

Validation of GWAS Loci for Atopic Dermatitis in a Singapore Chinese Population

Journal of Investigative Dermatology advance online publication, 9 February 2012; doi:10.1038/jid.2011.471

TO THE EDITOR

Atopic dermatitis (AD) is a complex trait resulting from an interaction between a large variety of environmental and genetic factors (Irvine and McLean, 2006; Rodríguez *et al.*, 2009; van den Oord and Sheikh, 2009). Research on the filaggrin gene (*FLG*) in Asian populations including Singapore has confirmed that allelic variants in this gene influence risk to AD (Akiyama, 2010), as in Europe, while also strengthening the evidence for other factors involved in the disease etiology (Chen *et al.*, 2011). Recently, Sun *et al.* (2011) reported a genome-wide association study on AD in which they identified two new susceptibility loci at 5q22.1 (rs7701890) and 20q13.33 (rs6010620), and one suggestive locus at 10q21.2 (rs2393903) in the Chinese population. The previously unreported associations were validated in Northern and Southern Chinese, but only the 20q13.33 (rs6010620) locus was validated in a German population, supporting the presence of ethnic differences or environmental influences on the observed genetic associations.

Here, we analyzed these three loci in 827 AD cases and 1,104 controls (Supplementary Information online), all of which were Singaporean Chinese (Andiappan *et al.*, 2011). All three single-nucleotide polymorphisms (SNPs) were directly assayed by Taqman genotyping and were found to be in Hardy-Weinberg equilibrium in controls ($P > 0.05$). The chromosome (chr)10 and chr20 SNPs showed significant association in our population (odds ratio (OR) = 1.26 (95% confidence interval (CI) 1.11–1.44), $P = 0.0004$; and OR = 1.18 (95% CI 1.02–1.36),

$P = 0.024$, respectively). The effect sizes of these associations are similar to that reported in Sun *et al.* (Table 1). However, the chr5 SNP rs7701890 showed no evidence of association (OR = 0.96 (95% CI 0.81–1.13), $P = 0.59$). With our current sample size and the minor allele frequency of 20% in Singaporean Chinese, we estimate that we had close to 80% power to detect the effect observed by Sun *et al.* (OR 1.24; Purcell *et al.*, 2003).

The well-known association of atopy with allergic phenotypes (Bousquet *et al.*, 2001, 2008) prompted us to investigate the association of the three SNPs with positive skin prick test (SPT) results in our cohort. We analyzed a total of 2,627 SPT-positive and 389 SPT-negative individuals, which included both AD cases and controls. There was no association with positive SPT status (Supplementary Table S1 online), and adjustment for this SPT status had little or no impact on the AD association results for the chr10 and chr20 SNPs. We then tested the three above-mentioned SNPs (rs7701890, rs2393903, and rs6010620) for association with allergic rhinitis (AR; 472 cases) and asthma (323 cases), in the absence of AD. The rs6010620 SNP on 20q13.33 was associated with AR ($P = 0.008$), although further validation of this result is required. Although the 5q22.1 locus has recently been reported to be associated with AR (Ramasamy *et al.*, 2011), the SNP we tested was not in linkage disequilibrium with any of the reported SNPs ($r^2 = 0$ with rs17513503 and rs1898671).

The AD cases were separated into two groups (i) AD only and (ii) AD with other associated atopic conditions

(asthma, AR, or both). We found the risk C allele of the chr10 locus showed a significantly stronger association with the group with AD only compared with the group with AD and other atopic conditions (OR = 1.45 (95% CI 1.21–1.79) vs 1.19 (1.03–1.38), $P = 0.038$; Table 1). We then performed a quantitative trait analysis in a subset of 397 AD cases whose disease severities were determined by the SCORing Atopic Dermatitis (SCORAD) index (Oranje *et al.*, 2007). Consistently, we found the CC genotype of rs2393903 at chr10 was significantly associated with a lower SCORAD score (mean 33.99 ± 1.568 ; $n = 94$), compared with the CT (mean 39.48 ± 1.043 , $n = 197$; t -test $P = 0.0034$) or TT genotypes (mean 40.66 ± 1.491 , $n = 98$; t -test $P = 0.0023$; Table 1 and Figure 1). Our findings suggest that the chr10 risk allele is associated with a milder form of AD, whereas the chr20 SNP (rs6010620) showed no effect on AD severity.

