Pediatric Endocrinology Diabetes and Metabolism
Endokrynologia, Diabetologia i Choroby Przemiany Materii Wieku Rozwojowego

Original paper | Praca oryginalna

Pediatr Endocrinol Diabetes Metab 2020; 26 (2): 73–78
DOI: https://doi.org/10.5114/pedm.2020.95617

HLA-A gene variation modulates residual function of the pancreatic β-cells in children with type 1 diabetes

Zmienność genu *HLA-A* wpływa na resztkową insulinosekrecję u dzieci chorych na cukrzycę typu 1

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Abstract

Aim of the study: The study aimed to analyze an association between the *HLA-A* gene variation and a risk of type 1 diabetes development and to evaluate the association of HLA class I and class II alleles with β-cell destruction.

Material and methods: A group of 108 children with type 1 diabetes were genotyped in *HLA-A*, *-DRB1*, and *-DQB1* genes using hybridization with oligonucleotides probes. Plasma C-peptide concentration was assessed by radioimmunoassay method.

Results: No differences in allele *HLA-A* distribution between type 1 diabetes patients and healthy individuals were found. Among "low C-peptide"(< 0.28 pmol/ml) individuals, the frequency of HLA-A*02 allele was 41.3%, whereas only one HLA-A*26 allele was detected in this group (0.7%). Conversely, among "high C-peptide"(≥ 0.28 pmol/ml) probands the prevalence of *A*02* allele was 19.7% (Pc = 0.008, OR = 1.4, 95% CI: 1.2–1.7) and A*26 10.5 % (Pc < 0.007, OR = 0.15, 95% CI: 0.02–0.9). Genotype analysis showed that *A*02/*02* and *A*02/X* children were more likely to have "low" C-peptide at the onset compared to those with *non-A*02/non-A*02* genotype (p = 0.008, OR = 1.6, 95% CI: 1.3–2.0 and p = 0.015, OR = 1.4, 95% CI: 1.1–1.9, respectively). A02 phenotype individuals had lower median C-peptide (0.17 pmol/ml) than non-A02 patients (0.26 pmol/ml, p = 0.008). Median C-peptide was higher in the A26-positive group comparing to A26-negative (0.40 and 0.20, respectively, p = 0.04). No association between HLA class II and C-peptide levels was observed.

Conclusions: HLA-A alleles are not associated with disease development nevertheless strongly influence a residual pancreatic β-cell function. The results suggest a different role of *HLA* class I and class II in type 1 diabetes pathogenesis.

Key words: type 1 diabetes, HLA-A HLA-DR, HLA-DQ, C-peptide, genetic predisposition, disease progression, β-cell destruction.

Streszczenie

Cel pracy: Celem pracy była analiza związku między zmiennością genu *HLA-A* a ryzykiem rozwoju cukrzycy typu 1 oraz analiza wpływu obecności odpowiednich alleli HLA klasy I i II na szczątkowe wydzielanie insuliny.

Materiał i metody: Badaniem objętych zostało 108 dzieci chorujących na cukrzycę typu 1, u których wykonano analizę genów *HLA-A*, *-DRB1*, and *-DQB1* z użyciem sond oligonukleotydowych. Stężenie C-peptydu w osoczu oznaczono metodą radioimmunologiczną.

