Brief Genetics Report

# The Variable Number of Tandem Repeats Upstream of the Insulin Gene Is a Susceptibility Locus for Latent Autoimmune Diabetes in Adults

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**The etiopathological relationship between latent autoimmune diabetes in adults (LADA) and classical type 1 (insulin dependent) diabetes remains unclear. Variation at the insulin gene variable number of tandem repeats (VNTR) minisatellite influences susceptibility to type 1 diabetes, but studies in LADA have been small and inconsistent. We examined the role of insulin gene variation (using flanking variants as surrogates for VNTR subtypes) in the largest case-control study of LADA to date (400 case and 332 control subjects). Highly significant associations were identified with disease, with dominant protective effects of the T allele at −23*HphI* (odds ratio [OR] 0.42 [95% CI 0.31–0.58], $P = 2.4 \times 10^{-8}$), A allele at +1,404*Fnu4HI* (0.50 [0.36–0.70], $P = 3.2 \times 10^{-5}$), and C allele at +3,580*Msp*I (0.55 [0.35–0.85], $P = 0.0046$). As with type 1 diabetes, the −23*HphI* variant (a surrogate for the subdivision of VNTR into class I and III alleles) most clearly defined susceptibility in LADA. However, there was no association with age at diagnosis or requirement for insulin therapy 6 years postdiagnosis. This study establishes that variation within the insulin gene region does influence susceptibility to LADA, with the direction and magnitude of effect indistinguishable from that previously reported for type 1 diabetes. In conclusion, differences in VNTR-encoded susceptibility do not explain the differences in clinical presentation that distinguish classical type 1 diabetes and LADA. *Diabetes* 55:1890–1894, 2006**

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HWE, Hardy-Weinburg equilibrium; LADA, latent autoimmune diabetes in adults; LD, linkage disequilibrium; PH, protective haplotype; SNP, single nucleotide polymorphism; UKPDS, U.K. Prospective Diabetes Study; VNTR, variable number of tandem repeats; VPH, very protective haplotype; W2 Repository, Warren 2 Repository; Exeter YT2D, Exeter Young-Onset type 2 Diabetes study.

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Latent autoimmune diabetes in adults (LADA) and type 1 (insulin dependent) diabetes are both characterized by islet autoimmunity, indicated by the presence of circulating islet autoantibodies. However, the later age of onset (after the peak of type 1 diabetes incidence) and less aggressive clinical course (no immediate requirement for insulin therapy) of LADA result in substantial clinical overlap with type 2 diabetes (non–insulin dependent) (1–3). Qualitative or quantitative differences in genetic susceptibility may underlie the marked variation in disease course and clinical presentation between LADA and type 1 diabetes.

The insulin gene region (*IDDM2*) accounts for ~10% of familial risk for type 1 diabetes (4). Most evidence indicates that the variable number of tandem repeats (VNTR) minisatellite (which maps upstream of the translation initiation site) is the probable etiological variant, though certain flanking single nucleotide polymorphisms (SNPs) that are highly correlated with VNTR variation may also play a role (5,6). In non-African populations, much of the variation in VNTR length (repeat unit number) and composition (repeat element sequence) can be captured by dichotomizing VNTR alleles into two classes: the short class I alleles (26–63 repeats) and longer class III alleles (141–209 repeats) (7). In type 1 diabetes, class I alleles confer susceptibility to disease and class III, protection (8). Because of strong linkage disequilibrium (LD) between VNTR allele structure and haplotypes spanning the insulin gene, this major division in VNTR structure can be assayed using the −23*HphI* SNP (9).

Further refinement of type 1 diabetes susceptibility is possible by examining the finer structure within major VNTR classes. A discrete subgroup of class I alleles, termed "ID−," has been reported to confer protection rather than susceptibility (7). Additionally, class IIIA and IIIB alleles may differ in the degree of protection they offer, an observation reflected in the designation of the extended haplotypes on which these alleles lie as the protective haplotypes (PHs) and very protective haplotypes (VPHs), respectively (9). However, recent reexamination of these subclass associations in additional samples has cast significant doubt on these latter observations (5).

It is unclear whether these susceptibility effects of the insulin gene are evident in LADA and/or whether differences in VNTR-encoded susceptibility contribute to the clinical differences between LADA and type 1 diabetes.

