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Three microsatellites from the T1DGC MHC data set show highly significant association with type 1 diabetes, independent of the *HLA-DRB1*, *-DQA1* and *-DQB1* genes

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Abstract

Aim—The aim of this study was to test the microsatellites in the Type 1 Diabetes Genetics Consortium major histocompatibility complex (MHC) data set for association with type 1 diabetes (T1D) independent of the *HLA-DRB1*, *-DQA1* and *-DQB1* genes.

Methods—The data set was edited to contain only one affected child per family, and broad ethnic subgroups were defined. Genotypes for *HLA-DRB1*, *-DQA1* and *-DQB1* were replaced by a haplotype code spanning all three loci, with phase inferred based on common haplotypes. The final data set contained 8190 samples in 2301 families, 59 microsatellites and the *DRB1-DQA1-DQB1* haplotype code. Statistical analyses consisted of conditional logistic regression and haplotype estimations and linkage disequilibrium calculations.

Results—The data set was screened using a main effects test approach adjusted for *DRB1-DQA1-DQB1*, and significant results tested for validity. After these procedures, four markers remained significant at the Bonferroni-corrected threshold: *D6S2773* ($p = 0.00014$), *DG6S185* ($p = 0.00015$), *DG6S398* ($p = 0.00043$) and *D6S2998* ($p = 0.00015$). These results were supported by allelic tests conditioned on *DRB1-DQA1-DQB1* haplotypes, except for *DG6S185*, which may contain artefacts.

Conclusions—We have identified three microsatellites that mark additional risk factors for T1D at highly significant levels in the MHC. Further analyses are needed to establish the relationship with other possible genetic determinants in this region.

Keywords

conditional analysis; genetic association; major histocompatibility complex; microsatellites; type 1 diabetes

Introduction

The major histocompatibility complex (MHC) is a unique genomic area containing hundreds of genes at high density, of which an unusually high amount are involved in immunological processes [1]. It therefore comes as no surprise that genetic determinants for many diseases, including type 1 diabetes (T1D), are located in this region. The main genetic risk factors for T1D are known to be conferred by certain haplotypes of the *HLA-DRB1*, *-DQA1* and *-DQB1* loci within the MHC class II region [2–4]. However, previous studies have shown that additional loci within the MHC remain unidentified [5–9]. Still, mapping of this region has proved exceedingly difficult because of strong and extensive linkage disequilibrium (LD) with the *HLA-DRB1*, *-DQA1* and *-DQB1* loci. Controlling for the effects of these loci is therefore essential in any attempt to identify additional risk loci within this region. In addition, the effect sizes of additional risk factors are expected to be relatively small as is often the case for complex diseases. Therefore, large data sets are needed to obtain significant results, which are recognized in the efforts of the Type 1 Diabetes Genetics Consortium (T1DGC). The first main project under the auspices of the T1DGC is the MHC Fine Mapping Workshop. In this study, we have tested the microsatellite data gathered for this project for association with T1D independent of the *HLA-DRB1*, *-DQA1* and *-DQB1* loci.

Materials and Methods

The T1DGC MHC data set (February 2007 release) consists of a large collection of multiplex families (N = 2321), mostly Caucasians from Europe, USA and Australia, typed for 2957 SNPs, 66 microsatellites and all classical HLA loci. Microsatellite marker names starting with ‘DG6S.’ are internal nomenclature to deCODE genetics, and allele names are raw data calls.

Sample Selection

The data set was edited to contain only two generations per family. Twenty families were removed because of unlikely high numbers of recombinations between windows of two markers analysed using FAMHAP [10] on the complete T1DGC MHC data set. To avoid the problem of statistical dependence between siblings in the association analyses, we edited the data set to contain only one affected child (the proband) per family. Proband was specified as the child with the youngest age of onset, if available, other-wise as the affected child with the highest analytic ID. Unaffected siblings were retained to improve haplotype inference, whereas siblings diagnosed with T1D or without genotyping data were removed. The above procedures resulted in a total of 8190 samples in 2301 families.

