The Protein Tyrosine Phosphatase Nonreceptor 22 (*PTPN22*) Is Associated With High GAD Antibody Titer in Latent Autoimmune Diabetes in Adults

Non Insulin Requiring Autoimmune Diabetes (NIRAD) Study 3

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OBJECTIVE — We previously demonstrated the presence of two different populations among individuals with adult-onset autoimmune diabetes: those having either a high titer or a low titer of antibodies to GAD (GADAs). Protein tyrosine phosphatase nonreceptor type 22 (*PTPN22*) has been identified as a new susceptibility gene for type 1 diabetes and other autoimmune diseases. The aim of the present study was to evaluate whether the phenotypic heterogeneity of adult-onset autoimmune diabetes based on the GADA titer is associated with the *PTPN22* C1858T polymorphism.

RESEARCH DESIGN AND METHODS — Analysis for the C1858T polymorphism using the TaqMan assay was performed in 250 subjects with adult-onset autoimmune diabetes, divided into two subgroups with low (≤32 arbitrary units) or high (>32 arbitrary units) GADA titers and 450 subjects with classic type 2 diabetes (from the Non Insulin Requiring Autoimmune Diabetes [NIRAD] Study cohort of 5,330 subjects with adult-onset diabetes) and in 558 subjects with juvenile-onset type 1 diabetes and 545 normoglycemic subjects.

RESULTS — Genotype, allele, and phenotype distributions of the *PTPN22* C1858T variant revealed similar frequencies in autoimmune diabetes with high GADA titer and juvenile-onset type 1 diabetes. An increase in TT and CT genotypes was observed in individuals with a high GADA titer compared with a low GADA titer, those with type 2 diabetes, and control subjects ($P < 0.002$ for all comparisons). The *PTPN22* 1858T allele and phenotype frequencies were increased in high GADA titer compared with a low GADA titer, type 2 diabetic, and control subjects ($P < 0.001$ for all comparisons, odds ratio 2.6).

CONCLUSIONS — In adult-onset autoimmune diabetes, the *PTPN22* 1858T variant is associated only with a high GADA titer, providing evidence of a genetic background to clinical heterogeneity identified by GADA titer.

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A consistent fraction of subjects (4–10%) with adult-onset non–insulin-requiring diabetes at diagnosis, also referred to as latent autoimmune diabetes in adults or non–insulin-requiring autoimmune diabetes, has autoimmune features, specifically the presence of GAD autoantibodies (GADAs). These patients do not initially require insulin treatment and are extremely heterogeneous in terms of clinical presentation, ranging across the whole spectrum between classic phenotypes of type 1 and type 2 diabetes (1–4).

We have recently demonstrated the presence of two different populations among individuals with adult-onset autoimmune diabetes (5); analysis of GADA titers showed a bimodal distribution that identified two subgroups of patients with either a low or a high GADA titer. Compared with patients with a low GADA titer, patients with a high GADA titer had more prominent traits of insulin deficiency and a profile of more severe autoimmunity, resulting in a higher prevalence of protein tyrosine phosphatase IA-2, thyroid peroxidase antibodies, and DRB1*03-DQB1*0201 and a decreasing frequency of DQB1*0602 and DRB1*0403.

A new susceptibility gene to type 1 diabetes has been recently identified outside the HLA region, protein tyrosine phosphatase nonreceptor type 22 (*PTPN22*) (6), which encodes a lymphoid-specific phosphatase known as LYP, a powerful inhibitor of T-cell activation (7). Several studies showed that a missense single nucleotide polymorphism, C1858T, in the *PTPN22* gene is

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Abbreviations: GADA, GAD autoantibody; NIRAD, Non Insulin Requiring Autoimmune Diabetes; *PTPN22*, protein tyrosine phosphatase nonreceptor type 22.

