

ORIGINAL ARTICLE

Sex-specific effect of IL9 polymorphisms on lung function and polysensitization

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Sex differences in asthma-associated phenotypes are well known but the genetic factors that may account for these differences have received little attention. This study aimed to characterize sex-specific and pleiotropic genetic factors underlying four quantitative phenotypes involved in the main asthma physiopathological pathways: immunoglobulin E levels, a measure of polysensitization (SPTQ), eosinophil counts and a measure of lung function FEV₁/H₂ (forced expiratory volume in one second divided by height square). Sex-stratified univariate and bivariate linkage analyses were conducted in 295 families from the Epidemiological study on the Genetics and Environment of Asthma study. We found genome-wide significant evidence for a male-specific pleiotropic QTL (quantitative trait loci) on 5q31 ($P = 7 \times 10^{-9}$) influencing both FEV₁/H₂ and SPTQ and for a female-specific pleiotropic QTL on 11q23 underlying SPTQ and immunoglobulin E ($P = 2 \times 10^{-5}$). Three other sex-specific regions of linkage were detected for eosinophil: 4q24 and 22q13 in females, and 3p25 in males. Further, bivariate association analysis of FEV₁/H₂ and SPTQ with 5q31 candidate genes in males showed a significant association with two single-nucleotide polymorphisms within IL9 gene, rs2069885 and rs2069882 ($P = 0.02$ and $P = 0.002$, respectively, after Bonferroni's correction). This study underlies the importance of taking into account complex mechanisms, such as heterogeneity according to sex and pleiotropy to unravel the genes involved in asthma phenotypes.

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Introduction

In model organisms, many quantitative traits show sex-specific genetic architecture and the same is likely to be true for complex diseases in humans.¹ Asthma is a complex and heterogeneous disease that is associated with various quantitative phenotypes related to atopy, inflammation and lung function and displays sex differences that vary over the life span.² Asthma prevalence is higher in boys than in girls in childhood but this reverses around puberty, with higher prevalence in adult women than in men, and it is again higher in men than in women after menopause.³ Asthma and atopy-related phenotypes also show sexual dimorphism in humans. These sex differences may result from many factors including genetic, developmental, immunological, hormonal, cultural and environmental factors, but little is known about the actual role of these factors.^{3,4}

Despite the numerous genetic studies conducted to date to identify the genetic determinants of asthma and asthma-related phenotypes,⁵ only a few of them have been stratified by sex. Sex-specific effects of a few candidate genes associated with asthma and atopy phenotypes have been reported recently.⁴ Among the many genome-wide (GW) linkage scans published to date, only two scans conducted in isolated populations (the Hutterites and a genetic isolate from Costa Rica), have searched for sex-specific genetic factors.^{1,6–8} In the Hutterites, sex-specific linkages were found for immunoglobulin E (IgE) levels, lymphocyte counts and measures of lung function.^{1,7} In Costa Rican families sex-specific linkages and associations were reported for total IgE and specific IgE to cockroach.^{6,8}

Although taking into account factors such as sex that modify gene expression may lead to the detection of new genetic factors; multivariate linkage analysis of complex traits can also increase power to detect genes with small effects.⁹ This is of particular relevance for asthma-related phenotypes for which a given chromosomal region is often linked to several phenotypes, suggesting the presence of genes with pleiotropic effects.¹⁰

Following our previous GW scan conducted for asthma-related phenotypes in the Epidemiological study on the Genetics and Environment of Asthma (EGEA)

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¹⁰Details of the EGEA cooperative group are given in the appendix. Received 18 December 2008; revised 3 April 2009; accepted 12 May 2009

study,¹¹ the aim of this study was to characterize sex-specific and potentially pleiotropic genetic factors underlying four quantitative phenotypes involved in the main asthma physiopathological pathways: total serum IgE levels, a quantitative measure of allergen sensitization, eosinophil (EOS) counts and a measure of lung function. We conducted sex-stratified univariate GW linkage analyses for each of these phenotypes followed by bivariate analyses. These analyses were followed by association analyses with candidate genes belonging to the 5q31 region that showed the strongest GW significant linkage signal.

