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HLA class II associations with Type 1 diabetes mellitus: a multivariate approach

Key words:
HLA class II; insulin-dependent diabetes mellitus; multivariate analysis

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Abstract: The association of HLA class II phenotype with the development of insulin-dependent (Type 1) diabetes mellitus (IDDM) is well established but the contribution of the various HLA-DR and -DQ alleles and haplotypes to disease predisposition is not fully understood. We have determined haplotype and genotype odds ratios, and further employed multivariate tree analysis to explore the contribution of individual HLA-DRDQ haplotypes to the genetic risk for developing IDDM in the Dutch population. Next to haplotype and genotype odds ratios, multivariate tree analysis techniques provide overall risk calculations for each modeled parameter, and offer insight in the interaction of the model parameters via tree-shaped reports, in which subsequent stratifications on the data can easily be followed. We compared 206 Dutch IDDM patients with 840 serologically typed random healthy unrelated Dutch Caucasoid controls. The multivariate tree analysis showed that the HLA-DR7DQ9 and DR15DQ6 haplotype were strongly associated with disease protection (OR=0.04, P=0.0003, and OR=0.07, P=<0.0001, respectively). The highest ORs were found for the DR4DQ8/DR8DQ4 genotype (OR=21.04, P=0.001), followed by DR4DQ8/DR17DQ2 (OR=12.45, P< 0.0001) and DR9DQ9/DR17DQ2 (OR=10.87, P=0.02). DR4DQ8 homozygous and DR17DQ2 homozygous individuals have a disease OR of 9.0 and 3.0 (P=0.01 and 0.03), respectively. In conclusion, the results from haplotype, genotype, and tree analyses provide insight into the disease associations for combinations of HLA-DRDQ haplotypes. We confirm that the DR9DQ9/DR17DQ2 genotype is associated with susceptibility in the Dutch population, which has previously been noticed as a HLA risk genotypes in Asian populations only.

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The human major histocompatibility complex (MHC), HLA, has been shown to be the major genetic component (IDDM1) associated with the development of diabetes mellitus type 1 (IDDM) (1–4). The strongest association is with the HLA-DQB1 locus, implying the highest genetic risk for the development of IDDM as well as protection against this disease (5, 6). However, in recent years it has become evident that IDDM1 constitutes a joint interaction of DQB1, DQA1, and DRB1 (5, 7–12). Due to the strong linkage disequilibrium

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between these genes, it is sufficient to consider only the DRB1-DQB1 (DRDQ) haplotypes for assessment of the risk for type 1 diabetes, which has been determined in IDDM1 patients and controls from several different populations (13–18). Collectively, these studies show that susceptibility is most strongly associated with the DR4DQ8 and DR17DQ2 haplotype, and that dominant protection is associated with DR15DQ6 haplotype. Furthermore, the highest susceptibility is associated with the DR4DQ8 / DR17DQ2 genotype.

Due to the observed intra- and interchromosomal interactions (19), the complex association between alleles of the HLA class II genes and the development of IDDM requires multi-variate statistical techniques. The multivariate method that is most widely used is logistic regression (LR) (20). Although the mathematical principles of LR are sound, interpretation of LR results in genetic studies is not straightforward. Without stratification the resulting odds ratio (OR) for a factor (an allele or haplotype) and its significance are calculated over all cases under evaluation. A stepwise LR procedure will build a multivariate model by repeatedly selecting factors using score-tests. The factor that emerges from the score-test most significantly is added to the model. To allow interpretation of the effect of a secondary factor, stratification for each superimposed factor is required. Due to interactions with the superimposed, a factor may be significantly associated with the disease within one stratum, while it is not so in other strata. The score-test of the stepwise LR calculates the significance of a factor over all strata and may, because of weak or opposite effects of the factor in different strata, not lead to selection of such a factor in the model. Moreover, LR may fail to converge to a model, because of limitations of the method. Multivariate tree analysis (MTA) offers a solution to these drawbacks (21). Factor selection is carried out per stratum rather than overall and interpretation of the results is unambiguous. Iterative stratification is the basis of both LR and MTA, but the latter will test all candidate factors within each stratum and calculate the stratified OR for the selected factor in that particular stratum. The results can conveniently be organized into a tree-shaped report. Moreover, in prognostic decision trees, the HLA markers can be subdivided according to the MTA results, rather than pooling the HLA markers into a single susceptibility factor, as was done for example by Bingley and colleagues (22). Prognostic decision trees serve to categorize individuals for their risk of developing disease by applying combinations of markers and can be used in population screening.

