Online Data Supplement

Extended Haplotype in the TNF Gene Cluster Is Associated with Asthma and Asthma Related Phenotypes

Adrienne G. Randolph, MD, MSc

Christoph Lange, PhD

Edwin K. Silverman, MD, PhD

Ross Lazarus, MBBS, MPH

Scott T. Weiss, MD, MS

CAMP Population

The diagnosis of asthma was based upon methacholine hyperreactivity (PC_{20} no greater than 12.5 mg/ml) and one or more of the following criteria for at least 6 months in the year prior to recruitment: (1) asthma symptoms at least two times per week; (2) at least two usages per week of an inhaled bronchodilator; and (3) daily asthma medication. Spirometry was performed according to the American Thoracic Society recommendations using a volume-displacement spirometer, and airway responsiveness was assessed by methacholine challenge using the Wright nebulizer tidal breathing technique [E1]. Serum eosinophil counts were performed at each enrolling center. Allergy testing was performed using skin prick tests for 10 common allergens as well as locally relevant allergens. Total serum immunoglobulin E (IgE) was measured using the ImmunoCAP assay (Pharmacia) on blood samples collected during the screening sessions.

Choice of SNPs

SNPs were chosen from resequencing data reported on SeattleSNPs, NHLBI Program for Genomic Applications, UW-FHCRC, Seattle, WA (URL: http://pga.mbt.washington.edu) [March 2003 accessed]. This data was derived from 23 Caucasian individuals from the CEPH collection (DNA from the Coriell Cell Repository). We identified the previously reported LTA *Nco*I polymorphism as SNP LTA 2374. The previously reported TNF SNP that was reported as being significantly overtransmitted to Japanese atopic asthmatics [E2] was found on the SeattleSNPs website in LTA beyond the 3' UTR region. This SNP was very close to another SNP, which could interfere with genotyping accuracy using the Sequenom system. Therefore, we chose a SNP in TNF (SNP 1078) that was in 100% linkage disequilibrium in the CEPH sample with the SNP found to be positively associated in the Japanese. We also genotyped the

LTA 4371 SNP so that we could replicate the analysis of the haplotypes reported in the Japanese study. The TNF -308 and LTA 4371 SNPs are in the flanking regions of the gene and the TNF 1078 and LTA *Nco*I SNPs are in the intronic regions.

We ran the combined TNF and LTA sequencing data reported in the CEPH European population from the SeattleSNPs website (URL above) through Phase [E3] using the bioinformatics tools on the Innate Immunity Website, NHLBI Program for Genomic Applications, Channing Laboratory, Boston, MA (URL: http://innateimmunity.net/) [October 2002 accessed]. The Phase output was then put into the BEST program [E4] from the same Innate Immunity Website to identify haplotype tagging SNPs for the European population. The four SNPs chosen for genotyping should have been able to distinguish all of the common (>5%) haplotypes identified in the European population (See Table 3).

Molecular Methods

SNP genotyping in the CAMP cohort was performed using the Sequenom system. The forward and reverse PCR primers used are shown in Table E1. Samples of genomic DNA were subjected to standard polymerase chain reactions (PCR) to amplify genomic DNA flanking the target polymorphism. We amplified 2.5 ng of genomic DNA in a 5 μl reaction containing 0.1 U HotStar Taq polymerase and 1x HotStart Taq PCR Buffer (Qiagen Inc., Valencia, CA), 2.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dNTPs) (USB, Cleveland, OH), and 50 nM of each PCR primer. Samples were incubated at 95°C for 15 minutes followed by 45 cycles of 95°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, followed by 3 minutes at 72°C on a 384-well DNA Engine Tetrad (PTC225, MJResearch Inc., South San Francisco, CA). Excess dNTPs were then removed from the reaction by addition of 0.3 U shrimp alkaline