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Published studies have yielded conflicting results (10,11). One small study from South America ($n$ = 44 LADA and 138 control subjects) reported a significant class I association with LADA (10). However, another larger study, performed in 95 LADA case subjects and 172 control subjects from Finland, found no association of the −23*HphI* site with disease (11). The present study uses the largest collection of patients yet studied to determine whether the *IDDM2* locus is associated with LADA.

The LADA group ($n$ = 400) comprised autoantibody-positive patients from three sources, the U.K. Prospective Diabetes Study (UKPDS), the Warren 2 Repository (W2 Repository), and the Exeter Young-Onset type 2 Diabetes study (Exeter YT2D); 332 normoglycemic autoantibody-negative subjects from the Diabetes in Families study served as control subjects (Table 1). All were genotyped for three SNPs known to be surrogate markers for minisatellite lineages: −23*HphI* (A→ T), +1,404*Fnu4HI* (C→ A), and +3,580*Msp*I (C→ T) (7,9).

Comparisons of genotype frequency distributions revealed borderline evidence for heterogeneity between the different case groups (−23*HphI*, $P$ = 0.045; +1,404*Fnu4HI*, $P$ = 0.033; +3,580*Msp*I, $P$ = 0.062) (Table 2). Accordingly, results are presented for the case groups combined as well as for individual case groups with the control group. Genotype frequencies at all sites were in Hardy-Weinburg equilibrium (HWE) in the control subjects, but there were modest departures in some of the case groups (Exeter YT2D, −23*HphI*, $P$ = 0.008; W2 Repository, +1,404*Fnu4HI*, $P$ = 0.01; UKPDS, +3,580*Msp*I, $P$ = 0.024). Only the last of these departures was in a direction consistent with the observed association effect. We attribute the other deviations to stochastic variation, since extensive regenotyping and resequencing (and failure to detect unexpected haplotypic combinations) effectively excluded genotyping error.

All three SNPs were associated with LADA in the combined group, with dominant protective effects of the T allele at −23*HphI* (odds ratio [OR] 0.42 [95% CI 0.31–0.58], $P = 2.4 \times 10^{-8}$), A allele at +1,404*Fnu4HI* (0.50 [0.36–0.70], $P = 3.2 \times 10^{-5}$), and C allele at +3,580*Msp*I (0.55 [0.35–0.85], $P$ = 0.0046). Patterns of associations were similar under the additive model (data not shown). Comparisons of the individual case groups with the control subjects (Table 3) confirmed that these associations were not an artifact of case-group heterogeneity: all case-control comparisons showed similar ORs, although sample size differences meant that not all such comparisons were significant. Step-wise logistic regression analysis in the combined LADA group versus control subjects identified the −23*HphI* site (indicating VNTR class) as the primary disease-associated variant (Wald $\chi^2$, $P = 2.8 \times 10^{-8}$); the other sites had no additional independent effect.

Given recent discussion over the appropriate definition of LADA (12,13), we repeated these analyses, excluding those patients in the UKPDS ($n$ = 45), W2 Repository ($n$ = 4), and Exeter YT2D ($n$ = 6) groups diagnosed under the age of 30 years and in whom the interval between diagnosis and insulin therapy was between 3 and 6 months. The strength of the association was unchanged in this analysis ($OR_{-23HphI}$ = 0.42 [95% CI 0.30–0.58], $P = 5.6 \times 10^{-8}$).

All three SNPs were, as expected, in strong LD: pairwise D′ values exceeded 0.94, although $r^2$ values ranged from 0.06 to 0.89. Given the modest departures from HWE in the case groups, the expectation-maximization algorithm was

TABLE 1
Clinical characteristics of study subjects

| Study ($n$) | Ascertainment criteria | Autoantibody positivity* | Range of age at onset (years) | Age at onset (years) | Duration of diabetes (years) | BMI (kg/m$^2$) | Male sex (%) | Insulin requiring by 3 years postdiagnosis (%) |
|---|---|---|---|---|---|---|---|---|
| UKPDS (232) | Newly diagnosed type 2 diabetes: not insulin requiring within 3 months of diagnosis, ketonuria <3 mmol/l | GADA and/or IA-2A† | 25–65 | 46.7 ± 10.6 | Studied at diagnosis | 25.2 ± 4.9† | 53 | 51 |
| W2 Repository (131) | Clinical diagnosis of type 2 diabetes: not insulin requiring within 12 months of diagnosis | GADA‡ | 26–68 | 47.4 ± 10.0 | 8.9 ± 6.7‡ | 28.6 ± 5.3‡ | 57 | 41 |
| Exeter YT2D (37) | Clinical diagnosis of type 2 diabetes: not insulin requiring within 3 months of diagnosis | GADA and/or IA-2A‡ | 29–45 | 38.4 ± 5.4 | 11.9 ± 7.7‡ | 27.5 ± 5.8‡ | 43 | 57 |
| Control subjects (332) | Spouses/friends of probands collected in the DIF study, normoglycemic | Negative for GADA and IA-2A‡ | 20–91‡ | 55.5 ± 19.8‡ | N/A | 25.4 ± 4.0‡ | 45 | N/A |

