Effects of Non-HLA Gene Polymorphisms on Development of Islet Autoimmunity and Type 1 Diabetes in a Population With High-Risk HLA-DR,DQ Genotypes

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We assessed the effects of non-HLA gene polymorphisms on the risk of islet autoimmunity (IA) and progression to type 1 diabetes in the Diabetes Autoimmunity Study in the Young. A total of 1,743 non-Hispanic, white children were included: 861 first-degree relatives and 882 general population children identified as having high-risk HLA-DR/DQ genotypes for type 1 diabetes. Of those, 109 developed IA and 61 progressed to diabetes. Study participants were genotyped for 20 non-HLA polymorphisms, previously confirmed as type 1 diabetes susceptibility loci. *PTPN22* and *UBASH3A* predicted both IA and diabetes in regression models controlling for family history of type 1 diabetes and presence of HLA-DR3/4-DQB1*0302 genotype. In addition, *PTPN2* predicted IA whereas *INS* predicted type 1 diabetes. The final multivariate regression models for both IA and type 1 diabetes included *PTPN22*, *UBASH3A*, and *INS*, in addition to family history of type 1 diabetes and HLA-DR3/4. In general population children, the most frequent combinations including these five significant predictors conferred hazard ratio of up to 13 for IA and >40 for type 1 diabetes. Non-HLA susceptibility alleles may help estimate risk for development of type 1 diabetes in the general population. These findings require replication in different populations. *Diabetes* 61:753–758, 2012

The strongest genetic association for type 1 diabetes is with the HLA class II genes (odds ratio >6). It is estimated that 30–50% of the genetic risk for type 1 diabetes can be attributed to the HLA region (1). However, first-degree relatives of a person with type 1 diabetes carrying the HLA-DR3/4-DQ8 or DR4/DR4 genotypes have a >20% risk for developing islet autoantibodies during childhood (2), compared with only a 5% risk if they do not have a relative with type 1 diabetes (3). This points to a strong effect of non-DR,DQ susceptibility genes within or outside the HLA region. Non-HLA loci have a modest effect on disease risk relative to the HLA region, with *INS* on chromosome 11p15 (4) and *PTPN22* on chromosome 1p13 (5) showing the strongest association (odds ratio ~2). With the advent of single nucleotide polymorphism

(SNP) analysis and genome-wide association studies, >50 non-major histocompatibility complex loci have been described and are currently being validated and confirmed (http://www.t1dbase.org) (6,7). Genome-wide association studies have validated previously discovered associations (*INS*, *PTPN22*, and *CTLA4*) and discovered novel loci (e.g., *IFIH1*, *CD25/IL2RA*, *PTPN2*, *GLIS3*, *SH2B3*, *BACH2*, and *UBASH3A*) (8–12). Cohort studies following children with high-risk HLA genotypes to islet autoimmunity (IA) and diabetes offer a valuable tool to further validate the independent predictive value of novel non-HLA markers and to explore the genetic architecture of type 1 diabetes in specific populations. In the largest such cohort available from the U.S. population (the Diabetes Autoimmunity Study in the Young [DAISY]), we analyzed the additional effects of non-HLA susceptibility polymorphisms on the risk of IA and diabetes, controlling for the effects of HLA-DR,DQ genotypes.

RESEARCH DESIGN AND METHODS

Study population. Since 1993, DAISY has followed two cohorts of young children at increased risk of type 1 diabetes: first-degree relatives of type 1 diabetes patients and general population children found through a newborn screening to carry high-risk HLA-DR,DQ genotypes. The details of screening and follow-up have been previously published (13). Children included in the current cohort are all non-Hispanic white (NHW), including 861 first-degree relatives (FDRs) and 882 general population children. Of those, 109 developed IA and 61 progressed to diabetes during the 9-year mean prospective follow-up; 5 subjects developed diabetes without having antibodies. In both the IA and diabetes groups, 70% of the subjects were FDRs, i.e., 76 of 109 and 43 of 61 were FDRs, respectively. Informed consent was obtained from the parents of each study subject. The Colorado Multiple Institutional Review Board approved all study protocols.

