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The MHC type 1 diabetes susceptibility gene is centromeric to \textit{HLA-DQB1}

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Abstract

\textit{HLA-DQB1} is widely considered to be the major histocompatibility complex (MHC) susceptibility gene for type 1 diabetes (T1D). However, since inheritance of the gene in T1D is recessive, the presence of the protective \textit{HLA-DQB1*0602} allele with normal nucleotide sequence in some patients raises the question of whether \textit{HLA-DQB1} is not the susceptibility locus itself but merely a good marker. \textit{HLA-DQB1*0602} is part of a conserved extended haplotype (CEH) [HLA-B7, SC31, DR2] (B7, DR2) with fixed DNA over more than 1 Mb of genomic DNA that normally carries a protective allele at the true susceptibility locus. We postulated that, in patients with \textit{HLA-DQB1*0602}, the protective allele at the susceptibility locus has been replaced by a susceptibility allele through an ancient crossover at meiosis centromeric to \textit{HLA-DQB1}. We analyzed single nucleotide polymorphisms (SNPs) distinguishing the \textit{HLA-DQA2} (the first expressed gene centromeric to \textit{HLA-DQB1}) allele on the normal HLA-B7, DR2 CEH from those on susceptibility CEHs in T1D patients and controls with \textit{HLA-DQB1*0602}. All but 1 of 20 healthy control \textit{HLA-DQB1*0602} haplotypes had identical (consensus) first intron \textit{HLA-DQA2} 5-SNP haplotypes. Fifteen of 19 patients with \textit{HLA-DQB1*0602} were homozygous for 1 or more \textit{HLA-DQA2} SNPs differing from consensus \textit{HLA-DQA2} SNPs, providing evidence of crossover involving the \textit{HLA-DQA2} locus. The remaining 4 patients were heterozygous at all positions and therefore uninformative. The loss of dominant protection usually associated with \textit{HLA-DQB1*0602} haplotypes is consistent with a locus centromeric to \textit{HLA-DQB1} being a major determinant of MHC-associated susceptibility, and perhaps the true T1D susceptibility locus.

Keywords: Major histocompatibility complex; Single nucleotide polymorphism; Susceptibility gene; Type 1 diabetes

1. Introduction

It is clear from family studies to define disease susceptibility haplotypes \cite{1} that all of the known MHC markers for both

Abbreviations: CEH, conserved extended haplotype; MHC, major histocompatibility complex; SNPs, single nucleotide polymorphisms; T1D, type 1 diabetes.

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susceptibility to and protection from type 1 diabetes (T1D) are parts of large (1–4 Mb or longer) fixed stretches of DNA called conserved extended haplotypes (CEHs) \cite{2–4}. CEHs account for \textasciitilde{}50\% of all normal European Caucasian MHC haplotypes \cite{3,4} and for \textasciitilde{}65\% of European Caucasian T1D patient haplotypes. The identity of DNA on independent examples of individual CEHs, postulated by us in 1983 \cite{2}, was supported by evidence of identical alleles reported in many publications by us and by others \cite{3,4}. Recently, \textasciitilde{}99\% identity of single nucleotide polymorphisms (SNPs)
over 2.9 Mb of the [HLA-B8, SC01, DRB1*0301] CEH was shown [5-7]. Similar >99% conservation of SNPs over 4 Mb of the [HLA-B18, F1C30, DRB1*0301] CEH has also been documented by us [7]. In general, patients carry the complete CEHs (not just the markers). This hinders identification of the MHC T1D susceptibility gene in such large stretches of identical DNA. Therefore, approaches that study genes on protective HLA-DR, -DQ haplotypes or on non-CEHs in T1D patients are most likely to be informative for susceptibility gene localization and identification.

