

The Role of HLA Class I Gene Variation in Autoimmune Diabetes

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■ Abstract

The use of DNA-based genetic typing has enabled the identification of type 1 diabetes mellitus (T1DM) susceptible and protective major histocompatibility complex (MHC) class II alleles and haplotypes. The application of this approach has also progressed to locate MHC class I alleles that contribute to the clinicopathology of T1DM. Recent studies have shown a widespread involvement of genes from the MHC class I gene region in the clinicopathology of T1DM. These genes are demonstrated to be involved in contributing to progression from the preclinical stage of the disease, which is characterized by the occurrence of islet-specific antibodies, to clinical disease and also to the occurrence of autoimmunity. They can either contribute directly to disease development or indirectly in concert with other susceptible MHC class II alleles or haplotypes via linkage disequilibrium. Class I alleles may also be negatively associated with T1DM. These findings are useful for the development of future

strategies in designing tolerogenic approaches for the prevention or even reversal of T1DM. In this article, the latest evidence for the different kinds of participation of HLA class I genes in the etiology of T1DM is reviewed. A meta-analysis which included existing association studies was also carried out in order to re-assess the relevance of class I genes in diabetes development. The analysis of an enlarged heterogeneous sample confirmed the involvement of previously detected serotypes in the etiology of T1DM, such as A24, B8 and B18, and revealed hitherto unknown associations with B60 and B62. The analysis points out that much of the conflicting results of previous association studies originate from inadequate sample sizes and accentuate the value of future investigations of larger samples for identifying linkage in multigenic diseases.

Keywords: type 1 diabetes · HLA class I · cross-study analysis · serotype association

Introduction

Type 1 diabetes mellitus (T1DM) is a multifactorial and multigenic autoimmune organ disease characterized by progressive T cell-mediated destruction of the pancreatic β -cells. The major determinants of this disease are genes of the human leucocyte antigen (HLA) region, which are highly polymorphic. In a multiplicative model, they account for between 20 and 53% of T1DM susceptibility markers [1]. Gene products of HLA class I genes function as antigen present-

ing molecules for CD8⁺ cytotoxic T lymphocytes (CTL) and determine the antigen specificity of the CTL-mediated immune response against pathogens and self-antigens. T cell reactions with islet-specific self-antigens may lead to clonal proliferation of autoreactive CTLs directed against pancreatic β -cells provoking destruction of these organ cells.

Initial genetic analyses clearly rejected a dominant major locus. In contrast, a series of other genes, such as the coding sequences for TAP (transporters associated with antigen processing), LMP (large multifunc-

tional proteases) and TNF- α , the MIC-A (MHC class I chain-related A) gene, as well as identified (the insulin gene on chromosome 11p15) and largely unidentified genes on other chromosomes (e.g. 6q and 11q) have been linked to the disease corroborating its polygenic nature [1-5]. This has invoked an additive model, from which it was possible to predict that a maximum of only 0.15% of the general population is at 100% risk of T1DM, about 5% is at intermediate risk, while the remaining population has a risk of almost 0 [6]. The onset of the disease is most common in the young, but is also known to occur at any age, which reflects the heterogeneity of the disease. Early age of onset (≤ 10 yr) is genetically associated with highly susceptible HLA class II alleles from DQA1, DQB1 and DR3 subgroups e.g. the DQA1*0301/DQA1*05011 genotype and the DQA1*0302 allele in a Chinese [7, 8], the DQB1*02/*0302 genotype in a Finnish [9] and the DR3 serotype and A30-B18-DR3 haplotype in a Caucasian population [10] as well as A*2402 [11]. In contrast, later age of onset (≥ 15 yr) carries haplotypes, which also include protective alleles [9, 12-14]. Generally, young age at onset is associated with rapid progression of disease [15], and very early onset (between the ages of 1 and 2 yr) seems to have a greater genetic and smaller environmental contribution to the initiation of the autoimmune process than at later ages of onset [16].

Clinical appearance is preceded by a preclinical period that may vary from weeks to several years and which involves the occurrence of autoantibodies specific against insulin and other target antigens, such as glutamic acid decarboxylase (GAD) and the tyrosine phosphatase-like islet associated antigen (IA-2), detected in the serum of individuals at risk of developing T1DM. The occurrence of these autoantibodies is considered as evidence for the concomitance of islet-specific autoimmunity and they serve as surrogate markers for T1DM development. In a clinical study including 758 children with newly diagnosed diabetes, 90.7% were positive for two of the four tested antibodies and only 2.1% negative for all four [17]. The induction of antibodies against these self antigens is a T cell-dependent response. In preclinical auto-immune responses antigen presenting cells (APCs) are potentially enabled to present pancreatic self-antigens via MHC class II molecules to CD4⁺ T cells. These class II/peptide complexes stimulate them by expressing gene products encoded by disease susceptible HLA class II genes [18]. Progressive damage of β -cells is largely attributed to the cytotoxic activity of diabetogenic CD8⁺ T cells that infiltrate the pancreatic islets in

the latter stages of the cell-mediated immune response [18].

Genetic linkage analyses have also revealed that HLA class I (A, B and C) alleles contribute to T1DM etiology. HLA-A, B and C genes are attributed to conferring risk as well as development of clinical disease with or without the existence of risk DR and DQ genes [19]. It is hypothesized and has recently been verified in NOD mouse models and human genetic case control studies that class I gene products present particular peptides of autoantigen origin to trigger the generation of diabetogenic CD8⁺ T cells which mediate the progression to T1DM from its preclinical phase to clinical relevance [18, 20, 21]. Although CD8⁺ T cells seem to participate substantially in the pathogenesis of T1DM, the pathogenic role of certain MHC class I alleles has largely been disregarded [22]. In this article, we therefore aim to discuss the recent findings on classical HLA class I genes and the encoded antigen presentation molecules to re-assess their role in the etiology of T1DM.