Islet autoantibodies. Measurement of islet autoantibodies to insulin, GAD65, IA-2, and ZnT8 was performed in the laboratory of Dr. George Eisenbarth at the Barbara Davis Center for Childhood Diabetes using previously described radioimmunoassays (14). IA was defined as presence of one or more of the autoantibodies to insulin (GAD65, IA-2, or ZnT8) on at least two consecutive visits, 3–12 months apart.

Genotyping. *INS*-23Hph1 (rs689), *CTLA-4* T17A (rs231775), and *PTPN22* R620 W (rs2476601) polymorphisms were genotyped using a linear array (immobilized probe) method essentially as described in Mirel et al. (15). The following SNPs were genotyped in the laboratory of Dr. Cisca Wijmenga using Illumina GoldenGate Beadexpress assays (veracode 48-plex): *IL2RA* (rs12251307), *SH2B3* (rs3184504), *PTPN2* (rs1893217), *C10orf59* (rs10509540), *IL18RAP* (rs917997), *BACH2* (rs11755527), and *TAGAP* (rs1738074). Genotype calling was performed in BeadStudio.

Taqman SNP genotyping assays (Applied Biosystems, Carlsbad, CA) were used to obtain genotype information on the following SNPs: *CD69* (rs4763879), *GAB3* (rs2664170), *GLIS3* (rs7020673), *IL10* (rs3024496), *SIRPG* (rs2281808), *PRKD2* (rs425105), *UBASH3A* (rs11203203), *IFIH1* (rs1990760), and *SLC30A8* (rs13266634). For each 12.5-μL volume assay, 20 ng of amplified gDNA template (1 μL) was used with 6.35 μL Taqman Genotyping master mix (Applied Biosystems), 0.15 μL SNP assay probe mix, and 5 μL PCR-grade water. PCR cycling conditions were according to manufacturer protocol. Genotype results were obtained using an AB7000 Sequence Detection System and analysis software.

CCR5 genotypes were determined using a fluorescent-based method. PCR fragments were generated using primers that differentiate between the wild-type genotype (*CCR5/CCR5*) at 225 base pairs and the homozygous mutant (Δ32/Δ32) at 193 base pairs. Reactions (25 μL) were assembled using FailSafe PCR PreMix J, 2.5 units MasterAmp Taq polymerase (Epicentre), 10 nmol each primer, and 100 ng genomic template. The PCR product was amplified by 35 PCR cycles of 94°C for 30 s, 57°C for 35 s, and 72°C for 1 min and a final extension of 72°C for 45 min. Products were diluted 1:60 and separated by capillary electrophoresis on an ABI3100-Avant Genetic Analyzer (Applied Biosystems). Alleles were identified using GeneMapper version 3.5 (Applied Biosystems).

Statistical analysis. All analyses were performed in SAS version 9.2 (SAS Institute, Cary, NC). Cox proportional hazards models were used to test the effect of each genetic polymorphism on time to development of IA and, separately, time to progression to diabetes. Multivariate Cox models included family history of type 1 diabetes in first-degree relatives (yes/no) and the presence of the HLA-DR3/4-DQB1*0302 genotype (yes/no); independently significant non-HLA polymorphisms were identified by backward selection at a critical level of 0.05. Because our analyses were based on a priori hypotheses, *P* values were not corrected for multiple testing. Finally, we analyzed the effect of all combinations of the significant and independent predictors of IA or diabetes present in >2% of the DAISY NHW population. The analyses were based on genotypes using the additive model except for *PTPN22*, which was found to deviate from linearity and was therefore included as a 2df variable in a multivariate model. The SNPs were in Hardy-Weinberg equilibrium except for *C10orf59*, *PRKD2*, and *GAB3*, which were therefore not included in multivariate models. To determine whether inclusion of multiple siblings per family in this cohort affected our findings, we performed analyses accounting for the clustering of patients within a family (using the robust sandwich estimate for statistical inference) and found similar results (data not shown).