We also analyzed effects of these three SNPs on serum measurements of total IgE and specific IgE to *Blomia tropicalis* and *Dermatophagoides pteronyssinus* mites in a subset of 151 individuals from the genotyped cohort. The difference in the level of specific IgE towards *Blomia tropicalis* was observed among different genotype groups of rs2393903 ($P = 0.014$), but did not survive correction for multiple testing (Supplementary Table S2 online and Supplementary Figure S1 online).

To test whether the lack of association on chr5 in our Singaporean Chinese AD samples could be attributed to interaction with environmental factors, we conducted gene-environment interaction analysis using a logistic regression model in a subset of our samples (up to 432 cases and 700 controls) for which data on alcohol

Table 1. Association test results on the three SNPs with AD and AD severity

Chr	SNP	MAF controls	Power to detect effect in Sun <i>et al.</i> (2011) ¹	All AD		AD only		AD + others		Association with severity	SCORAD
				827 Cases, 1,104 controls		251 Cases, 1,104 controls		572 Cases, 1,104 controls		251 AD only, 572 AD + others	n=397
				OR	P-value	OR	P-value	OR	P-value		
5	rs7701890	0.20 (G)	79.0%	0.956 (0.809–1.129)	0.594	1.107 (0.870–1.416)	0.411	0.887 (0.732–1.073)	0.218	0.109	0.861
10	rs2393903	0.42 (C)	57.7%	1.264 (1.109–1.440)	0.0004	1.447 (1.210–1.791)	0.0002	1.190 (1.028–1.378)	0.021	0.038	0.003
20	rs6010620	0.28 (G)	60.7%	1.179 (1.022–1.360)	0.024	1.045 (0.842–1.303)	0.691	1.244 (1.061–1.459)	0.007	0.159	0.990

Abbreviations: AD, atopic dermatitis; Chr, chromosome; MAF, minor allele frequency; OR, odds ratio; SCORAD, SCORing Atopic Dermatitis; SNP, single-nucleotide polymorphism.

¹Genetic power calculator ($\alpha=0.05$) based on current sample size and MAF in the controls.

²Comparing association with AD only versus AD+other atopic phenotypes (allergic rhinitis, asthma, or both).

³One-way analysis of variance (ANOVA) comparing SCORAD scores across the three genotype groups (Kolmogorov-Smirnov normality test $P>0.05$).

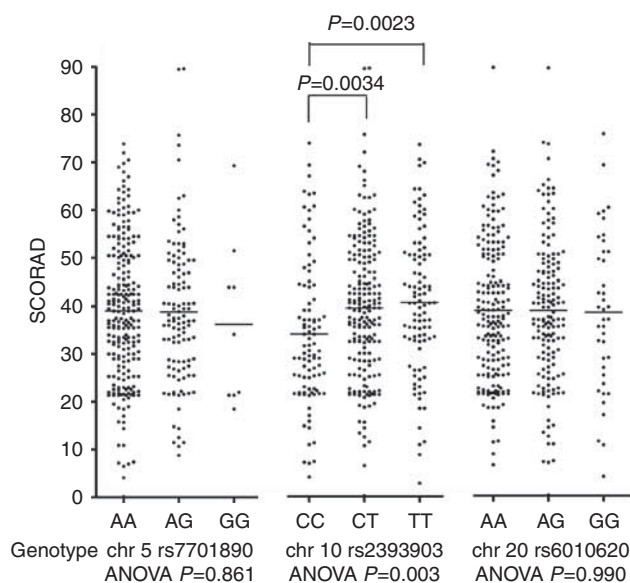


Figure 1. Distribution of SCORing Atopic Dermatitis (SCORAD) scores for atopic dermatitis (AD) subjects ($n=397$) grouped according to their genotypes at rs7701890 (chromosome (chr)5), rs2393903 (chr10), and rs6010620 (chr20). The solid line indicates the mean of each group. One-way analysis of variance (ANOVA) test P -values comparing the three genotype groups, as well as Student's t -test P -values comparing the CC with CT and TT genotype groups at rs2393903 are shown.

