Brief Genetics Report

Association of Non-HLA Genes With Type 1 Diabetes Autoimmunity

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Approximately 50% of the genetic risk for type 1 diabetes is attributable to the HLA region. We evaluated associations between candidate genes outside the HLA region–INS, cytotoxic T-lymphocyte–associated antigen (CTLA)-4, interleukin (IL)-4, IL-4R, and IL-13 and islet autoimmunity among children participating in the Diabetes Autoimmunity Study in the Young (DAISY). Children with persistent islet autoantibody positivity (n = 102, 38 of whom have already developed diabetes) and control subjects (n = 198) were genotyped for single nucleotide polymorphisms (SNPs) in the candidate genes. The INS-23Hph1 polymorphism was significantly associated with both type 1 diabetes (OR = 0.30; 95% CI 0.13–0.69) and persistent islet autoimmunity but in the latter, only in children with the HLA-DR3/4 genotype (0.40; 0.18–0.89). CTLA-4 promoter SNP was significantly associated with type 1 diabetes (3.52; 1.22–10.17) but not with persistent islet autoimmunity. Several SNPs in the IL-4 regulatory pathway appeared to have a predisposing effect for type 1 diabetes. Associations were found between both IL-4R haplotypes and IL-4–IL-13 haplotypes and persistent islet autoimmunity and type 1 diabetes. This study confirms the association between the INS and CTLA-4 loci and type 1 diabetes. Genes involved in the IL-4 regulatory pathway (IL-4, IL-4R, IL-13) may confer susceptibility or protection to type 1 diabetes depending on individual SNPs or specific haplotypes. *Diabetes* 54:2482–2486, 2005

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CTLA, cytotoxic T-lymphocyte–associated antigen; DAISY, Diabetes Autoimmunity Study in the Young; IA-2, insulinoma-associated protein 2; IAA, insulin autoantibodies; IL, interleukin; NHW, non-Hispanic white; SNP, single nucleotide polymorphism.

Type 1 diabetes is associated with susceptibility genes in the HLA region on chromosome 6p21 that account for 50% of the familial clustering of type 1 diabetes in Caucasians (1). Candidate genes outside the HLA region are being identified. The insulin gene on chromosome 11p15 confers ~10% of genetic susceptibility to type 1 diabetes (2). The polymorphic, cytotoxic T-lymphocyte–associated antigen (CTLA)-4 gene on chromosome 2q33 has also been associated with susceptibility to type 1 diabetes (3), although not consistently (4). At least 10 additional susceptibility genes are under investigation (5,6).

Interleukin (IL)-4 and IL-13 are multifunctional cytokines that play a critical role in the regulation of immune responses. The IL-4 and IL-13 genes have been mapped to chromosome 5q31 and the major component of their receptor, the IL-4Rα gene, to chromosome 16p11.2–12.1 (7). IL-4R polymorphisms have been associated with immune-related diseases, including asthma, atopy (8,9), and recently type 1 diabetes (10,11).

We evaluated associations between candidate genes outside the HLA region (INS, CTLA-4, IL-4, IL-4R, and IL-13) and islet autoimmunity among children participating in the Diabetes Autoimmunity Study in the Young (DAISY).

RESEARCH DESIGN AND METHODS

DAISY is a prospective study that has followed, since 1993, two cohorts of young children at increased risk of type 1 diabetes (12): the siblings and offspring cohort and the general population newborn cohort, screened for diabetes high-risk HLA class II alleles. The details of the newborn screening (12) and follow-up (13) have been previously published.

For this nested case-control study, all subjects were selected from the DAISY cohort. Cases included children who converted to persistent autoantibody positivity, i.e., children having one or more islet autoantibodies (insulin autoantibody [IAA], GAA, or insulinoma-associated protein 2 [IA-2] above the 99th percentile) in serum samples collected on at least two consecutive visits 3–6 months apart (n = 102); 38 of these children have already developed type 1 diabetes. Type 1 diabetes was defined by a random blood glucose >200 mg/dl and/or an HbA_{1c} (A1C) >6.2% in the presence of typical symptoms of diabetes. The average age of diagnosis in this study was 5.9 years (range 0.9–14.7). The control subjects (n = 198) were children who have never been positive for any antibody and were selected for each case by matching on age and HLA frequency; the oldest antibody-negative–matching general population newborn cohort was selected preferentially. Most control subjects (91%) have been selected from the general population newborn cohort, whereas 60% of cases come from the siblings and offspring cohort. Sex (male-to-female ratio, 1.17) and average follow-up from birth (7.0 years) did not differ by

