Evidence of Gene-Gene Interaction and Age-at-Diagnosis Effects in Type 1 Diabetes

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The common genetic loci that independently influence the risk of type 1 diabetes have largely been determined. Their interactions with age-at-diagnosis of type 1 diabetes, sex, or the major susceptibility locus, HLA class II, remain mostly unexplored. A large collection of more than 14,866 type 1 diabetes samples (6,750 British diabetic individuals and 8,116 affected family samples of European descent) were genotyped at 38 confirmed type 1 diabetes-associated non-HLA regions and used to test for interaction of association with age-at-diagnosis, sex, and HLA class II genotypes using regression models. The alleles that confer susceptibility to type 1 diabetes at interleukin-2 (IL-2), IL2/4g27 (rs2069763) and renalase, FAD-dependent amine oxidase (RNSL)/10q23.31 (rs10509540), were associated with a lower age-at-diagnosis ($P = 4.6 \times 10^{-6}$ and $2.5 \times 10^{-5}$, respectively). For both loci, individuals carrying the susceptible homozygous genotype were, on average, 7.2 months younger at diagnosis than those carrying the protective homozygous genotypes. In addition to protein tyrosine phosphatase nonreceptor type 22 (PTPN22), evidence of statistical interaction between HLA class II genotypes and rs3087243 at cytotoxic T-lymphocyte antigen 4 (CTLA4) was obtained ($P = 7.90 \times 10^{-6}$). No evidence of differential risk by sex was obtained at any loci ($P \geq 0.01$). Statistical interaction effects can be detected in type 1 diabetes although they provide a relatively small contribution to our understanding of the familial clustering of the disease. 

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Knowledge of the genetic architecture of type 1 diabetes has increased recently owing to large-scale genome-wide association (GWA) studies (1–3). Estimates of the contributions of the HLA region and numerous non-HLA loci across the genome now account for a sizeable proportion of familial clustering of the disorder (4–6). However, there remains substantial familial clustering that is not explained by the known loci (likely to be in excess of 40%) (4–6). Interactions between risk loci beyond that of a multiplicative model on the odds ratio (OR) scale (or additive on the log odds scale $7$) could account for some of the “missing heritability.” In addition, the existence of differential effects according to age-at-diagnosis and sex remains relatively unexplored.

The HLA region on chromosome 6p21 is the major source of familial clustering in type 1 diabetes (4). HLA-DRB1 and HLA-DQB1 are associated with ORs in excess of 10 for susceptible genotypes (or less than 0.1 for protective genotypes) (8). The risk genotype HLA-DRB1*04/HLA-DRB1*04- HLA-DQB1*0302 (referred to as DR3/DR4-DQ302) with greatest effect has been shown to have the highest frequency in the individuals with youngest onset (9). An age-at-diagnosis interaction has also been reported for HLA-DRB1*04 (10) and the HLA class I alleles HLA-A*24 and HLA-B*39 (11,12).

In contrast, reports of age-at-diagnosis interaction effects at non-HLA loci are contradictory, with positive reports largely confined to studies involving small sample sets (3,13–15). Similarly, reports of gene–gene interaction of type 1 diabetes–associated regions are also mainly conflicting (16–19), we presume due to inadequate sample sizes, with most positive reports likely to be false because the false-discovery rate would be high in these underpowered studies. The only convincing gene–gene interaction reported, is between a major non-HLA locus, protein tyrosine phosphatase non-receptor type 22 (PTPN22) and DR3/DR4-DQ302 genotypes (20–23).

The incidence of childhood type 1 diabetes is similar in males and females, unlike other autoimmune diseases such as Graves disease,eliac disease, or multiple sclerosis. Despite similar frequencies of childhood type 1 diabetes by sex, there have been reports of genetic risk factors differing between males and females (22,24).

Given that most studies of gene–gene interaction, age-at-diagnosis effects, and sex effects on type 1 diabetes risk have not been addressed in sufficiently well-powered studies, the Type 1 Diabetes Genetics Consortium (T1DGC) has collected more than 16,000 type 1 diabetes–affected samples.
and tested them for interaction effects with sex and age-at-diagnosis at 38 non-HLA type 1 diabetes–associated regions (Supplementary Table 2). Gene–gene interaction was also tested between HLA class II and the 38 non-HLA loci. With this very large sample set, the study had at least 80% power to detect effects as small as an interaction OR = 1.12 for sex and 1.19 for interactions with age-at-diagnosis or HLA. These calculations assume a multiplicative (log additive) effects model, an OR = 1.15 for association with type 1 diabetes for the test locus and a minor allele frequency of 0.2 and α = 0.0004. In contrast, with 5,000 samples, which is twice as large as any other study testing for interaction effects in type 1 diabetes published to date, the study would only be powered at 80% to detect interaction effects larger than an OR = 1.3 with sex (with the same assumptions as above). For age-at-diagnosis interaction, an OR ≥ 1.37 could be detected; for HLA interaction, an OR ≥ 1.38 could be detected (Supplementary Figs. 1–6).