Results

Sample characteristics

The total sample contained 727 genotyped siblings of whom 386 were male and 341 were female siblings. Descriptive characteristics of these sibs are presented in Table 1. The mean age at examination was higher in females (16.9 ± 9 years) than in males (15.4 ± 7.4 years) ($P = 0.004$). There was a higher proportion of asthmatics among males (58%) than among females (46%) ($P = 0.003$). Males had, on average, significantly higher

values of atopy-related phenotypes and lower values of lung functions (Table 1).

Genome-wide univariate linkage analyses and test for heterogeneity

Results of sex-stratified linkage analyses of the four asthma-related phenotypes (FEV_1/H^2 , SPTQ, EOS and IgE) are shown in Table 2. We found GW suggestive evidence ($P \leq 0.0007$)¹² for seven linkage signals belonging to five chromosomal regions. The two male-specific regions were 3p25 for EOS ($LOD = 2.65$, $P = 0.0002$) and 5q31 for both FEV_1/H^2 (forced expiratory volume in one second divided by height square) ($LOD = 2.87$, $P = 0.0001$) and SPTQ ($LOD = 4.58$, $P = 2 \times 10^{-6}$). The three female-specific regions included 4q24 ($LOD = 2.59$, $P = 0.0003$) and 22q13 ($LOD = 2.22$, $P = 0.0007$), each linked to EOS, and 11q23 linked to both SPTQ ($LOD = 2.86$, $P = 0.0001$) and IgE ($LOD = 2.98$, $P = 0.0001$). Two of these five regions met GW significance, as computed by simulations: 5q31 for both FEV_1/H^2 and SPTQ in males ($P_{GW} = 0.04$ and $P_{GW} = 0.01$, respectively) and 11q23 for both SPTQ and IgE in females ($P_{GW} = 0.04$ and $P_{GW} = 0.03$, respectively). None of these sex-specific regions were significant in the whole sample (results not shown).

Tests for linkage heterogeneity according to sex showed that the two GW significant sex-specific linkage regions also reached GW significant evidence for heterogeneity: 5q31 for SPTQ ($P_{GW \text{ heterogeneity}} = 0.001$) and 11q23 for IgE ($P_{GW \text{ heterogeneity}} = 0.04$). Linkage heterogeneity at the GW level of 10% was also obtained for FEV_1/H^2 in 5q31 ($P_{GW \text{ heterogeneity}} = 0.08$) and SPTQ in 11q23 ($P_{GW \text{ heterogeneity}} = 0.08$), as well as for EOS on 3p25 ($P_{GW \text{ heterogeneity}} = 0.1$).

Bivariate linkage analysis

We conducted sex-stratified bivariate linkage analysis in the 5q31 and 11q31 regions, which were each found linked to two asthma-related phenotypes. Bivariate linkage analysis (Table 3) led to a substantial improvement of the male-specific linkage signal in 5q31 ($MLB_{comb} = 35.23$, $P = 7 \times 10^{-9}$) at the same marker position (D5S2115) as the univariate analyses. However, in the 11q23 region, the increase in evidence for the bivariate female-specific linkage was more modest ($MLB_{comb} = 19.19$, $P = 2 \times 10^{-5}$).

Table 1 Descriptive characteristics of offspring in male and female sibs

	Females (n = 341)	Males (n = 386)	P-value
Age at examination, mean (s.d.)	16.9 (7.9)	15.4 (7.4)	0.004
Asthma, n (%)	158 (46.3)	223 (57.8)	0.003
Age at onset (s.d.)	7.3 (5.8)	5.9 (5.4)	0.02
SPTQ, n (%)			
0	138 (41.1)	96 (25.5)	<0.001
1	66 (19.6)	94 (24.9)	
2	53 (15.8)	69 (18.3)	
3	34 (10.1)	58 (15.4)	
≥4	45 (13.4)	60 (15.9)	
IgE, mean (s.d.)	2.07 (0.75)	2.29 (0.68)	0.0002
EOS, mean (s.d.)	2.33 (0.38)	2.47 (0.36)	<0.0001
FEV_1/H^2 , mean (s.d.)	1.18 (0.24)	1.12 (0.17)	0.0004