Despite the fact that it has been known for over 30 years that the MHC contains a susceptibility gene (or genes) for T1D, the specific locus (loci) and responsible alleles have not been identified [3]. It is widely believed that HLA-DB1 is that locus (or that alleles of the HLA-DRB1, -DQA1, -DQB1 block together confer susceptibility or protection). However, a brief review of the available facts suggests that this is not likely to be so. Since there is evidence for recessive inheritance of the T1D MHC susceptibility gene(s) [8-12], no protective marker at the true susceptibility locus should occur in a patient. Nevertheless, the protective markers HLA-DRB1*1501 and/or HLA-DQB1*0602 (among others) do occur in T1D patients, although rarely. This supports the view, but does not prove, that HLA-DRB1, -DQB1 provide good markers for T1D but are not themselves the true susceptibility loci.

In our view, DNA fixity on the protective CEH [HLA-B7, SC31, DRB1*1501, DQB1*0602] in normal subjects almost always includes the usual protective allele of the susceptibility locus. The MHC T1D susceptibility locus is in the class II region and HLA-DRB1 and HLA-DQB1 are fixed on CEHs that are marked by these alleles. Therefore, if HLA-DQB1 is not the susceptibility locus, the latter must be centromeric to HLA-DQB1. In T1D patients who carry these markers, this view holds that there has been an ancestral (in some prior generation) meiotic crossover centromeric to HLA-DQB1 but telomeric to the susceptibility locus so that the usual protective allele is replaced by a susceptibility allele. In 1 T1D patient with HLA-DRB1*1501 [13], it was shown that the HLA-DQB1 allele was *0402 (susceptibility) rather than *0602 (protection), consistent with HLA-DQB1 or a gene centromeric to HLA-DQB1 being the susceptibility locus. However, in 14 T1D patients with HLA-DQB1*0602, the nucleotide sequences were entirely normal [14,15], consistent with the view that the true T1D MHC susceptibility gene is centromeric to HLA-DQB1.

In this report, many of these T1D patients and others with HLA-DQB1*0602 were studied at HLA-DQA2, the first locus centromeric to HLA-DQB1 known to be expressed (Fig. 1) [16], in order to detect and localize ancient crossovers.

2. Methods

Patients were ketosis-prone, insulin-dependent since diagnosis and presented before age 30. Mean onset of diabetes was 14.8 years (n = 11; SD = 7.4 years) and patients tested (n = 12) were GAD65 and ICA512 autoantibody-positive. All subjects gave informed consent. Genomic DNA was obtained from peripheral blood mononuclear cells, EDTA-treated whole blood or lymphoblastoid cell lines and was isolated using the QIAamp DNA mini kit (Qiagen, Valencia, CA). Patients carried HLA-DQB1*0602 as determined by direct sequencing of group-specific second exon class II amplicons [17]. Other typing was as described previously [4].

We analyzed HLA-DQA2 first intron SNPs distinguishing the [HLA-B*0701, DRB1*1501, DQB1*0602] CEH from other haplotypes determined by the Sanger Centre [18]. We determined comparable sequences in a T1D patient homozygous for the [HLA-B60/62, SB42, DRB1*0701, DQB1*0302] CEH. HLA-DQA2 SNPs (and the corresponding refSNP IDs) were at positions 899 (rs5018343), 1150 (rs9276408), 1157 (rs9276409), 1176 (rs9276410) and 3446 (rs9276434) [19]. Patients homozygous for SNPs not found in consensus normal HLA-DRB1*1501, -DQB1*0602 CEHs defined crossover.

Eleven HLA-DQB1*0602-bearing haplotypes in 6 unrelated normal homozygotes established phase. Phase was determined in heterozygotes through SNPs of known CEHs when they were the other haplotype or by pedigree analysis.

Primers used were: F1: 5’-AAATGGGCACTGTAGATAGCTTT-3’; R1: 5’-TTCCAGTTTCACCTTCCCCCTTTTTT-3’; R1a: 5’-TCAGATATTGGGAGCAGCAGTATG-3’; F2: 5’-CAGGATCCATCTCTGACTCTCC-3’; R2: 5’-TGCCCAACACTGTCTTCTAACTGG-3’; F3: 5’-CGTTCTCTACATGTCTTTTCTTGG-3’; and R3: 5’-GGCAAGTCCTCCTGAAACATC-3’.