RESEARCH DESIGN AND METHODS

Subjects. All subjects were of white European ancestry and are described in Table 1.

Single nucleotide polymorphism genotyping. Samples were genotyped using TaqMan assays or, where available, existing genotyping data from two GWA studies in a subset of the British individuals was used and has been described elsewhere (1,2). All TaqMan genotyping was performed at the University of Cambridge, blinded to disease status, and double-scored. In this sample set, 18% of British case genotypes were common to both TaqMan and single nucleotide polymorphism (SNP) chip platforms. Concordance across genotypes was excellent (99.63%), and therefore, no further samples were double-genotyped. A total of 26.9% of genotypes in the British samples were from SNP chips that used a SNP call rate of 0.95, minor allele frequency of 0.01, and Hardy-Weinberg equilibrium cutoff of P < 5.7 × 10−8. All SNPs tested in the current study were in Hardy-Weinberg equilibrium in unaffected parents (P > 0.01) and controls (P = 0.01).

Statistical methods. Cases and affected offspring were both used to test for age-at-diagnosis effects in a regression model, with age-at-diagnosis as the outcome variable and genotype as the predictor, stratified by geographic region (cases) and collection (family samples). Robust variance estimates, which relax the assumption of independent observations, were used for all gene–gene interaction tests to allow for nonindependence within families. SNP genotype was also regressed on class II genotypes using a linear regression model adjusted for geographic region or collection. Joint effects of SNP and HLA genotype were estimated using the method of Umbach and Weinberg (25) for case–control data, as detailed in Smyth et al. (20), and the method of Cordell et al. (26) for family data. Combined effects from family and case–control data were computed by the sum of effects (the vector of coefficients from the regression models), weighted by their variances, and divided by the sum of the weights (inverse of the variances). Quanto (http://hydra.usc.edu/GxE/) was used for power calculations. All other statistical analyses were performed in Stata (www.stata.com) or R (www.r-project.org) software. P < 0.0004 was considered significant, which equates to a Bonferroni correction for the number of loci and interactions tested.

RESULTS

Two of the 38 susceptibility loci tested were associated with age-at-diagnosis, rs2069763 at interleukin 2 (IL2)/4q27 (P = 4.6 × 10−6) and rs10509540 at renalase, FAD-dependent amine oxidase (RNL5)/10q23.31 (P = 2.5 × 10−5, Supplementary Table 2). Consistent with a true biologically plausible age-at-diagnosis effect, the protective allele was more frequent with increasing age-at-diagnosis and the

| Cohort | Country/region of recruitment | Families N | Individuals N (% males) | Average age-at-diagnosis (max)/years |
|--------|-------------------------------|------------|-------------------------|-------------------------------------|
| Isolated case samples | | | | |
| British | Great Britain | | 8,512 (52) | | 7.7 (16) |
| Danish | Denmark | | 1,789 (51) | | 9.0 (18)† |
| Family samples | | | | |
| T1DGC | Europe | 1,180 | 2,444 (51) | 12.5 (52) |
| T1DGC | Asia-Pacific | 277 | 571 (48) | 10.9 (40) |
| T1DGC | North America | 1,017 | 2,135 (54) | 10.0 (49) |
| T1DGC | U.K. | 154 | 336 (46) | 8.3 (33) |
| Diabetes UK, Warren | Great Britain | 470 | 997 (53) | 11.6 (50) |
| Yorkshire | Great Britain | 80 | 84 (51) | 9.1 (15) |
| Belfast | U.K. | 262 | 283 (54) | 7.3 (16) |
| Romanian | Romania | 423 | 446 (48) | 12.5 (52) |
| Finnish | Finland | 1,230 | 1,418 (54) | 9.0 (46) |
| HBDI | U.S. | 335 | 706 (52) | 10.3 (51) |

Most cases were diagnosed with type 1 diabetes in childhood; however, across the family collections, 1,216 were aged older than 18 at diagnosis, and 196 of those were aged older than 30 years at diagnosis. Minor allele frequencies for each SNP within each cohort for type 1 diabetes samples are reported in Supplementary Table 1. HBDI, Human Biological Data Interchange. *The UK Genetic Resource Investigating Diabetes childhood cases (www.childhood-diabetes.org.uk/grid.shtml). †Age-at-diagnosis for individual Danish cases was unknown, so information applies to the whole cohort.
The protective alleles for both loci are most common in the older age-groups. ORs with 95% CI were calculated for the protective allele using the British and Danish cases and controls. Age-at-diagnosis category represents the quartiles of the age-at-diagnosis distribution for cases and affected offspring.

