Clin Exp Immunol 1997; **110**:98–103

CTLA-4 gene polymorphism confers susceptibility to insulin-dependent diabetes mellitus (IDDM) independently from age and from other genetic or immune disease markers

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(Accepted for publication 10 July 1997)

SUMMARY

Apart from genes in the HLA complex (*IDDM1*) and the variable number of tandem repeats in the 5′ region of the insulin gene (*INS* VNTR, *IDDM2*), several other loci have been proposed to contribute to IDDM susceptibility. Recently, linkage and association have been shown between the cytotoxic T lymphocyte-associated protein 4 (*CTLA-4*) gene on chromosome 2q and IDDM. In a registry-based group of 525 recent-onset IDDM patients < 40 years old we investigated the possible interactions of a *CTLA-4* gene A-to-G transition polymorphism with age at clinical disease onset and with the presence or absence of established genetic (*HLA-DQ*, *INS* VNTR) and immune disease markers (autoantibodies against islet cell cytoplasm (ICA); insulin (IAA); glutamate decarboxylase (GAD65-Ab); IA-2 protein tyrosine phosphatase (IA-2-Ab)) determined within the first week of insulin treatment. In new-onset IDDM patients, G-allele-containing *CTLA-4* genotypes (relative risk (RR) = 1·5; 95% confidence interval (CI) = 1·2–2·0; $P < 0{\cdot}005$) were not preferentially associated with age at clinical presentation or with the presence of other genetic (*HLA-DR3* or *DR4* alleles; *HLA-DQA1*0301-DQB1*0302* and/or *DQA1*0501-DQB1*0201* risk haplotypes; *INS* VNTR I/I risk genotype) or immune (ICA, IAA, IA-2-Ab, GAD65-Ab) markers of diabetes. For 151 patients, thyrogastric autoantibodies (anti-thyroid peroxidase, anti-thyroid-stimulating hormone (TSH) receptor, anti-parietal cell, anti-intrinsic factor) were determined, but association between *CTLA-4* risk genotypes and markers of polyendocrine autoimmunity could not be demonstrated before or after stratification for *HLA*- or *INS*-linked risk. In conclusion, the presence of a G-containing *CTLA-4* genotype confers a moderate but significant RR for IDDM that is independent of age and genetic or immune disease markers.

Keywords insulin-dependent diabetes mellitus CTLA-4 HLA-DQ genes insulin gene autoantibodies

INTRODUCTION

Both multigenic predisposition and environmental factors are important in the pathogenesis of IDDM. The major genetic locus (*IDDM1*) is situated in the HLA class II region on chromosome 6p21, and a second locus (*IDDM2*) is identified as the polymorphic variable number of tandem repeats in the 5′ flanking region of the insulin gene (*INS* VNTR) on chromosome 11p15. Whole-genome searches have identified at least 15 additional chromosomal regions with putative linkage to IDDM [1,2]. So far only a minority of these candidate regions have been confirmed as true susceptibility loci in independent linkage and/or association studies [3]. Recently, linkage to and/or association with IDDM of an A-to-G transition polymorphism at position 49 in the first exon of the *CTLA-4* gene on chromosome 2q33 (designated *IDDM12*) have been shown in data sets from several countries, including Italy, Spain, Belgium and Germany, but not in others [4,5]. CTLA-4 is

