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Genetic variation in ORM1-like 3 (*ORMDL3*) and gasdermin-like (*GSDML*) and childhood asthma

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Abstract

Background—A genome-wide association study identified ORM1-like 3 (orosomucoid 1-like 3, *ORMDL3*) as an asthma candidate gene. Single nucleotide polymorphisms (SNPs) in the region including *ORMDL3* on chromosome 17q21 were related to childhood asthma risk and *ORMDL3* expression levels in Europeans.

Objective—We examined whether polymorphisms in *ORMDL3* and the adjacent gasdermin-like (*GSDML*) gene associated with asthma in the genome-wide association study are related to childhood asthma and atopy in a Mexico City population.

Methods—We genotyped rs4378650 in *ORMDL3* and rs7216389 in *GSDML* in 615 nuclear families consisting of asthmatic children aged 4–17 years and their parents. Atopy was determined by skin prick tests to 25 aeroallergens.

Results—Individuals carrying the C allele of rs4378650 or the T allele of rs7216389 had increased risk of asthma [relative risk (RR) = 1.73, 95% con.- dence interval (CI) 1.19–2.53, $P = 0.003$ for one or two copies of rs4378650 C, and RR = 1.64, 95% CI 1.12–2.38, $P = 0.009$ for one or two copies of rs7216389 T). Linkage disequilibrium between the two SNPs was high ($r^2 = 0.92$). Neither of the SNPs was associated with the degree of atopy. A meta-analysis of five published studies on rs7216389 in nine populations gave an odds ratio for asthma of 1.44 (95% CI, 1.35–1.54, $P < 0.00001$).

Conclusions—Our results and the meta-analysis provide evidence to confirm the finding from a recent genome-wide association study that polymorphisms in *ORMDL3* and the adjacent *GSDML* may contribute to childhood asthma.

Keywords

allergy; asthma; genetic predisposition to disease; meta-analysis; single nucleotide polymorphism

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Asthma is a complex disease caused by multiple genetic and environmental factors. Moffatt et al. recently identified ORM1-like 3 (orosomucoid 1-like 3, *ORMDL3*) as a potential asthma candidate gene using genome-wide association and microarray approaches (1). Multiple single nucleotide polymorphisms (SNPs) on chromosome 17q21 were statistically significantly associated with childhood asthma in German and British populations (1). In a microarray analysis, SNPs that were related to asthma in the region containing the *ORMDL3* gene on 17q21 were associated with expression levels of *ORMDL3* in Epstein–Barr virus-transformed lymphoblastoid cell lines from asthmatic children (1).

ORMDL3 belongs to a novel evolutionarily conserved gene family with unknown function (2). Genes in the *ORMDL* family encode transmembrane proteins located at the endoplasmic reticulum membrane (2). *ORMDL3* is ubiquitously expressed in human tissues (2). Yeast double knockouts of the *ORMDL* genes show slower growth and higher sensitivity to toxic compounds (2). The function of *ORMDL3* in humans is unclear (2).

The gasdermin-like (*GSDML*) gene belongs to the cancer associated gasdermin-domain containing protein family, which has been related to cancer pathogenesis (3). *GSDML* is adjacent to *ORMDL3* on chromosome 17. Moffatt et al. found that polymorphisms in *GSDML* were statistically significantly associated with childhood asthma and transcript levels of *ORMDL3* (1) suggesting that *GSDML* SNPs may also modify *ORMDL3* expression and thus contribute to asthma susceptibility.

Several subsequent studies have confirmed associations between variants in *ORMDL3* and *GSDML* and asthma (4–7). Given that these are the only published associations with asthma from genome-wide association studies to date and that the original study was modest in size relative to the magnitude of the association, confirmation in other populations remains crucial (8). We examined associations of polymorphisms in *ORMDL3* and *GSDML* with childhood asthma and atopy in a case– parent triad study in Mexico City. Given that the asthma involves interactions between genetic susceptibility and environmental exposures, we also explored possible effect modification by two environmental risk factors for childhood asthma – ambient ozone concentrations (9), which are especially high in Mexico City and parental tobacco smoking (10). Evidence of gene by environment interaction in relation to asthma phenotypes exists for these two exposures (11–14). In addition, to put our findings and previous publications in perspective, we performed a meta-analysis of all published data on SNP rs7216389 which was associated with asthma and *ORMDL3* expression with the highest degree of statistical significance in the original report (1) and examined in all subsequent studies (4–7).