Functional impact of HLA class I genes and molecules on the retention of a balanced immunity and their role in T1DM pathogenesis

The primary function of HLA class I molecules is to present peptide fragments derived from cleavage of native antigens in the cytosol. Thus, an explanation for the positive association between T1DM and the gene products encoded by susceptible disease conferring HLA class I genes is their inefficiency to present antigenic peptides required to mediate negative selection for the deletion of autoreactive T cells in the thymus and the periphery. This can result from the architecture of their peptide binding grooves which are in turn determined by their primary sequences. In this regard, the disease susceptible class I molecule A*2402 is related to its non-susceptible A24 counterparts. Their conformational differences may create variability in the peptide binding properties for presentation to CD8⁺ T cells resulting in the induction of tolerance or self-reactivity [23]. Diversity in the CTL repertoire induced by variations in MHC genes is a critical trait of the immune system to promote powerful anti-pathogenic defense, but it also hazards the consequence of capsizing into an over-aggressive and self-destructing pitfall. Thus T cell selection is a complex system of antagonizing mechanisms equilibrating immune defenses against self- and foreign antigens. In this regard, for instance A24-restricted T cells may be involved in β -cell destruction supported by mechanisms acting in the selec-

tion of specific T cell clones. Such mechanisms are benefited by conformational alterations in the antigen-binding pockets due to amino acid substitutions in MHC class I molecules preventing negative selection of autoreactive T cells [23]. The observation that

HLA-A24 is associated with rapid progression of T1DM from its preclinical (antibody positive) to its clinical stage characterized by the almost complete destruction of the pancreatic β -cells [24, 25] supports this notion.

The functional mechanisms that may lead to disturbances in the selection of T cells and that direct them to become diabetogenic are still not fully understood. Our presumption that the participation of MHC class I gene variation in the pathogenesis of T1DM has emerged from the observation that enhanced levels of MHC class I heavy-chain RNA are present in pancreatic islets before overt inflammation and the onset of T1DM in the spontaneously diabetic BB rat, but there are no detectable levels of class II antigens [26]. The question of the nature of class I molecules that provide the epitopes restricted to islet-specific autoantigens is of major interest in the understanding of pathways responsible for the activation of β -cell specific autoreactive T cells. Although this question is largely unanswered, it was recently shown that CD8⁺ CTLs can recognize a peptide from a leader sequence of preproIAPP in A*0201-positive T1DM patients of recent onset [27]. The use of HLA assembly assay has led to the identification of HLA-A*0201-binding peptides that activate GAD-specific CD8⁺ CTLs in individuals with preclinical

Table 1. Frequencies and reported results of HLA class I serotypes obtained from association studies

| Locus | Sero-type | T1DM (n) | Controls (n) | p | Study | Confirming studies | Contradicting studies |
|-------|-----------|----------|--------------|--------------------------|--------------------------|---|---|
| A | A1 | 144 | 1925 | n.s. | Tait <i>et al.</i> | | Pitkäniemi <i>et al.</i> |
| | A2 | 205 | 3120 | n.s. | Tait <i>et al.</i> | Nakanishi <i>et al.</i> Pitkäniemi <i>et al.</i> | |
| | A3 | 82 | 1544 | n.s. | Tait <i>et al.</i> | Pitkäniemi <i>et al.</i> | |
| | A11 | 23 | 740 | < 0.0005* | Tait <i>et al.</i> | Pitkäniemi <i>et al.</i> | |
| | A23 | 10 | 205 | n.s. | Tait <i>et al.</i> | | |
| | A24 | 93 | 928 | < 0.00001 | Tait <i>et al.</i> | | Pitkäniemi <i>et al.</i> |
| | A25 | 12 | 216 | n.s. | Tait <i>et al.</i> | | |
| | A26 | 10 | 308 | n.s. | Tait <i>et al.</i> | Nakanishi <i>et al.</i> | |
| | A28 | 16 | 498 | < 0.01* | Tait <i>et al.</i> | | Pitkäniemi <i>et al.</i> |
| | A29 | 36 | 434 | n.s. | Tait <i>et al.</i> | | |
| | A30 | 21 | 296 | n.s. | Tait <i>et al.</i> | | |
| | A31 | 19 | 359 | n.s. | Tait <i>et al.</i> | | |
| | A32 | 18 | 438 | n.s. | Tait <i>et al.</i> | | |
| | A33 | 25 | 35 | n.s. | Nakanishi <i>et al.</i> | Tait <i>et al.</i> | |
| B | B7 | 70 | 1682 | < 0.0001* | Tait <i>et al.</i> | Nejentsev <i>et al.</i> Pitkäniemi <i>et al.</i> | |
| | B8 | 171 | 1420 | < 0.00001 | Tait <i>et al.</i> | Pitkäniemi <i>et al.</i> | |
| | B13 | 13 | 261 | n.s. | Tait <i>et al.</i> | | |
| | B14 | 16 | 513 | < 0.005* | Tait <i>et al.</i> | | |
| | B15 | 88 | 802 | n.s. | Tait <i>et al.</i> | | |
| | B18 | 65 | 487 | < 0.00001 | Tait <i>et al.</i> | Pitkäniemi <i>et al.</i> | |
| | B27 | 22 | 519 | n.s. | Tait <i>et al.</i> | Pitkäniemi <i>et al.</i> Nejentsev <i>et al.</i> | |
| | B35 | 36 | 846 | < 0.025* | Tait <i>et al.</i> | | Pitkäniemi <i>et al.</i> Nejentsev <i>et al.</i> |
| | B39 | 32 | 212 | < 0.00001 | Tait <i>et al.</i> | Nejentsev <i>et al.</i> | |
| | B40 | 54 | 756 | n.s. | Tait <i>et al.</i> | | |
| | B44 | 82 | 1733 | n.s. | Tait <i>et al.</i> | Pitkäniemi <i>et al.</i> Nejentsev <i>et al.</i> | |
| | B50 | 15 | 120 | n.s. | Tait <i>et al.</i> | | |
| | B51 | 20 | 556 | < 0.025* | Tait <i>et al.</i> | Nejentsev <i>et al.</i> | |
| | B56 | 1 | 74 | n.s. | Tait <i>et al.</i> | Nejentsev <i>et al.</i> | |
| | B57 | 6 | 418 | < 0.0001* | Tait <i>et al.</i> | | |
| | B60 | 110 | 54 | n.s. | Pitkäniemi <i>et al.</i> | Nejentsev <i>et al.</i> | |
| B62 | 217 | 83 | < 0.05 | Pitkäniemi <i>et al.</i> | | Nejentsev <i>et al.</i> | |

Legend: n: number of subjects in the study. p: reported p-value. * Decreased frequency in T1DM subjects.

