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SHORT COMMUNICATION

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Impact of *IDDM2* on disease pathogenesis and progression in children with newly diagnosed type 1 diabetes: reduced insulin antibody titres and preserved beta cell function

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Abstract *Aims/hypothesis:* The insulin-dependent diabetes mellitus 2 gene (*IDDM2*) is a type 1 diabetes susceptibility locus contributed to by the variable number of tandem repeats (VNTR) upstream of the insulin gene (*INS*). We investigated the association between *INS* VNTR class III alleles (–23*Hph*IA/T) and both insulin antibody presentation and residual beta cell function during the first year after diagnosis in 257 children with type 1 diabetes. *Materials and methods:* To estimate C-peptide levels and autoantibody presentation, patients underwent a meal-stimulated C-peptide test 1, 6, and 12 months after diagnosis. The insulin –23*Hph*IA/T variant was used as a marker of class III alleles and genotyped by PCR-RFLP. *Results:* The insulin antibody titres at 1 and 6 months were significantly lower in the class III/III and class I/III genotype groups than in the class I/I genotype group (p = 0.01). Class III alleles were also associated with residual beta cell function 12 months after diagnosis and independently of age, sex, BMI, insulin antibody titres, and HLA-risk genotype group (p = 0.03). The C-peptide level was twice as high among class III/III genotypes as in class I/I and class I/III genotypes (319 vs 131 and 166 pmol/l, p=0.01). Furthermore, the class III/III genotype had a 1.1% reduction in HbA_1c after adjustment for insulin dose (p = 0.04). *Conclusions/interpretation:* These findings suggest a direct connection in vivo between *INS* VNTR class III alleles, a decreased humoral immune response to insulin, and preservation of beta cell function in recent-onset type 1 diabetes.

Keywords Beta cell function · *IDDM2* · Insulin antibodies · Type 1 diabetes

Abbreviations GADA: GAD antibodies · IA-2A: antibodies against insulinoma-associated antigen · ICA: islet cell antibody · VNTR: variable number of tandem repeats

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Introduction

Four genetic loci have been confirmed as being involved in the development of type 1 diabetes: HLA class II region (insulin-dependent diabetes mellitus 1 [*IDDM1*]), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*), insulin-dependent diabetes mellitus 2 (*IDDM2*), which is contributed to by the variable number of tandem repeats (VNTR) region upstream of the insulin gene (*INS*), and *LYP/PTPN22* [1–4].

The *INS* VNTR class III alleles were previously described as a dominant trait with relative protection against developing type 1 diabetes. However, recent studies have questioned this observation [5]. It has been suggested that association of the class III alleles with higher thymic insulin mRNA expression and lower titres of insulin autoantibodies are the protective mechanisms behind reduced autoimmunity directed against beta cells [6]. It has also been reported that, in comparison with a class I allele, an *INS* VNTR class III allele exerts higher transcriptional activity on the insulin gene in vitro [7].

We analysed the effect of *INS* VNTR class I and III alleles on insulin antibody titres in relation to residual beta cell function during the first 12 months after diagnosis in children with type 1 diabetes.

Subjects and methods

Subjects This observational study was conducted in 18 centres representing 15 countries in Europe and Japan. Clinical information on demographics and anthropometry, insulin and infusion therapy, hospital admission and severe hypoglycaemia, as well as blood samples for centralised measurement of HbA_1c and boost-stimulated C-peptide, were collected prospectively. Exclusion criteria were: definitive non-type-1 diabetes (MODY, secondary diabetes etc.), rejection of enrolment into the study by patients or parents, and patients initially treated outside the centres for more than 5 days. Diagnosis was according to the World Health Organization criteria.

The cohort included 144 girls and 131 boys, 84% of whom were white Caucasians. The age at clinical diagnosis was 9.1±3.7 years (mean±SD), BMI was 16.5±3.2 kg/m^2, and HbA_1c was 11.2±2.1%. Diabetic ketoacidosis was present in 20.7% of the cases at the time of diagnosis (HCO_3 <15 mmol/l and/or pH <7.30).

The study was performed according to the criteria of the Helsinki II Declaration and was approved by the local ethic committee in each centre. All patients and their parents or guardians gave informed consent.

Genotyping The –23*Hph*IA/T polymorphism (rs689) was used as a marker for the VNTR region as described earlier [8].

Typing of the HLA-class II *DRB1* locus was performed by direct sequencing of exon 2 of *DRB1* according to the Immuno Histocompatibility Working Group [9].

Clinical biochemistry HbA_1c and C-peptide were analysed centrally by ion-exchange high-performance liquid chromatography and a fluoroimmunometric assay (AutoDELFIA C-peptide; PerkinElmer Life Sciences Wallac, Turku, Finland) respectively.