Methods

Study design and subject enrollment

We used the case–parent triad design (15,16). The cases were children aged 4–17 years with asthma diagnosed by a pediatric allergist at the allergy referral clinic of a large public pediatric hospital in central Mexico City (Hospital Infantil de México, Federico Gómez). Children and parents provided blood samples as sources of DNA. The study population included 615 families consisting of asthmatic children and their parents with adequate DNA samples for genotyping either of the two SNPs. A parent, nearly always the mother, completed a questionnaire on the child's symptoms and risk factors for asthma including current parental smoking, parental smoking during the first 2 years of the child's life, maternal smoking during pregnancy, and residential history.

Ozone has been related to development of asthma; Mexico City has the highest ozone concentrations in North America (9). We obtained measurements of ambient ozone from the

Mexican government's air monitoring stations. Ozone levels were measured via UV photometry (analyzer-model 400; API, San Diego, CA, USA). The residence of each child who participated in this study was located using a map and the closest monitoring station was assigned to that residence (17). The ozone exposure data were collected for the year prior to the time of entry into the study. The parameter we used was the annual average of the daily maximum 8 h averages. We dichotomized this variable at the median of 67 ppb for stratified analyses.

The protocol was reviewed and approved by the Institute Review Boards of the Mexican National Institute of Public Health, the Hospital Infantil de México, Federico Gómez, and the U.S. National Institute of Environmental Health Sciences. Parents provided the written informed consent for the child's participation. Children also gave their informed assent.

The diagnosis of asthma was based on clinical symptoms and response to treatment by pediatric allergists at a major referral hospital (18). The severity of asthma was rated by a pediatric allergist according to symptoms in the Global Initiative on Asthma schema as mild (intermittent or persistent), moderate or severe (19). At a later date, for research purposes, pulmonary function was measured using the EasyOne spirometer (ndd Medical Technologies, Andover, MA, USA) according to ATS specifications (20). The best test of three technically acceptable tests was selected. Spirometric prediction equations from a Mexico City childhood population were used to calculate the percent predicted forced expiratory volume in 1 s (FEV_1) (21). Children were asked to hold asthma medications on the morning of the test.

Atopy was determined using skin prick tests (SPT). The following battery of 25 aeroallergens (IPI ASAC, Mexico) common in Mexico City was used: *Aspergillus fumigatus, Alternaria* species, *Mucor* species, *Blattella germanica, Periplaneta americana, Penicillium* species, cat, dog, horse, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae, Ambrosia* species, *Artemisa ludoviciana, Cynodon dactylon, Chenopodium album, Quercus robur, Fraxinus* species, *Helianthus annus, Ligustrum vulgare, Lolium perenne, Plantago lanceolata, Rumex crispus, Schinus molle, Salsola* species and *Phleum pratense*. Histamine was used as a positive control and glycerin as a negative control. Children were considered atopic if the diameter of the skin reaction to at least one allergen exceeded 4 mm. The test was considered valid if the reaction to histamine was ≥6 mm according to the grading of the SPT recommended by Aas and Belin (22).

SNP selection and genotyping

The original genome-wide association study showed tight linkage disequilibrium in and around *ORMDL3* on 17q21 in European populations (1). The SNPs highly statistically significantly associated with both asthma and *ORMDL3* expression were located in *ORMDL3* as well as the adjacent *GSDML*. Other than the association with gene expression, there are no data on the functional significance of any of the previously studied SNPs. Among the many linked SNPs associated with asthma and *ORMDL3* expression in this region with the highest degree of statistical significance in the study of Moffatt (1), we selected rs4378650 in *ORMDL3*, which gave the smallest *P* value for association among SNPs inside that gene in the original study, and rs7216389 in neighboring *GSDML*, which is the only SNP analyzed in all subsequent studies, for genotyping in our Mexican population. SNP rs7216389 was very strongly ($P < 10^{-22}$) associated with expression of *ORMDL3* (1).