T1DM [28]. These CTLs were able to lyse A*0201-positive target cells presenting GAD65 peptide fragments, as pancreatic β -cells do, indicating that class-I-restricted recognition is a critical mechanism even in early stages of the diabetogenic process [28]. A*0201 was found to be positively associated with disease ($p = 0.029$) in the enlarged sample analyzed within the scope of the meta-study, as shown in the latter section of this article.

Table 2. Significant serotypes in control, antibody-positive and T1DM individuals detected by Tait *et al.*

| Locus | Sero-type | controls vs. Ab ⁺ | Ab ⁺ vs. T1DM | controls vs. T1DM |
|-------|-----------|------------------------------|--------------------------|-------------------|
| A | A1 | < 0.0001 | < 0.05* | n.s. |
| | A11 | n.s. | n.s. | < 0.0005* |
| | A24 | n.s. | < 0.0005 | < 0.00001 |
| | A26 | < 0.05** | n.s. | n.s. |
| | A28 | n.s. | < 0.005* | < 0.01* |
| | A30 | < 0.05** | < 0.025 | n.s. |
| B | B7 | n.s. | n.s. | < 0.0001* |
| | B8 | < 0.00001 | n.s. | < 0.00001 |
| | B14 | n.s. | < 0.05* | < 0.005* |
| | B18 | n.s. | < 0.005 | < 0.00001 |
| | B35 | n.s. | n.s. | < 0.025* |
| | B39 | n.s. | n.s. | < 0.00001 |
| | B51 | n.s. | n.s. | < 0.025* |
| | B57 | < 0.05** | n.s. | < 0.0001* |

Legend: Data represent p-values. Ab⁺: antibody-positive. *Decreased frequency in T1DM group. **Decreased frequency in Ab⁺ group.

Results obtained from genetic studies conducted in the fashion of patients vs. controls have demonstrated that HLA class II genes are clearly associated with the occurrence of high levels of autoantibodies against antigens, such as the 65 kDa isoform of GAD, GAD65 [29], and that these genes contribute decisively to the onset of autoimmunity [7, 9, 13]. The role of class I genes in the early stages of the disease is disputed. In the context of GAD recognition, another indication for the participation of these genes is suggested by the observation that peripheral blood mononuclear cells (PBMC) may respond to GAD peptides with a sequence similar to a protein of Coxsackie B virus. This responsiveness of PBMCs to GAD is certainly not restricted to persons at risk of developing T1DM, but PBMCs of these persons respond significantly more frequently to a sequence encompassing the amino acid residues 247-279 of GAD than healthy persons [30].

Since amino acids 247-279 are encoded by a region that has significant sequence similarity to the P2-C protein of Coxsackie B virus, the clinicopathology of T1DM has long been suspected to be due to molecular mimicry, which in turn substantiates the role of MHC class I genes in preclinical stages of T1DM.

Interestingly, HLA-A2 subgroup molecules were earlier demonstrated to feature high cross-binding propensities to different peptides due to their structural conformity with various residue motifs [31]. Cross-reactivity is a critical phenomenon in cellular immunity where specific MHC epitopes become capable of loading an array of antigenic fragments for the selection and education of T cells for defense against pathogens. In situations where T cells are generated by cross-reactivity of this kind, they become capable of recognizing and reacting even with antigenic peptides other than the original ones that have been used to select these T cells [32]. Indeed peptide cross-binding phenomena were observed for gene products of several MHC class I alleles, such as those of the A2 subgroup and B*2705 [31, 33]. Molecular mimicry and T cell cross-reactivity to β -cell autoantigens and environmental agents with sequence similarities are thus proposed mechanisms that may influence the pathogenesis of type 1 diabetes [34, 35], but has still not been proven to be causal and the mechanism is criticized because T cells could not be shown to cross-react with GAD *in vitro* [36].

Today's leading concept is that distinct combinations of MHC alleles interact synergistically to induce the disease when further environmental and genetic factors are present [37]. Genetic predisposing alone does not explain the etiology of T1DM as only 40% of monozygotic twins develop the disease concordantly [38]. Thus, the existence of predisposing factors in terms of specific allele variations more or less randomly results in the generation of autoreactive β -cell-specific T cells, namely if other factors, in particular viruses, appear accessorially to induce disturbances of the immunologic equilibrium. In this regard, ex post analyses of diabetic patients, where several factors have already acted in concert to advance the outbreak of the disease, have limited meaning when identifying certain predisposing alleles. It is necessary to also include those persons who are only antibody positive before the clinical manifestation of the disease to identify specific outbreak markers. There is only one study that has tracked the clinical history of antibody positive persons in the context of HLA class I gene susceptibility [18] and the results are reported and re-examined in this article.

Positive association of HLA class I serotypes and alleles with T1DM

Approximately 25 years ago, HLA-B8, B15 and B18 were first found to exist at higher frequencies in diabetic patients of Caucasian origin [39, 40]. Further investigation of these early studies has shown that the B8 serotype association occurs in Caucasians more widely than the B15 and B18 type (Table 1) [18]. Among individuals of Caucasian origin, B8 is mainly associated with patients in Northern Europe, in particular in Britain, while B15 is more prevalent in patients from Southern Europe. Apparently, individuals carrying both the B8 and B15 serotypes are twice as susceptible to developing T1DM, as those with either the B8 or B15 serotype alone [6, 39, 40]. Regardless of the B8 dominance, Tait *et al.* have reported that B18 can be found significantly more frequently in Caucasian patients with clinical T1DM than in antibody positive individuals, and that this association exists in the absence of any class II gene contribution (Table 2) [18]. In contrast, B8 and A1 could be observed at more than twice the frequency in antibody positive individuals ($p < 0.0001$ for both), while no further increase in frequency from antibody positive to T1DM probands could be found. Surprisingly, A1 was even found significantly less frequently ($p < 0.05$) in patients than in antibody positive individuals (Table 2) [18]. B8 was also reported in an earlier study as being associated with a significantly increased CD4⁺/CD8⁺ ratio indicating that B8 may even be involved in the preclinical stage of diabetes, i.e. the emergence of autoantibodies and the adverse immune response against self-antigens [41]. It is not entirely clear whether class I genes contribute in a more direct or indirect way to the activation and proliferation of autoreactive CD4⁺ T cells. Evidence for a direct role engaged by class I genes arose from the observation that alloreactive CD4⁺ T cells are capable of recognizing self-peptides loaded on MHC class I epitopes [42, 43]. In support of the indirect role of class I genes, is

the finding that their gene products expressed on dendritic cells prime naïve CD8⁺ T cells that secrete IFN- γ and TNF- α to polarize the development of CD4⁺ cells [44]. These results suggest that MHC class I molecules are not only contributing to disease progression but also to the occurrence of autoimmunity.

