Original article

Association and interaction analyses of eight genes under asthma linkage peaks

Background: Linkage studies have implicated the 2q33, 9p21, 11q13 and 20q13 regions in the regulation of allergic disease. The aim of this study was to test genetic variants in candidate genes from these regions for association with specific asthma traits.
Methods: Ninety-five single nucleotide polymorphisms (SNP) located in eight genes (*CD28*, *CTLA4*, *ICOS*, *ADAM23*, *ADAMTSL1*, *MS4A2*, *CDH26* and *HRH3*) were genotyped in >5000 individuals from Australian (n = 1162), Dutch (n = 99) and Danish (n = 303) families. Traits tested included doctor-diagnosed asthma, atopy, airway obstruction, total serum immunoglobulin (Ig) E levels and eosinophilia. Association was tested using both multivariate and univariate methods, with gene-wide thresholds for significance determined through simulation. Gene-by-gene and gene-by-environment analyses were also performed.
Results: There was no overall evidence for association with seven of the eight genes tested when considering all genetic variation assayed in each gene. The exception was *MS4A2* on chromosome 11q13, which showed weak evidence for association with IgE (gene-wide $P < 0.05$, rs502581). There were no significant gene-by-gene or gene-by-environment interaction effects after accounting for the number of tests performed.
Conclusions: The individual variants genotyped in the 2q33, 9p21 and 20q13 regions do not explain a large fraction of the variation in the quantitative traits tested or have a major impact on asthma or atopy risk. Our results are consistent with a weak effect of *MS4A2* polymorphisms on the variation of total IgE levels.

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Allergic asthma is a heritable chronic disease that affects >10% of children in developed countries (1), many of whom do not respond to the few established treatments (2). In spite of a large public health burden, our knowledge of the aetiological mechanisms underlying asthma, both genetic and environmental, is still very limited. One of the most promising approaches to further our understanding of the disease mechanisms involved is the identification of genetic variation that contributes to asthma risk.

As demonstrated for other complex diseases (3), genome-wide association studies (GWAS) are the most successful gene mapping approach currently available to identify systematically all pathways at which genetic variation contributes to disease risk. However, given the multiple testing burden associated with GWAS and the

small effect sizes that are predicted for most risk loci, the success of this approach rests on the ability to analyse data for many thousands of samples, for both the initial discovery and replication stages.

While adequately powered datasets are being collected for such large-scale hypothesis-free analyses, smaller, more focused association studies can still provide a valuable framework to investigate specific genetic hypotheses. In this report, we screened eight genes that were selected based on their potential functional relevance from four regions previously reported to be linked to asthma traits. Amongst these were known candidate genes for asthma, namely the *CD28*, cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and inducible T-cell co-stimulator (*ICOS*) genes, but also new genes not previously tested in the literature, including ADAM

metallopeptidase domain 23 (*ADAM23*) and histamine receptor H3 (*HRH3*). We employed novel association methods and tested for gene-by-gene and gene-by-environment interaction effects to asses the influence of genetic variants in these genes with both self-reported and clinical measures of asthma in 5470 individuals.

Material and methods

Selection of genes for analysis

Our aim was to investigate the association between specific asthma traits and genes located in four chromosomal regions identified through previous linkage studies (Table 1). Genes were selected from these regions for single nucleotide polymorphism (SNP) genotyping based on their potential functional relevance and included *CD28*, *CTLA4*, *ICOS* and *ADAM23* on chromosome 2q33; ADAMTS-like 1 (*ADAMTSL1*) on 9p21; membrane-spanning 4-domains, subfamily A, member 2 (*MS4A2*, previously named β chain of the high-affinity IgE receptor, *FCER1B*) on 11q13; and cadherin-like 26 (*CDH26*) and *HRH3* on 20q13.

Samples

We genotyped samples drawn from two studies conducted at the Queensland Institute of Medical Research (QIMR), Australia, a study conducted at the VU University (VU), the Netherlands, and a study conducted at the Bispebjerg Hospital (BH), Denmark. The first QIMR study (AS) included data for 735 families that are part of a larger collection of pedigrees ascertained through an asthma twin proband and described elsewhere (4). A subset of this sample was used in the analysis that reported linkage of multiple asthma traits to 20q13 (5). For the QIMR second study (TM), data were available for 427 families that were recruited as part of an ongoing study of melanocytic naevi (moles). Data from this panel were included in the genome-wide linkage scan that implicated chromosome 2q33 in the regulation of basal eosinophil levels (6). The VU sample consisted of 99 families ascertained as part of an ongoing survey study of health-related behaviour (7), whereas the BH sample included data for 303 families ascertained through an asthma twin proband as described previously (8). The characteristics of the study participants are summarized in Supporting Information Table S1. This study was performed with the ethical approval of all institutions involved and with the written informed consent of all participants.