‡The following members of the Belgian Diabetes Registry have also contributed to the present study: E. Balasse (Brussels), H. Becq (Wilrijk), J. Beirinckx (Izegem), L. Claeys (Zoersel), M. Coeckelberghs (Antwerp), J.-L. Coolens (Hasselt), W. Coucke (Roeselare), E. Couturier (Brussels), R. Craen (Gent), J.-C. Daubresse (Charleroi), J. De Schepper (Brussels), C. Delvigne (Antwerp), H. Dorchy (Brussels), M. Du Caju (Antwerp), F. Féry (Brussels), M. Gaham (Braine-l'Alleud), J. Gérard (Liège), C. Gillet (Brussels), J. Guiot (Seraing), C. Herbaut (Mons), B. Keymeulen (Brussels), G. Krzentowski (Jumet), M. Letiexhe (Liège), J. Monballyu (Ekeren), G. Moorkens (Antwerp), D. Nicolaij (Kortrijk), F. Nobels (Aalst), M.-C. Pelckmans (Duffel), A. Purnode (Brussels), R. Rooman (Antwerp), R. Rottiers (Gent), A. Scheen (Liège), J. Schutyser (Kortrijk), J.-C. Sodoyez (Liège), G. Somers (Brussels), L. Terriere (Hoboken), J. Teuwen (Kapellen), J. Tits (Genk), K. Van Acker (Antwerp), P. Van Crombrugge (Aalst), L. Van Gaal (Antwerp), E. Vandenbussche (Herentals), S. Vanneste (Zoersel), C. Vercammen (Bonheiden), J. Vertommen (Antwerp).

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involved in the B7 costimulatory pathway and is a candidate gene for T cell-mediated autoimmune disease because it is a powerful negative regulator of T cell proliferation and immune responses [6–11], possibly acting through inhibition of CD28-dependent IL-2 production [11].

The aim of the present study was to investigate the possible interactions of this *CTLA-4* marker with age at clinical IDDM onset, with other genetic markers such as *IDDM1* and *IDDM2* and, finally, with immune disease markers including islet autoantibodies and markers of polyendocrinopathy. Such investigation is warranted in view of the reported age-dependent clinical and biological heterogeneity of IDDM. For instance, the high-risk HLA genotype *DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201* is associated with younger age at IDDM onset [12–15]. Moreover, autoantibodies against conformational epitopes of islet cell cytoplasm (ICA), insulin (IAA), the 65-kD isoform of glutamic acid decarboxylase (GAD65-Ab) or the transmembrane protein tyrosine phosphatase IA-2 (IA-2-Ab) occur preferentially in IDDM patients or their siblings carrying certain combinations of *HLA-DQ* and *INS* VNTR risk alleles [15–25]. The reported association of the *CTLA-4* G allele with other autoimmune disorders such as Graves' disease [4,5,26] prompted us also to test the association of *CTLA-4* genotypes with thyrogastric autoantibodies as markers of polyendocrine autoimmunity in a subgroup of patients.

SUBJECTS AND METHODS

Patients and control subjects

Caucasian IDDM patients under 40 years old ($n = 525$) were consecutively recruited by the Belgian Diabetes Registry at clinical disease onset, and blood was sampled before or within 7 days of insulin treatment. In agreement with previous observations [15,27], the male/female (M/F) ratio was 1·0 in the age group 0–19 years and 1·4 in the age group 20–39 years. The mean age (± s.d.) of the 525 patients was 20 ± 10 years. IDDM was diagnosed according to the National Diabetes Data Group [28]. The control group comprised 530 healthy subjects who were recruited among blood donors, seemingly healthy (para)medical personnel, and children with minor local traumata attending emergency departments. The age range was 0–39 years (mean age ± s.d. 20 ± 11 years; median age 22 years) with a M/F ratio of 1·1. Ethical approval was obtained from the Ethical Committees of the universities participating in the Belgian Diabetes Registry.

Genotyping

HLA-DQA1, -DQB1 polymorphisms and the *INS* VNTR were analysed as described previously [27,29]. In 209 control subjects, the insulin gene polymorphism at position –23 from the transcription initiation site was determined by *Hph*I digestion of polymerase chain reaction (PCR) products as described [30]. In agreement with published data [31], the + allele of the *Hph*I polymorphism is in complete linkage disequilibrium with the class I VNTR alleles in our hands (B. J van der Auwera; unpublished results for 40 subjects). The *CTLA-4* exon 1 position 49 A-to-G polymorphism was typed using standard PCR allele dot-blot hybridization and detection was performed as described [4]. This transition polymorphism has been shown to be in linkage disequilibrium with the microsatellite marker located in the 3′ untranslated region of the *CTLA-4* gene [26,32]. *HLA-DR3* and *DR4* phenotypes were derived from known linkage disequilibria with *HLA-DQA1*-DQB1** haplotypes in the Belgian population (*DR3* with *0501–0201* and *DR4* with *0301–0301* and *0301–0302* [33]). The prevalence of *CTLA-4* genotypes was previously determined in both subject groups [4]. We have now completed *HLA-DQ* and *INS* VNTR genotyping on the patient groups. The results for the different genetic markers are summarized in Table 1 and are in agreement with previous results on other patient groups [14,15].