consumption, smoking and/or smoking in subject's presence, father's and mother's education level, and household income were available. We observed a significant interaction with household income ($P=0.017$), in which the minor allele G showed a risk effect (OR = 1.22 (95% CI 0.92–1.61), $P=0.17$) in the low-income group (<\$4,000 a month) but a protective effect (OR = 0.71 (95% CI 0.49–1.01), $P=0.06$) in high-income group (>\$4,000 a month; Supplementary Table S3 online). In previous studies prevalence of allergic diseases

has generally been shown to vary with income level, with reduced prevalence in lower income groups in Singapore (Goh *et al.*, 1996) and Hong Kong (Lau *et al.*, 1995), but increased prevalence with lower income in countries like the United States (Hanifin and Reed, 2007). Environmental factors might therefore be an important confounder, for which income is potentially a surrogate marker in our study. We speculate that this may explain why our study did not validate the Chinese finding on chr5, as there is likely to be higher percentage

of subjects from a high-income environment (38.9%) in the Singaporean Chinese population compared with the subjects of Chinese Han studied in Sun *et al.* However, more extensive and well-designed studies will be needed to further dissect these interactions between diseases, and social and environmental factors.

Finally, having genotyped a subset of our samples for *FLG* mutations (397 cases, 400 controls) and demonstrated strong association with AD in these samples (Chen *et al.*, 2011), we performed gene-gene interaction analyses between the three SNPs and *FLG*-null mutation carrier status. No evidence of interaction was observed at any of the loci ($P>0.05$; Supplementary Table S4 online), suggesting that these SNPs act independently of *FLG*-null mutations towards the contribution of atopic disease. However, we note that our power to detect interaction may be limited by insufficient sample numbers (Purcell *et al.*, 2003).

In summary, our findings validate a strong association at chr10q21.2 and a weaker one at 20q13.33 with AD among Singaporean Chinese subjects, but suggests that genetic association at 5q22.1 might be influenced by the interaction with environmental factors. The identification of new validated AD loci is important to further dissect the genetic basis of this complex disease and account for the large number of AD cases that do not have *FLG*-null mutations. Clinically this is also vital as more genome-targeted therapies are developed.

URLS

Genetic Power Calculator: <http://pngu.mgh.harvard.edu/~purcell/gpc/>

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Chang Hua Wong (GIS), Wing Shan Ng (NUS), Nancy Liew (IMB), Winnie Sia, Doreen Tay, and Veron Lu (NSC) for sample processing and data collection. We also thank the affected individuals for their participation, which made this research possible. The study was supported by the grants from the Singapore Immunology Network (SIgN-06-006 and SIgN-08-020); National Medical Research Council (NMRC/1150/2008), Singapore; Biomedical Research Council, Singapore; and National University of Singapore (NUS) for the Graduate Research Scholarship for students involved in the study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Validation of GWAS loci for atopic dermatitis in a Singapore Chinese population

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SUPPLEMENTARY DATA

			Atopy 2627 SPT (+), 389 SPT (-)		All Allergic rhinitis 884 cases, 704 controls		Allergic rhinitis only 472 cases, 704 controls		All Asthma 687 cases, 704 controls		Asthma only 323 cases, 704 controls	
Chr	SNP	MAF* controls	OR	P-value	OR	P-value	OR	P-value	OR	P-value	OR	P-value
5	rs7701890	0.20 (G)	1.069 (0.877- 1.303)	0.511	0.865 (0.722- 1.037)	0.118	0.885 (0.715- 1.096)	0.264	0.943 (0.781- 1.140)	0.546	0.855 (0.671- 1.090)	0.207
10	rs2393903	0.42 (C)	0.937 (0.804- 1.091)	0.402	1.017 (0.882- 1.174)	0.814	0.992 (0.838- 1.174)	0.925	1.065 (0.916- 1.239)	0.414	1.010 (0.835- 1.221)	0.917
20	rs6010620	0.28 (G)	0.966 (0.816- 1.143)	0.687	0.906 (0.774- 1.062)	0.223	0.771 (0.636- 0.934)	0.008	1.043 (0.884- 1.231)	0.620	1.047 (0.851- 1.288)	0.663