RESULTS

In univariate analyses, *UBASH3A* (rs11203203) was the only non-HLA SNP that predicted significantly both IA (hazard ratio [HR] 1.52 [95% CI 1.16–2.00], *P* = 0.0024) and type 1 diabetes (2.02 [1.40–2.91], *P* = 0.0002). The *PTPN22* and *PTPN2* polymorphisms were both associated with IA (1.82 [1.27–2.61], *P* = 0.001, and 1.46 [1.05–2.02], *P* = 0.03, respectively), whereas the *INS* polymorphism was significantly associated with diabetes (1.75 [1.09–2.83], *P* = 0.02) (data not shown). There were no significant interactions between any of the SNPs and HLA-DR3/4-DQB1*0302. Multivariate analyses adjusting for family history of type 1 diabetes and HLA high-risk genotype are shown in Table 1. *PTPN22* and *UBASH3A* were significantly associated with both IA and type 1 diabetes. *INS* remained significantly associated with diabetes outcome, whereas *PTPN2* remained associated with IA. Stratified analyses by cohort are shown in Supplementary Table 1. Overall, results are similar, although some of the SNPs do not reach statistical significance, which is likely due to smaller numbers. GAB3 shows a borderline significant association with diabetes in only FDRs; the rs2664170 G allele, previously associated with diabetes, is negatively associated with diabetes. These results should be interpreted with caution because of small sample size.

Multivariate regression analyses including all SNPs with *P* value <0.05 were performed for both IA (Fig. 1) and type 1 diabetes (Fig. 2). Genes that remained significantly associated with IA, adjusting for family history of type 1 diabetes and HLA-DR3/4-DQB1*0302, included *PTPN22*, *UBASH3A*, and *INS*. The final model for diabetes included the same five variables as for IA (*PTPN22*, *UBASH3*, *INS*, family history of type 1 diabetes, and HLA-DR3/4-DQB1*0302).

The most frequent combinations of the above five significant variables are shown in Fig. 3. Hazard ratios for the development of IA ranged from 1.6 to 13 for general population children and from 3.8 to 16 for FDRs, for those combinations that were present in >2% of the DAISY NHW population (Fig. 3A). For the same combinations, HRs for type 1 diabetes ranged from 2.4 and 6.7 for general population and FDRs, respectively, to >40 for both (Fig. 3B). These 15 combinations included 67% of the population studied but only half of the cases. Only 82 subjects (4.7%) of the DAISY NHW population were FDRs with the HLA-DR3/4-DQB1*0302 genotype. When adding the three non-HLA genes (*PTPN22*, *UBASH3*, and *INS*), none of the specific combinations in this high-risk group were present in >2% of the population.

TABLE 1
Association of non-HLA genes with IA and type 1 diabetes in DAISY NHW population (*N* = 1,743)*