Ethnic Groups

Ethnic groups were specified on the basis of self-reported ethnicity. The following groups were used in analyses: All (2301 families); Caucasian (2262 families): all families (including those where no ethnicity information was available) except those of Asian, African or indigenous ancestry; Northern Europe (781 families): families from the UK and Ireland, Scandinavia, Germany and the Netherlands; Eastern Europe (228 families): families from Poland, the Czech Republic, Slovenia, Hungary, Serbia, Romania, Bosnia and Slovakia; Southern Europe (161 families): families from Italy, Spain, Portugal, France and Malta.

Recoding of Markers

Genotypes for *HLA-DRB1*, *-DQA1* and *-DQB1* were replaced by a haplotype code spanning all three loci, with phase inferred based on common haplotypes. Mendelian consistency (tested using PEDCHECK [11]) was evaluated for quality control. To limit the number of input variables in the regression analyses, we grouped alleles with frequencies below 1% (calculated among parents), with the exception of the *DRB1*0403-DQA1*0301-DQB1*0302* haplotype

(frequency 0.48%). For microsatellites, alleles with frequency below 0.1% were grouped in a common allele for each marker. One microsatellite (*D6S2830*) was removed altogether because all minor alleles were below this frequency threshold.

Microsatellite Marker Quality Checks

Mendelian inheritance of the microsatellites was evaluated using PEDCHECK, and no errors were detected. Hardy–Weinberg equilibrium was calculated in the above-described ethnic groups in the complete T1DGC MHC data set using the exact test implemented in the program PEDSTATS [12]. Microsatellites showing disequilibrium with $p < 10^{-5}$ in any one of these populations, or $< 10^{-3}$ in at least two separate populations, were removed ($N = 6$). This resulted in a final data set containing 59 microsatellites.

Regression Analysis

Conditional logistic regression, adjusted for *DRB1–DQA1–DQB1* haplotypes, was used to test the overall effect of each microsatellite modelled by genotypes. We used the conditioning strategy 4 (with a likelihood ratio test) described in Cordell & Clayton [13], implemented in STATA (STATA Corp., College Station, TX, USA).

Allelic Tests on Haplotypes and LD Analyses

Maximum-likelihood haplotype frequency estimates combining markers with *DRB1–DQA1–DQB1* haplotypes were computed using FAMHAP. Based on these estimates, haplotype transmission/non-transmission (T/NT) tables were constructed as described in Becker & Knapp [10] and organized in separate groups for each *DRB1–DQA1–DQB1* haplotype. Statistically significant deviations of an allele within each *DRB1–DQA1–DQB1* group were tested by Pearson's Chi-squared test, and odds ratios were calculated. LD patterns were analysed using FAMHAP and the GOLD software package [14] based on the generalized D' measure.

Results

Main Effects Tests

Single-point main effects tests of microsatellites, adjusted for *DRB1–DQA1–DQB1* genotypes, were performed in the All data set and the three European subpopulations. Tests were also performed on the Caucasian data set, but results did not differ significantly from the All data set and are therefore not shown. The likelihood ratio test is affected by how rare genotypes are handled. For the initial screen, we used joint frequency cut-offs for all markers, adjusted for population size. To ensure the validity of our results, we further tested all results $p < 0.05$ for each microsatellite that showed significant results below the Bonferroni-corrected threshold ($p = 0.05/59 = 0.00085$) in any of the populations (treating the All data set as a separate population) using genotype cut-off frequencies adjusted for each microsatellite marker and population size. Because grouping of the *DRB1–DQA1–DQB1* haplotype codes are more likely to have statistically adverse effects (because of potentially divergent functional properties of different haplotypes), the cut-off frequency for this marker was kept constant at a low level ($p < 0.001$).

The results of these tests are presented in figure 1 (all results $p < 0.05$, with validity corrected p values where applicable) and table 1 (significant results after validity testing and Bonferroni correction). All validity tested results from the Southern European population were removed as counts turned out to be too small to perform meaningful tests. Also, results that initially were exclusively significant in the Northern and Eastern European populations (*D6S2843*, and *DG6S182* and *DG6S745*, respectively) rose above the Bonferroni-corrected threshold after

validity testing. The results in the All population, however, withstood these tests and were retained: *D6S2773* ($p = 0.00014$), *DG6S185* ($p = 0.00015$), *DG6S398* ($p = 0.00043$) and *D6S2998* ($p = 0.00015$). Except for *D6S2773*, which also showed nominal significance in the Northern European population ($p = 0.040$), results in the subpopulations for these markers were not significant.