Autoantibody assays

IA-2-Ab, IAA and GAD65-Ab were determined by liquid phase

Table 1. Prevalence of genetic markers in IDDM patients and control subjects

Genetic marker	Patients ($n = 525$)	Control subjects ($n = 530$)	RR (95% CI)	P
CTLA-4				
AG or GG	344 (66)	294 (55)	1·5 (1·2–2·0)	< 0·005
AA	181 (34)	236 (45)	0·1 (0·1–0·2)	< 0·005
HLA-DR phenotypes				
DR4-positive	318 (61)	106 (20)	6·1 (4·6–8·2)	$< 10^{-6}$
DR3-positive	295 (56)	131 (25)	3·9 (3·0–5·1)	$< 10^{-6}$
HLA-DQA1*-DQB1*				
(DR4)-0301–0302/(DR3)-0501–0201	147 (28)	4 (1)	51·1 (19·2–191·4)	$< 10^{-6}$
(DR4)-0301–0302/non*(DR3)-0501–0201*	151 (29)	64 (12)	2·9 (2·1– 4·1)	$< 10^{-6}$
non*(DR4)-0301–0302/(DR3)-0501–0201*	148 (28)	127 (24)	1·3 (0·9–1·6)	0·13
non*(DR4)-0301–0302*/non*(DR3)-0501–0201*	79 (15)	335 (63)	0·1 (0·08–0·14)	$< 10^{-6}$
***INS* VNTR**				
I/I	373 (71)	221 (51)*	2·4 (1·8–3·2)	$< 10^{-6}$
I/III or III/III	152 (29)	216 (49)*	0·6 (0·5–0·8)	$< 10^{-6}$

Data are *n* (%). *P* values are considered significant whenever $P < 0{\cdot}05/10$ or $P < 0{\cdot}005$ (Bonferroni).

**INS* VNTR data were determined in 437 control subjects.

radiobinding assay [23] and ICA (expressed in JDF units) by indirect immunofluorescence [15]. These four antibodies were determined in all 525 patients at disease onset, except for IA-2-Ab, which were only available in 483 subjects. The cut-off for antibody positivity was determined by receiver-operating characteristics (ROC) curve analysis [34] and found to be $\geq 0{\cdot}5\%$ of tracer bound for IA-2-Ab, $\geq 1{\cdot}7\%$ for GAD65-Ab, $\geq 0{\cdot}7\%$ for IAA, and ≥ 12 JDF units for ICA based on the analysis of over 300 control subjects and over 300 IDDM patients [23]. These assays repeatedly performed well in external quality control programs (Immunology of Diabetes Workshops, University of Florida Proficiency Tests, European Nicotinamide Diabetes Intervention Trial serum exchanges). Thyrogastric autoantibodies were determined in a subset of 151 patients. Thyroid peroxidase (cut-off >100 U/ml), thyroid-stimulating hormone (TSH) receptor (cut-off >9 U/l), and anti-intrinsic factor antibodies (cut-off ratio ((ct/min in patient serum)/(ct/min in normal serum)) $>1{\cdot}1$) were measured by radioimmunoassay and antigastric parietal cell antibodies (cut-off titre $>1{:}20$) by indirect immunofluorescence assay [35].