Table 1—*Clinical characteristics of the groups of subjects investigated*

	Type 1 diabetes	High GADA titer autoimmune diabetes	Low GADA titer autoimmune diabetes	Type 2 diabetes	Control
n (male/female)	296/262	65/58	66/61	234/216	278/267
Age at diagnosis (years)	14.9 ± 7.8	49.3 ± 12.6	51.8 ± 13.3	51.6 ± 10.8	30 ± 5
BMI (kg/m^2)	18.33 ± 3.6	26.1 ± 5.03	28.2 ± 5.12	29.4 ± 5.01	21.8 ± 2.2
Fasting glucose (mg/dl)	256 ± 137	170.6 ± 62.8	165 ± 55	144 ± 48.9	78.15 ± 10
A1C (%)	10.62 ± 2.49	7.6 ± 1.7	7.1 ± 1.6	6.5 ± 1.4	—

Data are means ± SD.

associated with type 1 diabetes (6) and other autoimmune diseases (8–10); so far it is unclear how the 1858T allele can influence the activity of the LYP phosphatase. In a recent study Vang et al. (11) demonstrated that the Arg620Trp variant (which corresponds to the C1858T polymorphism: the 1858T variant changes codon 620 from arginine [Arg] to tryptophan [Trp]) is a gain-of-function form of the protein, but the mechanism by which the *PTPN22* Trp20 variant exerts the disease-promoting effect has yet to be established. Evidence has also been provided regarding a permissive role played by the *PTPN22* C1858T variant on disease progression from pre-diabetes to clinical disease (12). Finally, it has been hypothesized that the *PTPN22* polymorphism is associated primarily with autoantibody-positive autoimmune diseases (13). The aim of the present study was to study whether the phenotypic heterogeneity of adult-onset autoimmune diabetes based on the GADA titer is supported by the genetic analysis, evaluating whether the *PTPN22* C1858T polymorphism is associated with a high GADA titer instead of antibody positivity per se.

RESEARCH DESIGN AND METHODS

— Four groups of patients and one of control subjects were investigated. All subjects were unrelated and of exclusively Italian origin (with parents and grandparents of Italian origin).

Adult-onset autoimmune diabetic subjects (n = 250) (mean ± SD age at onset 50.3 ± 12.8 years) and age- and sex-matched GADA-negative type 2 diabetic subjects (n = 450) (aged 51.6 ± 10.8 years) were selected from the Non Insulin Requiring Autoimmune Diabetes (NIRAD) Study cohort of 5,330 type 2 diabetic subjects recruited between February 2001 and January 2006 (4,250 from February 2001 to June 2004 [5] and an additional 1,080 from July 2004 to January 2006; the NIRAD study is an ongoing project aimed to identify autoimmune diabetic subjects for a series of clinical studies). Adult-onset autoimmune diabetic subjects were selected using the following inclusion criteria: *1*) an initial diagnosis of type 2 diabetes according to the American Diabetes Association (14), *2*) documented antibody positivity for GADAs (15), *3*) no insulin requirement and no evidence of ketosis from diagnosis to screening time, and *4*) disease duration between 6 months and 5 years. Type 1 diabetic subjects (n = 558) (age at onset 14.9 ± 7.8 years) were recruited by participating centers of the Immunotherapy Diabetes (IMDIAB) group in the Lazio region of central Italy (16). The control group comprised normoglycemic subjects (n = 545) with no family history of autoimmune disorders (aged 30 ± 5 years), collected from the Blood Transfusion Service of Sapienza University of Rome. The clinical characteristics of the five groups investigated are reported in Table 1. The study was approved by all local ethics committees of participating centers, and written informed consent was obtained from all patients.