DG6S185, *DG6S398* and *D6S2889* showed deviations from Hardy–Weinberg equilibrium but not enough to be excluded by our criteria ($p = 0.000034$, 0.00020 and 0.0012 , respectively, in the Caucasian data set, *DG6S185* in addition $p = 0.019$ in the Northern European data set, otherwise not significant (n.s.)).

Allelic Associations on Haplotypes

To further explore the results from the main effects tests, we tested the individual alleles of each of the four significant microsatellites, conditioned on haplotypes with the *DRB1–DQA1–DQB1* haplotype codes. Significant results at $\alpha = 0.05$ of these analyses are given in table 2. Because *D6S2773* also showed some association in the main effects test in the Northern European data set, this marker was also tested in this population. Results generally showed tendencies for risk associations in the same direction and/or for the same alleles as in the All population (some results were not significant and are therefore not shown).

Significant results at $\alpha = 0.01$ were consistent for each of the microsatellite alleles, that is odds ratio (OR) values were either above or below 1 independent of *DRB1–DQA1–DQB1* haplotypes. However, results at $\alpha = 0.05$ showed some inconsistencies, especially for *DG6S185* (e.g. *DG6S185*268* and *DG6S185*275* and *DG6S185*291* showed both protective and predisposing effects depending on the haplotype). Also, *DG6S398*167* and *D6S2889*153* showed protective effects on the *DRB1*1302–DQA1*0102–DQB1*0604* haplotype, in contrast to the predisposing effect seen on other haplotypes. These last two results could, however, be because of false positives as cell counts were low and results only nominally significant ($T + NT = 12$, $p = 0.033$ and $T + NT = 17$, $p = 0.048$, respectively).

*DG6S185*293*, *DG6S398*165* and *D6S2889*157* showed associations on the grouped rare haplotypes allele of *DRB1–DQA1–DQB1* ($p = 0.00026$, 0.045 and 0.0033 , respectively). This is a heterogeneous group and these associations should therefore be considered artefacts.

DG6S398 and *D6S2889* are located in introns of the same gene, *C6orf10*, and show significant associations on some of the same *DRB1–DQA1–DQB1* haplotypes (e.g., *DRB1*03–DQA1*0501–DQB1*0201*). We therefore tested both markers together on these haplotypes (table 3). The results only showed significant findings or tendencies for association of combinations of alleles that were associated for each marker alone (cf. table 2), with similar OR values.

LD Plots

LD plots (global D') of the four markers given in table 1 for the All, Northern European and Eastern European populations are shown in figure 2. The number of families from Southern Europe that could be incorporated in the analyses was low ($N = 25$), and results are therefore not shown. The two remaining subpopulations show similar patterns as the All data set, indicating that these microsatellites do not mark large population specific effects. The highest LD values were seen between *D6S2889* and *DG6S398* ($D' = 0.76$ in the All data set) and between these two markers and *DRB1–DQA1–DQB1* ($D' = 0.73$ and 0.70 , respectively), whereas the LD between *D6S2773*, *DG6S398* and the two other markers was low ($D' < 0.35$).

Discussion

In this paper, we tested 59 microsatellites from the T1DGC MHC data set for association with T1D independent of the *DRB1*, *-DQA1* and *-DQB1* genes. The tests consisted of two main steps: a screen using single-point main effects tests adjusted for *DRB1-DQA1-DQB1* genotypes and mapping of allele associations on the different *DRB1-DQA1-DQB1* haplotypes.