Phenotypes

The asthma traits measured in the AS, VU and BH studies that are relevant for the present analyses are doctor-diagnosed Asthma,

bronchial hyperresponsiveness (BHR), Atopy, skin prick test to house dust mite (Dpter) and the four continuous traits forced expiratory volume in 1 s (FEV_1), FEV_1 divided by forced vital capacity (FEV_1/FVC), total IgE and eosinophil (AS and VU only) levels. All traits were measured or defined using equivalent protocols in all studies. These are described elsewhere for the AS (4) and BH (8) studies and provided as Supplementary Methods for the VU study. Haematology data, including eosinophil levels, were collected for the TM study when twins were aged 12, 14 and 16 as described previously (6).

For the 2q33 region, the phenotypes selected for analysis were Dpter, FEV_1/FVC, IgE and eosinophil levels, as these were the traits previously reported to be linked (6, 9, 10) or associated (11, 12) to this region. For the TM study, we selected the age 12 eosinophil data as this was the time point at which significant linkage was detected to chromosome 2q33 (6). Similarly, we selected IgE for analysis with 9p21 (M. A. R. Ferreira, D. L. Duffy, N. G. Martin, unpublished data), IgE and Atopy with 11q13 (11, 13, 14), and Dpter, atopy, BHR, FEV_1, asthma and FEV_1/FVC for 20q13 (5).

Continuous traits were normalized and adjusted for significant covariates, which included age, gender, height (FEV_1 only) and regular smoking (FEV_1/FVC). As age showed a strong positive correlation with FEV_1 for individuals aged 6–20 years (younger cohort) and a negative correlation for those aged > 20 years, an interaction term with a cohort indicator was also used to correct for this effect.

SNP genotyping and quality control (QC)

A set of 108 SNPs reported to be highly polymorphic in the NCBI SNP Database and evenly spaced across each of the eight genes was originally selected for genotyping in the AS study using the Mass-ARRAY™ Sequenom system as described previously (15). A subset of these was also genotyped in the TM (n = 39), VU (n = 41) and BH (n = 41) studies. Genotyping was performed in five batches, with QC analyses performed for each batch separately (see Table S2 for details) using PLINK (16) and PEDSTATS (17). Twenty SNPs were genotyped in two or three (n = 1) batches to assess concordance between genotyping events. After individual QC, data from all batches were merged, resulting in a dataset with 5535 individuals genotyped for 95 SNPs. The concordance rate between different different genotyping events was 0.995. After data were merged, we compared genotype calls for self-reported monozygotic (MZ) twin pairs (n = 351, concordance 0.992) and (i) deleted all individuals (n = 65) from families (n = 22) with MZ twins having >1 discordant genotype and (ii) set to missing any discordant genotype for the remaining MZ pairs. We then used MERLIN (18) to detect and delete unlikely genotypes (n = 1227) and to infer missing genotypes using identical by descent (IBD) information (n = 17 755). Excluding 1250 ungenotyped founders for whom MERLIN inferred a small fraction of genotypes (a median of 5.3%), the final dataset consisted of 5470 individuals genotyped for 95 SNPs.

Table 1. Summary of study design

Region	Linkage reference(s)	Genes selected	N SNPs genotyped	Studies included	Phenotypes tested
2q33	6, 9, 10	*CD28, CTLA4, ICOS, ADAM23*	70	AS, VU, BH and TM	Dpter, FEV_1/FVC, IgE, eosinophils
9p21	Unpublished	*ADAMTSL1*	6	AS	IgE
11q13	13, 14	*MS4A2*	6	AS	Atopy, IgE
20q13	5	*CDH26, HRH3*	13	AS	Dpter, Atopy, BHR, FEV_1, Asthma, FEV_1/FVC

AS, QIMR asthma study; VU, VU University study; BH, Bispebjerg Hospital study; TM, QIMR twin moles study; Dpter, skin prick test to house dust mite; FEV_1/FVC, forced expiratory volume in 1 s (FEV_1) divided by forced vital capacity (FVC).