Statistical analysis

The significance of differences between IDDM patients and control subjects in the prevalence of alleles or genotypes was assessed by χ^2 test after Yates' correction, or by Fisher's exact test whenever appropriate. In order to correct for multiple comparisons, P values $<0{\cdot}05/n$ were considered significant, where n represents the number of comparisons (Bonferroni). The relative risk (RR) conferred by a given genetic marker was expressed as an odds ratio (95% confidence interval (CI)) as previously described [29]. Multivariate analysis of autoantibody levels was performed by stepwise linear regression using the statistical program SPSS version 7.0 for Windows (SPSS, Chicago, IL).

RESULTS

Prevalence of genetic risk markers according to age at clinical onset of IDDM

In IDDM patients, the frequency of *CTLA-4* genotypes was not significantly different among the three age categories considered (Table 2), and therefore *CTLA-4* genotypes were not stratified according to age in subsequent analyses. In contrast, the prevalence of *DR4*- and of *DR3*-positivity decreased with age at clinical onset. This decrease is mainly attributable to the declining frequency of the high-risk genotype *(DR4)-DQA1*0301-DQB1*0302/(DR3)-DQA1*0501-DQB1*0201*, decreasing from 47% in patients <10 years old down to 18% in patients >20 years old. The frequency of *HLA-DQ* genotypes with only one risk haplotype or without risk haplotypes, if anything, increased with age at onset, while the presence of the *INS* VNTR genotypes was age-independent (Table 2).

Interaction between CTLA-4 *genotypes and genetic (*HLA-DQ, INS *VNTR) or immune markers*

The possible association between *CTLA-4* genotypes and other genetic markers is shown in Table 3. The presence or absence of G alleles was not accompanied by differences in the prevalence of *DR3* or *DR4* phenotypes, of the high-risk genotype *DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201*, of *DQA1*-DQB1** genotypes containing only one type of risk haplotypes (*0301–0302* or *0501–0201*), or of the *INS* VNTR class I/I genotype, regardless of the *HLA-DQ*-linked risk. Neither did the presence or absence of AG or GG genotypes influence the occurrence of ICA, IAA, GAD65-Ab or IA-2-Ab, alone or in combination (Table 4). Similar negative results were obtained after stratification of the patients according to positivity or negativity for *HLA-DR3* or *DR4*, and to the four categories of *HLA-DQ* genotypes, in the absence or presence of the *INS* VNTR class I/I genotype (results not

Table 2. Prevalence of genetic risk markers in 525 IDDM patients according to age at clinical onset

Genetic marker	Age at clinical onset		
	0–9 years ($n=98$)	10–19 years ($n=167$)	20–39 years ($n=260$)
CTLA-4			
GG	16 (16)	29 (17)	30 (12)*
AG	47 (48)	91 (55)	131 (50)*
GG or AG	63 (64)	120 (72)	161 (62)*
***HLA-DR* phenotypes**			
DR4-positive	70 (71)	108 (65)	140 (54)**
*DR*3-positive	68 (69)	98 (59)	129 (50)***
HLA-DQA1*-DQB1*			
(DR4)-0301-0302/(DR3)-0501-0201	46 (47)	54 (32)	47 (18)****
(DR4)-0301-0302/non*(DR3)-0501-0201*	20 (20)	50 (30)	81 (31)*
non*(DR4)-0301-0302*)/*(DR3)-0501-0201*	22 (22)	44 (26)	82 (31)*
non*(DR4)-0301-0302*)/non*(DR3)-0501-0201*	10 (10)	19 (11)	50 (19)*
***INS* VNTR**			
I/I	69 (70)	124 (74)	180 (69)*

Data are *n* (%).

*Overall P by age $>0{\cdot}05$; **overall P by age $=0{\cdot}004$; ***overall P by age $=0{\cdot}003$; ****overall P by age $<10^{-6}$; overall P values are considered significant whenever $P<0{\cdot}05/10$ or $<0{\cdot}005$ (Bonferroni).