Beta cell function At 1, 6, and 12 months after diagnosis a total of the 257 of the 275 patients underwent a Boost-stimulated C-peptide test. The test was performed in the morning after at least 8 h fasting, the morning insulin dose was given after the test. In agreement with the Diabetes Control and Complications Trial protocol, capillary glucose was measured at time 0 and venous C-peptide and glucose at 90 min after ingestion of Boost.

Antibodies Insulin antibodies were measured by a modified version of a method that has been previously described [10]. The cut-off limit for positivity was 1.56 relative units, representing the 99th percentile in a group of 371 non-diabetic subjects.

Statistical methods The two main hypothesis evaluated were that the *INS* VNTR class III alleles are associated (1) with reduced insulin antibody titres, and (2) with preserved beta cell function. Most evaluations were based on normal distribution, but C-peptide was considered on the logarithmic scale. The distribution of insulin antibody titres was so skewed that non-parametric tests (Kruskal–Wallis) were used to evaluate the effect of the *INS* VNTR genotype on insulin antibody levels. C-peptide and HbA_1c were analysed by means of multiple regression, using age, sex, BMI, HLA-risk group, ketoacidosis at onset, *INS* VNTR genotype, and insulin antibody levels (four groups: negative, low, medium, high) as explanatory factors in a compound symmetric repeated measurement model. A p value of 0.05 or less was considered significant.

Results

Antibody-negative patients Among the patients, 22 tested negative for GAD antibodies (GADA), islet cell antibodies (ICA), and antibodies against insulinoma-associated antigen (IA–2A) throughout the study period. As these patients might represent an idiopathic and non-autoimmune type of diabetes, they were excluded from the analysis of the effect of *INS* VNTR on insulin antibodies, C-peptide and insulin-dose-adjusted HbA_1c.

INS VNTR and allele frequency The *INS* VNTR class I allele frequency was 85.2% and the frequencies of class I/I (73.5%), class I/III (23.3%), and class III/III (3.1%) genotypes were in Hardy–Weinberg equilibrium. Patients with the class III/III genotype were equally distributed between the sexes, slightly older, and had a modestly and non-significantly higher BMI (p=0.59 adjusted for age) compared to the other two genotype groups. All patients with the class III/III genotype were positive for at least one

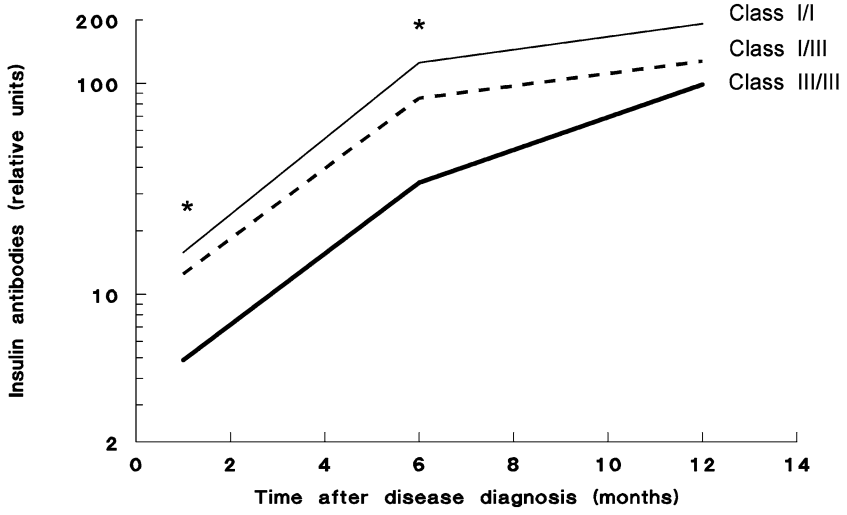

Fig. 1 Insulin antibody titres (medians) after 1 and 6 months were lower in the combined class III/III and III/I genotype groups (* p=0.01 and p=0.01 dominant model) and in the *INS* VNTR class III/III genotype group (* p=0.03 and p=0.02) compared to the class I/I genotype group

autoantibody (ICA, IA–2A, GADA) and were distributed equally among the three HLA-risk groups.

INS VNTR and insulin antibodies In a dominant model the *INS* VNTR class III alleles (class I/III and class III/III vs class I/I) were associated with lower titres of insulin antibodies at 1 and 6 months (p=0.014 and p=0.011, Fig. 1), and the *INS* VNTR class III/III genotype alone was associated with significantly lower insulin antibodies than the class I/I and class I/III genotypes at 1 month and 6 months after diagnosis (p=0.03 and p=0.02, Fig. 1). At 12 months, however, the differences were no longer significant (p=0.09).

INS VNTR and residual beta cell function Multiple linear regression showed that the *INS* VNTR geno- types after 12 months of clinical disease were significantly associated with residual beta cell function (p=0.03), and with C-peptide levels (geometric means) that were twice as high in the class III/III group (319 pmol/l) as in the class I/I and class I/III groups respectively (131 and 166 pmol/l, p=0.01, Fig. 2).