Wyniki: Nie stwierdzono różnic w rozkładzie alleli *HLA-A* między dziećmi z cukrzycą typu 1 a grupą kontrolną. W grupie z „niskim C-peptydem" (< 0,28 pmol/ml) częstość występowania allelu *HLA-A*02* wynosiła 41,3%, a allelu *HLA-A*26* – 0,7%. Odwrotna sytuacja była w przypadku grupy z „wysokim C-peptydem" (≥ 0,28 pmol/ml), gdzie częstość występowania allelu *A*02* wynosiła 19,7% (Pc = 0,008, OR = 1,4, 95% CI: 1,2–1,7), a allelu A*26 – 10,5% (Pc < 0,007, OR = 0,15, 95% CI: 0,02–0,9). Analiza genotypów wykazała, że dzieci z *A*02/*02* i *A*02/X* miały częściej „niski C-peptyd" na początku choroby w porównaniu z dziećmi *non-A*02/non-A*02* (odpowiednio: p = 0,008, OR = 1,6, 95% CI: 1,3–2,0 i p = 0,015, OR = 1,4, 95% CI: 1,1–1,9). Fenotyp A02 charakteryzował się niższą medianą stężenia C-peptydu (0,17 pmol/ml) w porównaniu z pacjentami z genotypem non-A02 (0,26 pmol/ml, p = 0,008). Mediana C-peptydu była wyższa w grupie A26-dodatniej w porównaniu z grupą A26-negatywną (0,40 *vs* 0,20, p = 0,04). Nie stwierdzono zależności między allelami *HLA* klasy II i stężeniem C-peptydu.

Wnioski: Allele genu *HLA-A* nie są związane z rozwojem choroby, jednak wpływają na szczątkowe wydzielanie insuliny. Wyniki te sugerują inną rolę genów HLA klasy I i II w patomechanizmie cukrzycy typu 1.

Słowa kluczowe: cukrzyca typu 1, HLA-A, HLA-DR, HLA-DQ, C-peptyd, predyspozycja genetyczna, progresja choroby, destrukcja komórek β.

Received: 7.01.2020
Accepted: 5.02.2020
Conflict of interests: none declared.
This study was supported by grants from the Polish State Committee for Scientific Research No 4PO5E 05516 and grant from the Medical University of Lodz.

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Introduction

Type 1 diabetes is an autoimmune T-cell-mediated disorder resulting from selective destruction of pancreatic β-cells and progressive decline in endogenous insulin secretion capacity. Both genetic and environmental factors are involved in disease process. Despite recent progress in our knowledge about the disease, cellular, and molecular events associated with the initiation and progression of the autoimmune reaction against -cells remain poorly understood [1].

In humans, as well as in animal models, type 1 diabetes has a multigenic basis. The Major Histocompatibility Complex (MHC) region located on the short arm of chromosome 6 contains the major genes predisposing to type 1 diabetes [2–4]. The HLA region accounts for approximately 50% heritability of T1DM [2].

Association of type 1 diabetes with HLA class I genes was first observed. However, higher relative risk associated with HLA class II DR/DQ compared to HLA-A and -B genes has led to the conclusion that the primary *locus* of susceptibility to type 1 diabetes is located within the class II region. In Caucasians, predisposition to type 1 diabetes is mostly associated with the DRB1*03-DQB1*0201 and/or DRB1*04-DQB1*0302 haplotypes, while the DRB1*15-DQB1*0602 haplotype confers a strong protection from the disease [4].

In our previous study of a Polish population, the highest risk for disease development was conferred by the DRB1*04-DQB1*0302 haplotype [5]. These genes, however, cannot completely explain the association between HLA and T1D development. Significant type 1 diabetes associations were observed at all class I HLA *loci* indicating that HLA class I alleles, in addition to and independently from HLA class II alleles, are associated with type 1 diabetes [6].

Recently, the well-established dogma about the origins of insulitis has changed as the solid evidence from histopathological studies such as DiViD or nPOD (7) confirmed that islet cell upregulation of HLA-I expression is a genuine pathological feature in type 1 diabetes in humans [8]. This "hyperexpression" of HLA-I antigens is critical for early disease progression, promoting the effective engagement of influent CD8+ cytotoxic T cells specific to defined islet antigen [8–10]. It was also discovered that islet cell hyperexpression of HLA-I can persist, at the protein level, beyond the initial phases of the disease [9]. This indicates the importance of some HLA class I alleles in T1D susceptibility and raises essential questions about the role of this phenomenon in disease progression and clinical presentation of the disease in humans.

A present study aimed to analyze the *HLA-A* gene association with a genetic predisposition to type 1 diabetes development and to evaluate the association of HLA class I and class II alleles with β-cell destruction.

