Sharma On-Line Supplement

Variants in *TGFB1*, dust mite exposure, and disease severity in children with asthma

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Online Data Supplement

METHODS

Study Populations: Genetics of Asthma in Costa Rica Study

Children between ages 6-14 years with asthma were recruited from 95 schools in the Central Valley of Costa Rica, as previously described (1). The Central Valley of Costa Rica is a genetically isolated population (2). Inclusion criteria for the Genetics of Asthma in Costa Rica study were physician-diagnosed asthma and either at least two respiratory symptoms (cough, wheeze, or dyspnea) or a history of asthma attacks in the year prior to enrollment. Patients recruited as part of the study had to have high probability of having at least six great-grandparents born in the Central Valley of Costa Rica (as determined by our study genealogist on the basis of the paternal and maternal last names of each of the child's parents). This requirement increased the likelihood that children would be descendants of the founder population of the Central Valley (3). Of the participating children, 91% had confirmation of having at least six great-grand parents born in the Central Valley (lack of confirmation was mostly due to adoption or birth out of wedlock).

For each child participating in the study, a parent completed a study questionnaire. Spirometry was performed according to American Thoracic Society standards for acceptability and reproducibility (4). Methacholine challenge testing utilized a modified version of the Chatham protocol (5).

Costa Rica Informed Consent

Parents provided written informed consent for themselves and for their children. The children provided written assent prior to study participation. The study was approved

by the Institutional Review Boards of the Brigham and Women's Hospital and the Hospital Nacional de Niños, San José, Costa Rica.

Study Populations: Childhood Asthma Management Program (CAMP) Genetics Ancillary Study

CAMP is a multi-center North American randomized controlled clinical trial designed to investigate the long-term effects of two asthma treatment regimens (inhaled budesonide or inhaled nedocromil compared to placebo) in children with mild to moderate persistent asthma (6,7). Following baseline evaluations which occurred over a 2-4 month period the 1,041 study participants were randomized to receive one of three treatment arms: (a) inhaled budesonide, 200 μg twice daily (311 children), (b) inhaled nedocromil sodium, 8 mg twice daily (312 children), and (c) inhaled placebo (418 children). All of the children used inhaled albuterol, delivered by two 90-μg actuations of a pressurized metered-dose inhaler, as rescue medication for relief of the symptoms of asthma (6,7).

Inclusion criteria for the CAMP trial included a diagnosis of asthma based on methacholine hyperresponsiveness (PC_{20} less than or equal to12.5 mg/ml) and one or more of the following criteria: (a) asthma symptoms at least two times per week; (b) at least two usages per week of an inhaled bronchodilator; and (c) use of daily asthma medication for at least six months in the year prior to recruitment. Children with evidence of severe asthma or other clinically significant medical conditions were excluded.

Of the 1,041 children originally enrolled, 968 children and 1,518 parents contributed DNA samples for an ancillary study of the genetic determinants of asthma.

DNA was sufficient for all family members for 483 nuclear families of self-reported non-Hispanic white ancestry studied previously (8).

CAMP Informed Consent

The Institutional Review Board of the CAMP study centers approved this study. Informed consent was obtained from the study participants and their parents and assent from the children was obtained prior to DNA collection for genetic studies.

Study Procedures: Costa Rica

Costa Rica: Questionnaire

Parents of participating children completed a slightly modified version of the questionnaire used in the Collaborative Study on the Genetics of Asthma (4), which was translated into Spanish.

Costa Rica: Pulmonary Function Tests

Spirometry was conducted with a Survey Tach Spirometer (Warren E. Collins, Braintree, MA) following American Thoracic Society recommendations (5). Height was measured to the nearest half inch at the time that spirometry was performed. Subjects were instructed to avoid use of short-acting bronchodilators for at least four hours before testing. As many as eight attempts were made to obtain three spirometric measures that met ATS criteria for acceptability and reproducibility. The best lung function measurements were used for data analysis.