DNA was extracted from peripheral blood lymphocytes using Gentra Puregene kits (Gentra System, Minneapolis, MN, USA). The rs7216389 and rs4378650 SNPs were genotyped using the TaqMan SNP Genotyping Assay. Primers and probes were purchased from Assay-on-Demand (Applied Biosystems, Foster City, CA, USA). All PCR amplifications were performed using 5′ exonuclease assay on GeneAmp PCR Systems 9700 (Applied Biosystems). The

fluorescence of PCR products was detected using ABI Prism 7900HT sequence detection system. All genotyping assays were performed by a researcher who was blinded to parent or child status of samples. Sixteen quality control samples were plated per 384-well plate along with 24 control samples with known genotype. An additional six blind replicate samples were included in the analyses. The quality controls and the blind replicates were 100% concordant for all genotyping methods. All genotyping data reflected more than 99% plate assay efficiency.

Non-parentage was ascertained with a set of short-tandem repeats (AmpFLSTR Profiler Plus; Applied Biosystems) analyzed using PEDCHECK software (University of Pittsburgh, Pittsburgh, PA, USA) (23).

Statistical analysis

We used a log-linear likelihood approach to analyze associations between asthma and individual SNPs (15). The log-linear likelihood- ratio test is a powerful and more flexible generalization of the transmission disequilibrium test (TDT) and has the advantage of providing estimates of the magnitude of associations rather than simply tests of significance (15). Similar to TDT based methods for the analysis of case–parent data, such as the family based association test (24), the log-linear model tests the same null hypothesis of no within-family relationship between variant and the disease and achieves robustness against genetic population structure through stratification on the possible parental mating types (15,25). The log-linear method thus gives comparable *P* values to TDT-based methods.

We calculated relative risks (RR) for individual SNPs without restricting to a specific genetic model and under the dominant genetic model. The dominant genetic model compares the combined group of one or two copies of the alternative allele to having no copies of that allele. We also calculated RR for associations under the additive model for comparison with previous studies. The log-linear models of case–parent data are inherently immune to confounding by demographic and lifestyle factors such as parental smoking or environmental exposures. However, we examined effect modification by gender, parental smoking, and level of ozone exposure. All SNP analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA) and STATA version 8.0 (Stata Press, College Station, TX, USA).

To evaluate whether the two SNPs influenced the degree of atopy, as assessed by the number of positive skin tests out of 25 performed, we used the polytomous logistic method of Kistner and Weinberg to estimate the linkage and association between polymorphisms and atopy (26). *P* values were calculated from likelihood ratio tests. We also used this method to analyze the relationship between SNPs and lung function, as assessed by percent predicted FEV_1.

We performed a PubMed database search on 10 June 2008 using the keywords ‘asthma’ and ‘genetic polymorphism’ together with ‘ORMDL3’ or ‘17q21’ to identify association studies on *ORMDL3* and asthma. In our meta-analysis, we included all studies with odds ratio [95% (confidence interval) CI] available for association between rs7216389 and asthma under an additive, or sometimes log-additive, genetic model. The odds ratios were not provided for the German and British populations in the genome-wide association study of Moffatt (1) and thus we calculated the odds ratios using the allele frequency data obtained from Moffatt and coworkers (1) by Galanter et al. (4), which provide nearly the same results as the log-additive model (27). For the Japanese population in the study of Hirota (5), the odds ratio under the log-additive model was used for the meta-analysis. We also included our data in the meta-analysis. In case– control studies, the odds ratio is given as an estimate of the RR. The log linear method we used to analyze our family data generates a RR. Therefore, for the meta-analysis, we calculated the odds ratio for the T allele for the additive model using the formula of Evangelou (28). We performed a fixed effects metaanalysis with inverse variance weighting using REVMAN 5.0 (http://www.cc-ims.net/RevMan).