Table 3. Prevalence of genetic markers in 525 IDDM patients at clinical onset according to the presence or absence of G-containing *CTLA-4* genotypes

Genetic marker	Prevalence of genetic marker	
	G allele present	G allele absent
	***n* = 344**	***n* = 181**
HLA-DR phenotypes		
DR4-positive	218 (63)	100 (55)*
DR3-positive	189 (55)	106 (59)*
HLA-DQA1*-DQB1*		
(DR4)-0301-0302/(DR3)-0501-0201	97 (28)	50 (28)*
(DR4)-0301-0302/non*(DR3)-0501-0201*	106 (31)	45 (25)*
Non*(DR4)-0301-0302/(DR3)-0501-0201*	92 (27)	56 (31)*
Non*(DR4)-0301-0302*/non*(DR3)-0501-0201*	49 (14)	30 (17)*
INS* VNTR I/I *in combination with		
Any *HLA-DQ* genotype or *HLA-DR* phenotype	244 (71)	129 (71)*
DR4-positive phenotypes	157 (46)	70 (39)*
DR3-positive phenotypes	130 (38)	79 (44)*
HLA-DQA1-DQB1*		
(DR4)-0301-0302/(DR3)-0501-0201	63 (18)	36 (20)*
(DR4)-0301-0302/non*(DR3)-0501-0201*	86 (25)	32 (18)*
Non*(DR4)-0301-0302/(DR3)-0501-0201*	67 (19)	43 (24)*
Non(*DR4*)-0301-0302/non(*DR3*)-0501-0201	28 (8)	18 (10)*

Data are *n* (%).
*$P > 0{\cdot}05$.

shown). Multivariate analysis confirmed the lack of association between levels of IDDM-specific autoantibodies and *CTLA-4* genotypes (results not shown).

In a subgroup of 151 patients, no association could be revealed between *CTLA-4* genotypes and positivity for thyroid peroxidase ($n = 26$), TSH receptor ($n = 0$), anti-parietal cell ($n = 10$), or anti-intrinsic factor antibodies ($n = 5$), alone (not shown) or in combination (Table 4). When considering thyroid peroxidase antibodies separately, subjects with or without *CTLA-4* G alleles did not differ in autoantibody prevalence, regardless of the presence or absence of *HLA-DR* phenotypes (Table 4).

Table 4. Prevalence of immune markers in IDDM patients at clinical onset according to the presence or absence of G-containing *CTLA-4* genotypes

Immune marker	Prevalence of immune marker	
	G allele present	G allele absent
	***n* = 344**	***n* = 181**
IDDM-specific autoantibodies		
IAA	135 (39)	79 (44)*
ICA	248 (72)	124 (69)*
GAD 65-Ab	283 (82)	140 (77)*
	***n* = 320**	***n* = 163**
IA-2-Ab**	187 (58)	93 (57)*
≥ 1 type of autoantibodies**	299 (93)	150 (92)*
≥ 2 types of autoantibodies**	268 (84)	124 (76)*
≥ 3 types of autoantibodies**	183 (57)	93 (57)*
All four autoantibodies**	67 (21)	43 (26)*
Thyrogastric autoantibodies*	***n* = 96**	***n* = 55**
≥ 1 type of autoantibodies***	24 (25)	11 (20)*
Thyroid peroxidase autoantibodies***		
Any *HLA-DR* phenotype	16 (17)	10 (18)*
DR4-positive	13 (14)	5 (9)*
DR3-positive	4 (4)	6 (11)*

Data are *n* (%).
*$P > 0{\cdot}05$; **$n = 483$; ***$n = 151$.

DISCUSSION

An A-to-G transition polymorphism at position 49 (codon 17) in the first exon of the *CTLA-4* gene has been linked to and associated with IDDM [4,5]. The G and A alleles code for, respectively, alanine (Ala) and threonine (Thr) at position 17. It is so far unknown whether this polymorphism is causally implicated in diabetes susceptibility, or rather represents an indirect marker of another mutation in the *CTLA-4* gene or in a nearby gene. How these genetic changes could be linked to IDDM susceptibility is a matter of speculation. Since CTLA-4 is a negative regulator of CD28 acting as a major repressor of the antigen-specific immune response [6–11], it is conceivable that certain *CTLA-4* gene polymorphisms could alter the function or expression level of the CTLA-4 protein and hence underlie differences in the individual proneness to (organ-specific) autoimmune diseases such as IDDM or Graves' disease [4,5,26]. The role of CTLA-4 in T cell development and in the pathogenesis of autoimmunity is so far