Linkage disequilibrium (LD) is known to exist across the HLA system. This region contains a multi-

Table 3. Frequencies and reported results of HLA-A alleles in association studies

| Allele | T1DM (n) | Controls (n) | p | Study | Confirming studies | Contradicting studies |
|--------|----------|--------------|-------------------|-----------------------|-----------------------|-----------------------|
| A*0101 | 116 | 60 | 0.05 | Noble <i>et al.</i> | | Bugawan <i>et al.</i> |
| A*0201 | 121 | 177 | n.s. | Noble <i>et al.</i> | Bugawan <i>et al.</i> | |
| A*0301 | 57 | 55 | 0.1 | Noble <i>et al.</i> | | |
| A*1101 | 28 | 100 | 0.01* | Bugawan <i>et al.</i> | Noble <i>et al.</i> | |
| A*2402 | 60 | 81 | 0.027 | Bugawan <i>et al.</i> | Noble <i>et al.</i> | |
| A*2403 | 5 | 4 | n.s. ¹ | Bugawan <i>et al.</i> | Noble <i>et al.</i> | |
| A*2407 | 17 | 53 | n.s. | Bugawan <i>et al.</i> | | |
| A*3002 | 14 | 3 | 0.06 | Noble <i>et al.</i> | | |
| A*3201 | 11 | 17 | 0.04* | Noble <i>et al.</i> | | Bugawan <i>et al.</i> |
| A*3301 | 1 | 3 | 0.09* | Noble <i>et al.</i> | | |
| A*3303 | 20 | 28 | n.s. | Bugawan <i>et al.</i> | Noble <i>et al.</i> | |
| A*3401 | 20 | 51 | n.s. | Bugawan <i>et al.</i> | | |

Legend: n: number of subjects in the (separate) study. p: reported p-value. * Decreased frequency in T1DM subjects. ¹ Significance of A*2403 was calculated in the study in combination with A*2402 yielding $p = 0.008$. Separately tested: not significant.

tude of genetic loci, which are considered to be jointly responsible for different stages of T1DM development in the context of various degrees of LD with other HLA genes [1]. The identification of genetic linkages to T1DM are thus more difficult given the polygenic character of the disease and the interaction of genetic and environmental factors. Fortunately, improved methods for detecting genetic linkages and hereditary patterns within the HLA system together with modern DNA-based genotyping have led to the identification of more detailed variations of susceptible class I genes and the existence of specific LD patterns [45-48]. In this regard, the early discovery of a significantly increased frequency of the B8 serotype in T1DM probands was detected predominantly in individuals carrying the high risk DRB1*03 allele [18]. B8 was also found to exist more frequently in association with the 8.1 ancestral haplotype, which consists of HLA-A1, C7, B8, C4AQ0, C4B1, DR3, DQ2, including the high risk DR3 serotype, in T1DM patients [49]. An increased CD4⁺ and decreased CD8⁺ T cell occurrence

Table 4. Frequencies and reported results of HLA-B alleles in association studies

| Allele | T1DM (n) | Controls (n) | p | Study | Confirming studies | Contradicting studies |
|--------|----------|--------------|-------------------|-----------------------|-----------------------|-----------------------|
| B*0702 | 45 | 66 | 0.0001* | Valdes <i>et al.</i> | | |
| B*0801 | 123 | 37 | < 0.0001 | Valdes <i>et al.</i> | | |
| B*1301 | 4 | 20 | n.s. | Bugawan <i>et al.</i> | | |
| B*1501 | 71 | 16 | < 0.0001 | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| B*1502 | 10 | 20 | n.s. | Bugawan <i>et al.</i> | | |
| B*1535 | 13 | 26 | n.s. | Bugawan <i>et al.</i> | | |
| B*1801 | 49 | 23 | n.s. | Valdes <i>et al.</i> | Bugawan <i>et al.</i> | |
| B*2703 | 24 | 9 | n.s. | Valdes <i>et al.</i> | | |
| B*3501 | 22 | 29 | 0.02* | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| B*3505 | 12 | 32 | n.s. | Bugawan <i>et al.</i> | | |
| B*3801 | 0 | 8 | n.s. ¹ | Bugawan <i>et al.</i> | Valdes <i>et al.</i> | |
| B*3802 | 16 | 49 | n.s. | Bugawan <i>et al.</i> | | |
| B*3906 | 22 | 3 | 0.003 | Valdes <i>et al.</i> | | |
| B*4001 | 12 | 24 | n.s. | Bugawan <i>et al.</i> | Valdes <i>et al.</i> | |
| B*4002 | 11 | 33 | n.s. | Bugawan <i>et al.</i> | Valdes <i>et al.</i> | |
| B*4402 | 39 | 30 | n.s. | Valdes <i>et al.</i> | Bugawan <i>et al.</i> | |
| B*4403 | 10 | 19 | 0.008* | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| B*5701 | 2 | 21 | < 0.0001* | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| B*5801 | 23 | 26 | n.s. ¹ | Bugawan <i>et al.</i> | | Valdes <i>et al.</i> |

Legend: n: number of subjects in the study. p: reported p-value. * Decreased frequency in T1DM subjects.

