Brief Genetics Report

Analysis of Polymorphisms of the Interleukin-18 Gene in Type 1 Diabetes and Hardy-Weinberg Equilibrium Testing

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Recently, the interleukin-18 cytokine gene (*IL18*) was reported to be associated with type 1 diabetes. In the present report, we calculated that the reported genotypes of the two 5′ region/promoter single nucleotide polymorphisms (SNPs), −607 (C→A) (rs1946518) and −137 (G→C) (rs187238), were not in Hardy-Weinberg equilibrium (HWE). We therefore investigated the association of the −607 and −137 SNPs in a U.K. type 1 diabetic Caucasian case-control collection (1,560 case and 1,715 control subjects tested at −607 and 4,323 case and 4,610 control subjects tested at −137) as well as a type 1 diabetic Caucasian collection comprised of families of European ancestry (1,347 families tested at −137 and 1,356 families tested at −607). No evidence for association with type 1 diabetes was found, including for the −607 A/A and C/A genotypes. To evaluate whether common variation elsewhere in the gene was associated with disease susceptibility, we analyzed eight *IL18* tag SNPs in a type 1 diabetic case-control collection (1,561 case and 1,721 control subjects). No evidence for association was obtained (P = 0.11). We conclude that common allelic variation in *IL18* is unlikely to contribute substantially to type 1 diabetes susceptibility in the populations tested and recommend routine application of tests for HWE in population-based studies for genetic association. *Diabetes* 55:559–562, 2006

Interleukin (IL)-18 is a strong candidate for association studies with type 1 diabetes, as prior biological information exists and a central role for this inflammatory cytokine in autoimmunity has been shown (1–6). Two single nucleotide polymorphisms (SNPs) in the gene promoter located at positions −607 (C→A) and −137 (G→C) are present in European American subjects at minor allele frequencies (MAFs) of 0.46 and 0.30, respectively (estimates obtained from the Innate Immunity Programs for Genomic Applications [IIPGA] database located at http://www.innateimmunity.net). These promoter SNPs have been associated with decreased *IL18* expression (7). Allele C at −137 (G→C) has been shown experimentally to disrupt a confirmed H4TF-1–binding site, while nucleotide substitution at −607 (C→A) may disrupt a potential cAMP-responsive element–binding site (7). In an *IL18* promoter transcription activity assay, Giedraitis et al. (7) demonstrated low promoter activity following stimulation for both the A and C alleles at positions −607 (C→A) and −137 (G→C) when present on the same haplotype. These results are suggestive of functionality, rendering these promoter SNPs attractive candidates in tests for genetic association with immune-mediated diseases.

These promoter SNPs have also been implicated as susceptibility loci for various diseases, including type 1 diabetes (8,9), rheumatoid arthritis (10,11), sarcoidosis (12), atopic eczema (13), adult-onset Still's disease (14), and seasonal allergic rhinitis (15). At −137 (G→C), for example, the C allele showed evidence for a positive association with atopic eczema, seasonal allergic rhinitis, and type 1 diabetes (8,13,15). At −607 (C→A), the A allele and the A/A genotype were reported to be negatively associated with sarcoidosis in a Japanese sample and with rheumatoid arthritis in a Chinese sample (10,12). In type 1 diabetes, Kretowski et al. (8) genotyped 201 case and 194 unrelated control subjects from Poland and reported that the C allele at −137 (G→C) was associated with type 1 diabetes (odds ratio [OR] 1.6; P = 0.002). Kretowski et al. also obtained evidence for a genotypic effect at −137 (P = 0.0015), noting an increase, in case subjects, in the proportion of G/C (62.2% case and 48.5% control) and C/C (6.5% case and 3.0% control) genotypes. In addition, they reported that disease risk conferred by a given genotype at −137 (G→C) varied according to the genotype at −607 (C→A); the presence of a G/C or C/C genotype at −137 and either a C/C or C/A genotype at −607 was found to be positively associated with type 1 diabetes, whereas the presence of the A/A genotype at −607 with either a G/G or G/C genotype at −137 was not associated with disease. Ide et al. (9) also tested these promoter SNPs in a Japanese type 1 diabetic case-control collection (116 case and 114 control subjects), noting at −607 (P = 0.023) that the C/A genotype was present in a higher proportion of case compared with control subjects (69% in case and 57.9% in control subjects), whereas the proportion of case subjects with the A/A genotype was decreased (12.1% in case and 26.3% in control subjects). In addition, they found evi-

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HWE, Hardy-Weinberg equilibrium; IIPGA, Innate Immunity Programs for Genomic Applications; IL, interleukin; MAF, minor allele frequency; SNP, single nucleotide polymorphism.

