.33; table 1). These include a study by our group, an English group, and a Japanese group.16–20 No genes have yet been investigated for an association with asthma and atopy in this region.

We chose a two step procedure using two Danish samples: (1) a genome-wide scan in one set of families followed by (2) fine scale mapping in a larger independent set of families in candidate regions with a maximum likelihood score (MLS) of ≥1.5 in the genome-wide scan. The results of the genome-wide scan have been published previously and suggest 12q24.21–q24.33 as a susceptibility locus for asthma.21

We also screened all coding single nucleotide polymorphisms (SNPs) in a functional gene (splicing factor, arginine/serine-rich 8 (SFRS8) [MIM 601945]) from the region showing linkage in our study (confidence interval maximum MLS-1) and carried out association studies on the SNPs which were polymorphic, as well as testing five SNPs (intronic and upstream) for an association with asthma. SFRS8 regulates the splicing of CD45, also known as leucocyte common antigen [MIM 151460].22 23 CD45 exists in different isoforms depending on alternative splicing of exons 4, 5, and 6. The glycoprotein is an essential molecule for activating T cells24 25 by mediating cell-to-cell contacts and regulating protein tyrosine kinases involved in signal transduction. The activation of T cells is dependent on the different CD45 splice variants,24 25 which are partly regulated by SFRS8. Mice and humans lacking CD45 expression are characterised by a block in T cell maturation.24 26 As T cells play a key role in the induction, regulation, and maintenance of the allergic inflammatory response,26 their activation could potentially be important in the development of atopic disease.
SFRS8, a possible asthma susceptibility gene

were ≥ class 1 for at least one of the specific allergens tested (same as sample 1 except for olive and *Paratetaria*) (CAP Trialtip; Pharmacia & Upjohn, Copenhagen, Denmark).

The study was approved by the Ethics Committee, Denmark, and informed written consent was obtained from all families before participation.

### Genotyping of microsatellite markers

Genomic DNA was prepared from whole blood by standard methods. The following microsatellite markers were genotyped in both samples: D12S2070, D12S2082, D12S1639, D12S367, D12S1045, D12S63, D12S392 and D12S357 to fine map a region on chromosome 12q24 that was indicated in a genome-wide scan using sample 1 only. The mean distance between the markers in the candidate region (the last six markers) was 2.1 cM (http://research.marshfieldclinic.org/genetics/). Primer sequences were obtained from the Genome Database (www.gdb.org). The amplified products were separated on a 4% polyacrylamide gel using an ABI PRISM 377 automated DNA sequencer and analysed using ABI software (Applied Biosystems, Stockholm, Sweden).

### Genotyping of SNPs

All SNPs were genotyped using the TaqMan method on ABI PRISM 7700. Primers and probes were designed using Primer Express software (Applied Biosystems); the sequences are shown in table 2. Primers were ordered from Invitrogen and probes from MWG and Applied Biosystems. Three SNPs (rs930863, rs1107871, rs755437) of SFRS8 were genotyped using Assays-on-Demand (Applied Biosystems) and primer/probe sequences are therefore not available.

### Statistical analyses

#### Linkage analysis

Data from the affected sib pair families were analysed by non-parametric multipoint linkage analysis using the MLS approach. The computer program MAPMAKER/SIBS was used for calculation of multipoint maximum likelihood identity by descent (IBD), allowing for dominance variance. An increment step setting of five facilitated computing of MLS scores between each marker was used. If a family consisted of more than one affected sib pair, only independent pairs were used for calculations. Values for locus specific sibling relative risk ratio (λs) for the susceptibility locus at peak MLS were calculated from the z0 parameter (λs = 0.25/z0).

Simulation to determine the empirical significance of the MLS values obtained in this study was performed using Allegro 1.1. One thousand replicates of the entire autosomal genome were generated assuming no linkage using pedigrees from sample 1 and a marker distance of 5.8 cM to mimic the original genome-wide scan. MAPMAKER/SIBS was used to analyse each of the replicates and regions with MLS values of ≥ 1.5 were selected. Genotypes were then simulated in samples 1 and 2 at the selected regions but with a dense marker set (marker distance 2.9 cM); in sample 1 conditional on the original genotype distribution leading to the MLS ≥ 1.5. MLS values were calculated for all the replicates and the number of replicates with MLS ≥ 3.27 was counted to obtain a genome-wide empirical p value.