incompletely understood. *CTLA-4*-gene knockout mice develop a lethal lymphoproliferative disease which is characterized by massive T lymphocytic infiltration and multiorgan tissue destruction, including heart and pancreas [9,10], revealing an immunosuppressive role for the CTLA-4 protein. These observations in CTLA-4-deficient mice also raise the possibility that mutations in the *CTLA-4* gene may lead to autoimmunity in humans. However, the A-to-G transition polymorphism is an unlikely candidate, because it is located in the coding region for the leader peptide whose function will probably not be affected by the Thr/Ala substitution [4]. Alternatively, the *CD28* gene which is located close to *CTLA-4* is another candidate gene for the alterations in the immune response.

By analogy with other intermediate-risk genetic IDDM risk markers (*INS* VNTR I/I and intermediate-risk *HLA-DQ* genotypes) [21], but in contrast to the heterozygous high-risk HLA genotype *DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201* [12–15], the risk conferred by G-allele containing *CTLA-4* genotypes does not decrease with age at clinical disease onset. Its diabetes association is not affected by the occurrence of *HLA-DR* phenotypes, of *HLA-DQ* susceptibility haplotypes and of the *INS* VNTR class I/I risk genotype, suggesting that the *CTLA4/IDDM12* region contains an independent risk factor for IDDM. This finding complements the observations of Donner *et al*. [5] and contrasts with reported interactions between the HLA gene complex and other loci including the *INS* VNTR region on chromosome 11p15, the IL-1 receptor type 1 gene (*IL1R1*) on chromosome 2q12-22, and the *HOXD8* locus on chromosome 2q31 [21,36,37]. Taken together, these findings indicate that the relative importance of various intermediate-risk genetic factors for IDDM susceptibility increases with age at clinical onset.

In contrast to the reported preferential occurrence of diabetes-specific autoantibodies with genetic risk as determined by *HLA-DQ* and *INS* VNTR genotypes [15–24], the *CTLA-4* G allele appears not preferentially associated with a higher frequency of islet or thyrogastric antibodies in the present patient group even after stratification for *HLA*- or *INS*-linked risk. This observation does not provide support to the assumption that the studied A-to-G *CTLA-4* polymorphism might determine changes in the humoral immune response. Disclosing such an association might, however, require longitudinal studies or much larger numbers of patients in the case of the *CTLA-4* G allele, in view of its relatively weak diabetes association compared with *HLA-DQ* risk genotypes. Moreover, its effects may only be detectable in the cellular immune response, or be non-specific in nature. Finally, the studied *CTLA-4* polymorphism may only represent an indirect genetic marker of IDDM susceptibility.

In conclusion, G allele-positive genotypes as determined by the A-to-G polymorphism in the first exon of the *CTLA-4* gene confer a moderate but significant relative risk for IDDM, independent of age and genetic or immune disease markers.

ACKNOWLEDGMENTS

Professor D. Pipeleers (Diabetes Research Centre, Vrije Universiteit Brussel, Belgium) and Professor J. A. Todd (The Wellcome Trust Centre for Human Genetics, University of Oxford, UK) are gratefully acknowledged for critical reading of the manuscript and useful suggestions. We are indebted to M. Bodson, V. Claessens, A. Demarré, L. De Pree, S. Exterbille, T. Ghysels, P. Goubert, C. Groven, A. Ivens, F. Lebleu, G. Schoonjans and H. Thomas for excellent technical assistance. JDF standards for the ICA assay and cDNA for preparation of ^{35}S-recombinant human GAD65 were kindly donated by Professor Å. Lernmark (University of Washington, Seattle, WA) and Dr A. Falorni (when at the Karolinska Institute, Stockholm, Sweden), human IA-2ic cDNA by Dr M. R. Christie (King's College, London, UK) and crystalline Humulin by Eli Lilly Co. (Brussels, Belgium). This work was supported by grants from the Belgian 'Fonds voor Geneeskundig Wetenschappelijk Onderzoek' (FGWO 3.9007.91 and 3.0113.97), the Belgian 'Nationale Loterij', the Ministries of Public Health of the Flemish and French Communities of Belgium, Johnson & Johnson, Novo Nordisk, and Weight Watchers. Presented in part at the Annual Meeting of the European Association for the Study of Diabetes, Vienna, Austria, 1996.