Autoantibody measurement

GADAs were measured by a radiobinding assay with in vitro translated [^{35}S]methionine-labeled GAD_{65} (15) and $IA\text{-}2_{IC}$ (amino acids 605–979) (15). Results for GADAs were converted into arbitrary units by extrapolation from a standard curve with a local standard designated 100 arbitrary units. The thresholds for positivity were determined from the 99th centile of control subjects and corresponded to 3 arbitrary units for GADAs. The following performances were obtained at the first, second, and third assay proficiency evaluations of the Diabetes Antibody Standardization Program (17) performed between 2002 and 2005: sensitivity 84, 86, and 88%; and specificity 97, 97, and 92%. The intra- and interassay coefficients of variation of GADA for control samples designated at 10 GADA arbitrary units were 9 and 17%, respectively. The distribution of GADA titer in patients with autoimmune diabetes was independent of diabetes duration and showed a bimodal distribution. Consistent with this observation, patients with autoimmune diabetes (GADA titer >3 arbitrary units) were divided into subgroups representing the two distributions, namely low (taken to be ≤32 arbitrary units) and high (>32 arbitrary units) GADA titer. Samples with low GADA titer were validated for GAD-specific binding by a competition assay with an excess of cold insulin (5). Based on the Diabetes Antibody Standardization Program as a reference (17), the threshold of 32 arbitrary units was equivalent to 300 World Health Organization units (5).

HLA and *PTPN22* genotyping

Genomic DNA was extracted using the salting out method (18). HLA-DRB1 and DQB1 typing was performed using allele group–specific amplifications. A reverse line blot method, kindly provided by H.A. Erlich and T. Bugawan (Roche Molecular Systems, Alameda, CA), was used as the detection system (19). The C1858T was genotyped using the fluorogenic 5′ nuclease assay application of the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The genotyping was performed using the following primers: forward, 5′-CAA CTGCTCCAAGGATAGATGATGA-3′; reverse, 5′CCAGCTTCCTCCTCAAC CAATAAATG-3′; and TaqMan MGB probes Fam TCAGGTGTCCGTACAGG and Vic TCAGGTGTGTCCATACAGG.

Of the 10 ng/μl stock of DNA, 4 μl was dispensed into 384-well PCR plates using a Biomek FX robot (Beckman Coulter, Fullerton, CA) to which 2 μl of a mix containing primers, MGB probes, and TaqMan Universal PCR Master Mix (Applied Biosystems) was added in accordance with the manufacturer's instruc-

Table 2—*Distribution of genotype, allele, and phenotype frequencies of the* PTPN22 *C1858T polymorphism in type 1 diabetic, autoimmune diabetic according to GADA titer, type 2 diabetic, and control subjects*

	Type 1 diabetes	High GADA titer autoimmune diabetes	Low GADA titer autoimmune diabetes	Type 2 diabetes	Control
n	558	123	127	450	545
Genotypes*					
C1858C	448 (80.3)	98 (79.7)	120 (94.5)	424 (94.2)	496 (91.1)
C1858T	105 (18.8)	24 (19.5)	7 (5.5)	25 (5.6)	47 (8.6)
T1858T	5 (0.9)	1 (0.8)	0 (0)	1 (0.2)	2 (0.4)
Alleles†					
C	1001 (89.6)	220 (89.5)	247 (97.3)	873 (97)	1039 (95.4)
T	115 (10.4)	26 (10.5)	7 (2.7)	27 (3)	51 (4.6)
Phenotypes‡					
CT/TT genotypes	110 (19.7)	25 (20.3)	7 (5.5)	26 (5.8)	49 (9)
CC genotype	448 (80.3)	98 (79.7)	120 (94.5)	424 (94.2)	496 (91)

Data are *n* (%). *χ^2 3 × 2 high GADA titer vs. low GADA titer, type 2 diabetic, and control subjects, $P \leq 0.008$ after Bonferroni correction. Type 1 diabetic vs. low GADA titer, type 2 diabetic, and control subjects, $P \leq 0.002$ after Bonferroni correction. †χ^2 2 × 2 high GADA titer vs. low GADA titer, type 2 diabetic, and control subjects, $P \leq 0.003$ after Bonferroni correction. Type 1 diabetic vs. low GADA titer, type 2 diabetic, and control subjects, $P < 0.0001$ after Bonferroni correction. ‡χ^2 2 × 2 high GADA titer vs. low GADA titer and type 2 diabetic and control subjects (OR 2.6 [95% CI 1.5–4.4]), $P \leq 0.002$ after Bonferroni correction. Type 1 diabetic vs. low GADA titer, type 2 diabetic, and control subjects, $P < 0.0001$ after Bonferroni correction.

tions. These were sealed with optical seals (Applied Biosystems) and incubated at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min before analysis on a 7900HT plate reader (Applied Biosystems).