Gene	SNP	Risk allele	Islet autoimmunity		Type 1 diabetes	
			HR (95% CI)	*P* value	HR (95% CI)	*P* value
BACH2	rs11755527	G	1.03 (0.76–1.38)	0.87	0.99 (0.65–1.51)	0.96
C10orf59	rs10509540	G	0.85 (0.69–1.05)	0.12	0.76 (0.57–1.02)	0.07
CD69	rs4763879	A	1.14 (0.87–1.50)	0.35	0.96 (0.65–1.40)	0.83
GAB3	rs2664170	G	0.94 (0.74–1.20)	0.62	0.76 (0.53–1.08)	0.13
GLIS3	rs7020673	C	0.79 (0.61–1.03)	0.08	0.74 (0.52–1.05)	0.09
IFIH1	rs1990760	C	1.05 (0.79–1.39)	0.75	1.28 (0.88–1.86)	0.21
IL10	rs3024496	A	1.22 (0.93–1.61)	0.14	1.24 (0.86–1.79)	0.25
IL18RAP	rs917997	A	1.13 (0.81–1.58)	0.46	1.05 (0.65–1.69)	0.84
IL2RA	rs12251307	A	0.67 (0.40–1.11)	0.12	0.73 (0.37–1.44)	0.36
INS	rs689	A	1.32 (0.94–1.85)	0.11	1.75 (1.08–2.83)	0.02
PRKD2	rs425105	C	0.90 (0.62–1.31)	0.59	0.95 (0.58–1.56)	0.85
PTPN2	rs1893217	G	1.42 (1.02–1.99)	0.04	0.99 (0.60–1.66)	0.98
PTPN22	rs2476601	T	1.87 (1.31–2.68)	<0.001	1.74 (1.04–2.90)	0.03
SH2B3	rs3184504	A	0.93 (0.70–1.24)	0.63	0.92 (0.62–1.37)	0.69
SIRPG	rs2281808	T	0.85 (0.63–1.14)	0.27	0.88 (0.60–1.30)	0.53
TAGAP	rs1738074	A	0.90 (0.67–1.20)	0.47	1.09 (0.75–1.61)	0.64
UBASH3A	rs11203203	A	1.46 (1.11–1.91)	0.01	1.83 (1.28–2.64)	0.001
SLC30A8	rs13266634	T	1.04 (0.77–1.41)	0.79	0.94 (0.61–1.44)	0.77
CTLA4	rs231775	G	1.19 (0.91–1.55)	0.21	1.00 (0.70–1.43)	1.00
CCR5	microsatellite	Del32	0.96 (0.61–1.53)	0.87	1.02 (0.55–1.88)	0.96

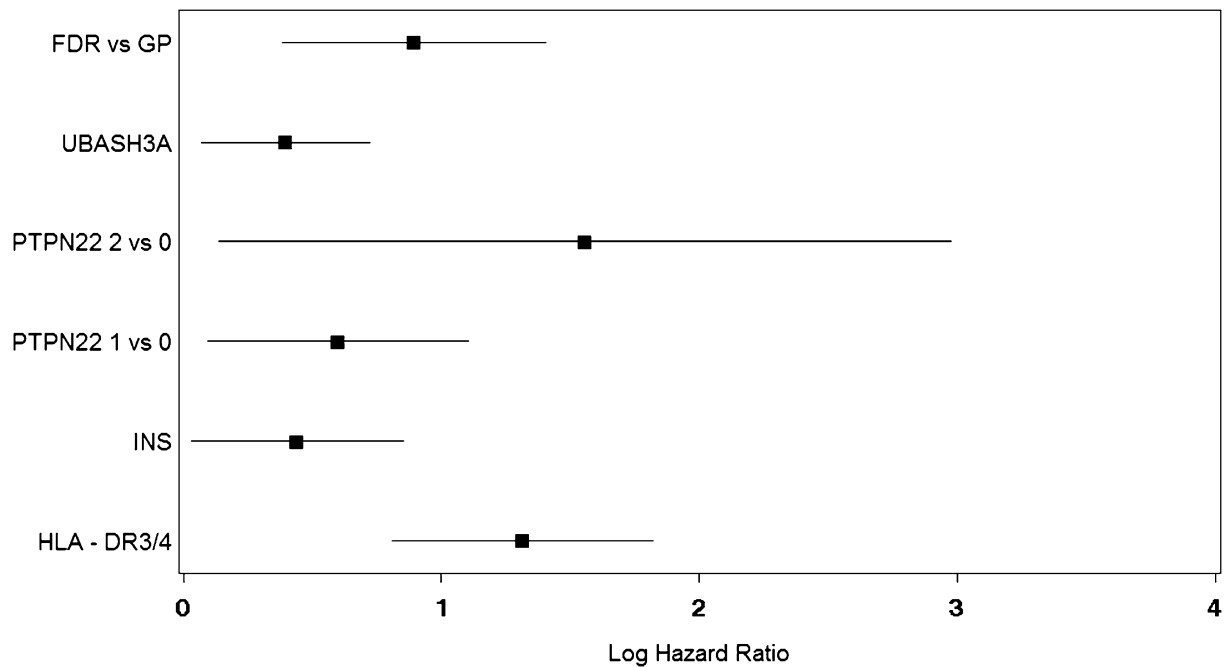*Multivariate analyses, adjusted for HLA-DR3/4,DQB1*0302 and family history of type 1 diabetes.