Mean age-at-diagnosis increased with the number of protective alleles (Table 2). At rs2069763 (IL2/4q27) the major allele G confers protection from type 1 diabetes (www.t1dbase.org). The average age-at-diagnosis for G/G homozygotes was 9.3 years compared with 8.7 years for T/T homozygotes (7.2 months later). Furthermore, the frequency of the G allele was 2.8% higher in the upper quartile of the age-at-diagnosis distribution (64.1%) compared with the lower quartile (61.3%; Table 2). Similarly, for rs10509540 of the age-at-diagnosis distribution (64.1%) compared with the upper quartile T/T homozygotes (7.2 months later). Furthermore, the frequency of the C allele was 2.8% higher in the upper quartile (64.1%) compared with the lower quartile (61.3%; Table 2). The protective rs3087243 on adult-onset type 1 diabetes, more loci may yet be involved SNPs in the IL-2 and RNLS genes. The focus here was on childhood-onset individuals, but with an emphasis on adult-onset type 1 diabetes (10).

HLA-SNP interaction results. HLA was modeled using the SNPs, rs2187668 and rs7454108, to capture the DR3/4-DQ302 protective alleles. Indeed, a recent study of 1,384 individuals with adult-onset autoimmune type 1 diabetes (10). The protective rs3087243 T allele conferred greatest protection in those who were DR3/4/X and DR3/X, but there was little effect in those with the DR3/DR4-DQ302 genotype (Table 3). All other regions had P > 0.007 for statistical interaction with the DR3/DR4-DQ302 genotype (Supplementary Table 2).

When SNPs were used to encode DR3/4/X genotypes rather than as presence or absence of the DR3/DR4-DQ302 genotype (RESEARCH DESIGN AND METHODS), PTPN22-D3/4/X deviated from multiplicative interaction on the OR scale ($P = 4.87 \times 10^{-4}$). In addition, there was evidence of nonmultiplicative statistical interaction on the OR scale with the cytotoxic T-lymphocyte antigen 4 (CTLA4) SNP, rs3087243 (P = 7.90 × 10^{-5}). The protective rs3087243 T allele conferred greatest protection in those who were DR4/X and DR3/X, but there was little effect in those with the DR3/DR3 genotype (Table 4). All other regions had $P > 0.006$.

**TABLE 2**

| Genotype | Age-at-diagnosis Mean (SD) | Type 1 diabetes association OR [95% CI] | Age-at-diagnosis category (16,663 cases) (years) | Frequency of the protective allele |
|----------|-----------------------------|----------------------------------------|-----------------------------------------------|----------------------------------|
| T/T      | 8.7 (5.7)                   | 1.00 reference                          | <5                                            | 0.613                           |
| G/T      | 9.0 (6.0)                   | 0.91 [0.84–0.99]                        | 5–8.2                                         | 0.625                           |
| G/G      | 9.3 (6.2)                   | 0.80 [0.74–0.88]                        | 8.2–12                                        | 0.633                           |
|          |                             |                                        | >12                                           | 0.641                           |

**TABLE 3**

| Genotype | Age-at-diagnosis Mean (SD) | Type 1 diabetes association OR [95% CI] | Age-at-diagnosis category (16,857 cases) (years) | Frequency of the protective allele |
|----------|-----------------------------|----------------------------------------|-----------------------------------------------|----------------------------------|
| T/T      | 8.9 (5.9)                   | 1.00 reference                          | <5                                            | 0.219                           |
| T/C      | 9.3 (6.1)                   | 0.80 [0.66–0.82]                        | 5–8.2                                         | 0.231                           |
| C/C      | 9.5 (6.3)                   | 0.73 [0.66–0.82]                        | 8.2–12                                        | 0.242                           |
|          |                             |                                        | >12                                           | 0.242                           |
TABLE 3
Joint effects of HLA class II genotypes, DR3/DR4-DQ302 and rs2476601 at PTPN22 and rs2290400 at GSDMB/ORMDL3

| HLA genotype | Affected offspring (frequency) | Cases (frequency) | RR [95% CI] for rs2476601 and HLA class II | OR [95% CI] |
|--------------|-------------------------------|------------------|------------------------------------------|-------------|
|              | C/T                           | C/T              | T/T                                      |             |
| DR3/DR4-DQ302| 2,964 (0.36)                  | 3,384 (0.34)     | 1.00 [reference]                         | 2.89 [2.54–3.29] |
| Non-DR3/DR4-DQ302| 5,359 (0.64)                  | 6,616 (0.66)   | 0.10 [0.09–0.11]                         | 0.39 [0.30–0.51] |
|              |                               |                  |                                          | 1.70 [1.59–1.81] |