Data are means ± SD unless otherwise indicated. *Antibody measurements were made in the accredited laboratory of Professor Bingley (University of Bristol) for the UKPDS, Exeter YT2D, and Diabetes in Families (DIF) cohorts and in the laboratory of Professor Bottazzo (Royal London Hospital) for the W2 Repository. †At diagnosis; ‡at time of sample collection.

TABLE 2
Genotype frequencies of the insulin gene polymorphisms in the sample groups

| Marker | Genotype | UKPDS LADA ($n$ = 232)* | W2 Repository LADA ($n$ = 131)* | Exeter YT2D LADA ($n$ = 37)* | All cases ($n$ = 400)* | Control subjects ($n$ = 332)* |
|---|---|---|---|---|---|---|
| −23*HphI* | | | | | | |
| | AA | 165 (0.71) | 81 (0.62) | 30 (0.81) | 276 (0.69) | 159 (0.49) |
| | AT | 58 (0.25) | 40 (0.31) | 4 (0.11) | 102 (0.26) | 141 (0.43) |
| | TT | 8 (0.04) | 10 (0.08) | 3 (0.08) | 21 (0.05) | 27 (0.08) |
| +1404*Fnu4HI* | | | | | | |
| | CC | 182 (0.80) | 89 (0.69) | 30 (0.81) | 301 (0.76) | 199 (0.61) |
| | CA | 42 (0.18) | 31 (0.24) | 5 (0.14) | 78 (0.20) | 105 (0.32) |
| | AA | 5 (0.02) | 10 (0.08) | 2 (0.05) | 17 (0.04) | 20 (0.06) |
| +3580*MspI* | | | | | | |
| | CC | 81 (0.35) | 50 (0.39) | 20 (0.54) | 151 (0.38) | 147 (0.45) |
| | CT | 97 (0.42) | 60 (0.46) | 12 (0.32) | 169 (0.42) | 143 (0.43) |
| | TT | 53 (0.23) | 20 (0.15) | 5 (0.14) | 78 (0.20) | 39 (0.12) |

Data are $n$ (frequency). *Individuals in whom genotyping was attempted.

reinitialized three times to ensure convergence. This, together with presence of high LD between the SNPs, enabled accurate haplotype estimation. Four haplotypes with estimated frequencies exceeding 1% were observed, two corresponding to the subdivision of class I alleles (into IC+/ID+ and ID−), the others to the major class III haplotypes PH and VPH (Table 4). Haplotype trend regression revealed highly significant differences in haplotype frequency distributions between the combined case subjects compared with the control subjects ($P = 7.9 \times 10^{-6}$). Class I haplotypes were more frequent in the LADA than in the control subjects, in keeping with classical type 1 diabetes.

Diplotype frequencies differed significantly between case and control subjects ($P < 10^{-4}$ overall). The OR (95% CI) for VNTR diplotypes estimated under the recessive model for class I haplotypes and the dominant model for class III haplotypes were as follows: ID− homozygotes versus all others 1.82 (1.18–2.84), $P$ = 0.0045; IC+/ID+ homozygotes versus all others 1.67 (1.10–2.58), $P$ = 0.016; PH-containing diplotypes versus all others 0.50 (0.36–0.69), $P = 2.0 \times 10^{-5}$; and VPH-containing diplotypes versus all others 0.49 (0.29–0.80), $P$ = 0.0028. Similar ORs were seen for the comparisons of the separate case groups against the control subjects (data not shown).

There was no association between age at diagnosis and insulin gene variation (class III positive mean ± SD 45.8 ± 10.1 years vs. class I homozygote 46.5 ± 10.5, $P$ = 0.54). Within the UKPDS group (the only group for which such data were available—insulin requirement was determined by a protocol-driven assessment [14]), there was no association with insulin therapy requirement at 6 years postdiagnosis (class III positive 35% vs. class I homozygote 34%, $P$ = 0.88).