FIG. 1. HRs and 95% CIs for predictors in multivariate model for IA. Model including all variables with an α level of <0.05. GP, general population.

Antibody levels for insulin, GAD65, and IA-2 were analyzed for correlation with *INS*, *PTPN22*, and *UBASH3A* polymorphisms. Children with the *INS* AA genotype had higher mean insulin autoantibody (IAA) levels compared with those carrying the AT/TT genotype ($P = 0.02$), whereas children with the *UBASH3A* AA genotype had higher mean IA-2 levels ($P = 0.03$) (Supplementary Fig. 1).

DISCUSSION

Multiple studies have recently linked type 1 diabetes to >50 non-HLA gene polymorphisms. This study tested the robustness of the associations for 20 previously confirmed non-HLA markers with IA and/or diabetes in a large cohort of NHW children at increased genetic risk for type 1 diabetes. The main advantage of this approach was the ability to
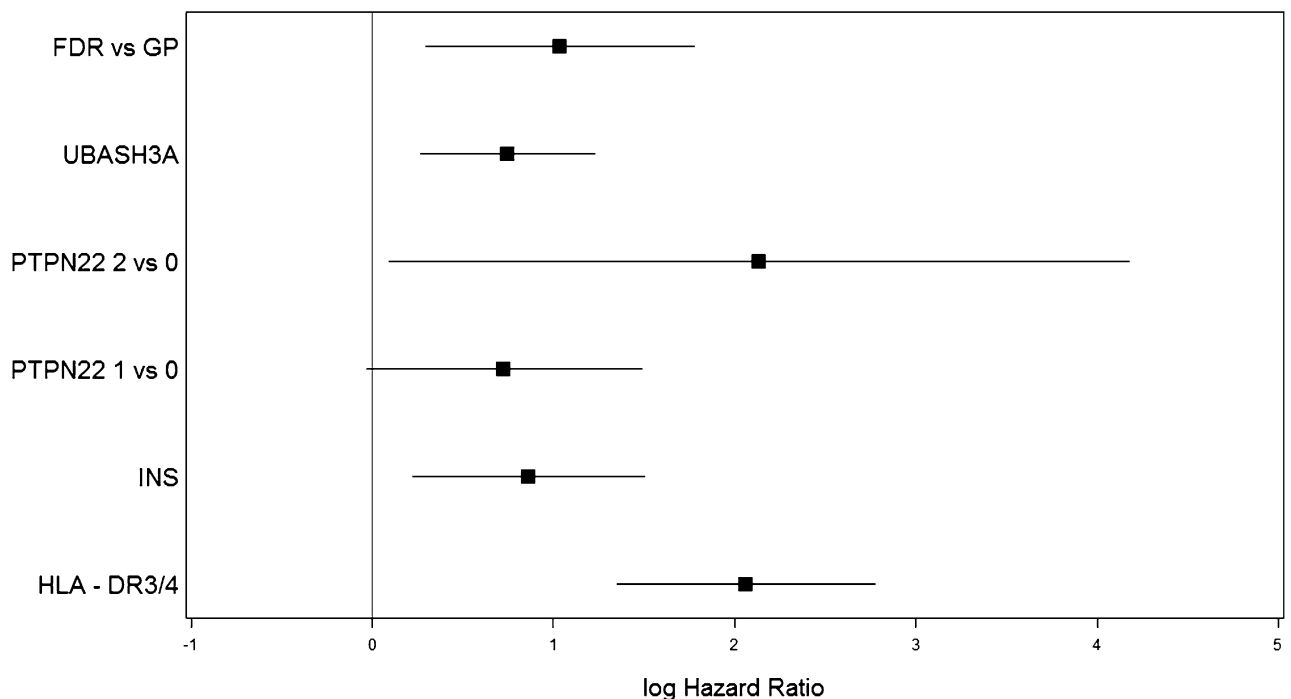