Association Analyses

In the main effects tests, the four most significantly associated microsatellites (*D6S2773*, *DG6S185*, *DG6S398* and *D6S2889* in the All data set) only showed nominally or non-significant findings in the subpopulations. Even though the All data set consists mainly of Caucasian families, population stratification may be a factor. This may also have contributed to the observed deviations from Hardy–Weinberg equilibrium. However, the conditional logistic regression method is not sensitive to such issues [15], and this is therefore not likely to have a large impact on the results. Moreover, the similar LD patterns in the Northern and Eastern European populations do not indicate large population specific differences for these markers. Also, the chosen test strategy, in combination with a high number of alleles, requires large populations. This was especially evident for the Southern European population, which consisted of only 161 families, and for which valid results were difficult to obtain. The absence of significant results in the subpopulations is therefore likely to be a question of statistical power.

Related to this is the fact that several markers that previously have been reported associated with T1D independent of the *HLA-DRB1*, *-DQA1* and *-DQB1* loci did not reach statistical significance in our tests. This might indicate a weakness with our approach using the somewhat heterogeneous All data set and conservative cut-offs for significance. Thus, more subtle, population specific effects may have gone unnoticed. For instance, *D6S2223* has repeatedly shown associations with T1D in Northern European populations [5,6,16–18]. In our tests, we did observe a tendency for this marker in the Northern and Eastern European populations ($p = 0.022$ and 0.023 , respectively), which might be stronger if looking at more specific subgroups of the material. Also, *D6S2843* and *DG6S184* (with no previously reported associations) showed interesting tendencies that should be investigated further.

Several characteristics of the results for *DG6S185* point to potential artefacts. First, the recurrent observation of alternating protective and predisposing effects for the same allele depending on the *DRB1-DQA1-DQB1* haplotype. Some of these inconsistencies could be attributed to false positives as results at $\alpha = 0.01$ were consistent. However, another explanation could be that the global result in the main effects test was the result of complex haplotype-specific effects, which may be attributed to underlying ancestral haplotypes and not real independent effects. Second, the highly significant association of allele 293 on the grouped rare haplotype allele of *DRB1-DQA1-DQB1* means that the result from the main effects test is likely to be artificially low. Lastly, this marker showed a large deviation from Hardy–Weinberg equilibrium, which may point to problems with the genotyping. Together, these observations question the status of this microsatellite as an independent genetic marker for T1D.

*DG6S398*165* and *D6S2889*157* also showed some effect on the grouped rare haplotype allele of *DRB1-DQA1-DQB1*. However, these results were either only nominally significant (*DG6S398*) or involved low cell counts compared with other haplotypes (*D6S2889*, 1.1% T + NT of total T + NT for associated haplotypes). In these cases, therefore, the results of the main effects tests are not likely to be significantly affected. Moreover, the observed deviation from Hardy–Weinberg equilibrium for these markers can be explained by the proximity and

relatively high LD with the *HLA-DRB1*, *-DQA1* and *-DQB1* loci, which are expected to show deviations [19], especially in a selected population like the present.

Judging from the LD plots, *D6S2773* and *DG6S185* appeared to represent largely independent effects of each other and of the two other markers. In contrast, *D6S2889* and *DG6S398* may reflect the same aetiological locus. These markers are located 57 kb apart in the same gene and showed similar patterns of OR values on different *DRB1-DQA1-DQB1* haplotypes. Moreover, haplotype estimates showed that in most cases, predisposing or protective alleles of each of the markers were located together on the same haplotypes, and the two markers therefore appeared to convey much of the same information. However, the global D' LD value indicates some independence, and *D6S2889*153* showed a significant association on the *DRB1*04-DQA1*0301-DQB1*0301* haplotype ($p = 0.0021$), which was not seen for any of the *DG6S398* alleles.

This last observation is related to a more general one that significant allelic associations only were seen on a subset of the *DRB1-DQA1-DQB1* haplotypes. For haplotypes present at low frequencies, this is likely to be a question of statistical power. However, none of the microsatellite alleles showed significant association on the high-risk *DRB1*0401-DQA1*0301-DQB1*0302* haplotype, which is common (16.5% among parents in the All data set). This could be explained by considering the high degree of conservation of haplotypes in this region [20,21] and the likely situation that the associated microsatellites are serving as genetic proxies for aetiological loci. Apparent specificity for certain *DRB1-DQA1-DQB1* haplotypes could then be the result of specific LD patterns, where aetiological variants in LD with the investigated markers only are picked up on certain haplotypes. An alternative or additional explanation could be that the aetiological loci have functional properties specific to certain variants of the MHC class II molecules. Answers to these questions are only possible after further fine mapping on the various *DRB1-DQA1-DQB1* haplotypes, including a greater number of markers in the immediate surrounding regions.