PCR reactions (50 µL) used AmpliTaq DNA polymerase (2.5 U), MgCl2 (1.5 mM), and dNTP (0.4 mM) with: 1 cycle at 94 °C (2 min); followed by 35 cycles at 94 °C (15 s), 60 °C (30 s), 68 °C (2 min) with a final extension at 72 °C for 10 min. The annealing cycle was 62 °C (30 s) for the PCR reactions 1F/1Ra and 2F/2R. Ethidium bromide-stained PCR products were excised from agarose gels, purified using the QIAEX II gel extraction kit (Qiagen, Valencia, CA) and sequenced by dyeideo sequencing using the Big Dye Terminator V3.0 chemistry (Davis Sequencing, Davis, CA). Sequences were compared using alignment software and the visual inspection of chromatograms.

3. Results

Table 1 presents the 20 independent instances of HLA-DQA2 first intron SNP haplotypes of normal unrelated healthy subjects who carried HLA-DQB1*0602-bearing CEHs. Nineteen of 20 or 95% of the independent normal examples of CEHs with HLA-DQB1*0602 were identical and fixed in the first intron of HLA-DQA2. A single individual (MOB) had 3 aberrant SNPs, at positions 1150, 1157 and 3446, suggesting an ancient crossover on 1 of her HLA-DQB1*0602-bearing haplotypes. Therefore, the DNA fixity that characterizes independent examples of the CEHs with HLA-DQB1*0602 in these subjects extends, in general, through HLA-DQA2, since virtually all independent examples had the same SNP haplotypes.

Analyses of the same SNPs in the T1D patients who carried the normally protective HLA-DQB1*0602 haplotype are shown in Table 2. One patient had no SNP in common with the consensus haplotype, 1 had only 1 in common and 1 had
In common. In 6 patients, there were 2 SNPs that differed from the consensus normal haplotype and in 6 other patients there was only 1 aberrant SNP. Thus, in the majority of patients with informative SNPs (9 of 15), there were 2 or more SNPs that differed from the consensus normal HLA-DQA2 first intron SNP haplotype on HLA-DQB1*0602-bearing CEHs. None of the informative patients (1–15) had the SNP haplotype that characterizes the HLA-DQA2 genes on the [HLA-B7, DR2, DQB1*0602] CEH in healthy subjects, as shown in Table 1. Four patients (16–19) were heterozygous at all 5 nucleotide positions and therefore uninformative for identity or non-identity with the consensus SNP haplotype since no first-degree relative was available to assign phase. The results indicate that another locus centromeric to HLA-DQB1 is a major determinant of genetic protection from and susceptibility to T1D associated with HLA-DQB1*0602 haplotypes, suggesting this may be the true major susceptibility locus within the MHC. Since HLA-DQA2 was the gene analyzed in all patients, the true MHC T1D susceptibility locus is HLA-DQA2 or at least a locus centromeric to HLA-DQB1.

Studies were also carried out in a healthy [HLA-B7, SC31, DR2, DQB1*0602] homozygous sib (CCO) of a patient with T1D who shared 1 of these haplotypes with his T1D-affected sib. We performed this experiment to test directly our hypothesis that a T1D susceptibility haplotype carrying HLA-DQB1*0602 (the haplotype shared with his T1D sib) would differ significantly in the class II region centromeric to HLA-DQB1 from a “normal” T1D susceptibility haplotype carrying HLA-DQB1*0602 (the haplotype not shared with his T1D sib nor any other T1D family member). The healthy sib homozygous for [HLA-B7, SC31, DR2, DQB1*0602] was heterozygous for all of the 5 SNPs studied, as shown in Table 3. Thus, the T1D and “normal” haplotypes differed at every SNP studied. Therefore, this subject appears to have inherited 1 HLA-DQB1*0602-bearing CEH with a normal protective HLA-DQA2 gene with consensus protective SNPs and 1 HLA-DQB1*0602 CEH that carried a completely different HLA-DQA2 gene produced by presumed ancient meiotic crossingover, resulting in that haplotype carrying susceptibility to T1D.