Main association analyses

We tested the following four hypotheses: (i) variants in the selected 2q33 genes are associated with Dpter response, FEV_1/FVC, total serum IgE or eosinophil levels; (ii) variants in *ADAMTSL1* (9p21) are associated with IgE levels; (iii) variants in *MS4A2* (11q13) are associated with Atopy or IgE levels; and (iv) variants in *CDH26* and *HRH3* genes (20q13) are associated with Dpter response, atopy, BHR, FEV_1, asthma or FEV_1/FVC. Hypothesis (i) was tested in the AS, VU, BH and TM (eosinophils) studies, whereas hypotheses (ii), (iii) and (iv) were tested in the AS study only.

For hypotheses (i), (iii) and (iv), the association between individual SNPs and all traits was tested using a recently described multivariate test of association implemented in PLINK (19). For these analyses, phenotype data for one twin from MZ pairs was discarded (that with milder asthma symptoms). Association between eosinophil levels and 2q33 SNPs in the TM study was tested with MERLIN while accounting for IBD allele sharing between relatives (20) and linkage disequilibrium (LD) between markers (21) because of the strong linkage signal present in this dataset (logarithm of odds, LOD > 3). The same approach was used to test hypothesis (ii).

Gene-wide significance thresholds and power for the main analyses

We adopted the concept of the gene as the unit of association (22). To assess the overall evidence for association with a particular gene given the genetic variation assayed, we used MERLIN to simulate 1000 datasets under the null hypothesis of no linkage or association, while preserving the marker informativeness, spacing, missing data patterns and LD between markers of the original dataset. Each dataset was analysed as described above and the most significant *P*-value of all SNPs tested in each gene and analysis was retained. The threshold for gene-wide significance (i.e. gene-wide $P = 0.05$) corresponded to the 95th percentile of the 1000 sorted *P*-values retained for each gene and analysis. Thresholds ranged between 0.002 and 0.028.

To estimate the power of the proposed analyses, we simulated 1000 datasets consisting of an SNP marker and an associated quantitative trait (40% heritable) with MERLIN, while preserving the missing data patterns of the original data. Based on these analyses, power ($\alpha = 0.001$) to detect univariate association with the AS (or AS + VU + BH) study was 0.034 (0.055) for a quantitative trait locus (QTL) explaining 0.1% of the trait variance, 0.500 (0.752) for a 0.5% QTL, 0.938 (0.981) for a 1% QTL and 1.00 (1.00) for a 2% QTL. Power for the TM study and the same QTL variances was respectively 0.01, 0.05, 0.18 and 0.55.

Secondary analyses

We performed gene-by-gene and gene-by-environment interaction analyses to address two additional hypotheses. The first considered the possibility that under specific genetic models with strong epistatic effects and weak or no main effects, risk loci could be detected through interaction but not association analyses (23, 24). Second, that an association between the eight genes tested and atopy might only be detected when considering individuals exposed to certain environmental factors.

To investigate whether strong gene-by-gene effects could be detected, we focused on the Atopy trait, as this was the general clinical phenotype reported to be linked with all four regions tested. We selected a subset of 350 unrelated cases and 184 unrelated controls from the AS study and tested pairwise SNP interactions

between the eight genes using logistic regression as implemented in PLINK. A total of 3733 pair-wise SNP interactions were tested. Given the sample size and number of tests performed, power was only adequate to detect strong interaction effects. For example, assuming a disease prevalence of 30%, a genotype relative risk of 0.6 for each of the four possible interaction parameters (additive × additive, additive × dominant, dominant × additive, dominant × dominant), no main effects and a minor allele frequency of 0.25 for both loci, power to detect epistasis was 0.80 (with $\alpha = 0.05/3733 = 1.3 \times 10^{-5}$), while power to detect association to either loci was 0.18 ($\alpha = 0.05/95 = 5.3 \times 10^{-4}$).

To investigate the presence of gene-by-environment effects, we specifically tested whether SNPs were associated with atopy status in individuals: (i) exclusively breastfed under the age of six months; (ii) exposed to parental smoking under the age of 2 years; (iii) with frequent chest infections under the age of two and (iv) who lived in a carpeted house under the age of two. After appropriately defining cases (e.g. breastfed atopics) and controls (e.g. breastfed nonatopics) within each family, association was tested using the DFAM test implemented in PLINK, with an adaptive permutation procedure used to assess significance.