Costa Rica: Methacholine challenge testing

After completion of baseline spirometry, subjects whose FEV_1 was at least 65% of predicted underwent methacholine challenge testing using a modified version of the Chatham protocol (6). The study protocol consisted of five breaths of saline solution

followed by one breath of a 1 mg/ml methacholine solution, one and four breaths of a 5 mg/ml methacholine solution, and one breath of a 25 mg/ml methacholine solution. All inhalations were taken from a DeVilbiss 646 nebulizer (Sunrise Medical, Carlsbad, CA) and lasted 6 seconds. Each inhalation was followed by a 2 second breath- hold. Spirometry was performed at 180, 210, and 240 seconds after each methacholine dose increase. The test was terminated if the FEV_1 declined by at least 20% from the patient's best baseline FEV_1 value (after inhalation of saline solution). For the statistical analysis, airway responsiveness was $\log_{10}$-transformed.

Costa Rica: Dust Mite Allergen Measurement

A Douglas vacuum cleaner (model 6735) was used to collect a global dust sample from different areas of the child's household: the upper mattress surface of the child's bed, a sample from upholstered furniture in the family room or the living room, a floor sample from the child's bedroom, the floor of the family room or living room, and the floor of the kitchen. Dust samples collected in Costa Rica were mailed to the Allergy and Immunology Reference Laboratory of Johns Hopkins Hospital, where the dust was weighed, sifted, and aliquoted for measurement of Der p 1 allergen by two-site monoclonal antibody ELISA assays (9).

Study Procedures: CAMP

CAMP: Questionaire

Phenotypic data were collected at baseline and during the course of the clinical trial as previously described (8,9). The research coordinator at each study site collected phenotypic data by interviewing the parents or guardians of the patients or the children themselves. Data collected included information regarding demographics, history of

asthma symptoms and severity, treatment of asthma, atopy history, characteristics of the home environment, and relevant family history(8).

CAMP: Pulmonary Function Tests

Spirometry was performed by certified pulmonary function technicians, at least 4 hours after the use of a short-acting bronchodilators and 24 hours after the last use of a long-acting bronchodilator. Spirometry was performed on a Collins Stead-Wells dry-seal Survey III spirometer and met or exceeded the American Thoracic Society (ATS) criteria for acceptability and reproducibility(5).

CAMP: Methacholine Challenge Testing

Airway responsiveness was determined by methacholine testing, which was performed by certified pulmonary function technicians. Methacholine challenge testing was performed with the Wright nebulizer technique using the method of Cockcroft and coworkers modified for methacholine administration by Juniper and coworkers (10,11) and described in detail elsewhere (12) . After a control diluent challenge, nine doubling doses of standardized methacholine (University of Iowa School of Pharmacy) ranging from concentrations of 0.098 to 25 mg/ml in 3 ml of saline solution were nebulized for 2 min each at 5-min intervals in a Wright nebulizer calibrated to a specific flow meter (9 L/min) (Western Enterprises, Westlake, OH) to deliver a final output of 0.13 ml/min. Spirometry was performed 90 s after each challenge until FEV_1 had fallen by 20% or more. Determinations of airway responsiveness were not made within 4 weeks of an upper respiratory tract infection, other viral illness, use of oral steroids, or if the FEV_1 at baseline was less than 70% predicted. The distribution of PC_{20} at all visits in the CAMP

study was skewed, with a long right tail. Thus, the PC_{20} values were log-transformed for all statistical analyses.

CAMP: Measurement of Dust-Mite Allergen Exposure

In CAMP, a total of five dust samples were collected in the same fashion as described in Costa Rica. Dust samples were analyzed for dust mite allergen using standardized mAB-based immunoenzymetric assays at a central laboratory (6).

Data Analysis

Genotyping

Nine SNPs, including three known functional variants, were genotyped in 416 parent-child trios from the Central Valley of Costa Rica. SNPs were selected from the HapMap (http://www. www.hapmap.org) and dbSNP (http://www.ncbi.nlm.nih.gov/SNP) databases to tag all common haplotype blocks in *TGFB1* and to include known functional variants. The nine SNPs genotyped in *TGFB1* capture $\geq$ 80% of the HapMap SNPs with minor allele frequency (MAF) $\geq$10% in *TGFB1* and its 10-kb flanks in CEU trios at an $r^2 \geq 0.97$. SNP genotyping was performed using the Illumina Golden Gate platform (Illumina Inc, San Diego, CA). Duplicate genotyping was performed on ~5% of the sample to assess the quality of genotyping. Genotype data quality was assessed by completion rates, discordance in duplicate genotyping, and evidence of Mendelian inconsistencies in the data. Genotype quality was high in both populations with an average completion rate of 97% and no discrepancies noted between the initial genotyping and the 5% of samples that underwent repeat genotyping.