GSDMB/ORMDL3/17q12 rs2290400 G>A, interaction \( P = 6.2 \times 10^{-4} \)

| HLA genotype | Affected offspring (frequency) | Cases (frequency) | RR [95% CI] for rs2290400 and HLA class II | OR [95% CI] |
|--------------|-------------------------------|------------------|------------------------------------------|-------------|
|              | C/T                           | C/T              | T/T                                      |             |
| DR3/DR4-DQ302| 2,995 (0.35)                  | 3,363 (0.34)     | 1.00 [reference]                         | 0.94 [0.90–0.99] |
| Non-DR3/DR4-DQ302| 5,451 (0.65)                  | 6,537 (0.66)   | 0.12 [0.11–0.13]                         | 0.09 [0.07–0.11] |
|              |                               |                  |                                          | 0.87 [0.84–0.90] |

Interaction was tested for the DR3/DR4-DQ302 genotype vs. the non-DR3/DR4-DQ302 genotype. The SNPs rs2187668 and rs7454108 were used to tag the HLA-DRB1*03 (DR3) and HLA-DRB1*04 (DR4) class II alleles. For samples with classical genotypes, the DR3/DR4 group was confined to those who were HLA-DRB1*0302-positive with the protective HLA-DRB1*0403 and *0407 included in the non-DR3/DR4 group. Effects were estimated in the cases and affected offspring separately and then combined to calculate relative risk (RR) and OR. A small \( P \) value is interpreted as evidence of deviation from multiplicative interaction on the OR scale. Note that cohort effects are accounted for in all tests. The current study included 2,409 cases analyzed previously by Smyth et al. (20); however, the interaction remained convincing in the 15,923 samples not previously analyzed \( (P = 1.97 \times 10^{-4}) \).

effects at \( IL2 \) \( (P = 0.026) \) and \( RNLS \) \( (P = 0.033) \) (10). The same study reported a suggestive association at interleukin 2 receptor \( \alpha \) (IL2RA)/10p15.1/rs2104286 \( (P = 0.027) \), a locus that was close to significant in the present sample set \( (P = 9.8 \times 10^{-5}) \). Regulator of G-protein signaling 1 (RGS1)/1q31.2, GLIS family zinc finger 3 (GLIS3)/9p24.2, approached significance in our study, and a further seven SNPs had \( P < 0.05 \) (Supplementary Table 2), but no support for \( RGS1 \) or \( GLIS3 \) was obtained previously (10).

The type 1 diabetes risk alleles at all 11 loci with \( P < 0.05 \) for age-at-diagnosis effects were associated with a younger age-at-diagnosis. The genetic risk score was strongly correlated with age-at-diagnosis, accounting for \( ~1\% \) of the variance in age-at-diagnosis \( (P = 9.2 \times 10^{-5}) \). Stratifying the risk score into quintiles demonstrated a trend toward an earlier age-at-diagnosis with increasing risk; average age-at-diagnosis from lowest risk to highest risk was 8.2, 8.2, 7.8, 7.4, and 7.0 years. Children carrying a higher dose of the earlier age-at-diagnosis alleles will probably have an earlier diagnosis of disease compared with individuals who do not carry these risk alleles, presumably due to a more rapid development of autoimmunity and/or progression from autoimmunity, as detected by being autoantibody-positive (10). Another possibility is that as the immune system matures and ages, alterations in its functions may nullify the effects of certain susceptibility alleles, for example, of the genes in the IL-2 pathway, and so cause these age-at-diagnosis–dependent associations.