Table 1

<table>
<thead>
<tr>
<th>Donor</th>
<th>Nucleotide position (in consensus haplotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Position relative to the first intron</td>
</tr>
<tr>
<td>1 (PGF)</td>
<td>899</td>
</tr>
<tr>
<td>2(1)C)</td>
<td>1150</td>
</tr>
<tr>
<td>3(2)C)</td>
<td>1157</td>
</tr>
<tr>
<td>4(3)B)</td>
<td>1176</td>
</tr>
<tr>
<td>5(4)B)</td>
<td>3446</td>
</tr>
</tbody>
</table>

* Nucleotide position from PGF transcription start site.
* PGF is from the Sanger Centre sequence [37].
* Includes the normal HLA-DQA2 SNP haplotype of CCO in Table 3.
* Normal HLA-DQA2 SNP haplotype from phase determination.

![Genetic map of the HLA-DQ region of chromosome 6p21.3 showing known genes and pseudogenes. Gene locations and distances were taken from the Sanger Centre MHC list for the PGF cell line [37] and are drawn to scale for the region from HLA-DQA1 at the telomeric (T) end to HLA-DQB2 at the centromeric (C) end. An expanded map of HLA-DQA2, also drawn to scale, shows its exon (E)–intron (I) structure and the regions of intron 1 amplified by forward (F) and reverse (R) primers for sequence analysis in this study.](image)
Table 2

<table>
<thead>
<tr>
<th>Subject</th>
<th>Other HLA-DR, DQB1</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>899</td>
</tr>
<tr>
<td>PGF DQB1*0602&lt;sup&gt;a&lt;/sup&gt;</td>
<td>A</td>
<td>T</td>
</tr>
<tr>
<td>1</td>
<td>5, 0301</td>
<td>T</td>
</tr>
<tr>
<td>2</td>
<td>5, 0301</td>
<td>T</td>
</tr>
<tr>
<td>3</td>
<td>4, 0302</td>
<td>T</td>
</tr>
<tr>
<td>4</td>
<td>4, 0302</td>
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<tr>
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<td>4, 0302</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>4, 0302</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>4, 0302</td>
<td>A</td>
</tr>
<tr>
<td>8</td>
<td>3, 0201</td>
<td>T</td>
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<tr>
<td>9</td>
<td>3, 0201</td>
<td>A</td>
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<tr>
<td>10</td>
<td>3, 0201</td>
<td>T</td>
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<td>11</td>
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<td>13</td>
<td>7, 0201</td>
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<td>14</td>
<td>4, 0301</td>
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<td>17</td>
<td>8, 0402</td>
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<tr>
<td>18</td>
<td>4, 0305</td>
<td>A</td>
</tr>
<tr>
<td>19</td>
<td>1, 0501</td>
<td>T</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide position from PGF transcription start site.

<sup>b</sup> Normal HLA-DQB1*0602. Homozygosity discordant with PGF sequence highlighted in gray.

4. Discussion

Although many reviews note that the genetics of T1D is far from well-understood [3,20,21], long-held but unsubstantiated assumptions about T1D and many other complex (including other autoimmune) genetic diseases are obstacles to understanding this increasingly important group of disorders. For example, in the absence of clear genetic models with precise predictive capacity, the environment is often implicated in the rising incidence of polygenic diseases and for the changing MHC haplotype frequencies among patients with T1D. We recently provided an alternative purely genetic explanation for these phenomena [22]. The present study now challenges a second assumption often made in the genetics of T1D; that HLA-DQB1 is the critical MHC susceptibility locus for T1D. We tested the hypothesis that HLA-DQB1 is not that gene, but that a gene centromeric to HLA-DQB1 is likely to be so.