Here, we determined the association of DRB1-DQB1 haplo-, and genotypes with type 1 diabetes in 206 Dutch IDDM patients and 840 controls. Woolf-Haldane odds-ratios and MTA were used to provide evidence for interaction between HLA-DR-DQ haplotypes in the protection against or susceptibility to development of IDDM.

Material and methods

The case group consisted of 206 unrelated IDDM patients that were collected consecutively upon diagnosis by pediatricians in the southwest of The Netherlands (23). The control group consisted of 2,441 random unrelated healthy Dutch Caucasoid blood donors (24), 840 of which were typed for both DQB1 and DRB1. HLA-DR and -DQ typing was carried out by serology, as described elsewhere (25). The control group was found to be in Hardy-Weinberg equilibrium (data not shown).

Woolf-Haldane analysis

Odds ratios (OR) were calculated from standard contingency table analysis using Haldane's modification of Woolf's method (26, 27). Two-tailed statistical significance of the ORs was tested with Fisher's exact test (28). We used Edwards method (29) for the correction of *P*-values rather than the widely used Bonferroni method, because the former calculates exact corrected probabilities through a simple probabilistic algorithm, while the latter simply multiplies the *P*-value by the number of tests performed leading to an over-conservative correction.

Haplotype assignment

Full HLA-DRDQ phenotype frequencies of patients and controls were established by direct counting. These frequencies were compared for all phenotypes with a frequency of 2% in either patients or controls. Haplotype assignment was carried out in patients and controls, using available maximum likelihood estimates (MLE) of the haplotype frequencies (24) to determine the relative probability of each combination of haplotypes (i.e. genotype). This method was previously validated (30). The resulting genotype distribution corresponded completely to the DRDQ phenotype frequency distribution. Homozygosity was added as a factor in the resulting patient and control genotype files used for MTA.

Multivariate tree analysis

Initially, a SAS macro supplied by the SAS Institute, employing the CHAID algorithm described by Kass (21), was used for MTA. Since this procedure ranks the haplotypes in each step on the significance of their effect, the dominance of protection against IDDM by certain

HLA specificities was not detected. Significance of an effect should be treated as a secondary criterion, the strength of the effect being of prime importance. Therefore, we developed a procedure which uses Woolf-Haldane (WH) ORs or, in case of protective haplotypes, the reciprocal of the ORs as the measure of effect strength. In the initial step, this procedure performs a WH analysis on all patients and control individuals. Subsequently, the HLA haplotype with the strongest significant effect is reported and stratifies the patient and control files on that haplotype. Analysis, haplotype selection and stratification are repeated until no significant haplotype effects remain in any stratum. The procedure calculates ORs and their significance both for the (haplotype) genotype as compared to all other genotypes (relative to the entire tree, OR_I) and for the haplotype (relative to the stratum, OR_{II}). The OR_I value indicates the disease risk for an individual with the corresponding genotype, while the OR_{II} indicates the contribution of the haplotype to the disease risk. The listed *P*-values are Fisher's exact before and after correction for the number of comparisons. There were 18 different genotypes with a frequency exceeding 2% in either patients or controls. This number was used as the number of comparisons for correction of the OR_I *P*-values. *P*-values for OR_{II} were corrected for the remaining informative haplotypes in the branch (NFac in Table 3).

Results

Haplotype analysis

The haplotype assignments had a mean probability of the genotype of 99.75% in patients and 99.91% in controls. The lowest probability in the cases was 54.3% in one patient, whose phenotype was DR4 DR12 DQ8 and the two possible genotypes were DR4DQ8/DR12DQ8 and DR4DQ8/DR12DQnull (missed DQ antigen), with a slightly higher probability for the former. This did not affect the remaining analyses, since the DR12DQ8 haplotype frequency is 0 in controls. The lowest probability in controls was 73.7% in one control subject, whose phenotype was DR4 DR8 DQ7 DQ8, assigned as DR4DQ8/DR8DQ7. The haplotypes involved were DR4DQ7 (haplotype frequency (HF)=0.05), DR4DQ8 (HF=0.11), DR8DQ7 (HF=0.001) and DR8DQ8 (HF=0.0007).

