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Lymphoid tyrosine phosphatase (LYP/PTPN22) Arg620Trp variant regulates insulin autoimmunity and progression to type 1 diabetes

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Abstract *Aims/hypothesis:* We analysed the contribution of the lymphoid protein tyrosine phosphatase (LYP) Arg620Trp variant (which corresponds to the *PTPN22* C1858T polymorphism) to the emergence of beta-cell-specific humoral autoimmunity and progression to type 1 diabetes in man. We also explored the heterogeneity in the disease-predisposing effect of this polymorphism in relation to known disease loci, sex and age at disease onset. *Subjects and methods:* A population-derived Finnish birth cohort with increased disease susceptibility conferred by *HLA-DQB1* was monitored for the appearance of islet cell autoantibodies, and individuals found to be positive were tested for autoantibodies against insulin (IAA), glutamic acid decarboxylase and islet antigen-2 (n=574; mean follow-up time 4.9 years). Gene interaction effects on disease susceptibility were analysed in case–control and family series (546 patients, 538 controls, 245 nuclear families). All subjects were typed for HLA *DR-DQ*, insulin gene (*INS*), *CTLA4* and *PTPN22* C1858T polymorphisms. *Results:* The *PTPN22* 1858TT genotype was associated with the appearance of IAA (adjusted hazard ratio=4.6, 95% CI 2.4–9.0; p=0.000013). *PTPN22*, *INS* and *HLA-DRB1* had an additive effect on the emergence of IAA. The 1858TT and CT genotypes conferred an increased risk of developing additional autoantibodies or clinical disease (hazard ratio=4.1, 95% CI 1.5–11.6; and 1.6, 95% CI 1.1–2.4, respectively; p=0.003). The strong effect of *PTPN22* on disease susceptibility ($p=2.1\times10^{-8}$) was more pronounced in males (p=0.021) and in subjects with non-*DR4-DQ8*/low-risk HLA genotypes (p=0.0004). *Conclusions/interpretation:* In the pathogenesis of type 1 diabetes the underlying mechanism of the *PTPN22* C1858T polymorphism appears to involve regulation of insulin-specific autoimmunity. Importantly, it strongly affects progression from prediabetes to clinical disease.

Keywords Autoantibodies · Autoimmune disease · Autoimmunity · *CTLA4* · *DQB1* · *DRB1* · HLA · Insulin · Insulin gene · *PTPN22* · Type 1 diabetes

Abbreviations DIPP: Type I Diabetes Prediction and Prevention Project · GADA: Glutamic acid decarboxylase autoantibodies · HR: Hazard ratio · IAA: Insulin autoantibodies · IA-2A: Islet antigen 2 antibodies · ICA: Islet cell antibodies · JDFU: Juvenile Diabetes Foundation unit

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Introduction

Genetic susceptibility to type 1 diabetes is defined by at least four gene loci. The HLA region is the major disease

locus and the primary determinant is *HLA-DQB1*, a class II gene, the effect of which is profoundly modified by the adjacent *HLA-DQA1* and *HLA-DRB1* genes and several other loci in the class I and class III regions [1–5]. In addition to the HLA region, the insulin gene (*INS*; *IDDM2* locus), the *CTLA4* region (cytotoxic T-lymphocyte-associated protein 4; *IDDM12* locus) and the gene *PTPN22* (lymphoid tyrosine phosphatase; 1p13, OMIM 600716) are confirmed to be associated with disease susceptibility [6–10].

The recent finding of association between the lymphoid protein tyrosine phosphatase (LYP) Arg620Trp variant (which corresponds to the *PTPN22* C1858T polymorphism) and type 1 diabetes has been reproduced in several white populations [10–17]. In addition, this variant appears to be a common predisposing factor for other autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Graves' disease, Hashimoto thyroiditis and autoimmune Addison's disease [11, 14, 18–20]. LYP is expressed in lymphocytes and acts by binding intracellular kinases such as C-terminal Src tyrosine kinase (Csk). LYP seems to be directly involved in setting thresholds for T cell receptor signalling [21, 22]. The Arg620Trp functional polymorphism may be the causative disease variant, as Bottini et al. [10] showed that this amino acid substitution disrupts interaction with the tyrosine kinase, which could lead to pathogenic T cell responses and may be the defect that underlies the development of autoimmunity.

In type 1 diabetes, the initiation of the beta-cell-specific immune process often occurs early in life, and several years later it results in clinical disease in most individuals. Early histological and functional studies indicate that the disease is caused by T lymphocytes infiltrating the pancreatic islets [23, 24]. The disease-specific immune events are, however, reflected in the appearance of beta-cell-specific autoantibodies, which are useful tools for the prediction of progression to clinical disease [25–27]. To understand the disease pathomechanism, it is crucial to explore the role of genetic factors in the emergence of beta cell autoimmunity. Previously, it has been shown that the *DR4-DQ8* haplotype is associated with the appearance of insulin autoantibodies (IAA) and islet antigen-2 autoantibodies (IA-2A), whereas glutamic acid decarboxylase autoantibodies (GADA) develop more frequently in individuals carrying the *DR3-DQ2* haplotype [28–31]. Recently, in a large Finnish birth cohort we observed a differential contribution of the *DRB1*04* alleles to the development of beta cell autoimmunity. Importantly, we also found that the *INS* locus plays a central role in the emergence of IAA and that the subsequent appearance of multiple autoantibodies is linked to IAA [32]. Our findings suggested that, in children who develop IAA, insulin autoimmunity may represent the primary event that is controlled, at least in part, by the *INS* locus.