REFERENCES

1 Davies JL, Kawaguchi Y, Bennett ST *et al*. A genome-wide search for human type 1 diabetes susceptibility genes. Nature 1994; **371**:130–6.
2 Hashimoto L, Habita C, Beressi J *et al*. Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q. Nature 1994; **371**:161–4.
3 Todd JA, Farrall M. Panning for gold: genome-wide scanning for linkage in type 1 diabetes. Hum Mol Genet 1996; **5**:1443–8.
4 Nisticò L, Buzzetti R, Pritchard LE *et al*. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Hum Mol Genet 1996; **5**:1075–80.
5 Donner H, Rau H, Walfish PG *et al*. CTLA4 alanine-17 confers genetic susceptibility to Graves' disease and to type 1 diabetes mellitus. J Clin Endocrinol Metab 1997; **82**:143–6.
6 Gribben JG, Freeman GJ, Boussiotis VA *et al*. CTLA-4 mediates antigen-specific apoptosis of human T-cells. Proc Natl Acad Sci USA 1995; **92**:811–5.
7 Linsley PS, Golstein P. Lymphocyte activation: T-cell regulation by CTLA-4. Current Biology 1996; **6**:398–400.
8 Walunas TL, Lenschow DJ, Bakker CY *et al*. CTLA-4 can function as a negative regulator of T cell activation. Immunity 1994; **1**:405–13.
9 Waterhouse P, Penninger JM, Timms E *et al*. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995; **270**:985–8.
10 Tivol EA, Boriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995; **3**:541–7.
11 Walunas TL, Bakker CY, Bluestone JA. CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med 1996; **183**:2541–50.
12 Wolf E, Spencer KM, Cudworth AG. The genetic susceptibility to type 1 (insulin-dependent) diabetes: analysis of the HLA-DR association. Diabetologia 1983; **24**:224–30.
13 Karjalainen J, Salmela P, Ilonen J, Surcel H-M, Knip M. A comparison of childhood and adult type I diabetes mellitus. N Engl J Med 1989; **320**:881–6.
14 Caillat-Zucman S, Garchon H-J, Timsit J *et al*. Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus. J Clin Invest 1992; **90**:2242–50.
15 Vandewalle CL, Decraene T, Schuit FC *et al*. Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical onset of type I (insulin-dependent) diabetes mellitus before age 10 years, but not at onset between age 10 and 40 years. Diabetologia 1993; **36**:1155–62.
16 Ziegler R, Alper CA, Awdeh ZL *et al*. Specific association of HLA-DR4 with increased prevalence and level of insulin-autoantibodies in first-degree relatives of patients with type 1 diabetes. Diabetes 1991; **40**:709–14.
17 Ziegler AG, Standl E, Albert E, Mehnert H. HLA-associated insulin autoantibody formation in newly diagnosed type 1 diabetic patients. Diabetes 1991; **40**:1146–9.
18 Serjeantson SW, Court J, Mackay IR *et al*. HLA-DQ genotypes are associated with autoimmunity to glutamic acid decarboxylase in