Woolf-Haldane (WH) analysis yielded significant associations for 8 haplotypes (Table 1). As expected, susceptibility was associated with haplotypes DR4DQ8, and DR17DQ2 (OR of 3.63 and 3.0). On the other hand, several haplotypes were found to be associated with a reduced risk (protection) for IDDM: DR7DQ9 (OR=0.04), DR15DQ6 (OR=0.08), DR14DQ5 (OR=0.14), and DR11DQ7 (OR=0.22).

Haplotype Woolf-Haldane analysis

Haplotype	Case	Control	OR	C.I.	*P*-value
DR4DQ8	127	185	3.63	2.8–4.7	4.05E-23
DR17DQ2	137	241	3.00	2.3–3.8	3.57E-18
DR8DQ4	13	33	1.67	0.9–3.2	n.s.
DR9DQ9	7	19	1.58	0.7–3.7	n.s.
DR1DQ5	46	210	0.89	0.6–1.3	n.s.
DR13DQ6	32	226	0.55	0.4–0.8	2.74E-02
DR4DQ7	10	91	0.46	0.2–0.9	n.s.
DR7DQ2	13	125	0.42	0.2–0.8	2.70E-02
DR10DQ5	3	33	0.42	0.1–1.3	n.s.
DR16DQ5	2	24	0.41	0.1–1.5	n.s.
DR11DQ7	6	116	0.22	0.1–0.5	3.77E-04
DR12DQ7	1	31	0.19	0.0–1.0	n.s.
DR14DQ5	1	43	0.14	0.0–0.7	n.s.
DR15DQ6	4	212	0.08	0.0–0.6	4.64E-03
DR7DQ9	0	52	0.04	0.0–0.2	5.73E-11

Counts of haplotypes in Dutch IDDM patients (Case) and controls are given. OR refers to Woolf-Haldane odds ratios, with 95% confidence intervals (C.I.), and corresponding *P*-value

Table 1

Genotype analysis

To evaluate genotype specific effects, Woolf-Haldane analysis was performed on genotypes rather than haplotypes (Table 2). Four genotypes in patients show pronounced increases (OR $\geq$ 9) as compared to controls (corresponding to 41% of the patients) and three show minor increases (OR $\geq$ 2, corresponding to 22% of the patients). Consequently, 37% of the patients did not have an increased genetic predisposition to IDDM. The highest significant risk was associated with DR4DQ8/DR8DQ4, consistent with other reports. The DR4DQ8/DR17DQ2, and DR4DQ8 homozygous associations were also significant and confirmatory of previous findings. The association of DR17DQ2/DR9DQ9 with IDDM susceptibility has not been reported in Caucasoid populations. The reduced ORs of genotypes that contain the DR15DQ6 haplotype confirmed the dominant nature of protection against IDDM.

Multivariate tree analysis

In MTA, most of the combinations (Fig. 1, Table 3) were consistent with the WH genotype analysis results (Table 2). MTA first reported two strongly protective haplotypes (branches 0 and 2), confirming the dominant nature of protection against IDDM associated with HLA. Strikingly, and in contrast to the WH genotype analysis,