In this study we examined the role of *PTPN22* in the development of type 1 diabetes-associated autoimmunity and its contribution to the genetic control of progression to clinical disease. For this purpose we employed a large population-based cohort of infants who are prospectively followed from birth in the framework of the Type I Diabetes Prediction and Prevention Project (DIPP). This study population carries risk HLA genotypes and is well characterised in terms of clinical data and for genetic and immunological disease markers [33].

In addition, we explored the heterogeneity of the disease-predisposing effect of *PTPN22* in relation to HLA genes, *INS* and *CTLA4* genotypes, sex and age.

Subjects and methods

Study design

Firstly, the effect of the LYP Arg620Trp (*PTPN22* C1858T; rs2476601) polymorphism on humoral beta cell autoimmunity and disease progression was evaluated in a population-based prospective follow-up cohort of young children with increased type 1 diabetes susceptibility—the DIPP cohort. In the framework of the DIPP, screening for risk-associated HLA genotypes is performed in all infants born at three university hospitals in Finland: Turku, Tampere and Oulu [33]. Infants (about 7500 by May 2004) with risk *HLA-DQB1* genotypes (*DR3-DQ2/DR4-DQ8*, *DR4-DQ8/X*, *DR3-DQ2/Y*, where *X≠DR3-DQ2*, *DQB1*0301*, *DQB1*0602* and *Y≠DR7-DQ2*, *DQB1*0301*, **0302*, **0602* or **0603*), were followed and sampled at intervals of 3–12 months, as described [33]. Islet cell antibodies (ICA) were analysed from all serum samples; if positive, IAA, GADA and IA-2A were tested in all samples available from that ICA-positive subject (Fig. 1).

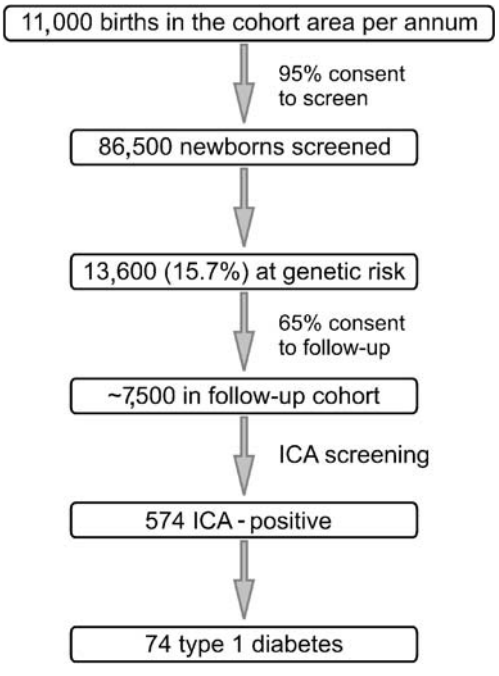


Fig. 1 Population screening and follow-up scheme in the Type I Diabetes Prediction and Prevention (DIPP) project (status in May, 2004). For the definition of the at-risk individuals, see Methods section in Subjects and methods

Secondly, we explored the heterogeneity of the disease-predisposing effect of *PTPN22* in relation to HLA, the insulin gene, *CTLA4* genotypes, sex and age at disease onset. To avoid population stratification effects we used both case–control and family-based designs.

The gene–gene interactions between *PTPN22*, HLA class II, *INS* and *CTLA4* were explored first in stratified data sets and then tested for significance using regression analyses.

The WHO criteria were used for the diagnosis of type 1 diabetes [34].

The local ethics committees approved the study and informed consent was obtained from the parents of the participating subjects. The study was conducted according to the principles of the Declaration of Helsinki.

Study populations

Antibody-positive children (DIPP cohort)

All children who had developed ICA by May 2004 (n=574, 180 with the *DR3-DQ2*/*DR4-DQ8* high-risk, 368 with the *DR4-DQ8*/*X* and 26 with the *DR3-DQ2*/*Y* moderate-risk genotypes; 53.5% boys) were tested for autoantibodies (IAA, GADA and IA-2A) and for genetic markers (*PTPN22* C1858T, *HLA-DRB1-DQA1-DQB1*, *INS* −23 *Hph*I and *CTLA4* CT60 polymorphisms). Two hundred and sixty-two infants developed biochemical autoantibodies, in addition to ICA, among whom 190 had multiple (≥2) autoantibodies. At the time of this analysis the mean (±SD) follow-up time in the *DR3-DQ2*/*DR4-DQ8* and *DR4-DQ8*/*X* groups was 4.9±2.2 years (range 0.5–9.3 years), and somewhat shorter in those carrying the *DR3-DQ2*/*Y* combination (mean 3.9±1.4 years, range 0.5–6.1 years). By May 2004, 74 of these children (12.9%) had progressed to clinical type 1 diabetes.

Case–control series

Samples from cases were collected from the Departments of Pediatrics at the Universities of Turku and Oulu (n=546, 55.7% boys; mean age at diagnosis 8.2±4.1 years). The control group comprised consecutive healthy infants born in the University Hospitals of Turku and Oulu (n=538, 51.2% boys).