Material and methods

Patients

The study was conducted in a group of 108 unrelated type 1 diabetes children (51 girls and 57 boys) admitted to the Department of Pediatrics, Medical University of Lodz in Poland for initial treatment of type 1 diabetes. Their median age was 10.5 years (range: 1.0–17.0 years). All children were hyperglycaemic at the time of blood sampling. Blood glucose value ranged 160–998 mg/dl (mean 433 ±112 mg/dl).

The control group consisted of 92 unrelated, healthy blood donors from Central Poland.

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Bioethics Committee of Medical University of Lodz.

HLA typing

All subjects were typed for *HLA-A*, *HLA-DRB1*, and *-DQB1* genes. Genomic DNA was extracted from peripheral blood cells using a conventional salting-out procedure. Genotyping was performed using hybridization with sequence-specific oligonucleotide probes following amplification of the corresponding gene by the PCR with 52 probes that were used for *HLA-A* typing, 25 for *DRB1* and 24 for *DQB1* as described previously [5].

Assessment of residual b-cell function in type 1 diabetes patients

The residual β-cell function at the clinical onset of the disease ("day 0") was assessed by analysis of plasma C-peptide concentration in all patients. Blood samples were drawn before the first dose of insulin and after ten days of insulin therapy ("day 10"). Plasma C-peptide level was estimated by the radioimmunoassay method (CIS-BioInternational, France).

Statistical analysis

Yates' corrected chi^2 or Fisher's exact tests were used to compute-two-tailed *p* values, which were considered significant if less than 0.05. The power of association with the predisposition and clinical parameters of the disease with 95% confidence intervals (95% CI) was calculated using odds ratios (OR) according to Woolf's formula.

An association between age, C-peptide concentration, and genetic factors was estimated by a t-test or Mann-Whitney U test according to the normality of data set. Comparison between "day 0" and "day 10" C-peptide levels was performed using the Wilcoxon signed-rank test. Correlation between C-peptide levels was assessed using the Spearman correlation test.

Pc indicates a *p*-value corrected by use of the Bonferonni inequality method, by multiplying p by the number of alleles compared.

Results

Association of HLA genes with type 1 diabetes

The distribution of *HLA-A* alleles among healthy controls corresponds to expected frequencies in the Caucasian population (Table I). 14 *HLA-A* alleles were found in our population. The most frequent allele in both control and type 1 diabetes groups

Table I. *HLA-A* allele frequencies in type 1 diabetes children and healthy controls. *Pc* value calculated with Fisher exact test with Bonferonni correction was more than 0.05 suggesting a lack of association of this gene with a predisposition to type 1 diabetes

HLA-A allele	T1D children *N* = 216 (%)	Control group *N* = 184 (%)
*01	26 (12)	24 (13)
*02	72 (33.3)	62 (33.7)
*03	19 (8.8)	32 (17.4)
*11	16 (7.4)	14 (7.6)
*23	2 (0.9)	1 (0.5)
*24	16 (17.4)	15 (8.2)
*25	27 (12.5)	10 (5.4)
*26	9 (4.2)	7 (3.8)
*29	5 (2.3)	0
*30	6 (2.8)	6 (3.3)
*31	3 (1.4)	3 (1.6)
*32	5 (2.3)	6 (3.3)
*33	2 (0.9)	1 (0.5)
*68	8 (3.7)	0
*69	0	1 (0.5)

N – number of alleles

Table II. *HLA-A* allele distribution in "high" and "low" C-peptide diabetic children. *Pc* value calculated with Fisher exact test with Bonferonni correction

HLA-A allele	C-peptide concentration [*N* (%)]		*Pc*	OR	95% CI
	< 0.28 [pmol/ml]	≥ 0.28 [pmol/ml]			
*01	17 (12.3)	10 (13.2)			
*02	57 (41.3)	15 (19.7)	< 0.01	1.4	1.2–1.7
*03	11 (8.0)	8 (10.5)			
*11	9 (6.5)	6 (7.9)			
*23	1 (0.7)	1 (1.3)			
*24	13 (9.4)	3 (3.9)			
*25	15 (10.9)	13 (17.1)			
*26	1 (0.7)	8 (10.5)	< 0.01	0.15	0.02–0.9
*29	2 (1.4)	3 (3.9)			
*30	4 (2.9)	1 (1.3)			
*31	1 (0.7)	2 (2.6)			
*32	4 (2.9)	1 (1.3)			
*33	1 (0.7)	1 (1.3)			
*68	4 (2.9)	4 (5.3)			