Results

Characteristics of the asthmatic children were described previously (13). Briefly, the mean age of cases was 9.0 years (range 4–17). Most had mild as opposed to moderate or severe asthma. Nearly all cases had used medication for asthma in the past 12 months. Among cases with spirometry data, the mean FEV_1 percent predicted was 97 (SD = 21). Ninety-two percent of cases had a positive skin test. The highest rates of skin test positivity were seen for dust mite and cockroach. Only 6% of mothers reported smoking during pregnancy, but 51% of children had a parent who currently smoked.

The frequency distributions of all mating types for the two SNPs are presented in Table 1. The minor allele frequency in parents was 0.32 for both SNPs. Both SNPs were in Hardy–Weinberg equilibrium ($P > 0.05$) in the parents. Linkage disequilibrium between the two SNPs was high ($r^2 = 0.92$).

Carrying the T allele of the rs7216389 SNP was associated with increased risk of childhood asthma (RR = 1.62, 95% CI, 1.11–2.37, $P = 0.013$ for one copy; RR = 1.74, 95% CI, 1.15–2.65, $P = 0.009$ for two copies). Individuals with either one or two copies of the T allele of rs7216389 had a RR of 1.64 (95% CI, 1.12– 2.38, $P = 0.009$). The results were similar for the linked rs4378650 SNP (RR = 1.73, 95% CI, 1.18–2.52, $P = 0.005$ for one copy of the C allele; RR = 1.76, 95% CI, 1.16–2.66, $P = 0.008$ for two copies; RR = 1.73, 95% CI, 1.19–2.53, $P = 0.003$ for one or two copies; Table 2). Results did not differ appreciably by gender, parental smoking status or ozone level (data not shown). Neither of the two SNPs was associated with the degree of atopy to aeroallergens, assessed by the number of positive skin tests out of a battery of 25 tests (data not shown). We also did not observe associations between the two SNPs and percent predicted FEV_1 (data not shown).

Meta-analysis of rs7216389 and asthma included nine populations (Table 3). Under a fixed effects model, the pooled odds ratio for asthma was 1.44 (95% CI, 1.35– 1.54, $P < 0.00001$). There was no evidence of heterogeneity among studies ($P = 0.59$). When the initial findings of Moffatt were excluded from the meta-analysis, the pooled odds ratio was 1.40 (95% CI, 1.29–1.52, $P < 0.00001$), indicating that the original report did not substantially bias the meta-analysis results.

Discussion

A recent genome-wide association study suggested *ORMDL3* as a potential asthma candidate gene (1). In our case–parent triad study in a Mexico City population, carrying the C allele of SNP rs4378650 in the *ORMDL3* gene or the T allele of SNP rs7216389 in the adjacent *GSDML* gene increased the risk of childhood asthma. We selected these two SNPs because they were associated with both asthma risk and *ORMDL3* expression in the original genome-wide association study with a high degree of statistical significance. Further, one of these SNPs has been studied in all subsequent papers enabling us to do a meta-analysis of results.

In a genome-wide association study with approximately 300 000 SNPs, multiple SNPs on chromosome 17q21 were highly statistically significantly associated with asthma in Europeans (1). A microarray analysis in Epstein–Barr virus-transformed lymphoblastoid cell lines from asthmatic children showed that transcript levels of the *ORMDL3* gene on 17q21 were correlated with asthma-associated SNPs in this region (1). These findings suggest that there may be unknown functional SNPs in potential *cis*-regulatory elements on the 17q21 locus that can modify *ORMDL3* expression and therefore contribute to asthma susceptibility (1).

Among asthma-associated SNPs on 17q21 in the initial study, the rs7216389 SNP gave the smallest *P* value for association with asthma and transcription levels of *ORMDL3* (1). The