Table 2 P-values from univariate sex-stratified multipoint linkage analysis of asthma-related phenotypes

Region	Marker	Location ^a	FEV_1/H^2		SPTQ		EOS		IgE	
			Female	Male	Female	Male	Female	Male	Female	Male
3p25	D3S1304	22.3	—	0.03	—	—	—	0.0002	—	0.003
4q24	D4S402	124.4	—	—	0.002	—	0.0003	—	0.02	—
5q31	D5S2115	138.6	—	0.0001	—	2×10^{-6}	0.09	0.05	—	0.06
11q23.3	D11S925	118.4	0.001	—	0.0001	—	0.01	—	0.0001*	—
22q13	D22S423	46.4	0.05	—	—	—	0.0007	—	0.08	—

Abbreviations: FEV_1/H^2 , forced expiratory volume in one second divided by height square; SPTQ, sum of positive skin test responses to 11 allergens; EOS, blood eosinophil counts; IgE, total serum immunoglobulin E levels.

^acM from pter position of linkage peak based on Marshfield map.

Results reaching the 5% genome-wide level of significance are shown in bold. The asterisk means that test for heterogeneity of linkage according to sex reached genome-wide significance level.

P-values > 0.1 are indicated with a dash sign.

Table 3 Results of univariate and bivariate sex-specific linkage analyses in 5q31 and 11q23 regions

Region	Sample	Marker	Location ^a	FEV ₁ /H ²		SPTQ		Bivariate analysis	
				LOD	P-value	LOD	P-value	MLB _{comb}	P-value
5q31	Male	D5S2027	111.2	—	—	0.80	0.03	—	—
		D5S471	119.1	—	—	2.04	0.001	7.50	0.009
		D5S2115	134.7	2.87	0.0001	4.58	2 × 10 ⁻⁶	35.23	7 × 10 ⁻⁹
		D5S436	145.2	—	—	2.04	0.001	10.42	0.002
		D5S410	152.8	—	—	0.97	0.02	6.49	0.02
		D5S422	162.1	—	—	—	—	—	—
11q23	Female			IgE		SPTQ		Bivariate analysis	
				LOD	P-value	LOD	P-value	MLB _{comb}	P-value
		D11S898	100.6	0.62	0.04	0.91	0.02	4.74	0.04
		D11S908	114.8	0.75	0.03	0.77	0.03	5.65	0.02
		D11S925	120.3	2.98	0.0001	2.86	0.0001	19.19	2 × 10 ⁻⁵
		D11S4151	125.8	1.26	0.008	0.68	0.04	5.97	0.02

Abbreviations: FEV₁/H², forced expiratory volume in one second divided by height square; IgE, total serum immunoglobulin E levels; SPTQ, sum of positive skin test responses to 11 allergens; MLB_{comb}, linkage test statistic constructed by summing the univariate statistics (MLB likelihood-ratio statistics) obtained with each of the two principal components computed from either FEV₁/H² and SPTQ (5q31) or IgE and SPTQ (11q23); LOD is equal to $\lambda_{\text{MLB}}/[2\text{Ln}(10)]$.

^acM from pter position of linkage peak based on Marshfield map.

Results with P-values >0.05 are indicated with a dash sign.

but was also reached at the same marker (D11S925) as the univariate linkage peak. These results provide significant evidence for a pleiotropic QTL (quantitative trait loci) influencing both SPTQ and FEV₁/H² in males, and suggestive evidence for such QTL underlying SPTQ and IgE in females.