Genotype Woolf-Haldane analysis *Table 2*

Genotype		Case	Control	OR	C.I.	*P*-value
DR4DQ8	DR8DQ4	7	1	21.15	3.64–122.90	4.00E-05
DR17DQ2	DR4DQ8	64	29	12.54	7.83–20.08	1.96E-34
DR17DQ2	DR9DQ9	6	2	10.93	2.52–47.38	2.47E-03
DR4DQ8	DR4DQ8	7	3	9.04	2.52–32.46	1.83E-03
DR17DQ2	DR17DQ2	16	23	3.03	1.58–5.80	1.98E-02
DR1DQ5	DR4DQ8	16	26	2.68	1.42–5.05	n.s.
DR4DQ8	DR13DQ6	14	30	2.01	1.06–3.83	n.s.
DR1DQ5	DR4DQ7	3	7	1.92	0.54–6.89	n.s.
DR1DQ5	DR7DQ2	4	10	1.77	0.58–5.39	n.s.
DR1DQ5	DR17DQ2	9	27	1.43	0.67–3.04	n.s.
DR17DQ2	DR13DQ6	10	35	1.22	0.60–2.47	n.s.
DR17DQ2	DR7DQ2	2	9	1.08	0.26–4.37	n.s.
DR4DQ8	DR7DQ2	3	15	0.92	0.29–2.97	n.s.
DR4DQ7	DR4DQ8	2	11	0.89	0.22–3.51	n.s.
DR1DQ5	DR1DQ5	3	16	0.86	0.27–2.77	n.s.
DR17DQ2	DR4DQ7	3	17	0.81	0.26–2.59	n.s.
DR1DQ5	DR11DQ7	1	9	0.64	0.11–3.62	n.s.
DR4DQ8	DR11DQ7	1	12	0.49	0.09–2.66	n.s.
DR1DQ5	DR13DQ6	3	32	0.43	0.14–1.31	n.s.
DR15DQ6	DR7DQ2	2	24	0.41	0.11–1.52	n.s.
DR17DQ2	DR11DQ7	1	15	0.39	0.07–2.10	n.s.
DR13DQ6	DR7DQ2	1	16	0.37	0.07–1.96	n.s.
DR4DQ7	DR13DQ6	1	16	0.37	0.07–1.96	n.s.
DR15DQ6	DR14DQ5	0	8	0.24	0.01–4.15	n.s.
DR15DQ6	DR4DQ8	1	27	0.22	0.04–1.13	n.s.
DR15DQ6	DR7DQ9	0	9	0.21	0.01–3.67	n.s.
DR17DQ2	DR14DQ5	0	9	0.21	0.01–3.67	n.s.
DR1DQ5	DR15DQ6	1	37	0.16	0.03–0.81	n.s.
DR15DQ6	DR4DQ7	0	14	0.14	0.01–2.33	n.s.
DR15DQ6	DR11DQ7	0	19	0.10	0.01–1.70	n.s.
DR15DQ6	DR17DQ2	0	23	0.08	0.01–1.40	n.s.
DR11DQ7	DR13DQ6	0	26	0.07	0.00–1.23	n.s.
DR15DQ6	DR13DQ6	0	34	0.06	0.00–0.93	n.s.

Counts of genotypes in Dutch IDDM patients (Case) and controls are given. OR refers to Woolf-Haldane Odds ratios, with 95% confidence intervals (C.I.), and corresponding *P*-value

protection associated with DR7DQ9 was selected as the strongest haplotype in the MTA and WH haplotype analysis. This is due to the relatively low frequency of that haplotype in controls (6.2% as opposed to 25% for DR15DQ6) with consequently no genotypes with a frequency greater than 2%. HLA-DR2 (DR15DQ6) associated protection was also confirmed by MTA. The MTA results further confirmed that the most important HLA class II haplotype in disease predisposition is HLA-DR4DQ8 (branch 4). In both DR4DQ8 positive and DR4DQ8 negative individuals, DR17DQ2 was strongly associated with susceptibility. The combinations DR4DQ8/DR8DQ4, DR4DQ8/DR17DQ2 and DR9DQ9/DR17DQ2 showed clear interaction in their association to IDDM.

HLA-DR9DQ9 is a good example of a haplotype that becomes apparent only after stratification for a primary haplotype. In order to include interaction between haplotypes in an LR model *a priori* hypotheses of such interactions are required, since LR does not in-