TABLE 1
SNPs and allele frequencies in control subjects and cases (persistent islet autoimmunity including type 1 diabetes)

		DR3/DR4 heterozygotes			Other HLA genotypes		
		Frequency (%)			Frequency (%)		
SNP	Allele Ref/Var	Control subjects (n = 75)	Cases (n = 40)	OR (95% CI)	Control subjects (n = 123)	Cases (n = 62)	OR (95% CI)
CTLA (−318)	C/T	7.3	11.2	1.60 (0.63–4.04)	9.3	9.7	1.04 (0.50–2.16)
CTLA exon 1	A/G	40.7	40.0	0.97 (0.56–1.69)	43.1	40.3	0.89 (0.57–1.38)
INS −23Hph1	A/T	32.7	20.0	**0.51 (0.27–0.98)**	25.6	23.4	0.89 (0.53–1.47)
IL-4R −3223	C/T	30.0	26.2	0.83 (0.45–1.53)	26.4	38.7	**1.76 (1.11–2.78)**
IL-4R −1914	C/T	44.0	47.5	1.15 (0.67–1.98)	50.4	36.3	**0.56 (0.36–0.87)**
IL-4R 50	A/G	50.7	40.0	0.65 (0.37–1.12)	43.1	54.8	**1.60 (1.04–2.48)**
IL-4R 142	C/T	9.3	7.5	0.79 (0.29–2.14)	11.0	9.7	0.87 (0.42–1.78)
IL-4R 375	A/C	12.0	11.2	0.93 (0.40–2.18)	11.0	14.5	1.38 (0.73–2.61)
IL-4R 389	G/T	12.0	11.2	0.93 (0.40–2.18)	11.0	14.5	1.38 (0.73–2.61)
IL-4R 406	T/C	11.3	11.2	0.99 (0.42–2.34)	11.0	12.9	1.20 (0.62–2.32)
IL-4R 478	T/C	16.0	17.5	1.11 (0.54–2.30)	17.5	18.5	1.07 (0.61–1.88)
IL-4R 551	A/G	18.7	21.2	1.18 (0.60–2.31)	26.0	23.4	0.87 (0.52–1.44)
IL-4R 761	T/C	0.7	1.2	N/A	0.8	0.0	N/A
IL-4 −524	C/T	19.3	11.2	0.53 (0.24–1.18)	17.9	23.4	1.40 (0.83–2.38)
IL-13 −1512	A/C	16.0	21.2	1.42 (0.71–2.83)	16.7	17.7	1.08 (0.61–1.91)
IL-13 −1112	C/T	19.3	21.2	1.13 (0.58–2.20)	17.5	18.5	1.08 (0.61–1.88)
IL-13 intron 3	C/T	25.3	18.7	0.68 (0.35–1.33)	21.1	19.4	0.89 (0.52–1.54)
IL-13 110	G/A	23.3	18.7	0.76 (0.38–1.49)	19.1	17.7	0.91 (0.52–1.60)

Variant allele frequencies for the SNPs are shown after stratification for HLA-DR3/4 status. Differences in allele frequencies between case and control subjects were tested by χ^2. ORs refer to the variant allele. Statistically significant OR values ($P < 0.05$) are indicated in bold. Ref, reference; Var, variant allele.

affected status. The majority of the cohort (80%, n = 241) was non-Hispanic white (NHW), although this proportion was higher in cases (88%) than in control subjects (76%).