phosphatase (SAP) (USB) in Thermosequenase RCTN Buffer (USB) at 37°C for 20 minutes followed by 5 minutes at 85°C. Amplified PCR product was used as a template in a second, modified single-primer minisequencing reaction, whereby either single-base extension and chain termination or two to three base extensions occurs at the variant allele. Extension reactions contained 600 nM of extension primer, 50 μM d/ddNTP in Thermosequenase RCTN Buffer and 0.126 U Thermosequenase (USB). Samples were at 94°C for 2 minutes followed by 45 cycles of 94 ^{o}C for 5 seconds, 52 ^{o}C for 5 seconds, and 72 ^{o}C for 5 seconds. The minisequencing reaction was then desalted by addition of SpectroClean resin (Sequenom). Using a nanoliter-plotting robot (SpectroPLOTTER, Sequenom), the purified minisequencing product was then spotted onto a chip (SpectroCHIP, Sequenom) containing matrix pads. The matrix aids in desorption and ionization of the DNA. Chips were individually analyzed using the Brukker Bi-flex MALDI-TOF mass spectrometer (Sequenom). The resulting spectra were converted to genotype data using SpectroTYPER-RT software (Sequenom), which interprets the spectral output based on information for expected allele-specific oligonucleotide lengths generated during the assay design phase. As a quality control measure, genotyping was repeated for at least 8% of the sample for each SNP and tested for discordance.

Time-Series Data

The time series data were obtained from the multiple visits before, at and after study entry from children in the CAMP population. The CAMP study took place over a four-year period. We tested the following four time-series: body mass index (BMI); logarithm of the provocative concentration of methacholine causing a 20% fall in FEV_1 from baseline (LNPC20);

baseline FEV1% predicted (PRE-FEV); post-bronchodilator FEV1% predicted (POS-FEV). See Table E2 for the mean values on each visit for the CAMP population.

References

E1. The Childhood Asthma Management Program (CAMP): design, rationale, and methods. Childhood Asthma Management Program Research Group. *Control Clin Trials* 1999;20:91-120.

E2. Noguchi E, Yokouchi Y, Shibasaki M, Inudou M, Nakahara S, Nogami T et al. Association between TNFA polymorphism and the development of asthma in the Japanese population. *Am J Respir Crit Care Med* 2002;166:43-46.

E3. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 2001;68:978-989.

E4. Sebastiani P, Lazarus R, Weiss ST, Kunkel LM, Kohane IS, Ramoni MF. Minimal haplotype tagging. *Proc Natl Acad Sci USA* 2003;100:9900-9905.

Table E1: PCR Primers and Extension Probes Used

TNF_-308
ACGTTGGATGGGTCCCCAAAAGAAATGGAG
ACGTTGGATGGATTTGTGTGTAGGACCCTG
GGAGGCTGAACCCCGTCC
(reverse)

TNF_1078
ACGTTGGATGCTTGCCACATCTCTTTCTGC
ACGTTGGATGGAAAGATGTGCGCTGATAGG
GGGAGGGATGGAGAGAAAAAAAC
(forward)

LTA-NcoI
ACGTTGGATGGAGAGACAGGAAGGGAACAG
ACGTTGGATGACTCTCCATCTGTCAGTCTC
CACATTCTCTGTTTCTGCCATG
(forward)

LTA-4371
ACGTTGGATGGGGAAGCAAAGGAGAAGCTG
ACGTTGGATGTACATGTGGCCATATCTCCC
CAGACCCTGACTTTTCCTTC
(reverse)

Table E2: Time series phenotype data in CAMP Population

I. Pre-bronchodilator FEV1% predicted (mean)

Baseline visit: 95.1

Year 1 (2, 4, 8, 12 month visits): 93.7; 95.7; 94.9; 95.2

Year 2 (4, 8, 12 month visits): 94.7; 94.9; 94.9

Year 3 (4, 8, 12 month visits): 94.9; 95.0; 94.5

Year 4 (4, 8 month visits): 95.9; 95.0

II. Post-bronchodilator FEV1% predicted (mean)

Baseline visit: 105.5

Year 1 (2, 4, 8, 12 month visits): 103.0; 104.6; 103.9; 103.8

Year 2 (4, 8, 12 month visits): 103.2; 103.1; 102.8

Year 3 (4, 8, 12 month visits): 102.8; 102.7; 102.7

Year 4 (4, 8, month visits): 103.5; 103.0

III. Body Mass Index (kg/m^2)

Baseline visit: 18.2

Year 1 (2, 4, 8, 12 month visits): 18.4; 18.7; 18.9; 19.2

Year 2 (4, 8, 12 month visits): 19.2; 19.5; 19.7

Year 3 (4, 8, 12 month visits): 19.9; 20.3; 20.5

Year 4 (4, 8 month visits): 20.8; 21.1

IV. Logarithm of PC20

Baseline visit: 0.48

Years 1-4 at 8 month visit: 0.68; 0.75; 0.90; 0.86