The IL-2 pathway is recognized as being important for the pathogenesis of type 1 diabetes. \( IL2 \) SNP genotypes are correlated with IL-2 gene expression (J. Yang, J.A.T., unpublished data), supporting \( IL2 \) as the causal gene in the chromosome 4q27 region for type 1 diabetes. Two IL-2 receptor genes, \( IL2RA \) and \( IL2RB \), are both associated with type 1 diabetes susceptibility (1). In the NOD mouse model, the major non-major histocompatibility complex locus, insulin dependent diabetes susceptibility 3 (Idd3), is the \( IL2 \) gene and its effect, via polymorphic gene expression, is age-dependent (27). The \( RNLS \) gene encodes renalase, a FAD-dependent amine oxidase secreted by the kidney that circulates in blood and modulates cardiac function and systemic blood pressure, perhaps through its ability to metabolize catecholamines (28). Little is known about its function, if any, in the immune system. Renalase RNA is expressed in monocytes (http://dil.tdbase.org/page/HaemAtlasView). We have found a correlation between the

TABLE 4
Joint effects of HLA class II genotypes and rs3087243 at CTLA4

| HLA genotype | Affected offspring (frequency) | Cases (frequency) | RR [95% CI] for rs3087243 and HLA class II | OR [95% CI] |
|--------------|-------------------------------|------------------|------------------------------------------|-------------|
|              | G/A                           | G/A              | A/A                                      |             |
| 3/4          | 3,211 (0.37)                  | 3,529 (0.35)     | 5.73 [5.22–6.28]                         | 4.20 [3.00–5.88] |
| 3/3          | 594 (0.07)                    | 798 (0.08)       | 2.30 [1.98–2.67]                         | 2.13 [1.81–2.52] |
| 4/4          | 855 (0.10)                    | 899 (0.09)       | 2.69 [2.23–3.27]                         | 2.05 [1.76–2.40] |
| 4/X          | 2,358 (0.27)                  | 2,686 (0.27)     | 1.00 [reference]                         | 0.62 [0.56–0.69] |
| 3/X          | 1,151 (0.13)                  | 1,433 (0.14)     | 0.52 [0.47–0.57]                         | 0.34 [0.28–0.41] |
| X/X          | 622 (0.07)                    | 786 (0.08)       | 0.09 [0.08–0.11]                         | 0.07 [0.06–0.08] |

The HLA genes were coded as DR3/4/X genotypes (3/4, 3/3, 3/X, 4/4, 4/X, X/X) where “X” represents the non-DR3 and non-DR4 alleles, and included HLA-DRB1*0403 and HLA-DRB1*0407 for samples with classical genotypes available. Effects were estimated in the cases and affected offspring separately and then combined in order to calculate relative risk (RR) and OR with 95% CI (see RESEARCH DESIGN AND METHODS). A small \( P \) value is interpreted as evidence of deviation from multiplicative interaction. Note that cohort effects are accounted for in all tests.
type 1 diabetes risk SNPs and SNPs associated with RNA levels of the RNLS gene in monocytes, implicating it as the causal gene in the chromosome 10q23.31 region (29).

We tested HLA non-HLA gene interactions because the HLA class II genes have the largest effects on type 1 diabetes in the genome. Hence, we expect the HLA to have the highest prior probability of showing a nonmultiplicative interaction on the OR scale with a non-HLA locus. The PTPN22–HLA class II genotype interaction is most convincing, probably because the main effect at PTPN22 is large compared with other non-HLA genes in type 1 diabetes with a genotype OR ≥ 3.5. The biological interpretation of a statistical interaction is difficult, but for PTPN22 and HLA class II, one can hypothesize that their coexpression and hence biological interaction in certain cell types critical for type 1 diabetes, such as T lymphocytes and antigen-presenting cells, could contribute to our observed statistical result. The CTLA4–HLA finding requires confirmation in future studies; however, the result is not surprising given the key role of HLA class II molecules and CTLA-4 in autoantigen presentation and autoreactive T-cell activation. Further insights into these molecules and their role in disease require detailed laboratory-based investigations.

Our findings here illustrate that statistical gene–gene interactions can be detected, and we can anticipate that evidence for many more interactions may be found (and those we report confirmed) with larger numbers of samples, and with the use of non-European samples. However, in keeping with another report (4), our data suggest that for common variants with ORs < 2, statistical interactions are unlikely to contribute substantially to the “missing heritability” in type 1 diabetes.

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J.M.M.H. analyzed and interpreted the data, and wrote, reviewed, and edited the manuscript. J.D.C. researched data and reviewed and edited the manuscript. D.J.S. performed TaqMan genotyping and reviewed and edited the manuscript. N.M.W. managed the data and reviewed and edited the manuscript. H.S. managed DNA samples and reviewed and edited the manuscript. J.X.-S., G.S.E., M.E., B.A., P.C., H.A.E., C.J., G.M., J.N., C.N., and S.S.R. reviewed and edited the manuscript. J.A.T. interpreted the data, and wrote, reviewed, and edited the manuscript. F.P. contributed Danish DNA samples and reviewed and edited the manuscript. J.M.M.H. is the guarantor of this work and as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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