Location of the Most Promising Markers

Although the most promising markers are not likely to be primary aetiological loci, some remarks can be made about their immediate genetic neighbourhood. *D6S2773* is located close to the *HLA-G* gene, which has been implicated in asthma [22]. However, this has not been replicated. Other possible candidates in this region include the *HLA-A* and *HLA-F* genes, of which the first has been implicated in T1D through functional studies [23].

DG6S398 and *D6S2889* are both located in introns of the *C6orf10* gene. The function of this gene is unknown, and it appears to be primarily expressed in testis (<http://www.ncbi.nlm.nih.gov/UniGene>). *C6orf10* is, however, located 23 kb downstream of the *BTNL2* gene, which recently was mapped as a susceptibility locus for sarcoidosis, independent of *HLA-DRB1* [24]. Sarcoidosis is an autoimmune disease, and the *BTNL2* gene has been implicated as a costimulatory molecule in T-cell activation. Although a recent study could not find an association independent of the *HLA-DRB1* and *-DQB1* genes with T1D in Dutch patients [25], the study population was small, and only one polymorphism was investigated. This gene may therefore still be a good candidate for further studies.

Conclusions

We have identified three microsatellites, *D6S2773*, *DG6S185* and *D6S2889*, that show highly significant association with T1D independent of the *HLA-DRB1*, *-DQA1* and *-DQB1* genes. These three markers with-stand Bonferroni correction and validity testing, and results are supported by haplotype estimates. In addition, *DG6S185* showed association in the conditional logistic regression analyses, but allelic tests on *DRB1-DQA1-DQB1* haplotypes indicated that

this, at least in part, may be because of artefacts imposed by the particular characteristics of the MHC and the grouping of rare *DRB1-DQA1-DQB1* haplotypes. To our knowledge, none of these microsatellites have been reported to be associated with T1D or any other autoimmune disease earlier. Further analyses are needed to establish the relationship with other possible genetic determinants in this region.

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Fig. 1.

Main effects tests of microsatellites adjusted for *DRB1-DQA1-DQB1* haplotypes; all results. Only results $p < 0.05$ are shown. Limits for statistical significance at $p = 0.05$ and $p = 0.05$ Bonferroni corrected for the number of markers tested ($p = 0.05/59 = 0.00085$) are indicated. Results that showed $p < 0.00085$ in any of the populations in the initial screen are marked with circles and marker names. The values given for these results are adjusted with results after validity testing (*DG6S745* was adjusted to >0.05 and removed). Grey areas did not contain any tested markers. Positional values are along chromosome 6, genome build 36, and locations of some HLA genes are indicated for reference.

Fig. 2.

LD of significant markers from the main effects tests. LD was measured as the generalized D' . The colour code is given on the right ($D' = 0$ to 1 in 0.1 increments).

Main effects test of microsatellites adjusted for *DRB1-DQA1-DQB1* genotypes; significant results after Bonferroni-correction and validity testing*

Marker name (deCODE)	Position	Closest public microsatellite (position)	Closest RefSeq gene (position, relative location)	p value	
				All	Northern Europe
D6S2773	29899213–29899413	—	<i>HLA-G</i> (29903497–29906856, 5')	0.00014	0.040
DG6S185	31153244–31153505	D6S2814 (31153263–31153505)	<i>C6orf15</i> (31186981–31188311, 3')	0.00015	—
DG6S398	32385920–32386075	D6S2734 (32386428–32386591)	<i>C6orf10</i> (32368453–32447634, intron)	0.00043	—
D6S2889	32442738–32442882	—	<i>C6orf10</i> (32368453–32447634, intron)	0.00015	—

* Only those markers that showed significant results (Bonferroni corrected for the number of markers tested; $p = 0.05/59 = 0.00085$) after validity testing, together with the only result in the European subpopulations with $p < 0.05$ for these markers, are shown. For microsatellites with marker names that are internal to deCODE, the closest public microsatellite is given with positions. The closest RefSeq gene to each marker is given with positions and relative location of the marker. Positions are on chromosome 6, genome build 36.