TABLE 1
IL18 −137 (4,323 case and 4,610 control subjects) and −607 (1,560 case and 1,715 control subjects) allele and genotype frequencies in the type 1 diabetes case-control collection

SNP	Minor allele	Case	Control	OR	95% CI	*P* value for association
−607	A	1,223 (39.2)	1,306 (38.1)	1.06	0.95–1.19	0.27
−137	C	2,281 (26.4)	2,451 (26.6)	1.00	0.93–1.07	0.94
SNP	Genotypes	Case	Control	OR	95% CI	*P* value for association
	CC	573 (36.7)	651 (38.0)	1.00	Reference	
−607	CA	751 (48.1)	822 (47.9)	1.08	0.91–1.27	0.54
	AA	236 (15.1)	242 (14.1)	1.12	0.89–1.41	
	GG	2,334 (54.0)	2,469 (53.6)	1.00	Reference	
−137	GC	1,697 (39.3)	1,831 (39.7)	0.98	0.90–1.07	0.86
	CC	292 (6.8)	310 (6.7)	1.02	0.86–1.22	

Data are *n* (%) unless otherwise indicated. *P* values are for overall test of association and not for specific genotypes. There was no evidence of deviation from a multiplicative model ($P > 0.05$).

dence ($P = 0.006$) of an increase in the frequency of the −607C/–137G haplotype in case subjects (87.9% in case and 73.7% in control subjects) (9). At −607, Kretowski et al. (8) also reported an increase in the proportion of the C/A genotype in case relative to control subjects (64.2% case and 56.7% control subjects) and a decrease in the proportion of the A/A genotype in case subjects (0% case and 5.7% control subjects). Thus, despite discrepant results at −137, data obtained by the two studies at −607 showed some consistency, a decreased proportion of case subjects carrying the A/A genotype.

Kretowski et al. (8) provided no Hardy-Weinberg equilibrium (HWE) information. Testing for deviations from HWE is an important quality control step in population genetic studies (16,17), and routine and appropriate testing of genotype data has been strongly advocated by many authors (18–21). Deviations from HWE can be very informative. In control subjects, this could indicate that one or more of the model assumptions have been violated or that a genotyping error has occurred. In case subjects, deviation from HWE, assuming sources of error have been eliminated, may indicate the association of a locus with disease.

Consequently, we tested the genotype distributions of Kretowski's control subjects for deviations from HWE and found that both −607 and −137 were out of HWE due to an excess of heterozygotes ($P = 0.0002$ and $P = 0.002$, respectively). These deviations could be due to control subjects being sampled from different ethnic groups. Kretowski et al. does not provide any ethnicity information and therefore may not have controlled for this. Alternatively, such deviations could indicate a systematic genotyping error; Kretowski makes no mention of the quality control measures used, such as double scoring or duplicate typing. Arguably, heterozygous excess is most likely to occur as a result of genotyping error. Hypernormal control subjects could also cause deviation from HWE, which may indicate that the alternative hypothesis may be true, although the control subjects used by Kretowski et al. were normally selected, so this is unlikely to be the cause of the observed deviation from HWE.

Some loci will, by chance, deviate from HWE, and both −607 and −137 could be examples of these. However, if these loci were perfectly correct and only deviated from HWE by chance, then it becomes important to use the most appropriate test of association. When deviations from HWE have been observed and they cannot be attributed to genotyping error or to selection or nonrandom mating and are in fact due to some unknown factor, then the Pearson χ^2 test applied to allele counts is not appropriate, and a test such as the Armitage trend test applied to counts of genotypes should be used (22–25) in order to reduce the chance of false-positive associations (24).