The variable fixity of DNA of the human MHC, including segments of 1–4 Mb as CEHs [2–7], is the reason HLA-DRB1, DQB1 alleles have been considered to be the actual susceptibility genes for T1D. European Caucasian CEHs with the greatest extent of fixity carry HLA-DR2, -DR3 and -DR4 and thus provide the best markers for T1D protection and susceptibility. Other haplotypes with presumably less or no fixity centromeric to HLA-DQB1, such as those marked by HLA-DR1, -DR5 or -DR6, are neutral (and therefore useless as markers) or only mildly protective or susceptibility-conferring. These poor markers for T1D susceptibility have not been explained. If HLA-DQB1 or HLA-DRB1, -DQB1 were in fact the susceptibility locus (loci) and the MHC susceptibility gene is recessive [8–12], there should be no weak or neutral haplotypes. Alleles at the true susceptibility locus should invariably either confer susceptibility or be protective (i.e., a protective allele should never occur in a patient). This concept is illustrated in Fig. 2, where it is shown that, if the fixity of a CEH includes the susceptibility locus, any of its marker alleles will mark susceptibility or protection. On a non-CEH or a protective CEH with an ancient crossover centromeric to HLA-DQB1, susceptibility and protective alleles are randomized.

The detailed study of HLA-DQA2 first intron SNPs revealed the remarkable general fixity of alleles on DQB1*0602-carrying CEHs in normal individuals, as predicted almost 25 years ago [2,4] and confirmed recently for the 2 HLA-DR3-carrying T1D susceptibility CEHs [5–7]. Since the few aberrant SNPs were in a single haplotype from 1 healthy individual, they could represent a single ancient crossover. The rarity of such non-consensus HLA-DQA2 SNP haplotype examples of HLA-DQB1*0602 haplotypes among normal individuals is consistent with the rarity of HLA-DQB1*0602 haplotypes among T1D patients in general.

In striking contrast, of the 19 HLA-DQB1*0602-bearing haplotypes from the T1D patients studied, all showed evidence of prior crossingover or were uninformative. The usual (consensus) protective 5-SNP haplotypes of the HLA-DQA2 first intron carried by [HLA-B7, SC31, DRB1*1501, DQB1*0602] but heterozygous for T1D susceptibility.

Table 3

<table>
<thead>
<tr>
<th>Haplotype 899 1150 1157 1176 3446&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HLA-DQA2 first intron</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>T</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nucleotide position from PGF transcription start site.
reason for these differences is crossover centromeric to HLA-DQB1. It is conceivable, but highly unlikely because of their multiplicity, that the nucleotide differences in the HLA-DQA2 first introns of T1D patients compared with controls were due to mutations. Nevertheless, whether the result of crossingover or mutation, these findings are consistent with HLA-DQB1 being a good marker for T1D susceptibility but not the true susceptibility gene. Rather, the data support the conclusion that the true T1D MHC susceptibility gene is HLA-DQA2 or another locus centromeric to HLA-DQB1. As this is a conclusion of critical significance, we considered several alternative explanations. The possibility that some or all of the patients did not have T1D is ruled out by the diagnostic criteria, the age of onset and the fact that many patients had autoantibodies typically associated with T1D. Another alternative explanation is that the patients do not carry HLA-DQB1*0602. However, the second exon of HLA-DQB1 was identical to the reference HLA-DQB1*0602 sequence, as determined by direct sequencing for all the patients studied (n = 6) [15]. Furthermore, all of the subjects were carriers of HLA-DQA1*0102, known to be strongly associated with HLA-DQB1*0602.

There are a number of open reading frames as well as recognized genes between HLA-DQB1 and HLA-DPB1 including HLA-DQB3, -DQA2, -DQB2, -DOB, -TAP-2, -LMP-7, -TAP-1, -LMP-2 and -DOA (Fig. 1) [16,23,24]. The literature on HLA-DQB3, HLA-DQA2 and HLA-DQB2 is contradictory. HLA-DQA2 has been variously described as non-polymorphic [25], polymorphic [26], non-expressed [27] and expressed in B cells and B cell lines [28,29]. It is now clear that HLA-DQA2 is expressed. A first intron HLA-DQA2 Taq I RFLP was described [30] and claimed to be a good T1D marker [31,32]. The Taq I site is apparently on some HLA-DRB1*04, DQB1*0302 (DR4, DQ8) haplotypes [33]. The 388 bp 5' UT region of HLA-DQA2 on HLA-B8, DR3 has been shown to have identical sequences in T1D patients and normal subjects [34]. This was also true of the 5' UT region SNPs in HLA-DQA2 on DR4, DQ8 haplotypes [34]. Although the authors claimed that this rules out HLA-DQA2 as a T1D susceptibility locus, the results only show that the fixity of DNA on these haplotypes extends through HLA-DQA2 in both patients and controls, as expected. Whether HLA-DQA2 or a locus telomeric or centromeric to it is the susceptibility locus is not answered by the observations. HLA-DQB3 and HLA-DQB2 are less controversial. They appear not to be expressed [23], although HLA-DQB2 is highly conserved among individuals and compared with non-human primates [35].