#### Association analysis

Transmission disequilibrium tests (TDTs) of polymorphisms and haplotypes in SFRS8 and power estimations of the associations were performed using the FBAT and PBAT (Family Based Association Test) programs. The TDT method makes statistical inferences by examining the conditional distribution of a score statistic, S, constructed to summarise the genotype and marker values in the affected

### METHODS

#### Participants

The combined sample consisted of 167 Danish families with asthma from two different asthma studies.

#### Sample 1

Sample 1 included 23 affected sib pairs and eight families with three affected children, a total of 39 independent sib pairs. For association studies, a further 13 families with one affected child were included. All probands were children suffering from allergic asthma. Allergic asthma was defined by clinical symptoms, the effect of standard asthma medication, and a positive specific immunoglobulin E (IgE) test. Clinical asthma was diagnosed with one affected child were included. All probands were

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<th>Marker</th>
<th>Position Mb*</th>
<th>cM†</th>
<th>Cytogenetic*</th>
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<td>D12S357*</td>
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*Position in megabases (Mb) and on cytogenetic map, UCSC (http://genome.ucsc.edu), May 2004 assembly.
†Position in centimorgan (cM) sex averaged, Marshfield Clinic (http://research.marshfieldclinic.org/genetics).
*Not tested in this study but markers suggest linkage in the study by Wilkinson et al. 19
†Markers suggesting linkage in studies by Wilkinson et al 18 and Shao et al. 19
siblings. Most importantly, the method offers calculation of the empirical variance for the test statistics in the case of presence of linkage to account for sibling genotype correlation for families with more than one child,34 which is the case for most of our data.

RESULTS

Linkage studies

A genome-wide scan of sample 1 suggested chromosome 12q24.21–q24.33 to be a candidate region for asthma in the Danish population.20 We therefore genotyped both samples 1 and 2 (total of 167 pedigrees) in this region, using additional markers. Figure 1 shows the results from multipoint analyses of the two samples combined and for samples 1 and 2 individually using MAPMAKER/SIBS. Four markers had an MLS value of >2.5; the highest MLS value of 3.27 occurred between markers D12S63 and D12S392 when the two combined samples were analysed. Linkage analyses of sample 1 alone resulted in an MLS of 2.15 at marker D12S392 and the 136 affected sib pair families in sample 2 had an MLS of 1.71 at D12S1045 supported by nearby markers.

At the location of the maximum MLS, the IBD sharing between affected siblings was 62% for the combined sample, compared with the null hypothesis of 50%. The information content in the point showing maximum MLS was 90% (95% information for the flanking markers). The locus specific sibling relative risk ratio (lsr) for the susceptibility locus at peak MLS was 1.9 in the combined sample. The results of the simulation studies showed that the probability of obtaining an MLS value of >3.27 by fine mapping the peak regions from the first genome scan was 0.018, given the pedigree structure of both data sets and assuming no linkage. The IBD sharing (61%) was maintained in the candidate region if samples 1 and 2 combined were subclassified into groups of sib pairs with a positive specific IgE test in addition to asthma (data not shown).

Association studies

Association analysis was performed on the microsatellite genotype data and resulted in a minimum p value of 0.009 (D12S1659). However, this result was not significant after correction for multiple testing of eight markers with an average of 10 alleles each.

Variations in SFRS8—a positional and functional candidate gene—were chosen for the association studies. An additional 13 families with one affected child from sample 1 and 24 families with one asthmatic child from sample 2 were included in the association studies. Association with asthma was tested in a family based TDT design, using all sibs from the families, and showed that three SNPs (rs1051219, rs1051233, rs755437) in SFRS8 (fig 2) were significantly associated with asthma (table 3). The strongest association

### Table 2 Primer and probe sequences for SNPs tested in SFRS8

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<th>Gene</th>
<th>SNP</th>
<th>SNP function</th>
<th>Primer</th>
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*Underlined nucleotides in the sequence of probes used for allele discrimination on real-time PCR represent the polymorphic site.
†MGB, Minor Groove Binder (Applied Biosystems).
‡SNP tested by Assays on Demand (Applied Biosystems) so the exact primer/probe sequence is only known by the company.
*SNPs are exonic SNPs in the long transcript of SFRS8 (NM_004592).