Nuclear family series

Families with one affected child were recruited mainly from the Departments of Pediatrics at the Universities of Turku and Oulu (n=245, 52.9% boys; mean age at diagnosis 8.6±4.4 years).

Methods

Genotyping

The *PTPN22* C1858T (LYP Arg620Trp; rs2476601) polymorphism was genotyped with a homogeneous genotyping method [35]. The oligonucleotide sequences and reaction conditions are available upon request. Genotyping accuracy was evaluated by DNA sequencing 140 samples (MegaBace1000; GE Healthcare, Chalfont St Giles, UK). The genotypes obtained with the two methods were 100% identical. The methods for the *HLA-DQB1* 'full house' and for *DQA1* and *DRB1*04* typing have been described [36–38]. For the gene–gene interaction analysis in the case–control material, HLA genotypes were grouped among cases according to the presence or absence of the two major HLA risk haplotypes, as follows: HLA *DR3-DQ2*/*DR4-DQ8*, *DR4-DQ8*/non-*DR3-DQ2*, *DR3-DQ2*/non-*DR4-DQ8*, non-*DR4-DQ8*/non-*DR3-DQ2*. In the ICA-positive DIPP cohort, the heterogeneity of *PTPN22* effects was tested between HLA genotype groups defined according to the screening classification criteria: *DR3-DQ2*/*DR4-DQ8*, *DR4-DQ8*/*X* and *DR3-DQ2*/*Y*. The *INS* −23 *Hph*I polymorphism was typed as described previously [32, 36, 39]. The *CTLA4* CT60 polymorphism was genotyped using minisequencing (SnuPe Kit, MegaBace1000; GE Healthcare).

Autoantibody assays

The antibody assay parameters used have been described in detail [40]. The detection limit for ICA was 2.5 Juvenile Diabetes Foundation units (JDFU; sensitivity 100%, specificity 98%). IAA levels were quantified with a microassay [41, 42]. GADA and IA-2A were measured using specific radioligand assays [43, 44]. The cut-off values for IAA, GADA and IA-2A positivity were 1.56, 5.36 and 0.43 RU (relative units), respectively, being the 99th percentiles in a series comprising more than 370 non-diabetic Finnish children and adolescents. The disease sensitivities of the IAA, GADA and IA-2A assays were 44, 82 and 62%, respectively, and the specificities were 98, 98 and 100%, respectively, based on the 2002 Diabetes Autoantibody Standardization Program Workshop.

Statistical analysis

Kaplan–Meier survival analysis was applied to evaluate genetic effects on the appearance of various autoantibodies. Differences between the survival curves were tested with the log rank test. Cox regression was employed to analyse the effects of various factors (antibody status, genotypes) on autoantibody-free survival. Disease associations of gene variants were evaluated by forward stepwise logistic regression analysis and, in some comparisons, using the χ^2

statistic with Yates' correction. All statistical analyses were performed using SPSS for Windows (version 11.0.1; SPSS, Chicago, IL, USA). The transmission disequilibrium test was performed using Unphased software [45]. Values of p lower than 0.05 were considered statistically significant.

Results

The effect of the *PTPN22* C1858T variant on the emergence of beta-cell-specific autoimmunity and progression to clinical type 1 diabetes in the DIPP cohort

Figure 2 shows the Kaplan–Meier survival analysis of progression to type 1 diabetes in the ICA-positive DIPP follow-up cohort. Clinical disease appeared at a higher rate in children with the TT and TC genotypes than in those carrying the CC variant (hazard ratio [HR]=5.5, 95% CI 2.3–13.2 and HR=1.6, 95% CI 1.0–2.7, respectively; p=0.0001). Using logistic regression analysis, we observed that the *PTPN22* C1858T variant showed association with progression of autoimmunity reflected by the number of autoantibodies appearing during follow-up. Children carrying the TT and CT genotypes had an increased risk of developing additional autoantibodies (HRs for developing an additional antibody were 4.1 [95% CI 1.5–11.6] and 1.64 [95% CI 1.1–2.4], respectively; p=0.003).

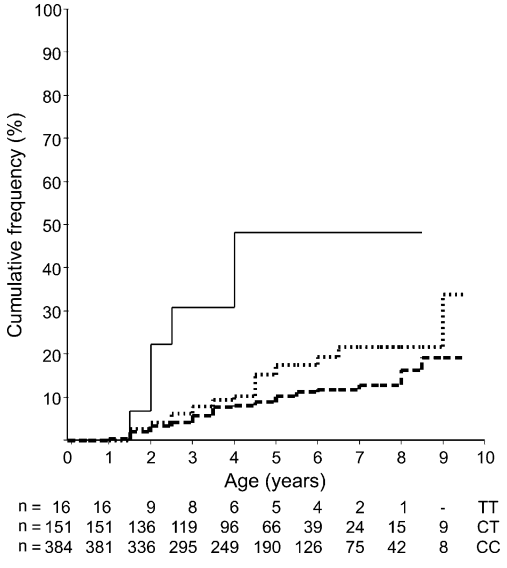


Fig. 2 The effect of *PTPN22* on progression to clinical type 1 diabetes in children with risk HLA genotypes. Cumulative disease frequencies are shown for different *PTPN22* C1858T genotypes. Median disease-free survival was shorter in individuals carrying the TT genotype (5.6 years; 95% CI 3.9–7.3 years; n=16) than in those with the TC or CC variant (8.0 years, 95% CI 7.6–8.6; n=151 and 8.4 years, 95% CI 8.2–8.7; n=384, respectively, p=0.0001). All children carried the HLA *DR3-DQ2/DR4-DQ8* or *DR4-DQ8/X* combination, in which $X \neq$ *DR3-DQ2*, *DQB1*0301* or *DQB1*0602*. *Solid line* subjects with the TT genotype; *dotted line* subjects carrying the CT combination; *dashed line* subjects with the CC variant