The functions of many of the genes centromeric to HLA-DQB1 are unclear. Moreover, there is evidence that the MHC susceptibility genes for deficiencies of IgA, IgG3, IgG4 and IgD are distinct from those for T1D [36]. Much work needs to be done to identify the T1D MHC susceptibility gene, to define its normal function and to determine its role in T1D pathogenesis. In a disease with many puzzling genetic features, these are not simple tasks.

In the present study, we used the protective CEHs [HLA-B7, SC31, DRB1*1501, DQB1*0602] and [HLA-B18, S042, DRB1*1501, DQB1*0602] in normal subjects to determine the 5-member SNP haplotype that defines the protective allele of the first intron of the HLA-DQA2 locus closely centromeric to HLA-DQB1 that is carried by the full CEH that includes protection. In all informative T1D patients who carry the complete CEH or the HLA-DQB1*0602 allele and fragments of the CEH, there has been ancient centromeric crossover at meiosis, which together with the associated loss of protection make a compelling case that HLA-DQB1 is not the T1D MHC susceptibility locus. In addition, the fact that all of the detected crossovers are telomeric to HLA-DQA2 suggests, but does not prove, that HLA-DQA2 may be a major determinant of MHC-associated susceptibility, and perhaps the true T1D susceptibility locus.

Susceptibility-conferring alleles of HLA-DQA2 (or some other gene centromeric to HLA-DQB1) may interact with HLA-DQB1 (directly or indirectly) to modify the protection otherwise afforded by HLA-DQB1*0602 haplotypes. Indeed, there are reports that critical residues of HLA-DQB1 play a role in susceptibility and functional evidence has been offered that both HLA-DR and HLA-DQ molecules influence susceptibility in transgenic mice. In humans, HLA-DRB1*04 subtypes modulate the susceptibility associated with HLA-DQB1*0302. This could be due to a direct role of HLA-DRB1 in T1D, as there is also evidence that autoreactive T cells are restricted by DRB1 and DQB1 molecules. However, the results presented here offer an alternative explanation of

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**Fig. 2.** Model to explain “protective” or “neutral” HLA-DQB1 alleles in T1D patients. Shown is the relationship of DNA fixity at the T1D susceptibility locus (HLA-DQA2 as the better susceptibility marker in this case) on independent examples of CEHs or fragments of CEHs that include a T1D susceptibility allele S on a susceptibility CEH (upper diagram) or a T1D protective allele P on a protective CEH (middle diagram). The bottom diagram shows a “neutral” CEH, in which the region of fixity does not include the susceptibility locus, and S and P are variably present. Results in the present study are consistent with rare T1D patient haplotypes containing the “protective” HLA-DQB1*0602 marker being the result of an ancient crossover centromeric to HLA-DQB1 and telomeric to HLA-DQA2 to incorporate a susceptibility allele at the true T1D susceptibility gene centromeric to HLA-DQB1.
the genetic “effect” of HLA-DB1 subtypes: these may be in strong linkage disequilibrium with specific alleles of the putative susceptibility gene centromeric to HLA-DB1. In any case, the possibility that HLA-DQA2 or some nearby gene abrogates the putative protective function of HLA-DQB1*0602 and conversely modifies HLA-DQB1/DR-associated susceptibility is a strong argument to investigate its (their) direct role(s) in T1D susceptibility.

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