Figure 1 Linkage results from multipoint analyses for asthma on chromosome 12q. The grey bars illustrate the approximate location (due to limited availability of data) of linkage reported by Wilkinson et al20 and Shao et al.34 The position of the linkage signals from these two studies were adjusted for marker position according to the genetic map from Marshfield to be comparable to the linkage result in the Danish population. MLS, maximum likelihood score.

Figure 2 Gene structure of SFRS8 indicating the location of all SNPs included in the association study.
was found with the only intronic SNP of the three—rs755437 (p < 0.002)—in a recessive model in sample 2, which means that more affected offspring were homozygous for allele C than was expected by chance. The association with asthma for this SNP remained (p = 0.0079) when calculated using the empirical variance (that is, correcting for linkage). Allele C of SNP rs755437 also showed significant association in an additive model (p = 0.0068, corrected p = 0.018). This association for allele C of rs755437 was also seen when the two samples were combined (p < 0.028). In the recessive model (for the combined sample) 88 homozygous probands for allele C were observed compared with 73 expected by chance (p = 0.028, table 3). A weak association with asthma was also seen for the SNPs rs1051219 and rs1051233, both of which are located in exons (p = 0.043 and p = 0.018, respectively) when testing the full sample.

The software PBAT estimates the conditional power of the results in the association study for every SNP. All significant associations in our study have a p value of ≥0.08 except the association of genotype CC for SNP rs1051219 in sample 2 only (PBAT power 0.05). The remaining SNPs tested in SFRS8 failed to show an association with asthma in our sample (table 3).

A p value of 0.0068 (corrected for linkage 0.013) was obtained when the three SNPs with significant associations were combined into haplotypes and tested for an association with asthma in sample 2 for the haplotype t-c-t. Significantly fewer t-c-t haplotypes were transmitted to asthmatics than was expected by chance. The opposite haplotype (c-g-c) was also associated with asthma, despite the very few informative families (H5, table 4). A significant association for haplotypes 2 and 5 was also seen in the combined sample. Analysing the association of the haplotype in a phenotypic subgroup of sample 2 (patients with both asthma and increased specific IgE) resulted in a slightly more significant association, despite the fact that fewer families were included (p < 0.0019, corrected p value 0.0040, fewer haplotypes transmitted compared with the null hypothesis; data not shown).

In addition to the seven SNPs, four additional SNPs in SFRS8 were genotyped in our study—rs1051314, rs1982528, rs1051207 (all giving rise to a non-synonymous codon change) and rs3759110 (upstream from gene). None of the four additional SNPs was found to be polymorphic in our sample and this was later confirmed by the SNP databases for rs1051314 and rs1051207.

**DISCUSSION**

In this study we investigated an asthma susceptibility locus at chromosome 12q24.32–q24.33 in the Danish population. Evidence for linkage in this region was obtained from a genome-wide scan of part of the samples and fine scale mapping was carried out with a denser set of markers in the full set of families including 136 additional sib pair families. A gene from the region was investigated for association by SNP genotyping using a total of 787 individuals including 379 asthmatics. Multi-point linkage analysis using MAPMAKER/SIBS on the whole data set yielded an MLS score of 3.27 between markers D12S63 and D12S392, which shows that the distal region on 12q harbours a risk gene for the development of asthma.

Several groups have obtained suggestive evidence for linkage between 12q and asthma and atopy, but most of these groups have focused on the central part of the long arm on chromosome 12 and have not investigated the most distal part of the chromosome in detail. Only two other groups reported suggestive evidence for linkage to 12q24.33. Shao and co-workers suggested possible linkage to asthma using 19 families in the same region as in our study (table 1). Wilkinson et al also reported linkage at 12q24.33 at marker
D12S97 using an asthma score.\textsuperscript{14} D12S97 is located in the same position as D12S1045 on the genetic map (table 1) used in our study and in the study by Shao et al.,\textsuperscript{19} and had an MLS value of 2.7 in our study. This indicates that our results support the results of these two earlier studies.\textsuperscript{18} Shao et al.\textsuperscript{19} also found evidence to support linkage to the marker D12S97 using an asthma score. D12S97 is located in the interdependent two step procedure, had a significance which is close to the borderline p value of 0.05. However, this conservative way, using the sample and obtained a genome-wide empirical p value of 0.064 for an MLS value of 3.27, which is close to the borderline p value of 0.05. However, this is too conservative as full genome fine mapping of the whole sample was not conducted. Our simulation, which mimics the interdependent two step procedure, had a significance level of p = 0.018 for an MLS value of $\geq 3.27$ by fine mapping peak regions from the first genome-wide scan.