This study has established that the *INS* gene is a susceptibility locus for autoimmune diabetes developing in adulthood as well as in children. The −23*HphI* site (or, equivalently, VNTR class) appears to be the primary disease-associated variant, as in classical type 1 diabetes. We conclude that the different clinical features of LADA and type 1 diabetes cannot be explained by an absence of *IDDM2*-encoded genetic susceptibility in adult-onset patients (11). From the data available, there is no suggestion that the magnitude of the *INS* VNTR effect size in LADA differs appreciably from that seen in type 1 diabetes. In the combined LADA group, the OR (95% CI) associated with dominantly protective class III alleles was 0.42 (0.31–0.58), a value comparable with that found in studies of classical type 1 diabetes (for example, OR 0.36 [95% CI 0.23–0.55] and 0.22 [0.12–0.39], as calculated from previous reports [9,15]).

Failure to detect a VNTR association with LADA in a previous study (11) may reflect several factors, including reduced power (a lower frequency of the class III allele in Finns (11,16) combined with a smaller sample size) and/or genuine differences arising through variation in genetic background or ascertainment.

Fine-structure analysis of the region demonstrated similarities in the association patterns of LADA compared with those reported for type 1 diabetes. Diplotype analyses showed that the ID− haplotype confers similar susceptibility to LADA to other class I haplotypes and that the class III PH and VPH had similar protective effects. Both are consistent with findings from a recent reevaluation of

TABLE 3
Single-point association analysis of the −23*HphI*, +1404*Fnu4HI*, and +3580*MspI* SNPs with disease in the LADA groups assuming dominance for the protective allele

| Sample group ($n$)* | −23*HphI* OR (95% CI) | −23*HphI* $P$ | +1404*Fnu4HI* OR (95% CI) | +1404*Fnu4HI* $P$ | +3580*MspI* OR (95% CI) | +3580*MspI* $P$ |
|---|---|---|---|---|---|---|
| UKPDS LADA (232) | 0.38 (0.26–0.55) | $9.3 \times 10^{-8}$ | 0.41 (0.27–0.62) | $6.8 \times 10^{-6}$ | 0.45 (0.28–0.73) | $7.2 \times 10^{-4}$ |
| W2 Repository LADA (131) | 0.58 (0.38–0.90) | 0.013 | 0.73 (0.46–1.15) | 0.16 | 0.74 (0.40–1.40) | 0.35 |
| Exeter YT2D LADA (37) | 0.22 (0.08–0.53) | 0.00019 | 0.37 (0.13–0.90) | 0.019 | 0.86 (0.31–3.00) | 0.79 |
| All cases (400) | 0.42 (0.31–0.58) | $2.4 \times 10^{-8}$ | 0.50 (0.36–0.70) | $3.2 \times 10^{-5}$ | 0.55 (0.35–0.85) | 0.0046 |

ORs (95% CIs) were calculated based on the dominant model of inheritance for the allele at each SNP known to be associated with protection from type 1 diabetes: 23*HphI*, dominant for T allele (VNTR class III alleles); +1404*Fnu4HI*, dominant for A allele; +3580*MspI*, dominant for C allele. All comparisons were carried out between the case group concerned and the common control set. *Individuals in whom genotyping was attempted.

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TABLE 4
Estimated haplotype frequencies in the sample groups

| Haplotype (type 1 diabetes defined) | Haplotype* | UKPDS LADA | W2 Repository LADA | Exeter YT2D LADA | All cases | Control subjects |
|---|---|---|---|---|---|---|
| $n$ | | 232 | 131 | 37 | 400 | 332 |
| Class ID− | ACT | 0.440 | 0.386 | 0.297 | 0.409 | 0.336 |
| Class IC+/ID+ | ACC and AAC | 0.401 | 0.385 | 0.568 | 0.411 | 0.364 |
| Class III PH | TAC | 0.115 | 0.190 | 0.122 | 0.140 | 0.222 |
| Class III VPH | TCC | 0.045 | 0.039 | 0.014 | 0.040 | 0.078 |
| $P$ | | $1.7 \times 10^{-6}$ | 0.056 | $1.0 \times 10^{-3}$ | $7.9 \times 10^{-6}$ | |

*Allelic combination at −23*HphI*, +1404*Fnu4HI*, and +3580*MspI* SNPs. $P$ = haplotype trend regression $P$ value to test for difference in haplotype frequency distributions between case and control subjects.

the insulin gene in type 1 diabetes (5), although the wide CIs indicate the need for cautious interpretation. The present study had >90% power to detect the dominantly protective association of class III alleles with OR ≤0.5 (for $\alpha = 0.05$). However, considerably larger numbers of subjects would be required to detect subtle differences in predisposition associated with specific class I or class III subgroups; the largest comparison of the protective effects associated with PH and VPH in type 1 diabetes featured meta-analysis of data from ~3,000 pedigrees (5).