Positional candidate gene association studies

We further explored the most significant 5q31 region by testing for association with polymorphisms of five candidate genes belonging to the 1.5 LOD-unit support interval of the linkage peak: interferon regulatory factor 1 (*IRF1*), interleukin-13 (*IL13*), interleukin-4 (*IL4*), interleukin-9 (*IL9*) and cluster of differentiation 14 (*CD14*). Bivariate association analyses for FEV₁/H² and SPTQ were carried out in 68 families that showed positive LOD scores with MLB_{comb} in male offspring. Of the 23 single-nucleotide polymorphisms (SNPs) belonging to the five genes investigated, two SNPs in *IL9* were found associated with FEV₁/H² and SPTQ, rs2069882 (FBAT_{comb} = 13.7, *P* = 0.001) and rs2069885 (FBAT_{comb} = 18.8, *P* = 0.00008), and remained significant after Bonferroni's correction (Table 4). Multi-marker analysis based on FBAT-MM statistic did not improve markedly the single marker association signals (results not shown).

Discussion

This is the first report of a male-specific pleiotropic QTL on 5q31 influencing both a measure of lung function (FEV₁/H²) and a measure of allergen polysensitization (SPTQ) in asthmatic families. Moreover, we found significant association of these phenotypes with polymorphisms of *IL9* gene. Four other sex-specific regions of

Table 4 Family-based bivariate association tests of FEV₁/H² and SPTQ with four IL9 SNPs in males of linkage families

SNP	Location (pb)	Minor allele	MAF	FBAT _{comb}	P-value
rs6596271	135248727	G	0.06	1.39	0.5
rs2069885	135256063	T	0.15	13.76	0.001
rs2069882	135257531	G	0.23	18.81	0.00008
rs31564	135258151	C	0.40	0.67	0.7

Abbreviations: FBAT_{comb}, association test statistic constructed by summing the univariate FBAT statistics obtained with each of the two principal components of FEV₁/H² and SPTQ; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

linkage were detected, 11q23 for two atopy-related phenotypes (IgE and SPTQ) in females, and three regions for EOS (3p25 in males, 4q24 and 22q13 in females).

Our linkage analyses used the maximum-likelihood-binomial (MLB) method rather than the classical variance-components method, as it does not require normality assumption of the trait distribution.¹³ The bivariate linkage analyses were based on an approach that combines univariate linkage test statistics applied to the principal components of the original phenotypes. This approach, first proposed by Mangin *et al.*,¹⁴ in the context of variance-component analysis was extended to other test statistics, including the MLB test statistic.¹⁰ We also extended that approach to bivariate association analysis based on the FBAT statistics. This study outlines the interest of this simple and flexible approach. We addressed the issue of multiple testing by computing GW significance levels based on simulations for linkage tests and using the Bonferroni's correction for association tests.

None of the sex-specific regions detected by this study have been found by previous GW linkage screens of these phenotypes in the whole EGEA sample.¹¹ When compared with the two sex-specific linkage scans performed in the Hutterites and Costa Ricans,^{1,6–8} none of the linkage signals detected here were previously reported, except for the 5q31 region. A male-specific linkage, as in this study, was found with a measure of lung function (FEV₁/FCV, FVC being the forced vital capacity) in the Hutterites at a position 15 cM telomeric from our linkage peak, whereas a female-specific linkage was reported with specific IgE to cockroach in Costa Ricans at a position 24 cM centromeric from our peak.⁶ These results suggest that there may be several genes with sex-specific effects in the 5q31 region. Both 5q31 and 11q23 regions have been previously reported to be linked and associated with several asthma phenotypes in a non sex-specific manner.^{5,15} Our other three regions of linkage are new regions, as only the 4q24 region has been reported once to be linked to allergic rhinitis.¹⁶ However, these regions contain several candidate genes potentially involved in asthma or asthma-related phenotypes.^{5,15,17}

Following these linkage findings, bivariate association analysis of SPTQ and FEV₁/H² with markers in 5q31 showed significant association with two SNPs, rs2069885 and rs2069882, within *IL9* gene in males. The rs2069885 SNP is a nonsynonymous variant (T361M) in exon 5, whereas the rs2069882 SNP lies in intron 4. Moreover, we checked that this association accounted for the linkage signal in 5q31 by partitioning LOD scores for a given microsatellite according to genotypes at SNP rs2069882.