¹ Reported as slight association with T1DM, but corrected p-value not significant.

as well as increased antibody titers have been described in association with this haplotype [41], which is consistent with the result derived by Tait *et al.* [18] and appears to be attributed to the A1 and B8 participation (Table 1). The B39 serotype is reported to exist with increased frequency in DRB1*0404-DQB1*0302-positive diabetic patients of Eastern European and Russian origin ($p < 0.0001$) [50]. Allele B*5801 was reported as being positively associated with disease only in the Filipino but not in another Caucasian cohort (Table 4) [12, 51].

Studies conducted with HLA-A locus antigens have confirmed that the A24 serotype is predominantly existent in antibody positive individuals [18, 21, 26, 27, 52] (Table 2). It is simultaneously associated with a young age of onset [11, 26, 53] and with almost complete loss of residual β -cell function [26, 52]. The relevance of A24 in T1DM was further confirmed in recent studies by Noble and co-workers in a Caucasian ($p = 0.042$) as well as by Bugawan *et al.* in a Filipino cohort ($p = 0.027$) demonstrating allele A*2402 being existent at more than twice the frequency in patients than con-

trols (Table 3) and being strongly associated with total β -cell destruction [11, 26, 51, 54]. The study by Noble *et al.* conducted with family-based association controls and known LD associations from CEPH families (Centre d'Etude du Polymorphisme Humain) revealed that A*2402 exists at a higher frequency in Caucasian diabetics in the absence of LD with several class II DR and DQ risk conferring haplotypes [54]. Other HLA-A locus alleles, such as A*3002 and A*0101, are also identified as being susceptible in promoting disease development. Their association with T1DM appears to have more of a secondary nature to disease due to LD with the high risk DRB1*0301-DQB1*02 haplotype found in T1DM patients of Caucasian origin [54]. However, several alleles, such as A*2403, A*3002, A*3301, B*2703,

B*3801, B*5701 and C*0802, that were reported as being negatively or positively associated with disease, exist rather infrequently (Tables 3, 4, 5). The frequency of these alleles in the two populations varies between 0 and 4.3% [51, 54]. Therefore, their appearance cannot be regarded as representative and the results must be interpreted carefully. Given the rarity of appearance, it is instructive to merge the frequencies of these alleles in a combined group in order to avoid selection biases, as was done in the cross-study analysis. The results of this analysis are presented in the latter section of this article.

The involvement of genes at the HLA-B and C loci in T1DM development has only recently been explored. In the Caucasian cohort, alleles B*0801, B*1501, C*0303, C*0304 and C*0701 were positively associated with T1DM (Table 4 and 5) [54]. Among these susceptible alleles C*0302 is reported to be in LD with the high risk class II allele DRB1*0301. Therefore, its direct contribution to diabetes progression is unclear, it may at most be accounted to serve as a subordinate marker for the disease. C*0102 has not

been found to be in LD with another class II allele or haplotype suggesting it has a more direct role in disease development.

B locus, B7, B14, B35, B51 and B57 serotypes have been reported to be present at lower frequencies in T1DM probands compared to non-diabetics (Table 1)

Table 5. Frequencies and reported results of HLA-C alleles in association studies

| Allele | T1DM (n) | Controls (n) | p | Study | Confirming studies | Contradicting studies |
|--------|----------|--------------|---------|-----------------------|-----------------------|-----------------------|
| C*0102 | 16 | 14 | 0.05 | Bugawan <i>et al.</i> | | Valdes <i>et al.</i> |
| C*0202 | 19 | 18 | n.s. | Valdes <i>et al.</i> | | |
| C*0302 | 22 | 25 | n.s. | Bugawan <i>et al.</i> | Valdes <i>et al.</i> | |
| C*0303 | 36 | 10 | 0.008 | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| C*0304 | 80 | 36 | 0.024 | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| C*0401 | 38 | 42 | 0.049* | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| C*0403 | 12 | 31 | n.s. | Bugawan <i>et al.</i> | | |
| C*0501 | 62 | 31 | n.s. | Valdes <i>et al.</i> | | |
| C*0602 | 22 | 40 | 0.0002* | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| C*0701 | 145 | 52 | 0.00002 | Valdes <i>et al.</i> | | Bugawan <i>et al.</i> |
| C*0702 | 39 | 126 | 0.018* | Valdes <i>et al.</i> | Bugawan <i>et al.</i> | |
| C*0801 | 25 | 41 | n.s. | Bugawan <i>et al.</i> | Valdes <i>et al.</i> | |
| C*0802 | 9 | 17 | 0.013* | Valdes <i>et al.</i> | | |
| C*1203 | 31 | 26 | n.s. | Valdes <i>et al.</i> | | |
| C*1502 | 5 | 31 | n.s. | Bugawan <i>et al.</i> | Valdes <i>et al.</i> | |
| C*1601 | 7 | 15 | 0.015 | Valdes <i>et al.</i> | | |

Legend: n: number of subjects in the study. p: reported p-value. *Decreased frequency in T1DM subjects.

Negative associations of HLA class I genes with T1DM

Linkage analysis has revealed that particular genes within all three HLA class I loci (A, B and C) can be significantly decreased in antibody positive individuals and patients. While serotypes A26 and A30 were found at decreased frequencies in individuals in the stage of progression to overt T1DM compared to controls, A1 appeared to occur negatively associated with overt disease but not with the antibody positive state (Table 2) [18]. The same study reveals that A11 and A28 are decreased in patients vs. controls (Table 1). Among the Filipino patients, the frequency of the A*1101 allele was found to be negatively associated with T1DM, which was in LD with both protective and susceptible haplotypes, DRB1*0803-DQB1*0601 and DRB1*0901-DQB1*0303, respectively [51]. A*2407 was more frequently found in healthy individuals and existed in weak LD with the neutral/weakly protective alleles DRB1*1101 and DRB1*1202 [51].

Serotypes and alleles negatively associated with disease are also present in the HLA-B and C loci. For the

LD with the protective DRB1*1502 allele [51]. In contrast, C*0602 was significantly decreased in patients only in the Caucasian but occurred more frequently in the Filipino cohort (Table 5) [51, 54]. Interestingly, despite the fact that C*1502 is in LD with the risk DRB1*0405 allele, it is still detectable at decreased frequency among the patients examined in the Filipino cohort [51].