rs7216389 SNP is in the first intron of the *GSDML* gene that is adjacent to *ORMDL3*. The sequence around rs7216389 is conserved across species and contains a region homologous to the pro-inflammatory transcription factor C/EBPB (1). Our study and the studies of four others (4–7) have confirmed association between rs7216389 and asthma. Of note, allele frequencies differ markedly among populations – the T allele was the minor allele in the European populations, but the C allele was the minor allele in the populations studied outside of Europe. In ours and previous studies, the T allele of rs7216389 conferred significantly increased risk for asthma under an additive model in the Puerto Rican (4), Japanese (5), French Canadian (6), European (1,7), and Mexican populations, but not in African-American populations (4) (Table 3). We calculated the RR for rs7216389 without restricting to a specific genetic model. In our data, although the additive model was associated with asthma (RR = 1.20, 95% CI, 1.01–1.43, $P = 0.043$) with a magnitude similar to the study of Galanter (OR = 1.26, 95% CI, 0.95–1.65), also conducted among Mexicans (4), our data gave a stronger association under the dominant model. Most populations have not reported results under the dominant model limiting comparison. Only the study of Hirota, conducted among Japanese, tested for different genetic models, giving a *P* value of 0.18 for the dominant model (5). There is only one additional study where the dominant model could be evaluated given the data presented. In that study (7), calculation of the crude OR under the dominant model gave a significant result (crude OR = 1.69, 95% CI, 1.42–2.02, $P < 0.001$). The meta-analysis of our study with the other four published studies (nine populations in total) confirmed that rs7216389 was significantly associated with asthma and showed no evidence for heterogeneity in the results across populations.

In addition to rs7216389 in *GSDML*, we studied rs4378650 in the first intron of *ORMDL3*, which showed a comparable degree of association with asthma in Europeans (1). Ours and the other two studies that have looked (4,6) have confirmed association between rs4378650 and asthma. SNPs associated with asthma and *ORMDL3* expression in the genomic region of *ORMDL3* and *GSDML* were in tight linkage disequilibrium with each other in Europeans (1) and French Canadians ($r^2 = 0.97$ between rs7216389 and rs4378650) (6). Similarly, the linkage disequilibrium between rs7216389 and rs4378650 was high ($r^2 = 0.92$) in our Mexican population enrolled from central Mexico City. Although the allele frequency for rs7216389 and rs4378650 in our Mexican population and in that of Galanter (4) are similar, Galanter and coworkers reported much lower linkage disequilibrium ($r^2 = 0.17$) in a Mexican population enrolled from Mexico City and San Francisco (4).

Our study has several strengths. The triad design and analysis protect against population stratification (15). The number of families examined in our study is relatively large. The demographic and clinical characteristics of our asthmatic children and their parents are well characterized. Our asthma cases were diagnosed by pediatric allergists at a pediatric allergy specialty clinic of a large public referral hospital. Consultation with this pediatric allergy clinic is a tertiary referral, and thus the children in our study had already been seen by a generalist and a pediatrician over time for recurrent asthma symptoms. Diagnoses were made on clinical grounds according to previous guidelines (18). We did not test for bronchial hyper-reactivity. However, physician diagnosis of asthma is a valid outcome compared to objective measurements (29). We had objective data on atopy; SPT revealed the vast majority of these children with asthma (92%) to be atopic to aeroallergens.

Only 8% of our cases were not atopic to aeroallergens. Therefore, we could not examine the association with non-atopic asthma. We also were unable to evaluate whether associations might differ among severe asthmatics because asthma in our population was predominantly mild. Pooled analyses are planned across US asthma studies to examine effect modification of genetic association by severity and other factors – much large numbers are needed. With respect

to age, most of our children are close to 9 years old. It might be interesting in future pooling exercises to examine variation by age.

We found no evidence of effect modification by parental smoking or ambient ozone. It should be noted that ozone concentrations in Mexico City are high compared with other North American locations. To evaluate fully whether ozone modifies the relationship between any SNP and asthma, one would need to include demographically similar populations in a variety of locations with markedly different ozone exposures.

We found that genetic variations in *ORMDL3* and neighboring *GSDML* genes were associated with childhood asthma in a Mexican population. Our meta-analysis of published results shows a high degree of consistency in this association across studies. These results confirm the main finding from a recent genome-wide association study conducted among European populations.