Table 2
Allelic tests of significant microsatellites from the main effects tests, conditional on *DRB1-DQA1-DQB1* haplotypes[†]

Conditional haplotypes			Microsatellites			
DRB1	DQA1	DQB1	D6S2773	DG6S185	DG6S398	D6S2889
01	0101	0501	—	2.84 (275) 1.68 (287) 0.40 (291)	—	0.16 (151)
03	0501	0201	—	0.75 (268) 1.57 (289)	1.73 (165) ^{**} 0.59 (169) ^{**}	0.56 (151) ^{**} 2.55 (153) [*]
04	0301	0301	5.04 (227) [*]	—	—	1.61 (163) [*] 0.23 (151) 5.62 (153) [*]
0404	0301	0302	3.11/7.50 (227) [‡]	—	—	—
0405	0301	0302	—	3.79 (268)	—	—
07	0201	0201	0.15 (222) [*]	2.25 (268) 0.19 (275)	2.06 (165) [*] 0.56 (171)	0.52 (151) 1.94 (153) [*]
08	0401	0402	0.31 (212) [*] 3.64 (227) [*]	5.13 (287) ^{**}	—	—
0901	0301	0303	—	—	3.13 (167)	3.61 (169)
1301	0103	0603	5.00 (227) [*]	3.97 (268)	—	3.11 (153) 0.14 (155)
1302	0102	0604	3.81 (227) [‡]	2.08 (268) 0T/0.099NT (285) ^{*,§}	0.21 (167)	0.33 (153)
16	0102	0502	—	0.25 (268) [*] 4.78 (291)	—	—
Grouped rare haplotypes (<1%)			—	7.15 (293) ^{**}	1.90 (165)	0.09 (157) [*]

[†] Results for All population are given. For each marker, significant odds ratio (OR) values (increased or reduced risk on the given *DRB1-DQA1-DQB1* haplotype) are given together with the responsible allele (in parentheses).

[‡] Results of the Northern European population.

§OR calculation not possible, values are frequencies T: transmitted and NT: non-transmitted. Cut-off for significance was set at $p = 0.05$, results

* $p < 0.01$

** $p < 0.001$ are marked. Results for cell counts < 10 (T + NT haplotypes) were omitted.

Table 3
Haplotype tests of DG6S398 and D6S2889 combined, conditional on *DRB1-DQA1-DQB1* haplotypes[†]

Conditional haplotypes			Microsatellite alleles		Two-locus results OR (95% CI)		Single-locus results	
DRB1	DQA1	DQB1	DG6S398	D6S2889			DG6S398	D6S2889
03	0501	0201	165	153	2.48 (1.33–4.62) [*]	1.73 ^{**}	2.55 [*]	
			165	163	1.50 (1.09–2.06)	1.73 ^{**}	1.61 [*]	
			169	151	0.58 (0.44–0.77) ^{**}	0.59 ^{**}	0.56 ^{**}	
07	0201	0201	165	153	1.95 (1.16–3.27)	2.06 [*]	1.94 [*]	
			171	151	0.62 (0.37–1.03)	0.56	0.52	
			167	169	3.09 (0.97–9.81)	3.13	3.61	
0901	0301	0303						
1302	0102	0604	167	153	0.27 (0.05–1.29)	0.21	0.33	

[†] Results shown are for the All population. Only *DRB1-DQA1-DQB1* haplotypes where significant results were obtained for both markers alone (cf. table 2) were tested. Odds ratio (OR) values from the single marker tests are included for reference. Results for cell counts <10 (T + NT haplotypes) were omitted.

^{*} Results p < 0.01

^{**} p < 0.001 are marked.