Statistical analysis

Statistical analysis was performed using SPSS (version 13; SPSS, Chicago, IL). Genotype, allele, and phenotype frequency distributions were compared using the χ^2 test or Fisher's exact test when the criteria of the χ^2 test were not fulfilled. Allele and genotype frequencies of the *PTPN22* C1858T were in Hardy-Weinberg equilibrium in all groups analyzed. We considered all $P < 0.05$ values as statistically significant. A Bonferroni correction of a factor 4 was applied. Based on an odds ratio (OR) of 2.31 conferred by the 1858T variant in a previous study (6) and on preliminary evaluations, the present study should be able to identify statistical differences between type 1 diabetic, high GADA titer, low GADA titer, type 2 diabetic, and control subjects with a power of 75% and *P* value of 5%. Conditioning analysis based on HLA risk genotypes was also performed. HLA genotypes were classified in three risk categories (high, moderate, and low) based on the absolute risk values for type 1 diabetes previously estimated in Italian population (18). HLA genotypes were introduced as a dichotomous variable in the analysis as follows: high and moderate risk = 1, low risk = 0 (high-risk genotype: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 [DRB1*04 different from DRB1*0403]; moderate risk genotypes: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302/X, and DRB1*03/X [X different from DRB1*03, DRB1*0403-DQB1*0302, and DQB1*0602/03]; and low-risk genotypes: all the other genotypes) (18).

RESULTS — Table 2 shows the genotype, allele, and phenotype frequencies of the *PTPN22* C1858T variant in type 1 diabetic, high GADA titer, low GADA titer, type 2 diabetic, and control subjects. The analysis of the *PTPN22* C1858T variant revealed similar frequencies in subjects with type 1 diabetes and high GADA titers regarding genotype, allele, and phenotype distributions; the risk conferred by the 1858T variant in type 1 diabetes (OR 2.48, 95% CI 1.7–3.5) resulted independently from age at disease onset (data not shown). No significant differences in the frequencies were observed between low GADA titer, type 2 diabetic, and control subjects. Conversely, a significant increase in T1858T and C1858T genotypes was observed in high GADA titer compared with low GADA titer, type 2 diabetic, and control subjects ($P \leq 0.002$ for all comparisons).

The *PTPN22* 1858T allele frequency was significantly increased in high GADA titer (10.5%) compared with low GADA titer (2.7%), type 2 diabetic (3%), and control subjects (4.6%) ($P < 0.0001$ for all comparisons). The frequency of 1858T carriers was significantly increased in high GADA titer (20.3%) compared with low GADA titer (5.5%), type 2 diabetic (5.8%), and control groups (9%) ($P < 0.001$ comparisons) conferring an OR of 2.6 (95% CI 1.5–4.4). No differences were observed in the C1858T genotype distribution between men and women (data not shown). Moreover, no significant differences were observed in the frequency of the 1858T allele between subjects carrying high and moderate HLA risk genotypes and those carrying low HLA risk genotypes either in high or low GADA titer subjects (Table 3).