The *IL9* gene encodes interleukin 9, a cytokine produced by T-helper cells, which plays an important role in immunological and allergic processes. Studies in mice have shown association of differences in expression of *IL9* gene with biological variability in bronchial responsiveness.^{18,19} In humans, *IL9* was found to influence chemokine release from bronchial epithelial cell^{20,21} and airway smooth muscle,²² and has also been shown to induce goblet cell hyperplasia during repair of human airway epithelia.²³ This pleiotropic effect of *IL9* is in agreement with our findings. To our knowledge, no association has been reported between *IL9* polymorphisms and asthma or asthma related-phenotypes except once, wherein a weak association was found between *IL9* variants and atopic asthma defined by positive-specific IgE against Der p (or Der f) and house dust.²⁴ *IL9* acts through binding to *IL9* receptor, located on the X chromosome. Linkage and association studies have suggested that *IL9* receptor polymorphisms may contribute to asthma.^{21,25} Of particular interest is the recent finding of a sex-specific protective effect of *IL9* receptor against wheezing in boys, but not in girls, in the Swedish BAMSE study.²¹ This gene had also a weaker effect against sensitization in both sexes.²¹ These results are in agreement with our findings.

Sexual differences for asthma and asthma-related traits reflect the marked differences regarding dimensional and immunological characteristics observed according to sex over the life span.³ It is of interest to note that in this study, the *IL9* polymorphism was associated both to lung function (FEV₁/H²) and to a marker of polysensitization. Sex differences in asthma-related traits may depend on genetic factors, but also on environmental factors and hormonal factors, and more likely on the complex

interactions between these factors. There is still limited information on hormonal effects (endogenous or exogenous) on asthma-related traits,^{26,27} as most studies on hormonal-related events, are focused on asthma.^{28,29} Sex-specific exposures, important in adolescence and in the working life, are primary candidates to explain phenotypic heterogeneity. Regarding lung function, increased vulnerability of females to environmental factors, such as smoking or ozone has been reported.³⁰ With respect to atopic sensitization, sex differences in association with breast-feeding have been observed.³¹

In conclusion, our results provide further evidence for the role of sex-specific genetic factors underlying asthma phenotypes. Further studies investigating sex-specific interactions between genetic and environmental factors would be worth considering. This study also outlines the importance of taking into account complex mechanisms, such as heterogeneity according to sex and pleiotropy to unravel genes involved in asthma phenotypes.

Materials and methods

Family sample

The protocol of the EGEA data collection has been described elsewhere.^{32,33} Proband and their first-degree relatives answered a detailed questionnaire regarding respiratory symptoms and treatment based on International Standardized Questionnaires.^{32,33} A total of 388 families were ascertained through at least one asthmatic proband.

Phenotypes analyzed

Four quantitative traits were examined, including total serum IgE levels (UI ml⁻¹, log transformed), blood EOS counts (number of cells mm⁻³, log transformed), a quantitative score measuring the degree of allergen polysensitization (SPTQ, being the sum of positive skin test responses to 11 allergens, a score with good biometric properties)³⁴ and FEV₁/H², which takes into account dimensional aspects of lung growth and size for males and females over the life span³⁵ and was previously used in the EGEA study.³⁶ Before linkage analysis, those phenotypes were adjusted for relevant covariates including age, sex and smoking habits using multiple regression and standardized as described in Bouzigon *et al.*³⁷

Genotyping

For the GW linkage screen, a total of 378 autosomal microsatellites markers distributed along the genome were genotyped in 307 families with at least two offspring with DNA available at Centre National de Génotypage (CNG, Evry, France) as already described.¹¹ The average intermarker distance was 10 cM and average heterozygosity over all loci was close to 75%. Before statistical analysis, rigorous genotype quality assurance was performed to ensure accurate binning of alleles. After excluding families with genotyping problems or families showing non-Mendelian transmission, the analyzed sample included 295 nuclear families with at least two genotyped offspring. Among these families, 97.8% of parents were genotyped. The total sample included 1317 individuals comprising 727 genotyped offspring.