FIG. 2. HRs and 95% CIs for predictors in multivariate model for type 1 diabetes. Model including all variables with an α level of <0.05. GP, general population.
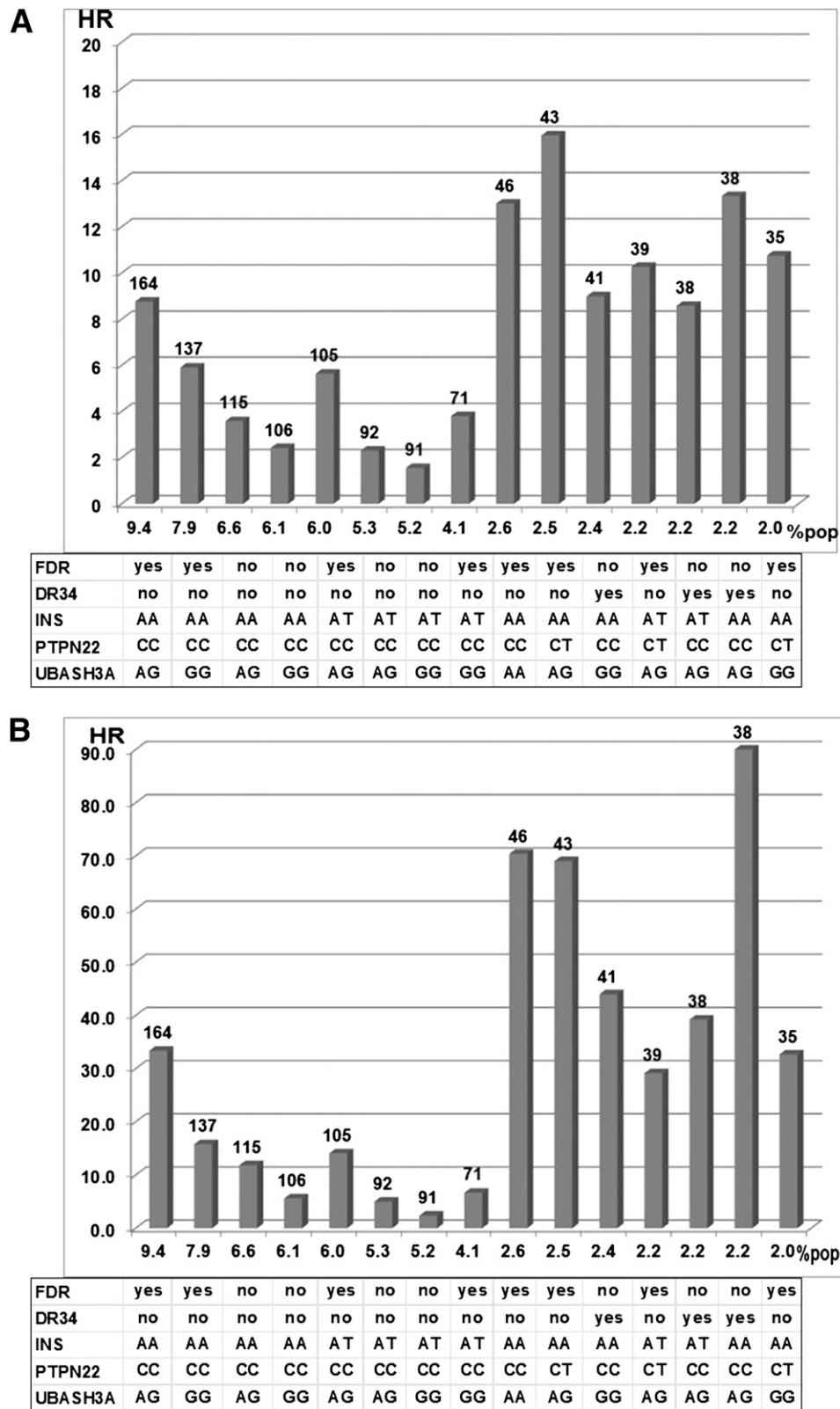
FIG. 3. HR estimates for the most frequent combinations for IA (*A*) and type 1 diabetes (*B*). The height of the bars indicates the magnitude of the HR for the group of patients described by the predictor variables displayed in the table below the bars. The *x*-axis displays the proportion of the population that falls into each group, and the value above the bars represents the number of patients in the group. DR34, HLA DR3/4-DQB1*0302 genotype; %pop, % population.