Islet autoantibodies. Measurement of biochemical islet autoantibodies was performed in the laboratory of Dr. George Eisenbarth at the Barbara Davis Center, Denver, CO. We used radioimmunoassays for autoantibodies to insulin, GAD_{65}, and IA-2. Insulin autoantibodies were measured by a micro-IAA assay with sensitivity of 58%, specificity of 99%, and interassay coefficient of variation (CV) 11% (14). The combined GAA (GAD65 autoantibodies) and IA-2A radioassay was done in duplicates on a 96-well filtration plate and radioactivity was counted on a TopCount 96-well plate β counter using methods previously described (15). In the 1995 Immunology of Diabetes Society Workshop, the GAA assay gave an 82% sensitivity and 99% specificity using sera from new-onset diabetic patients aged <30 years. The interassay CV was 6%. The IA-2A assay gave a 73% sensitivity and 100% specificity, and the interassay CV was 10%. Based on 198 nondiabetic control subjects ages 0.4–67.5 years, the 99th percentile for IAA (0.01), GAA (0.032), and the 100th percentile (single-highest value) for IA-2A (0.07) were used as the cutoffs for positivity.

Genotyping. One INS single nucleotide polymorphism (SNP) (−23Hph1), two CTLA-4 SNPs (the A49G site in exon 1 and the promoter C318T), ten IL-4R SNPs (including two in the promoter region), one IL-4 promoter SNP, and four IL-13 SNPs were genotyped using a linear array (immobilized probe) method essentially as described in Mirel et al. (10). Briefly, ~100 ng of genomic DNA was PCR amplified using 18 biotinylated primer pairs in a single PCR. The labeled amplicons were hybridized to an immobilized sequence-specific oligonucleotide probe (or SNP) array (36 probes) on a nylon membrane. The presence of bound amplicon to a specific probe is detected using streptavidin horseradish peroxidase and a soluble colorless substrate, tetramethylbenzidine, which can be converted in the presence of H_2O_2 to a blue precipitate.

Statistical analysis. Comparisons of allele and genotype frequencies between case and control subjects were assessed by χ^2 test with 2 × 2 contingency tables. P values exceeding 0.05 were denoted as not significant. Since our analyses were based on a priori hypotheses, P values were not corrected for multiple testing. The strength of the association was estimated by the OR. The SNPs were in Hardy-Weinberg equilibrium for both case and control subjects. The program PHASE version 2.02 was used to assign the most likely pair of haplotypes to each individual. PHASE implements a Bayesian statistical method for reconstructing haplotypes from population genotype data using the methods described by Stephens et al. (16). Allele and genotype frequencies were also subanalyzed by ethnicity and diabetes status. As a subanalysis including only one affected member per family (i.e., excluding six siblings) gave essentially identical results, we included all cases and present the results of the overall group. The Colorado Multiple Institutional Review Board approved all study protocols.

RESULTS

Individual SNPs. The distributions of alleles at the individual SNPs are shown in Table 1 after stratification by high-risk HLA genotypes. Overall, there were no significant differences between allele frequencies among case and control subjects (data not shown). However, a significant association was found between the INS-23Hph1 SNP and persistent autoimmunity in the DR3/4 heterozygotes. In the other lower-risk HLA subgroup, two IL-4R SNPs (promoter −3223 and the IL-4R50) had a predisposing effect, whereas the IL-4R promoter −1914 had a protective effect.

Results were similar in analyses restricted to NHW subjects (n = 241) (data not shown). Genotype frequencies were analyzed in the overall persistent autoimmunity group (online appendix 1 [available at http://diabetes.diabetesjournals.org]) and in the subgroup of only type 1 diabetic case and control subjects (Fig. 1). The INS-23Hph1 polymorphism was significantly associated with both type 1 diabetes (OR = 0.30; 95% CI 0.13–0.69) and persistent islet autoimmunity but in the latter only in children with the HLA-DR3/4 genotype (0.40; 0.18–0.89). CTLA-4 promoter SNP was significantly associated with type 1 diabetes (3.52; 1.22–10.17) but not with persistent islet autoimmunity. Several IL-4R SNPs (variant alleles) had a predisposing effect for persistent islet autoimmunity and type 1 diabetes except the promoter −1914, which showed a protective effect. Results were similar in analyses restricted to NHW subjects (n = 241) (data not shown).