insulin-dependent diabetes mellitus patients. Hum Immunol 1993; **38**:97–104.
19 Hagopian WA, Sanjeevi CB, Kockum I *et al*. Glutamate decarboxylase-, insulin- and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. J Clin Invest 1995; **95**:1505–11.
20 Genovese S, Bonfanti R, Bazzigalupi E *et al*. Association of IA-2 autoantibodies with HLA DR4 phenotypes in IDDM. Diabetologia 1996; **39**:1223–6.
21 Van der Auwera B, Schuit F, Lyaruu I *et al*. Genetic susceptibility for insulin-dependent diabetes mellitus in Caucasians revisited: the importance of diabetes registries in disclosing interactions between HLA-DQ- and insulin-gene-linked risk. J Clin Endocrinol Metab 1995; **80**:2567–73.
22 Vandewalle CL, Falorni A, Lernmark Å *et al*. Associations of GAD65- and IA-2-autoantibodies with genetic risk markers in new-onset IDDM patients and their siblings. Diabetes Care 1997, in press.
23 Gorus FK, Goubert P, Semakula C *et al*. IA-2-Autoantibodies complement GAD65-autoantibodies in new-onset IDDM patients and help predict the disease in their siblings. Diabetologia 1997; **40**:95–99.
24 Gorus FK, Vandewalle CL, Dorchy H *et al*. Influence of age on associations among insulin autoantibodies, islet cell antibodies and HLA-DQA1*0301-DQB1*0302 in siblings of patients with type 1 (insulin-dependent) diabetes mellitus. J Clin Endocrinol Metab 1994; **78**:1172–8.
25 Rowley MJ, Chen QY, Teoh KL *et al*. Diabetic sera react with the glutamic acid decarboxylase molecule in a dimeric-oligomeric form. Clin Exp Immunol 1996; **106**:323–8.
26 Yanagawa T, Hidaka Y, Guimaraes V, Soliman M, DeGroot LJ. CTLA-4 gene polymorphism associated with Graves' disease in a Caucasian population. J Clin Endocrinol Metab 1995; **80**:41–45.
27 Vandewalle CL, Falorni A, Svanholm S *et al*. High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. J Clin Endocrinol Metab 1995; **80**:846–51.
28 National Diabetes Data Group. Classification and diagnosis of diabetes mellitus and other categories of glucose intolerance. Diabetes 1979; **28**:1039–47.
29 Heimberg H, Nagy ZP, Somers G, De Leeuw I, Schuit F. Complementation of HLA-DQA and HLA-DQB genes confers susceptibility and protection to insulin-dependent diabetes mellitus. Hum Immunol 1992; **33**:10–17.
30 Julier C, Hyer RN, Davies J *et al*. Insulin-IGF2 region on chromosome 11p encodes a gene implicated in HLA-DR4-dependent diabetes susceptibility. Nature 1991; **354**:155–9.
31 Bennett ST, Lucassen AM, Goug SCL *et al*. Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. Nature Genet 1995; **9**:284–92.
32 Harper K, Balzano C, Rouvier E, Mattel M-G, Luciani M-F, Golstein P. CTLA-4 and CD28 activated lymphocyte molecules are closely related in both mouse and human as to sequence, message expression, gene structure, and chromosomal location. J Immunol 1991; **147**:1037–44.
33 Van der Auwera B, Van Waeyenberge C, Schuit F *et al*. DRB1*0403 protects against IDDM in Caucasians with the high-risk heterozygous DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 genotype. Diabetes 1995; **44**:527–30.
34 Zweig M, Campbell G. Receiver-operating characteristics (ROC) plots: a fundamental tool in clinical medicine. Clin Chem 1993; **39**:561–71.
35 Abrams P, De Leeuw I, Vertommen J, the Belgian Diabetes Registry. In new-onset insulin-dependent diabetic patients the presence of anti-thyroid peroxidase antibodies is associated with islet cell autoimmunity and the high risk haplotype HLA-DQA1*0301-DQB1*0302. Diabetic Med 1996; **13**:415–9.
36 Metcalfe KA, Hitman GA, Pociot F *et al*. An association between type 1 diabetes and the interleukin-1 receptor type 1 gene. Hum Immunol 1996; **51**:41–48.
37 Owerbach D, Gabbay KH. The HOXD8 locus (2q31) is linked to type I diabetes. Interaction with chromosome 6 and 11 disease susceptibility genes. Diabetes 1995; **44**:132–6.