evaluate the effect of candidate SNPs on the prospectively observed development of diabetes phenotypes. In addition to the non-HLA genes most strongly associated with type 1 diabetes in previous studies (*PTPN22* and *INS* [16,17]), we found a robust association of IA and diabetes with *UBASH3A* that has only recently been associated with type 1 diabetes (18). *PTPN2* only modestly predicted IA, but it has been previously associated with several autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, Crohn disease, and celiac disease (9,11). A couple of SNPs showed a possible association with diabetes: GLIS3 has been implicated in neonatal diabetes and pancreatic development, and GAB3 facilitates macrophage differentiation.

Only a few studies have explored the additional contribution of non-HLA genes to risk prediction of type 1 diabetes. Bjornvold et al. (19) looked at the joint effects of *HLA*, *INS*, *PTPN22*, and *CTLA4* genes and found that multiple susceptibility loci confer a very high risk of diabetes, but only a small proportion of the population carries all high-risk alleles. When assessing the predictive utility of these genetic risk markers by receiver operating characteristic curve, multiple susceptibility genotypes seemed to improve disease prediction only marginally compared with HLA genotype alone (19). In the Diabetes Prevention Trial-Type 1 (DPT-1) study, diabetes susceptibility loci were analyzed for their impact on progression from prediabetes to diabetes. Susceptibility alleles, including *PTPN22*, *CTLA4*, *INS*, and *IL2RA*, were not associated with progression to diabetes but were increased in both groups (progressors and nonprogressors) compared with general population; HLA-DQB1*0302 was increased in progressors whereas HLA-DQB1*0301 was decreased (20). In a previous DAISY study, our group showed that *PTPN22* was associated with progression to IA after adjusting for HLA-DR3/4, whereas *INS* had a more modest effect (21). IAA levels have been shown to be a significant predictor of progression to diabetes (22) and *INS* genotypes have been associated with IAA levels.

Additional risk conferred by non-HLA susceptibility alleles may help estimate risk for development of type 1 diabetes in the general population. In this study, combinations of the five significant predictors (*PTPN22*, *UBASH3*, *INS*, family history of type 1 diabetes, and HLA-DR3/4) can give HRs up to 16 for the development of IA and >40 for diabetes, although this study might over- or underestimate the risks for certain groups as we do not have a large enough number of cases to look at all possible interactions. So far, prediction of type 1 diabetes has mainly been based on family history, age of onset of proband, autoantibody number and levels, and genetic susceptibility markers such as *INS* and HLA-DR3/4-DQB1*0302 (2,22–25). Once children have developed two or more antibodies, the risk for diabetes is very high, regardless of family history of diabetes and high-risk HLA-DR3/4 genotype (22). A cost-effective strategy may include determination of high genetic risk at birth (by HLA and additional non-HLA genotyping) followed by antibody measurements at 1 year of age (with repeat antibody testing at 3) in those children at high genetic risk. This successive testing combination will likely be the best predictive model capable of identifying general population children who are at greatest risk for developing type 1 diabetes.

ACKNOWLEDGMENTS

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