Within the HLA-C locus, C*0702 is reported to appear significantly more frequently in healthy persons than in patients [51, 54], but this association was observed to be in

LD with the protective DRB1*1502 allele [51]. In contrast, C*0602 was significantly decreased in patients only in the Caucasian but occurred more frequently in the Filipino cohort (Table 5) [51, 54]. Interestingly, despite the fact that C*1502 is in LD with the risk DRB1*0405 allele, it is still detectable at decreased frequency among the patients examined in the Filipino cohort [51].

Cross-study analysis of HLA class I gene association with T1DM

Aim and background

The incidence of T1DM varies widely among different ethnic groups, as does the distribution of HLA class I and II genes in different populations. Therefore, the analysis of HLA patterns in association with the disease within a pooled population can be instructive in order to identify the most susceptible genetic predispositions by compensating environmental determinants driven by region and ethnology and to enlarge the sample in order to reduce the sample error.

Table 6. General parameters and reported results of studies incorporated in the cross-over comparison

| Studies | T1DM (n) ¹ | Controls (n) ¹ | Descent of subjects | Population | Genes examined | Reported significance ² |
|-------------------------------|-----------------------|---------------------------|---------------------|--------------------|--------------------|--|
| Nakanishi <i>et al.</i> 1993 | 111 | 171 | independent | Japanese | A serotypes | No significance |
| Nejentsev <i>et al.</i> 1997 | 204 | 121 | independent | Russian, Estonians | B serotypes | B7, B39, B51 |
| Noble <i>et al.</i> 2002 | 566 | 399 | AFBAC | Danish | A alleles | A*0101, A*1101, A*2402, A*3201 |
| Bugawan <i>et al.</i> 2002 | 180 | 382 | independent | Fillipino | A, B and C alleles | A*1101, A*2402, C*0102, C*0702, C*1502 |
| Tait <i>et al.</i> 2003 | 386 | 6204 | Multiplex families | Australian | A and B serotypes | A11, A24, A28, B7, B8, B14, B18, B35, B39, B51, B57 |
| Pitkaniemi <i>et al.</i> 2004 | 694 | 361 | AFBAC | Finnish | A and B serotypes | A1, A2, B8, B18, B60, B62 |
| Valdes <i>et al.</i> 2005 | 566 | 399 | AFBAC | Caucasian | B and C alleles | B*0702, B*0801, B*1501, B*3501, B*3906, B*4403, B*5701, C*0303, C*0304, C*0401, C*0602, C*0701, C*0702, C*0802, C*1601 |

Legend: n: number of included haplotypes, AFBAC: affected family-based control. ¹Number of individuals included in the cross-over study (allele number can be lower or higher dependent on occurrence). ² Either significantly increased or decreased in T1DM group.

Subjects and data selection

Data was pooled of an overall number of 2707 T1DM and 7638 healthy control haplotypes taken from seven separate studies that compared HLA class I allele occurrence between both these groups (Table 6) [12, 18, 26, 50, 51, 54, 55]. Individuals described in the previous studies were originally typed by sera or DNA samples using polymerase chain reaction. As some studies typed alleles on the basis of carriers (i.e. a single haplotype per carrier) and others on single allele level, carriers of matched characteristics as single haplotypes were regarded in order to yield consistency of the pooled data. No further differentiation of data by genotype or on subject level was considered. The frequencies of the identified characteristics differ from haplotype frequency due to multiple identification of alleles on identical haplotypes. Data was selected carefully by considering any frequencies of clearly identified alleles. Characteristics with infrequent appearance in both subject groups after data pooling were merged into a combined group in order to avoid selection biases.

The data are heterogeneous regarding ethnology, region and heredity. While controls of some studies are unrelated [26, 50, 51], other studies included affected family-based controls (AFBAC), i.e. either unaffected siblings or children of patients served as control individuals [12, 54]. The antibody-positive group in Tait *et al.* is pooled by subgroups of related and unrelated affected individuals. The transmission rate of specific DR-DQ haplotypes within the AFBAC can be as-

sumed to be higher than for unrelated controls. Differences in allele frequencies among the studies may thus be caused by ethnology as well as descent dependency, reflected by different haplotype transmission rates. A pooling of independent and descent dependent controls, as conducted in this analysis, balances the overall control group regarding this determinant. AFBAC analysis allows for examining genetic and environmental determinants of a disease, but is also afflicted with some weakness. It is well known that population association between a disease and a genetic marker can arise as an artifact of population structure, even in the absence of linkage to disease. On the other hand, linkage studies without affected sibling pairs may fail to detect linkage if there is a large linkage heterogeneity [56]. Due to the lack of relevant data, haplotype penetrances or LD patterns have not been determined and the control group is considered as being random, i.e. subjects are heterogeneous regarding susceptibility. This leaves an examination of overall risk of alleles and serotypes in randomly pooled individuals of different populations. The aim of this procedure is thus to test if class I alleles identified previously in smaller samples as being at risk of conferring disease can be confirmed in a larger sample.

Statistical methods

Data were pooled on serotype and single allele level and analyzed per locus and on each level separately in order to yield consistent allele matching. Pearson's χ^2 -test was applied to determine significance of the com-