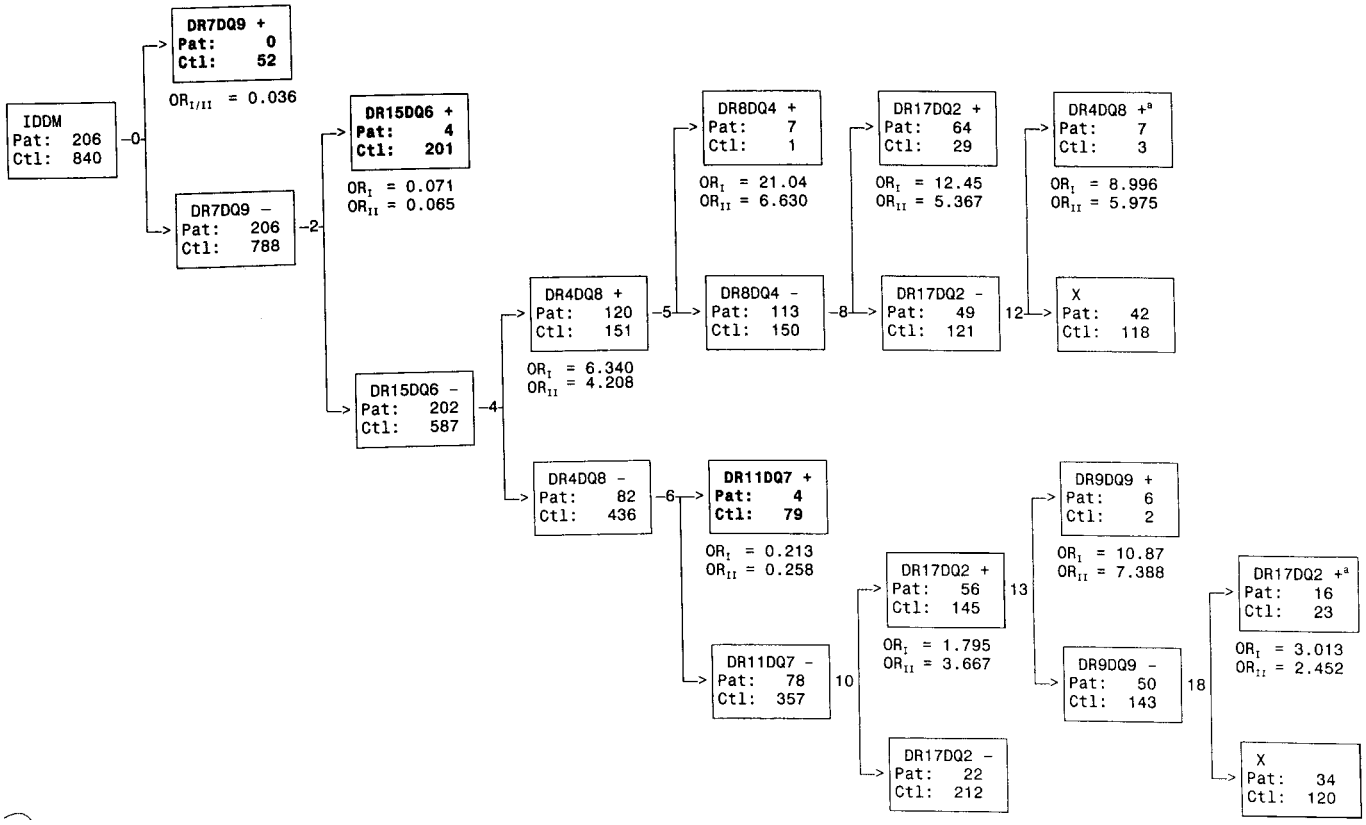

Fig. 1. **MTA result for IDDM patient (Pat) and controls (Ctl).** ORI=odds ratio for the factor, ORII=odds ratio for the genotype. Factors in bold type are protective. +a denotes homozygosity.

Results of MTA on HLA-DR-DQ haplotypes

Br	Factor	Genotype	OR_i	$1/OR_i$	95% C.I.	*Pu*	*Pc*	OR_{ii}	*Pu*	*Pc*	Nfac
0	DR7DQ9	DR7DQ9/X	0.04	27.47	1.69–454.54	<0.0001	0.0003	0.04	<0.0001	0.0003	17
2	DR15DQ6	DR15DQ6 X	0.07	14.18	5.50–36.63	<0.0001	<0.0001	0.07	<0.0001	<0.0001	16
4	DR4DQ8	DR4DQ8/X	6.34		4.57–8.80	<0.0001	<0.0001	4.21	<0.0001	<0.0001	15
5	DR8DQ4	DR4DQ8/DR8DQ4	21.04		3.62–122.28	<0.0001	0.001	6.63	0.02	n.s.	14
6	DR11DQ7	DR11DQ7/X	0.21	4.70	1.80–12.30	0.0001	0.002	0.26	0.002	0.02	14
8	DR17DQ2	DR4DQ8/DR17DQ2	12.45		7.78–19.93	<0.0001	<0.0001	5.37	<0.0001	<0.0001	13
10	DR17DQ2	DR17DQ2/X	1.80		1.26–2.56	0.0021	0.037	3.67	<0.0001	<0.0001	13
12	DR4DQ8	DR4DQ8/DR4DQ8	9.00		2.51–32.29	0.0007	0.01	5.98	0.007	n.s.	12
13	DR9DQ9	DR9DQ9/DR17DQ2	10.87		2.51–47.14	0.001	0.02	7.39	0.007	n.s.	12
18	DR17DQ2	DR17DQ2/DR17DQ2	3.01		1.58–5.77	0.002	0.03	2.45	0.02	n.s.	11