ICA

The effect of the *PTPN22* variant on the emergence of ICA was estimated by comparing the genotype frequencies of the children with ICA only (TT 1.3%, CT 24.1%, CC 74.6%) with those seen in healthy controls (see data in Table 3). No difference was observed.

IAA, GADA and IA-2A

Genetic effects on the appearance of these autoantibodies were estimated by survival analysis. The cumulative frequencies of IAA were higher and antibody-free survival was shorter among children with the TT genotype (76.0%, 3.0 years, 95% CI 1.3–4.6 years) compared with those with the genotypes CT (46.5%, 5.9 years, 95% CI 5.3–6.6 years) and CC (42.0%, 6.5 years, 95% CI 6.2–6.9; p=0.00007). The hazard ratio for the emergence of IAA in subjects with the TT combination was 3.7 (95% CI 2.0–6.9) and was 1.3 (95% CI 1.0–1.8) in those with the CT genotype. The effect of the T allele on the emergence of IAA was strongly significant also when the study group was stratified according to the presence of IA-2A and GADA (adjusted p=0.0001; Table 1). Accordingly, in Cox regression the effect of the *PTPN22* TT variant was significant after adjusting for GADA and IA-2A carrier status (adjusted HR for the appearance of IAA in the C1858T TT group was 3.2, 95% CI 1.7–5.9; reference genotype, CC, p=0.001).

When the effect of the *PTPN22* on the appearance of GADA was analysed, the TT variant appeared to be associated with a higher cumulative frequency of GADA; however, when adjusted for IAA status, the effect was not significant (Table 1; p=0.073). Similarly, the emergence of IA-2A was not significantly influenced by the *PTPN22* genotype after adjusting for IAA status (adjusted p=0.17).

The effect of the *PTPN22* was also analysed in relation to which autoantibody reactivity appeared first during the follow-up (Fig. 3). The 1858TT genotype was associated with higher cumulative IAA frequency and shorter IAA-free survival than the CC variant (median IAA-free survival with TT genotype was 3.2 years, 95% CI 1.5–5.0 versus 7.0 years, 95% CI 6.7–7.4 years in the group with CC combination; log rank 21.4, p=0.00008, df=2; Fig. 3a). However, no effect of *PTPN22* was seen on the emergence of GADA (p=0.27; Fig. 3b) or IA-2A (p=0.57; Fig. 3c).

We also evaluated how *PTPN22* influenced the transient appearance of autoantibodies (ICA n=84, 14.6%; IAA n=77, 13.4%; GADA n=37, 6.4%; IA-2A n=7, 1.2%). No significant effects were found, although, an increase in the cumulative frequency of transient IAA was suspected in subjects with the 1858TT genotype (p=0.11).

Table 1 Survival analysis of the effect of the *PTPN22* C1858T polymorphism on the appearance of autoantibodies in the ICA-positive DIPP cohort

Autoantibody analysed	Study subjects	*PTPN22* C1858T genotype (*n*)	Cumulative frequencies of autoantibodies (%)	Antibody-free survival (years): mean[a] (95% CI)	*PTPN22* effect: adjusted *p* value[b]
IAA	GADA/IA-2A-positive	TT (9)	100.0	1.2 (1.0–1.4)	0.0001
		TC (72)	83.5	3.2 (2.6–4.0)	
		CC (131)	78.2	3.5 (2.9–3.8)	
	GADA/IA-2A-negative	TT (6)	37.5	5.8 (2.9–8.7)	
		TC (80)	16.6	8.2 (7.7–8.8)	
		CC (254)	10.1	8.3 (8.0–9.0)	
GADA	IAA-positive	TT (11)	84.4	2.0 (1.4–2.7)	0.073
		TC (64)	89.0	3.0 (2.4–3.6)	
		CC (132)	76.0	4.1 (3.5–4.7)	
	IAA-negative	TT (4)	0	–	
		TC (87)	27.4	7.9 (7.4–8.5)	
		CC (253)	14.2	8.6 (8.3–8.8)	
IA-2A	IAA positives	TT (11)	87.5	2.2 (1.6–2.7)	0.17
		TC (64)	86.1	3.4 (2.7–4.1)	
		CC (132)	80.0	4.3 (3.7–4.8)	
	IAA negatives	TT (4)	0	–	
		TC (87)	21.4	8.5 (8.2–8.9)	
		CC (253)	13.9	8.8 (8.5–9.0)	

The number of children in each genotype category is shown in parentheses
[a]Median values for antibody-free survival could not be calculated in some cases because the cumulative antibody frequencies were lower than 50%; therefore, the mean values are reported
[b]Log rank test; *df*=2 for all *p* values