N – number of alleles; *OR* – relative risk; 95% CI – confidence interval

was *A*02* (33.2% and 33.7%, respectively). The second most frequent allele was *A*03* in controls (17.4%) and *A*25* in patients (12.0%). However, these differences were not statistically significant. The distribution of *HLA-DRB1* and *DQB1* alleles in our Polish population has previously been described [5].

Residual β-cell function in diabetic subjects

Evaluation of the residual β-cell function was assessed by plasma C-peptide concentration at the clinical onset of the disease. A strong positive correlation between serum C-peptide levels at "day 0" and "day 10" ($r = 0.71, p < 10^{-5}$) was found, with a median concentration of 0.21 pmol/ml at day 0 and 0.19 at day 10 ($p = 0.0004$). This observation confirmed that this parameter is useful for evaluation of β cell function at disease-onset.

The C-peptide level was analyzed in patients by comparison with the lowest value of the normal range in healthy individuals (0.28 pmol/ml). Among diabetic children, 35% had C-peptide above this level at the time of diagnosis. Median C-peptide concentration in this "high" C-peptide group was significantly higher than in the "low" C-peptide group (0.47 pmol/ml, range: 0.36–0.79 and 0.16 pmol/ml, range: 0.11–0.21, respectively, $p = 0.006$). The potential relationship between age and C-peptide concentration was investigated using the Spearman correlation test. Older children had a higher C-peptide level at the clinical onset ($r = 0.4, p < 10–5$).

To investigate the effect of HLA genes on beta-cell destruction, we analyzed the distribution of *HLA-A*, *-DR*, and *-DQ* alleles among "high" and "low" C-peptide diabetic children. No difference in *DRB1* or *DQB1* allele frequency was observed (data not shown). By contrast, analysis of *HLA-A* allele distribution revealed significant differences (Table II). Among "low C-peptide" individuals, *HLA-A*02* frequency was 41.3 %, compared to 19.7 % among "high C-peptide" patients ($Pc = 0.008$, OR = 1.4, 95% CI: 1.2–1.7). Conversely, the *HLA-A*26* allele was only detected once in the "high C peptide" group (0.7 %) compared to a frequency of 10.5 % in the other group ($Pc < 0.007$,

OR = 0.15, 95% CI: 0.02–0.9). Moreover, *HLA-A*02/*02* and *A*02/X* children were more likely to have "low" C-peptide value at disease onset compared to those with a non-A*02/non-A*02 genotype (p = 0.008, OR = 1.6, 95% CI: 1.3–2.0 and p = 0.015, OR = 1.4, 95% CI: 1.1–1.9, respectively). Analysis of C-peptide concentration according to HLA-A phenotype showed that median C-peptide levels at day 0 were significantly lower in A02 phenotype patients than in non-A02 (0.17 pmol/ml, range: 0.12–0.29, and 0.26 pmol/ml, range: 0.17–0.45, respectively; p = 0.008). Conversely, the C-peptide level was higher in *A*26*-positive compared to *A*26*–negative patients (median: 0.40 and 0.20, respectively, p = 0.04) (Table III). Similar results were obtained for genotype analysis (Table IV). Children with *A*02/*02* genotype had significantly lower C-peptide median (0.20 pmol/ml, range: 0.14–0.24) compared to those with *non-*02/non-*02* genotype (0.26 pmol/ml, range: 0.17–0.45, p = 0.008). In one patient A*26/*26 homozygote C-peptide level was 0.48 pmol/ml. In another patient,

*A*02/A*26* heterozygote, C-peptide concentration was 0.78 pmol/ml – far above the minimal value in healthy individuals.