Therefore, we sought to assess the validity of these reported associations at −607 and −137, including evaluation of the possibility that these SNPs show natural deviation from HWE, in a large sample collection consisting of a U.K. Caucasian case-control collection (1,560 case and 1,715 control subjects typed at −607 and 4,323 case and 4,610 control subjects typed at −137) as well as a type 1 diabetic Caucasian collection comprised of families of European ancestry from four countries, including the U.S., the U.K., Norway, and Romania (1,347 families tested at −137 and 1,356 families tested at −607). At the −137 (G→C) SNP, we had 94% power to detect a susceptibility allele (type 1 error, $\alpha = 0.01$) with an odds ratio of 1.15 and present at an MAF of 26% in our case-control collection (4,300 case and 4,600 control subjects). We had over 99% power to detect an effect size of 1.3. Similarly, we had 98% power in 1,465 families to detect a susceptibility allele (type 1 error, $\alpha = 0.01$) conferring a relative risk (RR) of 1.3 and present at an MAF of 0.26 and >45% power to detect a genetic risk factor present at a comparable MAF and conferring an RR of 1.15. At the −607 (C→A) SNP, our power increased, reflecting the increase in allele frequency for this SNP (MAF 0.40), giving 57% power to detect (type 1 error, $\alpha = 0.01$), in 1,600 case and 1,700 control subjects, a genetic risk factor with an OR of 1.15 and 99.6% power to detect a genetic risk factor with an OR of 1.3.

Genotypes at both loci were in HWE in both our control subjects and unaffected parents ($P > 0.05$). No evidence for association was obtained in the case-control collection either at −607 (OR 1.06 [95% CI 0.95–1.19], $P = 0.27$) or at −137 (1.00 [0.93–1.07], $P = 0.94$; Table 1). Likewise, association analysis in the family collection revealed no evidence of increased transmission of the minor allele to probands either at −607 (1.00 [0.91–1.09], $P = 0.96$) or at −137 (0.96 [0.87–1.05], $P = 0.37$; Table 2). We assumed a multiplicative model for both −607 and −137 in both the case-control collection and the families because this

TABLE 2
IL18 −137 and −607 transmission/disequilibrium test, and conditional logistic regression analyses in the type 1 diabetic family collection (1,347 families tested at −137 and 1,356 families tested at −607)

SNP	Minor allele (unaffected parental frequency)	Transmitted: untransmitted	Transmission (%)	RR	95% CI	*P* value for association
−607	A (39.4)	975:973	50.1	1.00	0.91–1.09	0.96
−137	C (26.3)	780:816	48.9	0.96	0.87–1.05	0.37

SNP	Genotype	Affected offspring	Pseudo-control	RR	95% CI	*P* value for association
−607	CC	762 (36.9)	2,258 (36.5)	1.00	Reference	
	CA	980 (47.5)	3,000 (48.4)	0.97	0.86–1.09	0.72
	AA	323 (15.6)	937 (15.1)	1.02	0.85–1.23	
−137	GG	1,122 (54.3)	3,369 (54.4)	1.00	Reference	
	GC	833 (40.3)	2,421 (39.1)	1.02	0.90–1.14	0.10
	CC	111 (5.4)	408 (6.6)	0.80	0.62–1.02	

Data are *n* (%) unless otherwise indicated. *P* values are for overall test of association and not for specific genotypes. There was no evidence of deviation from a multiplicative model ($P > 0.05$).

model was not shown to differ significantly from the full genotype model, which assumes no specific mode of inheritance. Importantly, the frequency of the −607 A/A genotypes did not differ between case and control subjects (15.1 and 14.1%, respectively) nor between affected offspring and pseudocontrol subjects (15.6 and 15.1%, respectively; Tables 1 and 2) and similarly for the C/A genotype. Our results are consistent with results from a recent study involving a small Northern Irish case-control collection (433 case and 426 control subjects) and 285 Northern Irish parent-offspring trios, 246 of which overlap with the U.K. component of our family collection (26).

Both Kretowski et al. (8) and Ide et al. (9) reported evidence for an associated haplotype. Ide et al. observed an increased frequency of the −607C/–137G haplotype in case subjects, while Kretowski et al. observed an increased frequency of the −607C/–137C haplotype in case subjects, a haplotype not found in the Japanese study. We tested for an associated haplotype in both the families and the case-control collection but found no evidence for an associated haplotype (online appendix Table 1 [available from http://diabetes.diabetesjournals.org]). Hence, no further subgroup analysis was undertaken, including a test for gene-gene interaction between an *IL18* haplotype and the susceptible *CTLA4* +49G/G genotype as that performed by Ide et al. (9).