Heterogeneity in the effect of *PTPN22* on the emergence of IAA according to HLA class II, *INS*, *CTLA4*, age and sex

The appearance of IAA in the three *PTPN22* genotype groups stratified according to HLA class II, *INS* and *CTLA4* genotypes is shown in Table 2. IAA appeared at a higher rate in children carrying the TT genotype both in the *DR3-DQ2*/*DR4-DQ8* and in the *DR4-DQ8*/*X* risk category (adjusted p=0.0003). In addition, Cox regression revealed a higher risk of developing IAA in the *DR3-DQ2*/*DR4-DQ8* category in all *PTPN22* genotype groups compared with the *DR4-DQ8*/*X* group (HR=1.5, 95% CI 1.1–2.1; p=0.015). The emergence of IAA could not be estimated reliably among boys with the *DR3-DQ2*/*Y* genotype because of the small numbers.

We analysed the effect of *PTPN22* on the development of IAA in data sets stratified according to the *DRB1* subtype on the *DR4-DQ8* haplotypes. We observed that the effect of *PTPN22* was present both in the *DRB1*0401* and **0404* groups (adjusted p=0.0002), with a higher cumulative IAA frequency in all *PTPN22* categories in the *DRB1*0401* group (p=0.00004). Only one child developed IAA in the *DRB1*0403* group.

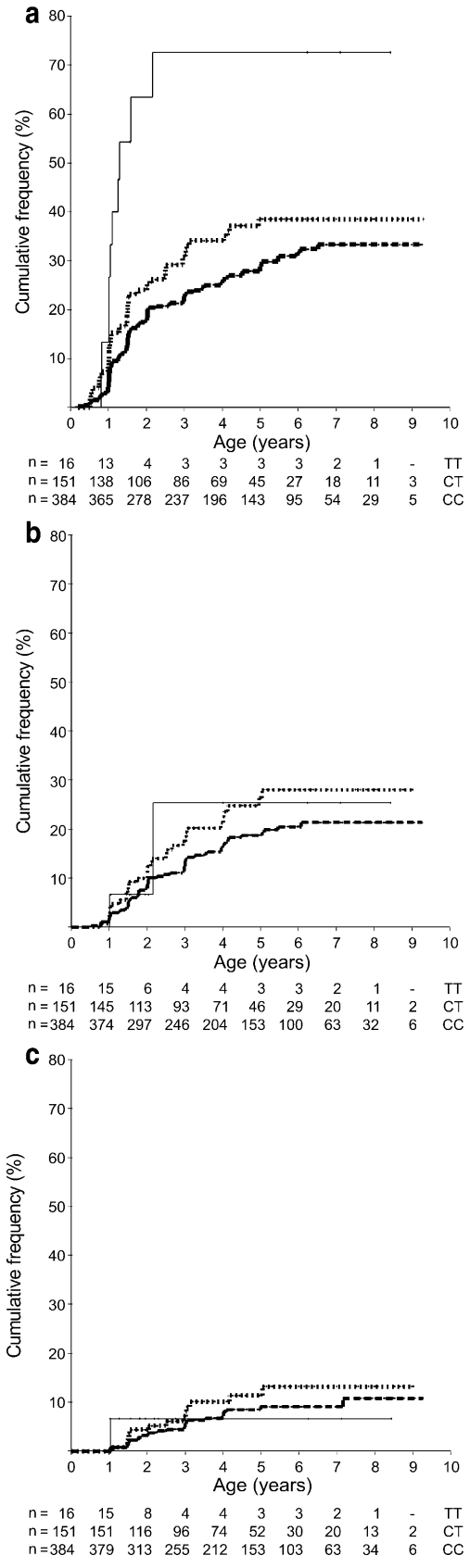
The strong effect of *PTPN22* on the emergence of IAA was present both in the *INS* (−23) *Hph*I AA and AT/TT *INS* genotype groups. In addition, IAA appeared at a higher rate in the *INS* (−23) *Hph*I AA group (HR=2.1, 95% CI 1.5–3.0; p=0.00007).

The *PTPN22* C1858T polymorphism affected the appearance of IAA in all three *CTLA4* CT60 genotype groups in a similar way (p=0.0005), and we did not observe any interaction between *PTPN22* and *CTLA4* in this respect (p=0.53).

Interestingly, sex influenced the appearance of IAA, as boys carrying the *PTPN22* 1858TT genotype had a higher risk of developing IAA than girls (HR=1.4, 95% CI 1.1–1.8; p=0.033).

Additionally, we observed that at the time of IAA seroconversion children with the TT genotype were younger (2.4±0.7 years) than those with CT or CC genotype (3.8±0.2 and 4.1±0.1 years, respectively; p=0.016). No age-dependent heterogeneity in the *PTPN22* genotype distribution was detected in the DIPP cohort (p=0.37).

Using Cox regression analysis, we estimated the effect of the *PTPN22* variant on the emergence of IAA adjusted for the effects of HLA, *INS*, *CTLA4* and sex. Individuals with the TT and CT genotypes had a strongly increased probability of developing IAA (adjusted HR=4.6, 95% CI 2.4–9.0; and HR=1.5, 95% CI 1.5–3.0, respectively; p=0.000013).