Linkage disequilibrium patterns were computed among the DAISY Caucasian control population (16a). Pairwise linkage disequilibrium between the 10 IL-4R SNPs on

SNP	Genotype		Frequency in controls (n=76)		Frequency in T1D (n=38)		P	OR (95% CI)
CTLA-318	CC	CT/TT	90.8	9.2	73.7	26.3	**0.02**	**3.52 (1.22-10.17)**
CTLA ex1	AA	AG/GG	27.6	72.4	39.5	60.5	0.20	0.58 (0.26-1.33)
INS Hph1	AA	AT/TT	39.5	60.5	68.4	31.6	**0.004**	**0.30 (0.13-0.69)**
IL4R-3223	CC	CT/TT	53.9	46.1	42.1	57.9	0.23	1.61 (0.73-3.53)
IL4R-1914	CC	CT/TT	28.9	71.1	44.7	55.3	0.09	0.50 (0.22-1.13)
IL4R 50	AA	AG/GG	31.6	68.4	23.7	76.3	0.38	1.49 (0.61-3.62)
IL4R 142	CC	CT/TT	86.8	13.2	84.2	15.8	0.70	1.24 (0.41-3.70)
IL4R 375	AA	AC/CC	81.6	18.4	63.2	36.8	**0.03**	**2.58 (1.07-6.21)**
IL4R 389	GG	GT/TT	81.6	18.4	63.2	36.8	**0.03**	**2.58 (1.07-6.21)**
IL4R 406	TT	TC/CC	84.2	15.8	68.4	31.6	**0.05**	**2.46 (0.98-6.18)**
IL4R 478	TT	TC/CC	72.4	27.6	55.3	44.7	0.07	2.12 (0.94-4.78)
IL4R 551	AA	AG/GG	61.8	38.2	47.4	52.6	0.14	1.80 (0.82-3.96)
IL4R 761	TT	TC/CC	94.7	5.3	97.4	2.6	0.52	0.49 (0.05-4.51)
IL4 -524	CC	CT/TT	60.5	39.5	63.2	36.8	0.79	0.89 (0.40-2.00)
IL13 -1512	AA	AC/CC	68.4	31.6	63.2	36.8	0.57	1.26 (0.56-2.86)
IL13 -1112	CC	CT/TT	68.4	31.6	60.5	39.5	0.40	1.41 (0.63-3.18)
IL13 intr.3	CC	CT/TT	52.6	47.4	57.9	42.1	0.59	0.81 (0.37-1.77)
IL13 110	GG	GA/AA	57.9	42.1	57.9	42.1	1.00	1.00 (0.45-2.20)

FIG. 1. Genotype frequencies in control and case subjects (type 1 diabetes [T1D] only). The figure shows genotype frequencies for type 1 diabetes only and control and case subjects (type 1 diabetes substudy). The first column in control subjects and in type 1 diabetes (shaded in gray) refers to homozygotes dominant. The second column in control and case subjects and type 1 diabetes (in white) refers to heterozygotes and homozygotes variant (these have been combined for analysis, since several cells had values <5 in the homozygote variant). ORs refer to this combined variant group. Statistically significant OR values ($P < 0.05$) are indicated in bold.

chromosome 16p11 and between the 5 IL-4/IL-13 SNPs on chromosome 5q31 confirmed that the SNPs are in strong linkage disequilibrium (see online appendix 2 and 3).

Haplotypes. We estimated five SNP haplotype (complex alleles) frequencies in Caucasian case and control subjects by using the program PHASE (16) (Table 2). We found that one haplotype (CACTA) seems to be protective both overall and in the type 1 diabetes subgroup ($P = 0.03$ and $P = 0.07$, respectively).

We analyzed the 10-SNP IL-4R haplotypes frequencies in Caucasian case and control subjects using two methods, which gave similar results: the EM haplotype frequency estimates (Table 3) and the most likely haplotype phase assignment. By taking the same nomenclature used in two earlier studies (10,11), we found that haplotype TC (H-2) is predisposing overall and in the type 1 diabetes subgroup. The same haplotype differing only at the second promoter SNP (TT [H-2]) appeared protective. Two other haplotypes CT (H-7) and CC (H-1) were protective and the CT (H-1) haplotype (differing from the latter only at the second promoter SNP) was predisposing in the overall group (haplotype data for type 1 diabetes subgroup not shown).