Table 7. HLA class I serotype frequencies obtained from cross-study data

| Locus | Serotype | T1DM (n) | Controls (n) | p _c | OR |
|--------------------|--------------------|----------|-----------------------|------------------------|-------|
| A | A1 | 284 | 1992 | 0.091 | 0.83 |
| | A2 | 662 | 3394 | 0.0016 | 1.25 |
| | A3 | 329 | 1675 | 0.043 | 1.21 |
| | A11 | 51 | 773 | 9.5x10 ⁻¹¹ | 0.38 |
| | A24 | 346 | 1114 | 2.5x10 ⁻²⁶ | 2.04 |
| | A26 | 30 | 351 | 0.0037 | 0.51 |
| | A28 | 89 | 536 | n.s. | |
| | A29 | 36 | 434 | 0.0004 | 0.03 |
| | A30 | 21 | 296 | 0.001 | 0.42 |
| | A31 | 19 | 359 | 2.3x10 ⁻⁶ | 0.03 |
| | A32 | 18 | 438 | 1.8x10 ⁻⁹ | 0.24 |
| | A33 | 25 | 35 | n.s. | |
| | Other ¹ | 25 | 35 | n.s. | |
| B | B7 | 175 | 1776 | 1.0x10 ⁻⁹ | 0.59 |
| | B8 | 395 | 1506 | 6.0x10 ⁻²¹ | 1.82 |
| | B13 | 13 | 261 | 0.0003 | 0.31 |
| | B14 | 16 | 513 | 7.0x10 ⁻¹² | 0.19 |
| | B15 | 88 | 802 | 0.013 | 0.68 |
| | B18 | 131 | 532 | 5.0x10 ⁻⁵ | 1.60 |
| | B27 | 116 | 590 | n.s. | |
| | B35 | 181 | 937 | n.s. | |
| | B39 | 68 | 218 | 9.0x10 ⁻⁶ | 2.01 |
| | B40 | 54 | 756 | 4.0x10 ⁻⁸ | 0.44 |
| | B44 | 187 | 1808 | 3.0x10 ⁻⁸ | 0.62 |
| | B50 | 15 | 120 | n.s. | |
| | B51 | 37 | 576 | 3.0x10 ⁻⁷ | 0.39 |
| | B60 | 122 | 67 | 4.0x10 ⁻⁹² | 12.20 |
| | B62 | 273 | 109 | 2.0x10 ⁻²⁴² | 18.22 |
| Other ¹ | 90 | 1819 | 4.0x10 ⁻³³ | 0.28 | |

Legend: n: number of subjects in the cross-over study per characteristic. p_c: corrected p-value. OR: odds ratio. ¹Combined characteristics including A34, A43, A66, B37, B38, B41, B45, B47, B49, B52, B53, B55, B58, B70, B75.

prised HLA alleles and serotypes for each locus. In order to improve approximation, frequencies of characteristics on serotype and allele level were merged that occurred at observed frequencies of less than 10 in at least one subject group, patients or controls. This makes the calculation robust against biases caused by small numbers. Bonferroni correction of p-values (p_c)

was applied for conservative calculation determined by the number of tests carried out per locus.

Association of class I serotypes with T1DM

The test for independence of class I serotypes between patients and controls confirmed the previously assumed high incidence of genetic variation. The overall significance for genetic variations in A and B serotypes between both groups of patients and controls was high (p < 0.0001). Among the A locus the analysis confirmed that A24 is positively associated with disease (p_c = 2.5x10⁻²⁶, Table 7), which is consistent with previous results [18, 21] and with observations of the age-related contribution to this serotype to disease onset [11, 53] and the association with complete loss of β-cell function [26]. However, in our enlarged sample, representing a heterogeneous population, A2 and A3 were also significant after correcting p values (Table 7). A2 was also found to be positively associated with the disease in a Finnish cohort (p = 0.05) from the “Childhood Diabetes in Finland” (DiMe) study, a prospective family study carried out between 1986 and 1989 [55]. Fennessey *et al.* reported an increased occurrence of A2 and A3 on the Cw-B56-DR4-DQ8 haplotype in the same Finnish population [21].

A11 was found to be decreased in patients (p_c = 9.5x10⁻¹¹, Table 7) confirming results previously reported [18, 55] but significance has been aggravated compared to these results. A28, which was decreased in Australian T1DM subjects [18], was not significant in our enlarged sample. This result corresponds to Pikäniemi *et al.* [55]. However, four other serotypes were found (A29, A30, A31 and A32) to be negatively associated with disease (Table 7). Most of these four serotypes were not conspicuous before being protective. A30 was even found to be positively associated with T1DM (p = 0.0001) on the A30-B18-DR3 haplotype [10], but this result may be due to the highly susceptible B18 and DR3 alleles (Table 7) [18, 55].

Beside B18 the susceptibility of B8, B39 and B62 in T1DM development was confirmed, as demonstrated previously [18, 50, 55]. Interestingly, a high significance for an increased occurrence of B60 in diabetic patients was also found (p_c = 4x10⁻⁹²), which was not observed in smaller samples [50, 55]. Moreover, seven serotypes in the B locus were existent with decreased frequency in patients (B7, B13, B14, B15, B40, B44 and B51, Table 7). Although allele B*1301 was observed to be decreased in patients previously, among serotypes only B7, B14 and B51 have been reported in previous studies to be protective, the other types were not found to be significantly decreased in these studies [18, 50, 55].

Table 8. HLA class I allele frequencies obtained from cross-study data

| Allele | T1DM (n) | Controls (n) | p _c | OR |
|--------------------|-------------|-----------------|-----------------------|------|
| A*0101 | 118 | 62 | 2.1x10 ⁻⁵ | 2.16 |
| A*0201 | 188 | 147 | 0.031 | 1.44 |
| A*0301 | 57 | 55 | n.s. | |
| A*1101 | 46 | 126 | 5.1x10 ⁻⁹ | 0.34 |
| A*2402 | 125 | 110 | n.s. | |
| A*2407 | 17 | 53 | 0.0002 | 0.32 |
| A*3303 | 21 | 29 | n.s. | |
| A*3401 | 20 | 51 | 0.003 | 0.39 |
| Other ¹ | 171 | 160 | n.s. | |
| B*0702 | 45 | 66 | n.s. | |
| B*0801 | 125 | 37 | 3.0x10 ⁻¹³ | 4.07 |
| B*1501 | 74 | 18 | 5.0x10 ⁻⁹ | 4.70 |
| B*1502 | 10 | 20 | n.s. | |
| B*1535 | 13 | 26 | n.s. | |
| B*1801 | 55 | 33 | n.s. | |
| B*3501 | 23 | 35 | n.s. | |
| B*3505 | 12 | 32 | 0.06 | 0.39 |
| B*3802 | 16 | 49 | 0.0011 | 0.33 |
| B*4001 | 53 | 50 | n.s. | |
| B*4002 | 15 | 37 | 0.055 | 0.42 |
| B*4402 | 40 | 30 | n.s. | |
| B*4403 | 12 | 23 | n.s. | |
| B*5801 | 23 | 29 | n.s. | |
| Other ¹ | 242 | 315 | 0.033 | 0.72 |
| C*0102 | 29 | 23 | n.s. | |
| C*0202 | 20 | 18 | n.s. | |
| C*0302 | 22 | 27 | n.s. | |
| C*0303 | 44 | 16 | 0.0018 | 2.98 |
| C*0304 | 85 | 61 | n.s. | |
| C*0401 | 57 | 89 | n.s. | |
| C*0403 | 12 | 31 | 0.075 | 0.39 |
| C*0501 | 64 | 31 | 0.0033 | 2.25 |
| C*0602 | 27 | 44 | n.s. | |
| C*0701 | 149 | 56 | 5.0x10 ⁻¹² | 3.20 |
| C*0702 | 110 | 200 | 2.0x10 ⁻⁶ | 0.50 |
| C*0801 | 26 | 41 | n.s. | |
| C*1203 | 32 | 28 | n.s. | |
| C*1502 | 11 | 41 | 0.0007 | 0.27 |
| Other ¹ | 72 | 85 | n.s. | |