◀ **Fig. 3** The effect of *PTPN22* on the appearance of IAA, GADA and IA-2A as first autoantibody during follow-up. **a** Appearance of IAA as the first autoantibody during follow-up in children carrying different *PTPN22* C1858T genotypes. Cumulative frequency of IAA was higher in the TT and CT groups than in children with the CC genotype (73.6 and 38.7 vs 33.5%; log rank 21.4, *df*=2, p=0.00008; n=168). **b** Emergence of GADA as the first autoantibody in subjects carrying different *PTPN22* C1858T genotypes (p=0.27; n=96). **c** Development of IA-2A as the first autoantibody in different *PTPN22* C1858T genotype groups (p=0.57; n=42). *Solid lines* subjects with the TT genotype; *dotted lines* subjects carrying the CT combination; *dashed lines* subjects with the CC variant

Heterogeneity of the effect of *PTPN22* on type 1 diabetes susceptibility

In the case–control series, the disease association of *PTPN22* and its interaction with HLA *DR-DQ*, *CTLA4*, *INS*, sex and age at disease onset were analysed using forward stepwise logistic regression analysis (Table 3). As expected, the *PTPN22* C1858T polymorphism showed a strong disease association ($p=2.1\times10^{-8}$). The odds ratios (OR) for the TT and CT genotypes were 3.1 (95% CI 1.4–5.2) and 2.9 (95% CI 2.0–4.2), respectively. The frequency of the T allele was 23.9% in cases and 13.9% in controls ($p=8\times10^{-6}$). In the cases, the exploratory analysis of the distribution of *PTPN22* C1858T genotypes stratified according to *HLA-DQB1*, *CTLA4* CT60 and *INS* (−23 *Hph*I) genotypes, age at disease presentation and sex indicated heterogeneity for HLA, *INS* and sex. The significance of these interactions was estimated using a case-only regression model (Table 3). A significant effect of sex on *PTPN22*-encoded disease susceptibility was observed, as affected boys with the TT genotype had higher disease risk than girls (OR 1.5, 95% CI 1.1–2.2; p=0.021). We found no significant effect of the age at diagnosis, although the TT genotype appeared to be more prevalent among patients at a young age. However, we detected heterogeneity in the disease-predisposing effect of *PTPN22* among different HLA class II genotypes, as the 1858TT and CT genotypes were found at higher frequencies in patients carrying the HLA *DR3-DQ2*/non-*DR4-DQ8* and non-*DR3-DQ2*/non-*DR4-DQ8* combinations compared with those with the *DR3-DQ2*/*DR4-DQ8* or *DR4-DQ8*/non-*DR3-DQ2* variants (p=0.0004). The data also suggested that the effect of *PTPN22* is stronger in children who carry *INS* (−23) *Hph*I AT or TT compared with the AA group (p=0.04). No significant heterogeneity in the effect of *PTPN22* was observed in relation to *CTLA4*.

In the controls, no heterogeneity was observed in the distribution of *PTPN22* genotypes, when stratified according to factors listed in Table 3.

In the nuclear family series, the T allele was preferentially transmitted to affected offspring (transmission disequilibrium test; 109 transmitted, 61.6%; p=0.001). The transmission rate to affected boys was higher (67.1%; p=0.0004) than that to girls (55.3%; p=0.317). This difference was valid both for paternal and maternal transmission (to boys, 70.2 and 65.3%; to girls, 50.83 and 55.6%, respectively). In the nuclear family series, the parental T allele frequency was 17.6%. There was no

Table 2 Survival analysis of the effect of the *PTPN22* C1858T polymorphism on the appearance of insulin autoantibodies in ICA-positive children carrying various *HLA-DQB1*, *INS* and *CTLA4* genotypes

	PTPN22 C1858T genotype (*n*)	Cumulative IAA frequency (%)	IAA-free survival in years: mean[a] (95% CI)	*PTPN22* effect: adjusted *p* value[b]
DR3-DQ2/DR4-DQ8[c]	TT (5)	100.0	1.3 (0.8–1.7)	0.0003
	TC (49)	52.9	5.3 (4.2–6.4)	
	CC (120)	52.5	5.9 (5.3–6.6)	
DR4-DQ8/X[d]	TT (10)	62.5	3.9 (1.6–6.2)	
	TC (95)	45.2	6.0 (5.2–6.7)	
	CC (250)	36.9	6.8 (6.3–7.3)	
*DRB1*0401*[e]	TT (9)	100	1.3 (0.9–1.52)	0.0002
	CT (96)	60.9	4.7 (4.0–5.5)	
	CC (241)	46.1	6.1 (5.6–6.6)	
*DRB1*0403*	TT (0)	–	–	
	CT (9)	0	–	
	CC (13)	11.1	7.9 (6.8–9.0)	
*DRB1*0404*	TT (4)	0	–	
	CT (36)	29.5	7.3 (6.2–8.3)	
	CC (106)	41.6	6.9 (6.2–7.5)	
INS (−23) *Hph*I AA[f]	TT (9)	77.8	2.9 (0.9–4.8)	0.0002
	TC (92)	51.7	5.4 (4.6–6.2)	
	CC (258)	47.7	6.1 (5.7–6.6)	
INS (−23) *Hph*I AT and TT	TT (4)	75.0	1.2 (1.0–1.4)	
	TC (49)	34.8	6.8 (5.8–7.9)	
	CC (108)	24.4	7.6 (7.1–8.2)	
CTLA4 CT60 AA[g]	TT (6)	50.0	4.8 (1.8–7.7)	0.0005
	TC (28)	59.1	5.4 (4.0–6.7)	
	CC (83)	41.3	6.7 (5.9–7.4)	
CTLA4 CT60 AG	TT (4)	75.0	2.8 (0.4–5.3)	
	TC (81)	45.0	5.7 (4.9–6.6)	
	CC (182)	44.8	6.2 (5.7–6.8)	
CTLA4 CT60 GG	TT (5)	100.0	1.2 (0.9–1.4)	
	TC (43)	40.6	5.7 (4.7–6.6)	
	CC (121)	40.0	6.6 (6.0–7.3)	