DISCUSSION

Whereas >15 diabetes-predisposing genes have been previously reported, only the HLA region and the insulin gene have been conclusively associated with susceptibility to

TABLE 2
Estimated IL-4 and IL-13 haplotype frequencies in control subjects and cases (persistent islet autoimmunity including type 1 diabetes)

Haplotype	Frequency (%) Control subjects	Cases	*P* value	OR (95% CI)
CACCG	65.7	68.3	NS	1.13 (0.78–1.62)
CACTA	4.5	1.0	**0.03**	**0.21 (0.05–0.92)**
CCTCG	5.6	5.9	NS	1.07 (0.51–2.22)
CCTTA	6.1	6.4	NS	1.06 (0.52–2.13)
TACCG	5.1	6.4	NS	1.29 (0.62–2.67)
TACTA	4.5	3.5	NS	0.76 (0.31–1.86)
TACTG	1.3	0.5	NS	0.37 (0.04–3.18)
TATCG	1.3	0.5	NS	0.37 (0.04–3.18)
TCTTA	4.5	6.4	NS	1.45 (0.69–3.05)

In the table and text, the haplotypes are designed with the SNPs in the following order: IL-4–524, IL-13–1512, IL-13–1112, IL-13 intron 3, and IL-13–110. The physical order of the SNPs on 5q31 is IL-13–1512, IL-13–1112, IL-13 intron 3, IL-13–110, and IL-4–524. Of the 12 inferred 5-SNP haplotypes, only those with an estimated frequency of >1% in either group are shown.

type 1 diabetes (6,17). We found that the INS-23Hph1 site was significantly associated with both type 1 diabetes and persistent islet autoimmunity but, in the latter, only in children with the HLA-DR3/4 genotype. Previously, INS polymorphisms were found to be associated only in type 1 diabetic subjects with HLA-DR4 genotype (18), those with non-DR3/non-DR4 genotype (19), or independent of HLA genotype (20). The discrepant findings in the previous reports might be explained by ethnic differences in the study populations. Spurious associations can arise due to population stratification in studies drawing case and control subjects from the heterogeneous U.S. population. Stratification bias may represent either recent admixture or incorrect matching of case and control subjects. Even though our Colorado population is heterogeneous, analyses restricted to NHW subjects only yielded essentially identical results. Although our Caucasian Colorado population might be a mix of ancestral groups, the association found shows external consistency. We, and a previous study (20), have shown that the association in type 1 diabetic patients is not dependent on HLA-DR3/4 genotype. Alternatively, age of diagnosis (very young in our type 1 diabetic cases) and severity of the disease (low in our nondiabetic cases) could explain the subgroup differences. Possible explanation for the association of INS-23Hph1 in only the DR3/4 subgroup in our overall autoimmunity study may be that not all persistent autoantibody positive subjects will develop type 1 diabetes, but most of those with HLA-DR3/4 will do so. Type 1 diabetes and persistent autoimmunity without diabetes may be different outcomes with potentially different genetic determinants.

CTLA-4 gene polymorphisms have been associated with type 1 diabetes (21), Graves' disease (21), celiac disease (22), and Addison's disease (23). This study showed an association between the CTLA-4 promoter and type 1 diabetes, nominally significant only in the type 1 diabetic subgroup. This association was not significant in persistent islet autoimmunity, possibly because the latter does not confer an absolute risk of subsequent development of type 1 diabetes. With two or more autoantibodies, the risk of type 1 diabetes is estimated at 68% within 5 years in first-degree relatives (24). Another possible explanation is that CTLA-4 gene polymorphisms are biologically important only in the late stages of development of type 1 diabetes.