We have made no corrections for multiple testing; application of a conservative Bonferroni correction to the lowest $P$ value obtained ($P = 2.4 \times 10^{-8}$) would still retain significance ($P < 10^{-4}$), indicating that the observed associations are likely to be real.

Class III alleles have been associated with a decreased level of transcription of *INS* in the thymus during development (17). It has been postulated that this difference may affect central tolerance mechanisms, promoting negative selection of autoreactive T-cells and decreasing the propensity to develop autoimmunity against insulin (17). Similar protective mechanisms are likely to operate in LADA. However, as yet unresolved differences in class I subgroup susceptibility (or, alternatively, within HLA or other type 1 diabetes–susceptibility loci) could, through their consequences on the development of tolerance, contribute to the differences in clinical phenotype between classical type 1 diabetes and LADA.

## RESEARCH DESIGN AND METHODS

All subjects were unrelated and of exclusively British/Irish Europid origin. LADA subjects ($n = 400$) came from three sources of type 2 diabetes–diagnosed patients: the UKPDS ($n = 232$ from the total cohort of 5,102) (14), the W2 Repository ($n = 131$ from the total sample of ~4,000) (18–20), and the Exeter YT2D study ($n = 37$ from the total cohort of 400) (21). LADA patients were selected using common criteria: an initial diagnosis of type 2 diabetes (age at diagnosis 25–68 years), documented antibody-positivity for GADA and/or IA-2, and no immediate requirement for insulin (within 3 months of diagnosis). The control group comprised normoglycemic, GADA/IA-2A–negative subjects recruited as part of the Diabetes in Families study ($n = 332$). Clinical characteristics of these subjects are given in Table 1. Requirement for insulin therapy in the UKPDS was protocol defined (14).

**Genotyping.** In non-African populations, low haplotype diversity in the insulin gene region enables flanking SNPs to act as surrogate markers for minisatellite lineages (7). The −23*HphI* SNP (rs689) denotes the class I/III subdivision; absence of the +3,580*Msp*I restriction site (rs2000993) distinguishes the ID− haplotype from other class I haplotypes (IC+/ID+), and the PH/VPH subdivision is detected through the +1,404*Fnu4HI* site (rs3842755) (7,9). All three SNPs were genotyped using the Amplifluor method (22). Based on extensive duplicate genotyping with a variety of methods including resequencing, we estimate an overall genotyping error of <0.5%. Genotyping success rates exceeded 95% in all cohorts. Primer sequences and additional genotyping details are available from the authors.

**Statistical analyses.** Deviations from HWE were sought using exact methods implemented in PEDSTATS (v6.0; University of Michigan) (23). Homogeneity (between LADA groups) and case-control association testing was performed by standard contingency table methods implemented in StatXact 6 (Cytel Software, Cambridge, MA). For each SNP, we tested for association under an additive model and assuming dominance for the protective allele (T at −23*HphI*, A at +1,404*Fnu4HI*, and C at +3,580*Msp*I) given previous evidence from studies of type 1 diabetes (5,15,24). Step-wise logistic regression (SPSS, v13.0 for Windows; SPSS, Chicago, IL) was used to ascertain which SNPs independently contributed to risk. Measures of pairwise LD and 3-point haplotype frequencies were estimated using the expectation-maximization algorithm implemented in HelixTree Genetics Analysis software (Golden Helix, Bozeman, MT). Haplotype frequencies were compared using haplotype trend regression in HelixTree (25). For diplotype analyses, the most probable pair of haplotypes was assigned to each individual. Though such direct assignment underestimates the associated variance, the consequences are modest: low rates of missing data and high local LD mean that >97% of assigned haplotypes had posterior probabilities >0.99. Association of age at diagnosis with genotype was assessed by linear regression modeling using SPSS. The relationship between insulin requirement 6 years postdiagnosis and genotype was determined by contingency table analysis in StatXact 6. Power calculations were performed using Quanto, v1.0 (http://hydra.usc.edu/gxe). A $P$ value <0.05 was deemed significant.

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