The number of children in each genotype category is shown in parentheses
[a]Median values for the antibody-free survival could not be calculated in some cases because the cumulative antibody frequencies were lower than 50%; therefore, the mean values are reported
[b]Log rank test; *df*=2 for all *p* values
[c]*p*=0.015 vs *DR4-DQ8/X* (*df*=1)
[d]*X≠DR3-DQ2*, *DQB1*0301*, *DQB1*0602*
[e]*p*=0.00004 vs *DRB1*0403* and *DRB1*0404* (*df*=2)
All *DRB1*04* subtypes carry *DQ8*
[f]*p*=0.00007 vs *INS* (−23) *Hph*I AT and TT (*df*=1)
[g]*p*=0.53 vs *CTLA4* CT60 AG and GG (*df*=2)

difference in parental T allele frequency between families with affected boys and girls.

Discussion

This study revealed several important novel findings on the effect of the *PTPN22* C1858T (LYP Arg620Trp) polymorphism on the development of humoral beta cell autoimmunity and disease progression.

Firstly, we observed that the *PTPN22* C1858T variant was strongly associated with progression of beta-cell-specific autoimmunity, as ICA-positive children carrying the TT genotype had a four-fold higher risk of developing an additional molecular autoantibody than those with the CC genotype. This implies that the *PTPN22* 1858TT genotype defines a more homogeneous high-risk population within the given HLA risk group, which is more suitable for future prevention trials than those identified by the genetic screening criteria used so far.

Table 3 Analysis of heterogeneity in the type 1 diabetes predisposing effect of *PTPN22* in the Finnish population

Subjects	*PTPN22* C1858T genotype *n* (%)			*p* value
	TT	CT	CC	
All cases	30 (5.5)	200 (36.6)	316 (57.9)	0.000000021
Male	17 (5.9)	118 (40.8)	154 (53.3)	0.021
Female	13 (5.1)	82 (31.9)	162 (63.0)	
Age at diagnosis				
0–4.99 years	9 (7.6)	48 (40.3)	62 (52.1)	0.305
5–9.99 years	11 (6.3)	64 (36.8)	99 (56.9)	
10–14.99 years	9 (4.0)	82 (36.8)	132 (59.2)	
HLA *DR3-DQ2*/*DR4-DQ8*	3 (3.1)	33 (33.7)	62 (63.3)	0.0004
HLA *DR4-DQ8*/non-*DR3-DQ2*	16 (5.4)	97 (33.0)	181 (61.6)	
HLA *DR3-DQ2*/non-*DR4-DQ8*	7 (7.3)	45 (46.9)	44 (45.8)	
HLA non-*DR3-DQ2*/non-*DR4-DQ8*	4 (6.9)	25 (43.1)	29 (50.0)	
INS (−23) *Hph*I AA	21 (5.3)	142 (36.1)	230 (58.5)	0.040
INS (−23) *Hph*I AT and TT	8 (7.4)	45 (41.7)	55 (50.9)	
CTLA4 CT60 AA	6 (13.3)	16 (35.6)	23 (51.1)	0.095
CTLA4 CT60 AG	7 (3.5)	76 (38.4)	115 (58.1)	
CTLA4 CT60 GG	15 (5.6)	94 (35.1)	159 (59.3)	
Controls	14 (2.6)	122 (22.7)	402 (74.7)	

The global effect of *PTPN22* was estimated using a logistic regression model comprising both cases and controls
Effects of sex, age at diagnosis, HLA, *INS* and *CTLA4* were estimated with a case-only regression model

Importantly, the emergence of IAA showed a strong association with the *PTPN22* TT genotype, which underlines the significance of this variant in the regulation of beta-cell-specific autoimmunity. In contrast, the development of GADA and IA-2A was not significantly influenced by this polymorphism. It should be noted that the analysis of genetic effects on various autoantibodies was complicated by the fact that most (81.7%) of the IA-2A- and GADA-positive children also tested positive for IAA. However, the Cox regression that enabled us to adjust for the presence of other autoantibodies, and also the analysis of the autoantibody reactivity that appeared first during follow-up, indicated that the *PTPN22* TT variant was primarily associated with the emergence of IAA (Fig. 3). Notably, individuals with the TT genotype had a considerably higher probability of developing IAA or other additional autoantibodies than those with the CT heterozygous genotype, which is consistent with a possible gene dose effect.