Table III. C-peptide concentration in type 1 diabetic children with different *HLA-A* phenotypes. *Pc* value calculated with the chi^2 test

HLA-A phenotype	C-peptide concentration [*N*] quartile range				*Pc*
	N	median	*25%*	75%	
02	56	0.17	0.12	0.29	< 0.02
non02	52	0.26	0.17	0.45	
26	8	0.40	0.30	0.80	< 0.05
non26	100	0.20	0.13	0.36	

N – number of patients

Table IV. Association of *HLA-A* genotypes with C-peptide level at the onset of type 1 diabetes. Pc value calculated with Fisher exact test with Bonferonni correction

HLA-A genotype	C-peptide concentration [*N*]		*Pc*	OR	95% CI
	< 0.28 [pmol/ml]	≥ 0.28 [pmol/ml]			
*02/X	42	14	< 0.02	1.4	1.1–1.9
*02/*02	15	1	< 0.006	1.6	1.3–2.0
*26/X	1	7	< 0.003	0.2	0.03–1.0

N – number of children; RR – relative risk; 95% CI – confidence interval

Discussion

Particular HLA class II haplotypes DRB1*04/DQA1*03/DQB1*03:02 have long been related to the risk of developing type 1 diabetes and/or severity of insulin deficiency in type 1 diabetes [11]. We have now investigated whether an HLA class I gene can modulate the presentation of type 1 diabetes. Our results indicate that some *HLA-A* alleles can affect β-cell function at the clinical onset of the disease. *HLA-A*26* phenotype was associated with higher, and *A*02* with lower C-peptide levels at disease onset. The strongest β-cell destruction was observed in *A*02/*02* patients, whereas *non-A*02/non-A*02* genotype was associated with the highest insulin secretion at disease-onset.

This finding is especially intriguing in the light of recent data showing that HLA class I alleles have a crucial role in insulitis. In the setting of type 1 diabetes (T1D), insulitis lesions are enriched for CD8+ T cells, which are held as the final mediators of islet destruction [8]. Interestingly, nearly all beta cell peptides identified as the antigenic targets of CD8+ T cells in type 1 diabetes patients (eg. native proteins, epitopes of proinsulin, glutamic acid decarboxylase (GAD), insulinoma-associated protein-2 (IA-2), and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), a highly immunoprevalent zinc transporter 8), are recognized in the context of HLA-A*0201 [12]. Moreover, hyperexpression of HLA class I on beta cells in T1D is associated with interferon response seen in tissues infected by viruses [13].

According to the molecular mimicry hypothesis, viral proteins could share a particular sequence with beta-cell proteins (examples: coxsackie and GAD [14], the rubella virus and GAD [15], rotavirus and IA-2 [16]. This model would at least partially explain the role of environmental factors in the pathogenesis of the disease.

Furthermore, CD4 helper T cells infiltrating pancreatic islets can enhance CTL-mediated destruction of the islets [17, 18]. The modulating effect of HLA class I alleles (HLA-A*02 and HLA-A*26) could modify antigen presentation to CTL and thus affect the rapidity of disease progression.

Another interesting hypothesis emerges from the studies of the thymus. Incomplete central tolerance mechanisms allow the survival of an islet reactive CD8+ T cell repertoire, which can be primed in the presence of defective peripheral immunoregulation and/or a proinflammatory islet microenvironment to progress toward T1D.

The thymic presentation of self-antigens to T cells also occurs via HLA class I molecules. Therefore it is reasonable to think that specific T1D-predisposing HLA class I alleles expressed in the thymus may contribute to insufficient thymic presentation of autoantigens to T cells [19, 20]. It has been shown

by Bulek *et al.* that weak interactions between a preproinsulin peptide and HLA-A2 lead to suboptimal presentation to the TCR of responding CD8+ T-cells, which may more easily survive thymic selection [21].

The influence of *HLA-A* alleles on C-peptide concentration has also been observed in the Japanese population. Nakanishi reported an association between the presence of *A*24* and complete β-cell destruction [22].