Supplementary Table 1: Association test results on the three SNPs with atopy (positive reaction to a skin prick test [SPT]), only allergic rhinitis and only asthma. *MAF refers to Minor Allele Frequency

Chr	SNP	Total IgE (kU/l)	IgE specific to <i>D. pteronyssinus</i> (kUA/l)	IgE specific to <i>B. tropicalis</i> (kUA/l)
Average (n = 151)		237±17(3.3 to 1591)	23 ± 5 (<0.35 to >100)	20 ± 4 (<0.35 to >100)
5	rs7701890	0.197 (0.468)	0.614 (0.923)	0.356 (0.635)
10	rs2393903	0.373 (0.317)	0.999 (0.753)	0.014 (0.097)
20	rs6010620	0.736 (0.687)	0.276 (0.201)	0.735 (0.653)

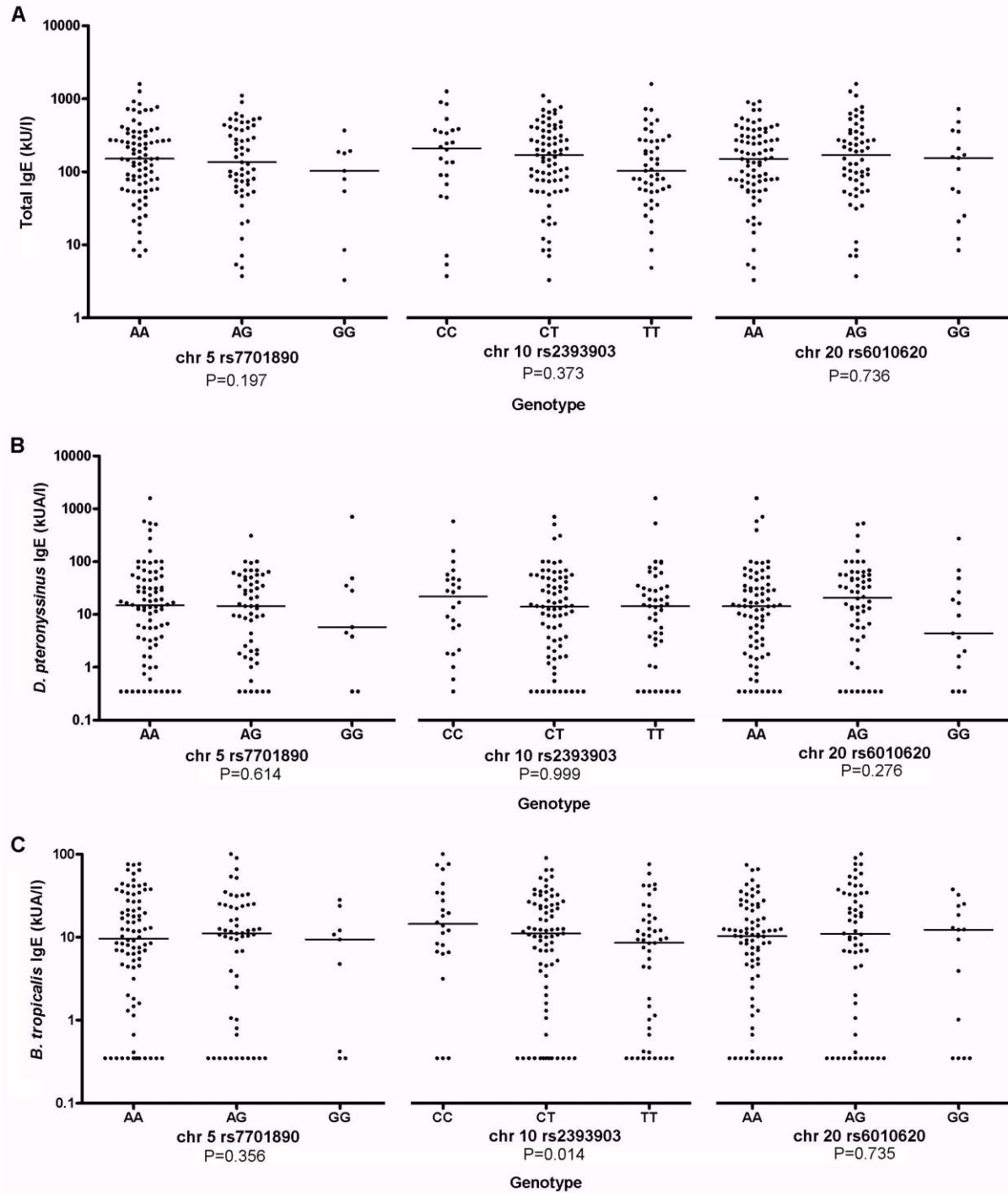
Supplementary Table 2: Linear regression *P*-values from comparisons of total serum IgE (log transformed to approximate a normal distribution) and IgE specific to *Dermatophagoides pteronyssinus* and *Blomia tropicalis* (square root transformed to approximate a normal distribution) across three genotype groups of each SNP in 151 subjects. The mean, standard error of mean and range of each measurement is given to the nearest whole number. *P*-values from non-parametric Kruskal-Wallis tests on the original measurements are also shown in brackets.