The effect of *PTPN22* on the appearance of IAA was more pronounced in the high-risk HLA *DR3-DQ2*/*DR4-DQ8* genotype group than in the medium-risk *DR4-DQ8*/*X* category, which could be explained by the higher number of disease-predisposing HLA heterodimer molecules, which promotes the autoimmune process in the former individuals. We also observed another HLA class II effect on *PTPN22*-encoded beta-cell-specific autoimmunity. The *DRB1*04* allele present in the *DR4-DQ8* HLA haplotype influenced the emergence of IAA in children with the TT and CT *PTPN22* genotypes, as those carrying the *DRB1*0401* variant had a higher probability of developing this autoantibody compared with subjects with the *DRB1*0404* and **0403* alleles. This effect could be caused by differences in the peptide binding specificity of the encoded DR molecule. The differential effect of *DRB1*04* alleles on the emergence of IAA is probably related to the different degrees of type 1 diabetes risk they confer [2, 46].

We have reported previously an association of IAA with the *INS* locus in the same cohort [32], and similar observations were made in two other studies on patients with newly diagnosed type 1 diabetes and on first-degree relatives [30, 47]. Therefore, we stratified the data set according to the *INS* genotype and observed that the effect of *PTPN22* was present in both *INS* risk categories. In addition, the appearance of IAA was more frequent in children in the *INS* (−23) *Hph*I AA risk group in all *PTPN22* genotype categories, which implicates an additive effect of these two loci on the emergence of IAA. Our data also suggest not only that *PTPN22* is associated with the emergence of IAA, but also that it seems to accelerate insulin-specific autoimmunity, as children with the 1858TT genotype developed IAA at an earlier age than others.

We found no evidence of heterogeneity in the effect of *PTPN22* on the appearance of IAA in relation to the *CTLA4*

genotype. This is in accordance with previous observations from us and from other groups indicating a lack of association of *CTLA4* with the emergence of diabetes-specific autoantibodies [30, 48].

Previously, we proposed the existence of two different pathways that lead to the emergence of beta cell autoimmunity [32]. In the *INS*-dependent pathway, insulin autoimmunity seems to play a central role in the initiation of the disease process, whereas in IAA-negative individuals the initiation of beta cell autoimmunity and the development of GADA and IA-2A are probably controlled by factors other than the *INS* locus. Data from the present study indicate that the *PTPN22* C1858T polymorphism, which corresponds to the LYP Arg620Trp variant, may be primarily involved in the *INS*-dependent pathway by modulating insulin-specific humoral autoimmunity. Since *PTPN22* is associated with other autoimmune diseases in addition to type 1 diabetes, such as Graves' disease and rheumatoid arthritis [11, 14, 19], it is likely that the LYP protein is involved in antigen-driven, disease-specific immune phenomena. Accordingly, our data suggest that insulin could be a target antigen for the action of *PTPN22* in type 1 diabetes.

We also observed in this study that the appearance of ICA alone was not associated with *PTPN22*. Most of the children who developed ICA had an antibody titre below 20 JDFU (median 5.0 JDFU; interquartile range 4.0–8.0 JDFU), which is much lower than the ICA titre in those who had additional autoantibodies (44 JDFU, 8.0–219.0 JDFU; $p<10^{-6}$). This observation is in accordance with our recent findings implicating that the emergence of low-titre ICA alone is not related to the diabetes-specific autoimmune process [32].

The analysis of the heterogeneity of the effect of *PTPN22* on type 1 diabetes susceptibility corroborated previous observations that the *PTPN22* 1858T allele is associated with type 1 diabetes [10–12, 14–16]. However, unlike in other studies, in both the case–control and the family series we observed that boys carrying the T allele were at higher risk of disease than girls. This sex-specific bias in the action of *PTPN22* was underlined by the findings in the antibody-positive cohort, which indicated that males carrying the TT genotype had a higher risk of developing IAA than females. In addition, the effect of *PTPN22* appeared to be stronger in individuals carrying non-*DR4-DQ8* genotypes. Such findings have not been reported before and suggest a particular molecular mechanism that may play a more important role in males, and involves *PTPN22* and HLA *DR3-DQ2* or neutral HLA haplotypes. A similar sex-specific HLA effect was implicated by our data in Finns [37, 49] and also by Cucca et al. [50], indicating that males with the *DR3-DQ2* haplotype have a higher disease risk than girls. The regression analysis also suggested heterogeneity in the effect of *PTPN22* on type 1 diabetes susceptibility in children with different *INS* genotypes, while no interaction between *PTPN22* and *CTLA4* was detected. Since the regression analysis in the case–control series had limited statistical power to detect gene–gene interaction phenomena between loci with such weak (OR<2) disease association, these findings require replication in larger series.

In conclusion, the present study provides strong evidence that the *PTPN22* C1858T polymorphism regulates diabetes-specific autoimmunity and is a marker of disease progression. In addition, it appears to be primarily associated with insulin-specific humoral autoimmunity, indicating an underlying mechanism for the LYP 620Trp variant protein. Importantly, this polymorphism seems to have an additive effect with the *INS* locus on the emergence of IAA. We also observed a complex interaction pattern of *PTPN22* with certain HLA class II genotype combinations and with sex. It should be noted that in the present analysis all children in the autoantibody positive cohort were ICA-positive; therefore, the role of the *PTPN22* C1858T variant on the emergence of humoral beta cell autoimmunity in ICA-negative subjects needs further investigation. Further functional studies are also required to refine our knowledge on the multifaceted molecular mechanisms by which *PTPN22* contributes to the development of type 1 diabetes.

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