Results

Previous linkage and association studies implicated the 2q33 region in the regulation of Dpter response, FEV_1/FVC, IgE and eosinophil levels. We tested whether variants in the 2q33 genes *CD28*, *CTLA4*, *ICOS* or *ADAM23* were associated with any of these four phenotypes in the combined data from the AS, VU and BH studies using a multivariate approach. The 70 SNPs genotyped tagged ($r^2 > 0.8$) between 44% and 79% of the common [minor allele frequency (MAF) > 0.05] variation reported for these genes in the HapMap CEPH population. No single SNP exceeded the respective threshold for gene-wide significance (Table 2). The most associated SNP was rs3181096 (multivariate $P = 0.028$), which is located in the *CD28* promoter region. The main phenotype associated with rs3181096 was FEV_1/FVC (Table S3). A subset of 30 SNPs was also tested for association with eosinophil levels in the TM study. This SNP set provided a similar coverage for the *CD28/CTLA4/ICOS* cluster (40–62%), but considerable less for *ADAM23* (7%). In spite of evidence for linkage to this region (best LOD = 3.77, $P = 1.5 \times 10^{-5}$, rs3732079), no single SNP was associated with eosinophilia at a gene-wide significant level (Table S4). Of note, the most associated SNP was rs3181098 (univariate $P = 0.02$), which is in near complete LD ($r^2 = 0.99$) with rs3181096 ($P = 0.034$).

Next, we investigated whether any of the six SNPs genotyped in *ADAMTSL1* (13% coverage) on chromosome 9p21 were associated with total serum IgE levels in the AS study. This region and gene were selected based on previous linkage results for a subset of samples from this study. There was some evidence for linkage (best LOD = 0.85, $P = 0.024$, rs1889007) but not association (best $P = 0.103$) with the markers tested (Table S5).

Table 2. Summary of multivariate association results between four genes in the 2q33 region and four asthma traits (Dpter, FEV$_1$/FVC, total IgE and eosinophils), in the combined analysis of the AS, VU and BH studies

	CD28	CTLA4	ICOS	ADAM23
Start position, kb*	204, 239	204, 401	204, 470	206, 977
Length target sequence, kb	111	86	105	254
SNP set				
N HapMap†	68	81	93	123
N tested (in HapMap)	15 (13)	9 (9)	19 (14)	27 (26)
Coverage‡	0.79	0.62	0.48	0.44
Gene-wide significance threshold§	0.004	0.006	0.003	0.003
N with multivariate P < 0.05	2	0	0	0
Top association result				
SNP	rs3181096	rs3087243	rs933988	rs3755224
Multivariate P-value	0.028	0.054	0.070	0.057
Associated trait(s)	FEV$_1$/FVC	–	–	–

SNP, single nucleotide polymorphism.

*Start position and length are based on the b36 coordinates for the first and last transcribed bases ±40 kb.

†Number of described common (minor allele frequency >0.05) SNPs located within the target region in CEU HapMap population. Also includes SNPs genotyped in this study that are not in the HapMap.

‡Proportion of CEU HapMap SNPs captured with $r^2 \geq 0.8$ by the SNPs tested.

§Estimated from 1000 simulated datasets using MERLIN. See *Methods* for details.

Chromosome 11q13 and the *MS4A2* gene in particular were amongst the first loci implicated by linkage studies in the regulation of IgE levels and the atopic response. We applied the same multivariate approach to test the association between *MS4A2* SNPs and variation in IgE levels or Atopy in the AS study. Of the six SNPs tested in this gene (42% coverage), five were significant at the $P < 0.05$ level (all in strong LD, $r^2 > 0.8$), with the most associated SNP (rs502581, multivariate $P = 0.010$) just reaching gene-wide significance ($P < 0.012$). The observed multivariate association signal to rs502581 was driven by variation in total serum IgE levels but not atopy status (Table S6), a result that we confirmed using standard univariate analysis ($P = 0.015$ and $P = 0.857$, respectively). This SNP explained approximately 0.4% of the phenotypic variation of IgE in the AS study. There were no significant maternal effects at this locus for either trait (not shown).