Chr	5
SNP	rs7701890
G x E interaction tests (PLINK)	<i>P</i> -value
Alcohol Consumption	0.250
Education (Father's)	0.672
Education (Mother's)	0.981
Gender	0.427
Household Income	0.017
Housing	0.599
Smoking in Sample's Presence	0.147
Stratified by household income	OR <i>P</i> -value
	1.218
Low income < \$4000 (245 cases, 424 controls)	(0.919-1.614) 0.171
	0.706
High income >\$4000 (167 cases, 260 controls)	(0.494-1.009) 0.055

Supplementary Table 3: Gene-environment interaction analyses on rs7701890

Genotype x Genotype		Interaction <i>P</i> -value	
Chr	SNP	<i>FLG</i> c.3321delA	All <i>FLG</i> -null mutations
5	rs7701890	0.888	0.899
10	rs2393903	0.291	0.148
20	rs6010620	0.697	0.303

Supplementary Table 4: Gene-gene interaction *P*-values between each of the three SNPs and the *FLG* c.3321delA mutation or all *FLG*-null mutation (Common *et al.*, 2011) in 397 cases and 400 controls.



Supplementary Figure1: Plots of the distribution of levels of a) total serum IgE and IgE specific to b) *Dermatophagoides pteronyssinus* and c) *Blomia tropicalis* for subjects in each genotype group at rs7701890, rs2393903 and rs6010620. Values are plotted on a log₁₀ scale and the solid lines indicate the median of each genotype group. *P*-values are based on linear regressions on log- or square root- transformed IgE levels.

Supplementary methods

NUHS patients and controls

Ethics Statement

This study has been performed with the approval of the Institutional Review Board of National University of Singapore (IRB, Reference - NUS07-023 and NUS10-343) and is also in compliance with the Helsinki declaration. DNA samples used in this study were collected from ethnic Chinese participants following standard protocols of informed consent. The consent obtained was a “written consent” collected using the Participant Information Sheet that had information about the study.

Samples

Study subjects were recruited at the National University of Singapore through multiple volunteer recruitment drives as described previously (Andiappan *et al.*, 2011).

Disease definition

Atopy is defined by a positive skin prick test (SPT) reaction to one of the common inhalant allergens (particularly dust mite allergens in our study) (Bousquet *et al.*, 2001; Bousquet *et al.*, 2008; Andiappan *et al.* 2011). In the current study, atopy was defined by a positive skin prick test (SPT) reaction to one of the common inhalant allergens, particularly dust mite allergens.