This apparent association of the *INS* VNTR genotypes with higher residual beta cell function was independent of age, sex, BMI, corresponding insulin antibody titres, and HLA-risk genotype group when these were included in our compound symmetric model.

INS VNTR and glycaemic control The preservation of beta cell function in the class III/III genotype subjects was associated with better glycaemic control than in the class I/I and class I/III genotypes, with an average reduction of insulin-dose-adjusted HbA_1c of 1.1% (p=0.04) during the first year of clinical disease (data not shown).

Discussion

In the present study we investigated the impact of the well-described susceptibility gene *IDDM2* on disease pathogenesis and disease progression by examining the humoral immune response to insulin and corresponding residual

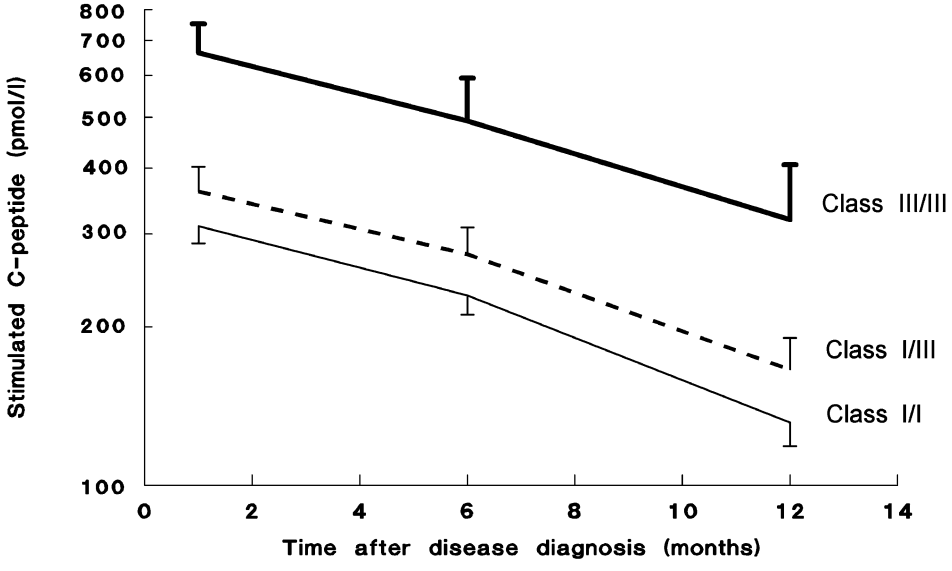

Fig. 2 C-peptide levels (geometric means) were higher (p=0.01) in the class III/III group than in the class I/I and class III/I groups during the first year of clinical disease. This also applied when accounting for age at diagnosis, sex, BMI, ketoacidosis at diagnosis, and HLA class II risk groups in a compound symmetric repeated measurement model. *Error bars* represent 1 SE

beta cell function during the first 12 months after diagnosis in 257 children with type 1 diabetes.

We found that insulin antibodies were lower in both the class I/III and class III/III genotypes than in the class I/I genotype, and confirmed previous findings [11, 12] that carriers of class III alleles inherited a higher tolerance towards endogenous insulin, thus possibly relieving the immunological attack on the beta cells. We also found that class III alleles are associated with higher residual beta cell function and that this putative beta-cell-protective effect of class III alleles seemed to be most evident in the class III/III genotype group who, although only eight patients, also seemed to benefit from a clinically evident improvement in glycaemic control (1.1% in insulin-dose-adjusted HbA_1c, p=0.04). The class III effect could not be explained by the association of class III alleles with lower titres of insulin antibodies, since inclusion of insulin antibody levels in the C-peptide analysis did not alter the association between preserved beta cell function and the class III/III genotype. In addition, we did not detect any influence of HLA-risk groups on the association between class III alleles and insulin antibodies or beta cell function.

Our data, therefore, suggest that the relative protective effects of class III alleles are caused by both a reduced insulin-directed immune autoreactivity and a possible direct beta-cell-specific effect. However, based on estimations of residual beta cell function, we were not able to determine whether the protective effect of class III alleles was inherited as a dominant, recessive or additive trait (p=0.08). This observation seems to be consistent with most recent genetic studies of the *IDDM2* locus [5].

Although it remains controversial, a direct effect of class III alleles on beta cells has previously been demonstrated in non-diabetic infants [13], adult patients with type 1 diabetes onset after 35 years of age [14] and obese non-diabetic women [15], while others have failed to show such an association [16].

The size of our present study group has clear limitations, but it is tempting to speculate that in type 1 diabetes, class III alleles have a synergistic effect on immune-directed insulin autoreactivity and on preservation of beta cell function, and that this synergy explains why the *INS* VNTR is only associated with type 1 diabetes, in contrast to the pathogenically less disease-specific *HLA*, *CTLA4*, and *LYP/PTPN22* loci, which are also associated with other types of autoimmune diseases.

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