In an attempt to investigate the effect of a narrower phenotype definition, we analysed the sib pairs from sample 2 who had a diagnosis of asthma and a positive specific IgE test. This reduced the total sib pair number to 112 but did not change the IBD sharing, indicating that the susceptibility locus is not specific for atopic asthma.

Several candidate genes for asthma susceptibility on chromosome 12q have been tested for association including IFNG, STAT6 and NOS1 with conflicting results.\textsuperscript{22} The linkage results of the present study point to genes located distal to previously tested genes. SFRS8 maps within the region showing linkage in our study (confidence interval of maximum MLS-1) and was thus a potential candidate gene in the Danish sample. The gene codes for a splice factor which not only regulates its own splicing but also the splicing of CD45,\textsuperscript{22} an important molecule in the activation process of T cells.\textsuperscript{22,23} The activation of T cells depends on different splice variants of CD45.\textsuperscript{22,23} T cells are involved in the pathogenesis of atopic diseases,\textsuperscript{24} but their exact role has still not been fully elucidated.

We tested 11 SNPs in SFRS8, seven of which were polymorphic in our samples and were tested in a family based TDT design. An association with asthma was found for three SNPs—rs1051219, rs1051233 and rs755437. The strongest association was seen for rs755437 where allele C was transmitted to affected offspring significantly more often than expected by chance in the combined sample ($p=0.0068$ and 0.028, respectively). Furthermore, homozygosity for allele C in sample 2 and the combined samples ($p=0.0068$ and 0.028) was unexpectedly high, which is close to the borderline p value of 0.05. However, this is too conservative as full genome fine mapping of the whole sample was not conducted. Our simulation, which mimics the interdependent two step procedure, had a significance level of p = 0.018 for an MLS value of $\geq 3.27$ by fine mapping peak regions from the first genome-wide scan.

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We tested 11 SNPs in SFRS8, seven of which were polymorphic in our samples and were tested in a family based TDT design. An association with asthma was found for three SNPs—rs1051219, rs1051233 and rs755437. The strongest association was seen for rs755437 where allele C was transmitted to affected offspring significantly more often than expected by chance in the additive model for both sample 2 and the combined samples ($p=0.0068$ and 0.028, respectively). Furthermore, homozygosity for allele C in sample 2 and the combined samples ($p=0.0068$ and 0.028) was unexpectedly high, which is close to the borderline p value of 0.05. However, this is too conservative as full genome fine mapping of the whole sample was not conducted. Our simulation, which mimics the interdependent two step procedure, had a significance level of p = 0.018 for an MLS value of $\geq 3.27$ by fine mapping peak regions from the first genome-wide scan.
When evaluating significant association studies, it is important to keep in mind that the literature reports numerous primary studies of significant associations but few associations have been reproduced. Additional studies are therefore necessary to determine whether SFRS8 is a weak susceptibility gene for asthma. However, we cannot rule out the possibility that the observed association could be due to linkage disequilibrium to a nearby gene.

Chromosome 12q24.32–q24.33 contains many other genes that could contribute to the susceptibility to asthma (fig 3), of which matrix metalloprotease 17 (MMP17) would be a plausible candidate. So too are other metalloprotease (ADAM33) has been reported to underlie asthma. Two other genes (PLAX2GB1 and SCARB1) were tested for association with asthma in our population because of their position in the region that earlier showed evidence of linkage to an asthma score. PLAX2GB1 has also shown association with high total IgE levels in another study. The SNPs tested (rs5634, rs5888, rs5892) are located in exons but do not give rise to an amino acid change. We did not find evidence for association for these SNPs.

In summary, both the simulations and the replication of previous positive linkage results in a larger set of subjects suggest that we have identified an asthma susceptibility region for the Danish population distal on chromosome 12q. Association was furthermore found between asthma and SFRS8, a positional and functional candidate gene for atopy and asthma.

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