Statistical Analysis

Hardy-Weinberg equilibrium was tested in parental data by using χ^2 goodness-of-fit test, and deviations from Mendelian inheritance were tested with PedCheck (13). Genotypes of families with Mendelian inconsistencies were set to missing. Estimates of D' and R^2 were obtained from Haploview v3.11(14)**.** Phenotypes analyzed included pulmonary function, airway responsiveness, and asthma exacerbations. All analyses were performed under a dominant genetic models , consistent with pre-existing data for *TGFB1* and to facilitate comparison of our results with those of previous publications (15). In both cohorts, SNPs and haplotypes were tested for association with asthma and its intermediate phenotypes using the family-based association test statistic implemented in PBAT version v3.2 (16). Association tests were adjusted for covariates for pulmonary function (age, gender, height), airways responsiveness (age, gender, and height), and asthma hospitalizations (age, gender, and use of inhaled steroids and/or leukotriene inhibitor). P values presented are nominal (unadjusted for multiple comparisons), as we defined significant associations in Costa Rica by independent replication in CAMP for a phenotype under the same genetic model and with the same direction of association and a Fisher's combined p value <0.001. Phenotypic residual values for asthma related phenotypes were generated in SAS version 9.1 SAS Institute, Cary, NC). The direction of each association was analyzed evaluating the phenotypic residuals in FBAT version 1.5 (18). A joint P value was calculated for replicated associations using Fisher's method (19).

In order to capitalize on the longitudinal nature of the CAMP data, we conducted association studies for *TGFB1* and another indicator of asthma severity: the number of asthma-related hospitalizations required during the four-year trial. These analyses were

performed using Poisson regression models and were adjusted for age, gender, and use of inhaled corticosteroids.

The analysis of interactions between *TGFB1* polymorphisms and dust mite allergen exposure on airway responsiveness and asthma-related hospitalizations was performed using FBAT_I. Given previous findings from studies of Der p 1 and asthma exacerbations, levels of Der p 1 in house dust were dichotomized at 10 μg/g for this analysis (21, 22). This dichotomization has been used in previous analyses from our group(23).

Online Supplement Tables:

Table E1. Family-based analysis of association between variants in *TGFB1* and lung function phenotypes in Costa Rica*

Phenotype	dbSNP rs#	Allele (frequency)	# Informative families	Z Score	P value
Post-bronchodilator FEV_1	rs6957	T (0.79)	71	2.49†	0.01
Post-bronchodilator FEV_1/FVC	rs6957	T (0.79)	71	2.20	0.03

*Under a dominant genetic model and adjusted for age, gender, and height
**No significant associations with lung function noted in CAMP
† Positive Z-score denotes that the allele is associated with a higher spirometric measurement of lung function.

Table E2a: Haplotype block associations for variants in *TGFB1* with airways responsiveness

Phenotype	Haplotype block	Haplotype	Costa Rica haplotype frequency	Costa Rica P Value	CAMP haplotype frequency	CAMP p value
Airways Responsiveness	Block 1	A:A:C	0.11	0.0002	0.08	NS†
		A:G:G	0.06	0.005	0.03	NS
		G:C:T	0.13	NS	0.49	0.03
		G:T:A	0.04	NS	0.52	0.009
	Block 1	A:T:C	0.12	0.02	0.79	NS
		A:C:C	0.14	NS	0.06	0.04
Asthma Exacerbations	Block 1	C:A	0.52	0.02	0.08	NS
	Block 2	C:A:C	0.12	0.02	0.06	NS

*Block definitions:
Block 1: SNPs rs, 2241718, rs6957, rs8179181
Block 2: SNPs rs1982073, rs2241712, rs1800469
†Non-significant (NS) p>0.05 for haplotype.

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