Allergic rhinitis (AR) is diagnosed based on the presence of atopic status and typical AR symptoms as defined by the ARIA guidelines (Bousquet *et al.*, 2001; Bousquet *et al.*, 2008) i.e., two or more AR symptoms (nasal congestion, rhinorrhea, nasal itching, sneezing) persisting for four or more days a week during the past year.

Asthma is diagnosed based on the presence of one or more symptoms (wheezing, troublesome cough especially at night, cough or wheeze after exercise, and tightness of chest) (Bousquet *et al.*, 2008), and a physician diagnosed asthma.

Controls were identified as subjects that are negative for all three allergic conditions (AD, AR and asthma) (N=704) (Andiappan *et al.*, 2011)

IgE measurement in Serum

Measurement of serum total IgE and specific IgE to *Dermatophagoides pteronyssinus* and *Blomia tropicalis* was performed using Immunocap based in vitro assays designed by Phadia Pvt. Ltd at the Allergy Lab, Singapore.

IMB patients

We collected saliva samples from 425 Singaporean Chinese patients treated at the paediatric dermatology and eczema outpatient clinic in the National Skin Centre or National University Hospital. At recruitment, each patient was examined for feature of AD, palmar hyperlinearity and keratosis pilaris. Questionnaire data collected included demographic characteristics, age of AD onset, and history of atopic diseases such as asthma or allergic rhinitis. All patients were diagnosed with AD by two paediatric dermatologists according to the UK Working Party's diagnostic criteria for AD (Williams *et al.*, 1994) and AD severity was graded as mild, moderate or severe according to the objective SCORing Atopic Dermatitis index (SCORAD) (Oranje *et al.*, 2007). The mean age of 425 Singaporean Chinese in the childhood-adolescent AD cohort was 10 years old (range 1-21 years old; standard deviation 5.18 years) and 68% of patients were male.

IMB controls

Genomic DNA from 400 Singaporean Chinese individuals were obtained from the Singapore Bio Bank and used as unselected population controls. These controls of unknown IV/AD status were specifically matched for Chinese ethnicity, with a mean age of 44 years (range 1-80 years old; standard deviation 14 years) and comprised of 44.1% males (Common *et al.*, 2011)

Combined controls

There were 2 set of controls (i) 704 controls from the previous publication by Andiappan *et al.*, 2011 and (ii) 400 controls from IMB cohort (Common *et al.*, 2011). Thus representing the total of 1104 controls mentioned in the main text of the letter.

Exclusion criteria and ethnicity of all cases and controls

Patients with known congenital and X-linked ichthyoses, equivocal diagnosis of AD or mixed ethnicities were excluded from the study. To avoid ethnic admixture due to inter-racial marriages, we only included cases where the patients, their parents and all four grandparents were recorded as ethnic Chinese. All patients satisfying the diagnostic and ethnic criteria described above were invited for study participation and included after informed written consent from all patients or their parents/guardians was obtained. This study was approved by the local

Domain Specific Review Board (DSRB) guidelines that are in full accordance with the Declaration of Helsinki Principles.

SNP genotyping

Samples from the NUHS cohort and the IMB cohort were genotyped for the 3 SNPs using Taqman assays (pre-designed) were obtained from Applied Biosystems. TaqMan reactions were carried out in 5 μ L volumes containing 10-20ng DNA according to the manufacturer's protocol. Fluorescence data was obtained in the ABI PRISM 7900HT machine and SDS 2.4 software (Applied Biosystems) was used to call genotypes. Cluster plots were visually inspected to ensure good quality genotype calls.

Statistical analyses

All statistical analyses were performed in PLINK v1.07 and GraphPad Prism 4. We used Cochran-Mantel-Haenszel tests for all case-control analyses combining the two studies and logistic regression models to test for SNP x covariate interactions. For quantitative traits, we used one-way ANOVA, linear regression or student's t-test to test for association across the genotype groups. A Kolmogorov-Smirnov goodness-of-fit test was used to test for normality and in cases where the assumption of normality was violated, we conducted non-parametric tests and analyzed log transformed or square root transformed values.

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