For the association study in the 5q31 region that showed the highest evidence for linkage, we examined 23 SNPs belonging to five candidate genes (*IFR1*, *IL13*, *IL4*, *IL9* and *CD14*), which were genotyped using a tagging approach, as part of a panel of candidate genes in the EGEA study. This set of SNPs allowed us to capture the majority of common haplotype variation of these five genes (that is, haplotype with frequency > 5%). Genotyping was done using either Taqman Probes (Applied Biosystems, Foster City, CA, USA) on an ABI7900HT Sequence Detection System or 1536-plex Illumina Golden Gate assay (Illumina, San Diego, CA, USA) at the Centre National de Génotypage (CNG, Evry, France). The consistency of the SNP data with Mendelian inheritance was evaluated using the PedCheck program.³⁸ Tests of Hardy–Weinberg equilibrium were performed using an exact test.³⁹ No SNP showed Hardy–Weinberg disequilibrium.

Univariate linkage analysis

Linkage analysis was performed using the MLB method,^{13,40} a model-free approach that can be applied to the whole sibship and is implemented in MLB-GENEHUNTER program.⁴⁰ The advantage of this method is that it does not require the assumption of normality of the distribution of the quantitative phenotype.¹³ The test for linkage is based on likelihood ratio test statistic $\lambda_{MLB} = 2\ln[L(\alpha)/L(\alpha = 0.5)]$, where α is the probability for an affected sib to receive from his/her parent the marker allele transmitted with the disease allele. λ_{MLB} is distributed asymptotically as a mixture distribution of $1/2\chi_0^2 + 1/2\chi_1^2$. This statistic can also be expressed as a LOD score criterion (equal to $\lambda_{MLB}/[2\ln(10)]$).

Sex-stratified linkage analyses were conducted by setting the sibs phenotypes of one sex to unknown and conversely. To take into account the multiple testing problem, GW significance levels for linkage were computed by simulations. For a given phenotype and a given sex-specific sample, 10 000 sets of simulated genotype data were generated by using the program SIPED, we developed (<http://sites.google.com/site/genepitools/>). Genome-wide significance levels were then estimated using the approach described by Sawcer *et al.*⁴¹

To test for heterogeneity of linkage according to sex, we computed, for each marker, the difference in *P*-values associated with the linkage test statistics obtained from the analysis of male and female siblings, respectively. Significance of these differences in *P*-values was obtained by permutation. The sex status was randomly permuted among the siblings, although keeping the same sibship size distribution as well the number of phenotyped and genotyped subjects in the observed sample. One thousand sets of sex-specific samples were generated for each phenotype and GW significance levels were estimated using the Sawcer *et al.* approach.⁴¹

Bivariate linkage analysis

The MLB method was extended to bivariate linkage analysis following the approach as proposed by Mangin *et al.*¹⁴ This bivariate analysis was applied to phenotypes showing significant linkage to the same region. In a first step, a PCA (principal components analysis) was applied to a given pair of phenotypes to obtain two uncorrelated PCs (principal components) using STATA 7.0. These

components were then subjected to independent univariate linkage analyses based on the MLB method. In a second step, a combined test statistic, MLB_{comb} , was constructed by summing the univariate MLB likelihood-ratio (LR) test statistics obtained for each of the two PCs: $MLB_{comb} = \sum_{i=1}^2 \lambda_{MLBi}$. As the two principal components are independent, the asymptotic distribution of MLB_{comb} is a mixture of $1/4\chi_0^2 + 1/2\chi_1^2 + 1/4\chi_2^2$.

Family-based association test

As the most significant linkage was obtained in the 5q31 region that contained a pleiotropic QTL linked to both FEV_1/H^2 and SPTQ, a bivariate association analysis was performed with 5q31 SNPs using the FBAT software.⁴² As for the bivariate linkage analysis, we constructed a combined FBAT statistic, $FBAT_{comb}$ by summing the two univariate FBAT statistics obtained with each of the two principal components of FEV_1/H^2 and SPTQ. This $FBAT_{comb}$ statistic follows a Chi-square with 2 d.f. We assumed an additive model and took into account the presence of linkage using the *-e* option. A multi-marker analysis was also conducted based on the FBAT-MM method.⁴³

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Appendix

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