"Br" refers to the tree branch in Fig. 1. The term 'X' in the genotype is defined as other haplotypes except the ones stratified for. All factors have $P<0.05$ for OR_i. OR_i=odds ratio for the genotype, OR_{ii}=odds ratio for the factor, conditional on the preceding stratification factors. 95% C.I. is the confidence interval for the effect strength

Pu=uncorrected *P*-value, *Pc*=*P*-value corrected for the number of comparisons. *P*-values for OR_i are corrected for the number of different informative genotypes (=18). *P*-values for OR_{ii} are corrected for the number of different haplotypes in the branch (Nfac)

Table 3

clude interaction parameters in the automatic (stepwise) building of models. MTA automatically includes interactions in the model.

Discussion

The method of assignment of haplotypes in unrelated individuals is essential for accurate risk assessment through WH analysis and MTA. In the original MTA selection of haplotypes is based on their *P*-values (or, in fact, on the LR score-test, which has a χ^2 distribution), which imposes the danger of overlooking the strongest haplotype (21). We suggest that the primary criterion for the selection of a haplotype should be its (WH) OR (or the reciprocal of the OR in case of protection) and that the significance should be used as the secondary criterion. A drawback of MTA is the possibility that an effect of a haplotype can be significant in one branch, while a less than borderline significant similar effect in another branch does not show. WH analysis of haplo- as well as genotypes is further needed for correct interpretation of the associations. However, WH analysis of haplotypes alone does not provide information about in which strata the haplotype contributed to the overall OR.

The results of multivariate analyses presented here provide the opportunity to include disease risks associated with different phenotypic combinations in prognostic decision trees as shown for example in Bingley et al. (22). Prognostic decision trees serve to categorize individuals for their risk of developing disease by applying combinations of markers and can be used in population screening.

In conclusion, we improved the MTA method by making use of Woolf-Haldane ORs, exact *P*-values and ranking of haplotypes on the strength of their effect. This method, combined with the increased discriminative power resulting from the use of haplotype assignment, shows higher power to detect protection against disease as well as association with development of disease for polymorphic HLA genotypes with various degrees of disease association. By exposing interactions between haplotypes, MTA improves interpretation of WH haplotype analysis.

The results of our analysis confirmed the identity of previously described susceptibility haplotypes, such as DR4DQ8 and DR17DQ2 (7, 21), but also provide information on the interaction between HLA class II markers, such as susceptibility strongly associated with DR4DQ8/DR8DQ4, DR4DQ8/DR17DQ2 and DR9DQ9/DR17DQ2 heterozygotes. The association between DR9 and development of IDDM and particularly of DR17/DR9 heterozygotes has been described in Asian populations (31–43), where DR9 has a population frequency (PF) between 20 and 30%, but in the population studied here, the PF of DR9 is 2.5%. In both Asiatic and Caucasoid populations, DR9 is associated with DQB1*0303(DR9) (35).

It should be noted that of the haplotypes that are associated with a significant increased or decreased risk, the 95% confidence intervals of the ORs are overlapping except for the DR17DQ2 homozygote and DR4DQ8/DR17DQ2 combinations, indicating that the final ranking of the haplotypes in the MTA tree is not the only possible ranking. This limitation also applies to the ranking of haplotypes in the standard Woolf-Haldane analysis.

We have used serological HLA typing in all analyses. Consequently, we could not distinguish between certain alleles, such as those of DQB1*06, which has alleles that are associated with protection (DQB1*0602/3) and neutral alleles (DQB1*0604) (36). This drawback is partly compensated by the use of the haplotype assignment technique, since many DRB1 and DQB1 alleles are found primarily or even exclusively in particular HLA-DRDQ haplotypes. Furthermore, in the samples of 206 patients and 840 controls, some HLA antigens or haplotypes (with low population frequencies) rarely occur, such as HLA-DRB1*0403, while most the most frequent DR4 subtypes display similar degrees of association with disease. By increasing the sample size, additional associations with IDDM such as DR13DQ6 are conceivable. Furthermore, although haplotype assignment offers high reliability, the certainty of pedigree-derived haplotypes is preferable.

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