Finally, following our earlier report of linkage between chromosome 20q13 and Dpter, Atopy, BHR, FEV$_1$, Asthma and FEV$_1$/FVC, we tested these six phenotypes for association with 13 SNPs genotyped in the *CDH26* (13% coverage) and *HRH3* (19%) genes in the AS study. There was no overall evidence for association with either gene after accounting for the number of SNPs tested (Table S7). The most associated SNP was rs6062144 (multivariate $P = 0.036$), located approximately 8 kb upstream of *HRH3*.

Given the largely negative results with most genes tested, we investigated whether strong gene-by-gene or gene-by-environment effects could be detected in the AS study. We focused on the Atopy trait, as this was the general clinical phenotype reported to be linked with the four regions tested. No significant pairwise interactions were detected between the eight genes, after accounting for the number of tests performed (Table S8). Similarly, no overall significant associations were detected with any gene after considering breastfeeding under six months and smoking exposure, the occurrence of chest infections or living in a carpeted house under the age of 2 years (not shown).

Discussion

Functional (25–27) and linkage studies (6, 9, 10) pointed towards a potential role of genetic variation in the *CD28/CTLA4/ICOS* gene cluster on the development of allergic disorders and many association studies have followed-up on this hypothesis (Table S9). However, most of these focused on a small set of variants that were tested in relatively underpowered samples. In fact, the only reported associations that would remain significant after a strict adjustment for multiple testing were between two independent variants ($r^2 < 0.1$) in *CTLA4* (rs5742909 and rs231775) and IgE levels (11, 12) and between a variant in *ICOS* (rs11883722) and cockroach skin prick response (28). In our analyses, there was no evidence for association between rs5742909 or rs11883722 and the four asthma traits tested (Dpter, FEV$_1$/FVC, IgE and eosinophils). Similarly, there was no association with a variant (rs231779) that is in complete LD with rs231775.

In addition to these negative results to specific variants previously reported in the literature, there was no overall evidence for association with seven of the eight genes tested and the respective phenotypes when we considered all genetic variation assayed in each gene. The only exception was for the gene encoding the β chain of the high-affinity IgE receptor (*MS4A2*) on chromosome 11q13, which showed weak evidence for association with total serum IgE at the gene-wide level. Although the observed association would not remain significant at the experiment-wide level (given the number of genes tested), it is consistent with the hypothesis that genetic variation in this gene may have an effect on the variation of IgE levels, as suggested by others (11, 14).

Given the power provided by the sample size used and analyses performed, we conclude that the individual genetic variation assayed in the 2q33, 9p21 and 20q13 regions does not explain a large fraction (i.e. > 1%) of the variation in the quantitative traits tested or have a major impact on asthma or atopy risk. However, given the caveats of the present study, namely, the small number of genes tested per region, the low SNP coverage provided for some genes, the low power to detect weaker but more realistic effect sizes and the limited number of environmental risk factors considered, the possibility remains that risk variants in these regions may only be identified using larger studies with improved power. Alternatively, it is possible that some or all of these regions represented false-positive results from previous linkage-analyses. In

this respect, combining data from published candidate-gene studies and emerging GWAS will provide ample opportunities to address some of these limitations.

Acknowledgments

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Supplementary Methods.
Table S1. Characteristics of the study participants for the four individual studies.

Table S2. Details of the QC filters applied to the SNP data.
Table S3. Multivariate association results between four asthma traits and *CD28*, *CTLA4* 119 and *ICOS* SNPs on chromosome 2q33, for the combined analysis of the AS, VU and BH studies.
Table S4. Univariate association results for the TM study between eosinophil levels and 128 *CD28*, *CTLA4* and *ICOS* SNPs on chromosome 2q33.
Table S5. Univariate association results for the AS study between IgE levels and 131 *ADAMTSL1* SNPs on chromosome 9p21.
Table S6. Multivariate association results for the AS study between *MS4A2* SNPs and 134 atopy and total IgE levels.
Table S7. Multivariate association results for the AS study between 20q13 SNPs and six 142 asthma traits.
Table S8. Gene-gene interaction results for atopy in the AS study between all eight genes 152 tested.
Table S9. Summary of main results from published 'candidate-gene' gene association studies of asthma traits and *CD28*, *CTLA4* and *ICOS* polymorphisms.
Supplementary References.

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