CONCLUSIONS — In the present study we have shown that the *PTPN22* 1858T variant is associated only with high GADA titer adult-onset autoimmune diabetes, giving important support to our previous findings in which the GADA titer identified two subgroups of subjects (with high or low GADA titers) with differences in clinical phenotype supported by a different HLA class II susceptibility (5). Our results further extend previous studies indicating an association between the *PTPN22* gene variant and classic type 1 diabetes in young subjects (6). The reported association of this variant with other autoimmune diseases such as rheumatoid arthritis (8), systemic lupus erythematosus (9), Graves' disease (10), and Hashimoto's thyroiditis (20) underscore the importance of the product of the *PTPN22* gene in the immune regulation. Kyogoku et al. (9) proposed that the C1858T polymorphism of the *PTPN22* gene may predispose individuals to auto-

Table 3—*Distribution of genotype, allele, and phenotype frequencies of the* PTPN22 *C1858T polymorphism in subjects carrying high and moderate HLA risk genotypes and in low HLA risk genotypes with either high or low GADA titer*

	High GADA titer autoimmune diabetes (n = 123)		Low GADA titer autoimmune diabetes (n = 127)	
	High and moderate HLA risk*	Low HLA risk†	High and moderate HLA risk*	Low HLA risk†
n	42	81	35	92
Genotypes				
C1858C	33 (78.6)	65 (80.2)	33 (94.3)	87 (94.6)
C1858T	8 (19)	16 (19.8)	2 (5.7)	5 (5.4)
T1858T	1 (2.4)	0 (0)	0 (0)	0 (0)
Alleles				
C	74 (88.1)	146 (90.1)	68 (97.1)	179 (97.3)
T	10 (11.9)	16 (9.9)	2 (2.9)	5 (2.7)
Phenotypes				
CT/TT genotypes	9 (21.4)	16 (19.8)	2 (5.7)	5 (5.4)
CC genotype	33 (78.6)	65 (80.2)	33 (94.3)	87 (94.6)

Data are *n* (%). *High-risk genotypes: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 genotype (DRB1*04 different from 0403); moderate-risk genotypes: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302/X, and DRB1*03/X (X different from DRB1*03, DRB1*0403-DQB1*0302, or DQB1*0602/03) genotypes. †Low risk genotypes: all the other genotypes.

immune disease by facilitating the generation of certain disease-associated autoantibodies, thereby contributing to disease progression. Thus, the existence of humoral abnormalities in the *PTPN8* knockout mouse, the ortholog mouse gene of *PTPN22*, is consistent with the fact that autoantibody production is a prominent feature of all human autoimmune diseases that are significantly associated with the *PTPN22* C1858T gene variant (20). Overall these data suggest that the *PTPN22* gene plays a role in autoantibody-related autoimmune diseases as recently supported by data in systemic lupus erythematosus (9).

In type 1 diabetes, early histological and functional studies indicate that the disease is caused by T lymphocytes infiltrating the β-cells (21). The disease-specific immune events are, however, reflected in the appearance of β-cell–specific autoantibodies, which are useful tools for prediction of progression to clinical disease (22,23). In this view, the present findings support and extend our previous study, demonstrating an association of *PTPN22* exclusively with high GADA titer, thus suggesting that the C1858T gene variant may be associated to autoimmune diseases only when autoantibodies are a marker of pathogenic cell destruction.

The *PTPN22* C1858T variant has been found to be a marker of disease progression and to regulate diabetes-specific autoimmunity (12). This progression was demonstrated by a fourfold higher risk of developing an additional autoantibody carried out by islet cell antibody–positive children possessing the *PTPN22* T1858T genotype compared with children with the CC genotype. Altered LYP function, codified by the 1858T variant in $CD4^+CD25^+$ T regulatory cells to make them less potent in suppressing immune response could explain more aggressive β-cell destruction and consequently a major loss in β-cell function in patients carrying this gene variant (24).

In summary, the association of this genetic variant with high GADA titer autoimmune diabetes supports the hypothesis that in these subjects the autoimmune process induces diabetes with no major contribution from other concomitant pathogenic processes. On the other hand, the lack of association with low GADA titer autoimmune diabetes, possibly reflecting a less intense autoimmune process, implies that the appearance of a low GADA titer alone seems not to be related to the diabetes-specific autoimmune process (5). Finally, our data suggest that the *PTPN22* 1858T variant controlling T cells is involved in humoral autoimmunity.

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