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Abbreviations

CI
: confidence interval

FEV_1
: forced expiratory volume in 1 s

GSDML
: gasdermin-like

OR
: odds ratio

ORMDL3
: ORM1-like 3

RR
: relative risk

SNP
: single nucleotide polymorphism

TDT
: transmission disequilibrium test

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Table 1

Distribution of case–parent triad genotypes for the two SNPs

	Triad counts	
Mother/father/child*	**rs7216389**	**rs4378650**
2/2/2	114	107
2/1/2	59	57
2/1/1	52	54
1/2/2	50	50
1/2/1	48	51
2/0/1	24	23
0/2/1	37	37
1/1/2	26	27
1/1/1	50	53
1/1/0	11	9
1/0/1	10	11
1/0/0	13	12
0/1/1	12	12
0/1/0	10	12
0/0/0	5	6
2/–/2	22	24
2/–/1	13	13
1/–/2	14	12
–/2/2	5	5
–/1/2	1	1
1/–/1	23	23
1/–/0	2	3
–/1/1	3	4
0/–/1	6	6
0/–/0	2	2
Total	612	614

*Number of copies of the T allele in mother/father/child for rs7216389 and number of copies of the C allele in mother/father/child for rs4378650. N-dash indicate the parent who was missing for incomplete triads.

Table 2

The two SNPs in relation to childhood asthma

SNPs	Model	Genotype	RR (95% CI)	*P*-value
rs7216389*	Unrestricted	CC	1.00	
		TC	1.62 (1.11–2.37)	0.013
		TT	1.74 (1.15–2.65)	0.009
	Dominant	TT + TC	1.64 (1.12–2.38)	0.009
rs4378650	Unrestricted	TT	1.00	
		CT	1.73 (1.18–2.52)	0.005
		CC	1.76 (1.16–2.66)	0.008
	Dominant	CC + CT	1.73 (1.19–2.53)	0.003

*For rs7216389, The T allele is the ancestral allele according to dbSNP and the allele frequency of the ancestral allele is 0.68 in the Mexican population. However, for comparability with all previous studies we used C as the reference allele.

Table 3

Studies examining association between rs7216389 in *GSDML* and asthma

Reference	Study population	Study design	No. of cases	Minor allele	MAF	Genetic model*	Relative risk (95% CI)†	*P*-value
Current study	Mexican	Family	612	C	0.32	TC *vs* CC	1.62 (1.11–2.37)	0.013
						TT *vs* CC	1.74 (1.15–2.65)	0.009
						(TT + TC) *vs* CC	1.64 (1.12–2.38)	0.009
						Additive	1.20 (1.01–1.43)	0.043
Moffatt, 2007 (1)	German	Case–control (GWAS)	728	T	0.47 (4)	Additive	N/A	<0.001
	British	Family (GWAS)	266	N/A	N/A	Additive	N/A	<0.001
	German	Case–control (replication cohort)	200	N/A	N/A	Additive	1.45 (1.17–1.81)	<0.001
Galanter, 2008 (4)	Mexican	Family	301	C	0.3	Additive	1.26 (0.95–1.65)	N/A
	Puerto Rican	Family	399	C	0.37	Additive	1.35 (1.07–1.70)	<0.05
	African-American	Case–control	261	C	0.22	Additive	1.21 (0.79–1.84)	N/A
Hirota, 2008 (5)	Japanese	Case–control	545	C	0.29	TT *vs* (TC + CC)	1.62 (1.30–2.03)	<0.001
						(TT + TC) *vs* CC	N/A	0.18
						T *vs* C	1.44 (1.20–1.73)	<0.001
Madore, 2008 (6)	French Canadian	Family	632	C	0.42	Additive	Risk	0.014
Tavendale, 2008 (7)	Northern European	Case–control	1054	T	0.46	CT *vs* CC	1.50 (1.24–1.81)	<0.001
						TT *vs* CT	1.41 (1.18–1.69)	<0.001
						TT *vs* CC	2.11 (1.71–2.61)	<0.001
						Additive	1.53 (1.34–1.75)	<0.001

GWAS, genome-wide association study; MAF, minor allele frequency; NS, not significant; N/A, data not available.

*C is the reference allele in the published studies although the T allele is more common in some populations.

†In case–control studies, the odds ratio is given as an estimate of the relative risk. In the log-linear analysis, the relative risk is calculated. For meta-analysis, we calculated the odds ratio for the T allele for the additive model using the formula of Evangelou (28) (OR = 1.26, 95% CI, 1.05–1.52).