Interestingly, in the Japanese as well as in our Polish population, the most frequent *HLA-A* allele is the one conferring accelerated β-cell destruction. This finding may support the alternative hypothesis that autoimmune diseases are a side effect of a natural selection process of HLA alleles that confer survival advantage by more efficient protection from infectious diseases [23].

In our study, *HLA-A* allele distribution did not show any difference between diabetic and control subjects, suggesting that this *locus* has no direct predisposing effect on type 1 diabetes development. This observation may result from the limited number of the patients studied. Honeyman and colleagues, based on analysis of families at risk for type 1 diabetes, showed an increased frequency of HLA-A*24 in relatives who developed diabetes compared to those who did not [24]. Kobayashi *et al.* also found a significant association between the presence of HLA-A*24 and Bw*54 alleles and the childhood-onset of the disease in Japanese patients [25].

More recently, significant type 1 diabetes associations were observed at all class I HLA *loci* indicating that HLA class I alleles, in addition to and independently from HLA class II alleles, are associated with type 1 diabetes [6]. Among predisposing alleles are B*5701, B*3906, A*2402, A*0201, B*1801, C*0501, while A*1101, A*3201, A*6601, B*0702, B*4403, B*3502, C*1601, and C*0401 seem to be protective. Some alleles, notably B*3906, appear to modulate the risk of all DRB1-DQA1-DQB1 haplotypes on which they reside, suggesting a class I effect that is independent of class II. Other class I, type 1 diabetes associations appear to be specific to individual class II haplotypes. Some apparent associations (e.g., C*1601) could be attributed to strong LD to another class I susceptibility *locus* (B*4403). A combination of HLA-A24, -DQA1*03, and -DR9 contributes to the acute-onset and early complete beta-cell destruction, whereas HLA-DR2 has a protective effect against complete beta-cell loss in type 1 diabetes [26]. These data indicate that HLA class I alleles, in addition to and independently from HLA class II alleles, are associated with type 1 diabetes [6].

In type 1 diabetes (T1D), autoreactive cytotoxic CD8+ T cells are implicated in the destruction of insulin-producing β-cells. The HLA-B*3906 and HLA-A*2402 class I genes confer increased risk and promote early disease onset, suggesting that CD8+ T cells that recognize peptides presented by class I molecules on pancreatic β-cells play a pivotal role in the autoimmune response [27]. Moreover, β-cell expression of class II molecules suggests that β-cells may interact directly with islet-infiltrating CD4+ T cells and may play an immunopathogenic role [28]. In our study, no significant difference was observed according to the age of patients with different genotypes. Furthermore, higher C-peptide levels were observed in older healthy children suggesting that insulin secretion level is age-dependant.

The association of some *HLA-A* alleles with β-cell damage raises a question about the possible participation of this gene in the development of diabetic complications. Indeed, in the Japanese population, *A*24* is associated with early development of diabetic retinopathy. As discussed above, *A*24* predisposes to β-cell destruction, hence to deprivation of endogenous C-peptide. On the other hand, C-peptide is thought to have a beneficial effect on some clinical parameters. There are pieces of evidence that diabetic patients with residual β-cell function are less prone to the development of diabetic microangiopathy [29], nephropathy [29], and neuropathy [30]. These biological effects do not depend on insulin level and do not influence patients' glucose blood levels [31]. In our study, 35% of children had normal C-peptide level at disease-onset. According to our results, this group would represent patients with low β-cell destruction or high insulin secretion capacity. Therefore, one could expect some clinical consequences, such as lower progression of the disease, lower initial insulin requirement, and fewer diabetic complications than patients with significantly lower plasma C-peptide at diagnosis. Confirming this hypothesis would require long follow-up and/or analysis of patients with the adult form of autoimmune diabetes (LADA).

In conclusion, our results suggest a different role of HLA class I and class II alleles in type 1 diabetes pathogenesis. *DRB1* and *DQB1* genes are involved in predisposition to the disease but play probably a minor role in β-cell damage. By contrast, specific *HLA-A* alleles influence a mechanism of pancreatic β-cell destruction. Such observation may be considered in trials evaluating treatments to maintain some β-cell function and induce remission at (or after) disease onset.

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