More than 13 SNPs have been identified in the coding region of the IL-4R gene, including 7 SNPs that result in amino acid substitutions and impact the biological activity

TABLE 3
Estimated IL-4R haplotype frequencies in control subjects and cases (persistent islet autoimmunity including type 1 diabetes)

Haplotype	Haplotype label	Frequency (%) Control subjects	Cases	*P* value	OR (95% CI)
CC-ACAGTTAT	CC (H-1)	21.9	12.4	**0.01**	**0.50 (0.31–0.82)**
CC-ACAGTTGT	CC (H-7)	1.4	0.2	NS	0.16 (0.01–3.26)
CC-ACCTCCGT	CC (H-5)	1.8	3.6	NS	2.06 (0.71–5.98)
CC-GCAGTTAT	CC (H-2)	3.3	2.0	NS	0.60 (0.19–1.86)
CC-GCCTCCGT	CC (H-3)	2.3	3.4	NS	1.49 (0.54–4.13)
CC-GTAGTCGT	CC (H-6)	1.9	2.0	NS	1.03 (0.30–3.54)
CC-GTAGTTAT	CC (H-4)	3.5	3.8	NS	1.08 (0.43–2.67)
CT-ACAGTTAT	CT (H-1)	18.9	28.3	**0.02**	**1.70 (1.14–2.53)**
CT-ACAGTTGT	CT (H-7)	4.3	1.2	**0.05**	**0.27 (0.07–1.07)**
CT-ACCTCCGT	CT (H-5)	3.7	1.5	NS	0.40 (0.11–1.39)
CT-GCAGTTAT	CT (H-2)	2.2	1.9	NS	0.84 (0.25–2.89)
CT-GCCTCCGT	CT (H-3)	1.4	0.9	NS	0.62 (0.11–3.46)
CT-GTAGTCGT	CT (H-6)	3.0	1.0	NS	0.34 (0.08–1.49)
CT-GTAGTTAT	CT (H-4)	1.7	0.6	NS	0.35 (0.05–2.44)
TC-ACAGTTAT	TC (H-1)	0.5	1.9	NS	3.77 (0.65–21.84)
TC-GCAGTTAT	TC (H-2)	12.8	23.2	**0.003**	**2.06 (1.32–3.22)**
TC-GCCTCCGT	TC (H-3)	0.9	2.0	NS	2.14 (0.50–9.15)
TT-GCAGTTAT	TT (H-2)	10.4	2.3	**0.001**	**0.20 (0.07–0.54)**
TT-GCCTCCGT	TT (H-3)	1.3	0.7	NS	0.55 (0.09–3.62)

The hyphen in each haplotype separates the two promoter SNPs from the eight coding-sequence SNPs. The haplotype label names the 10-SNP haplotypes by the identity of the −3223 and −1914 alleles, followed in parentheses by the 8-SNP haplotype ID as presented by Mirel et al. (see ref. 10). Only those haplotypes with an estimated frequency of >1% in either group are shown.

of the protein (8). We found significant differences in genotype frequencies between case and control subjects for several IL-4R SNPs. Apart from the promoter −1914, all of these individual SNPs (variant alleles) have a nominally predisposing effect for persistent islet autoimmunity and type 1 diabetes in this study. Furthermore, when looking at the 10-SNP IL-4R haplotypes, we found nominally significant associations for several haplotypes: the TT (H-2) and CC (H-1) appeared protective while the TC (H-2) and CT (H-1) (differing from the two former only at the second promoter SNP −1914) were predisposing. The promoter SNP −1914 may play a role in determining the positive or negative association of a particular haplotype with persistent islet autoimmunity and type 1 diabetes.

In a Filipino study done by Bugawan et al. (11), one specific 10-SNP haplotype (CC[H-5]) had a protective effect for type 1 diabetes. Another 8-SNP haplotype (H-3) has been shown to be protective both in the Filipino study and in a study of Human Biological Data Interchange families (10). This study does not replicate the protective effect of haplotypes (H-3) and (H-5); however, both of these haplotypes are relatively rare. Although the particular haplotypes involved are different from previously published studies, this report is consistent with the possibility that both IL-4R haplotypes and IL-4–IL-13 haplotypes may be involved with persistent islet autoimmunity and type 1 diabetes.

We investigated possible associations of several non-HLA polymorphisms with type 1 diabetes autoimmunity. Although we made several comparisons in a population of relatively modest size, we did not correct for multiple comparisons since we had a priori hypotheses regarding these candidate genes. In addition, our results mainly replicate others' findings; we confirm the association of the INS (2) and CTLA-4 (3) loci with type 1 diabetes and report possible associations in the IL-4 regulatory pathway, where associations have been found for both asthma (8,9) and type 1 diabetes (10,11), although not consistently (25).

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