Legend: n: number of subjects in the cross-over study per characteristic. p_c: corrected p-value. OR: odds ratio. ¹Combined characteristics including A*0203, A*2403, A*2601, A*2902, B*1301, B*1513, B*1521, B*2706, B*4801, B*5101, C*0704.

This suggests that even more genes within the B locus are involved in mediating protection against the disease.

Association of single class I alleles with T1DM

Table 8 shows the frequencies and results from the cross-study on single allele level. Corresponding to previous results, A*1101 is protective. A*3401 is also negatively associated with the disease, which has not been seen in smaller samples. Surprisingly, A*2407 belonging to the A24 serotype family appears to be protective in our enlarged sample. However, this result may be due to the low overall frequency of respective observations in the meta-data. In comparison, the overall frequency of A*2402 has increased after cross-over pooling of data and this allele has become insignificant, suggesting that sample enlargement had a balancing effect on possible selection biases regarding this allele. The converse effect could be observed for allele A*0101, its association with disease increased considerably after data pooling.

Two B alleles were found to be strongly associated with disease, B*0801 and B*1501 (p_c < 5x10⁻⁹, OR > 4, Table 8). While this result confirms the finding of Valdes *et al.* [12] regarding B*0801, B*1501 appeared to be significant only in the much larger cross-sample. The frequency amounting to (T1DM/controls) = (4/20) in the Filipino population (Table 4) [51] is too small to achieve conventional statistical power, which may explain the different results and emphasize the relevance of both alleles in mediating islet destruction by B8- and B15-restricted CTLs. Three B alleles, B*3505, B*3802 and B*4002, appeared protective for the first time in the cross-over sample. However, this result may be attributed to the frequency of the occurrences that remain small even after data pooling.

The high susceptibility of alleles C*0303 and C*0701 was confirmed in the study (Table 8). In addition, a third allele, C*0501, was identified to be positively associated with the disease. C*0702 was negatively associated analogously to the findings by others [12, 51], but the association appeared much more significant in the large sample analyzed here. C*0403 and C*1502 are also significantly decreased in the patient group, but the occurrence of these alleles is still rare.

Discussion

There are only few studies which hitherto analyzed the association of HLA class I genes with T1DM [12, 18, 26, 50, 51, 54, 55]. Most of them were only recently carried out following the growing insight that class I

genes may indeed be essentially involved in the clinicopathology of autoimmune diabetes. Apparently, the more recently a study is conducted, the larger the number of identified alleles associated with the disease (Table 6). However, these studies basically lack statistical power due to the small size of the samples analyzed. Several characteristics existed at a frequency of smaller than ten and these frequencies were used to carry out separate significance tests, which must be regarded as undependable and unbalanced (e.g. Bugawan *et al.* analyzed 41 HLA-B alleles in separate significance tests including 30 with a frequency of 0-8 just amounting 0-2% of overall appearance). Considering such small numbers would require the application of a simulation-based model, as applied in Pitkäniemi *et al.* [55]. Therefore in this study, the samples were enhanced by pooling separate samples and matching data exactly on serotype and allele level in order to reduce type 1 error accumulation. The larger sample is more robust against biases due to data selection while it revealed significant associations of several serotypes and alleles with T1DM.

The results presented in this enlarged sample revealed new hitherto unknown associations between HLA class I alleles and autoimmune diabetes. However, they should still be regarded as preliminary since many alleles occurred infrequently even after combining data of the seven existing studies. A larger sample would provide more power to identify markers more reliably. It would thus be instructive to carry out further studies. An alternative approach would be to test the data in a simulation model in order to obtain unbiased estimates.

Conclusion

In the process of preclinical T1DM conversion to overt clinical disease, it is commonly assumed that the

final destruction of pancreatic β -cells is mediated by the occurrence of diabetogenic CD8⁺ T cells. Recent linkage analysis has revealed that HLA class I alleles may contribute decisively to T1DM development dependently as well as independently of LD with other HLA class II alleles or haplotypes. Progression of disease seems to be largely influenced by alleles of the A24 and B18 superfamily [18]. A24 alleles were also observed at significantly increased frequency to participate in complete loss of β -cell function and disease onset at early ages [11, 26, 52]. The results of this cross-study obtained from an enlarged heterogeneous sample, suggested that only A*2407 is associated with T1DM but not A*2402. Family-based studies carried out to identify class I alleles which are preferentially transmitted to healthy family members have revealed that such heterogeneity applies for several alleles, such as those of the A11, A28 and B7 subgroups. Generally, this analysis clearly reveals that many serotype families may be involved in the clinicopathology of T1DM, but that a more detailed review is necessary to map detailed allele susceptibilities. Those studies, aimed at the location of class I-restricted disease markers, were only recently carried out. More studies are necessary to test genetic dependencies on the basis of larger samples which would increase statistical power. An accurate definition of disease susceptible alleles will improve our understanding of antigen presentation mechanisms prevailing in the etiology of autoimmune diabetes. This knowledge is necessary for the design of improved immune intervention strategies to halt T1DM progression in patients at risk of developing the disease or those who are already suffering from it.

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