We sought to evaluate, independently of previously reported associations with promoter SNPs, the possibility that common variation elsewhere in *IL18* was associated with disease susceptibility. Therefore, we undertook a further study using tag SNPs to test for regional associations across *IL18* in our U.K. type 1 diabetic case-control collection (1,561 case and 1,721 control subjects). *IL18* has five exons and has been mapped to chromosome 11q22 (27). Resequencing data from a panel of 23 Caucasian individuals was extracted from the IIPGA "SeattleSNPs" database (as previously described). Thirty-eight SNPs with allele frequencies >5% were used to select eight tag SNPs with a minimum allelic R^2 of 83.6% for the untyped SNPs. Allele frequencies and predicted R^2 values for the untyped SNPs are tabulated (online appendix Table 2). Genotype data were in HWE for the control subjects ($P > 0.05$). A multilocus test for an association of the whole gene region was performed (28), and no evidence for association was found ($P = 0.11$).

We conclude that common allelic variation in *IL18* is unlikely to contribute substantially (OR >1.15) to type 1 diabetes in the populations tested and recommend routine application of tests for HWE in population-based studies for genetic association.

RESEARCH DESIGN AND METHODS

All families were European Caucasian and comprised of two parents and at least one affected child. The families consist of 528 multiplex families from the Diabetes U.K. Warren 1 collection including 56 simplex families from Yorkshire, 336 multiplex families from the Human Biological Data Interchange (U.S.), 17 multiplex and 246 simplex families from Northern Ireland, 159 Norwegian simplex families, and 261 Romanian simplex families. The 4,323 type 1 diabetic case subjects tested were recruited as part of the GRID (U.K. Genetic Resource Investigating Diabetes) study, a joint project between the University of Cambridge Departments of Pediatrics and Medical Genetics and funded by the Juvenile Diabetes Research Foundation and the Wellcome Trust (29). The case subjects are Caucasian, and 99.6% are <16 years of age, with a mean ± SD age at onset of type 1 diabetes of 7.5 ± 4 years (http://www-gene.cimr.cam.ac.uk/ucdr/grid.shtml). Geographically matched Caucasian control subjects were obtained from the 1958 British Birth Cohort, a longitudinal study involving British citizens born in a particular week in 1958 (http://www.cls.ioe.ac.uk/Cohort/Ncds/mainncds.htm). Attempted and successful genotype counts for all SNPs are tabulated and may be found in online appendix Tables 4 and 5. Ethical approval by the relevant research ethics committees was obtained for all DNA samples collected, and written informed consent was obtained from the participants.

Genotyping. Four SNPs (rs1946518 [*IL18* −607], rs5744232, rs5744237, and rs5744241) were genotyped with Invader (Third Wave Technologies, Madison, WI). PCR optimization and subsequent DNA amplification were conducted for each SNP with primers and for product sizes listed in Table 3 of the online appendix. Four SNPs (rs187238 [*IL18* −137], rs360719, rs2043055, and rs5744281) were genotyped by TaqMan 5′ nuclease assays (Applied Biosystems, Warrington, U.K.). Design of the TaqMan probes and primers was outsourced to the supplier (Applied Biosystems). All genotyping data obtained was double scored to minimize error. A duplicate plate was typed to check genotyping quality; no mismatches were observed.

Statistical analyses. All statistical analyses were performed within STATA8 (http://www.stata.com), making specific use of the Genassoc package (http://www-gene.cimr.cam.ac.uk/clayton/software/). Genotype data for control subjects and unaffected parents were in HWE ($P > 0.05$); a modified test that made allowance for differences in allele frequencies between populations was used (D. Clayton, unpublished work). In the families, the transmission/disequilibrium test was used to test for allelic association under a multiplicative model. Genotype RRs were calculated by conditional logistic regression utilizing case and matched pseudocontrol subjects constructed from the untransmitted parental genotypes (30). In the case-control collection, tests for association and calculation of ORs were performed using logistic regression. To minimize geographical confounding, case-control analyses were adjusted for 12 geographical subregions of the U.K. (31,32). For both datasets, a likelihood ratio test was used to test for a difference between the multiplicative model and the

full genotype model, which assumes no specific mode of inheritance. There was no evidence of deviation from a multiplicative model ($P > 0.05$).

Conditional logistic regression was used to analyze haplotypes in matched sets of affected offspring and pseudocontrol subjects generated from the family data (30). Haplotypes were reconstructed separately in case and control subjects by imputing phase from the a posteriori distribution using an EM algorithm within the SNPHAP program (http://www-gene.cimr.cam.ac.uk/clayton/software/). A weighted logistic regression was used to compare haplotypic frequencies in case and control subjects. SNP and flanking sequence data for 23 Caucasian individuals were extracted from the IIPGA database (http://www.innateimmunity.net) and used for tag SNP selection.

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