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Increased risk of type 1 diabetes in Polish children — association with INS-IGF2 5′VNTR and lack of association with HLA haplotype

Zwiększenie ryzyka cukrzycy typu 1 u dzieci w populacji polskiej — zależność od regionu 5′VNTR INS-IGF2 oraz brak zależności od haplotypu HLA

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Abstract

Background: Human leukocyte antigens (HLA) complex and INS-IGF2 5′VNTR loci are principal determinants of the risk of type 1 diabetes mellitus (T1DM). Carriage of class III allele is protective, while class I/I homozygosity increases the risk of T1DM.

Material and methods: HLA and 5′VNTR allele frequencies were summarised and multivariate logistic regression models with interaction evaluation were employed to determine the presence and types of allele effect interdependency. The study group was planned to number 590 children who would undergo genotyping of 5′VNTR and HLA.

Results: 590 patients (302 with T1DM and 288 controls) were recruited. Frequencies of HLA risk alleles were: 117 carriers of DR3-DQ2; 130 carriers of DR4-DQ8 including 43 DR3-DQ2/DR4-DQ8 heterozygotes. In all cases, risk alleles were vastly overrepresented in the T1DM group compared to the controls ($p < 0.0001$ in all cases). The most frequent protective haplotype was DQB1 × 0602 observed in 24 controls and two T1DM cases ($p < 0.001$). Class I 5′VNTR homozygotes constituted 58% of the control group (n = 174) and 78% (n = 224) of T1DM patients [OR = 2.63 (95% CI: 1.79–3.57)]. Interactions between 5′VNTR and DR3-DQ2 or DR4-DQ8 variants did not reach statistical significance for risk of developing T1DM (p = 0.54 and 0.24) or age at its diagnosis (p = 0.14 and 0.67 respectively).

Conclusions: Interactions between HLA and 5′VNTR genotype are not of multiplicative character. Class I homozygosity at 5′VNTR is a significant risk factor of T1DM and acts independently from HLA haplotype in determining the actual risk of diabetes in children.
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Key words: *autoimmunity, human leukocyte antigens, proinsulin gene*

Streszczenie

Wstęp: Układ HLA oraz locus 5′VNTR INS-IGF2 uznaje się za główne determinanty genetyczne cukrzycy typu 1 (T1DM). Nosicielstwo allela klasy III 5′VNTR uznaje się za czynnik ochronny, a homozygotyczność klasy I/I jest silnym czynnikiem predysponującym do wystąpienia T1DM. W niniejszej pracy przeanalizowano interakcje genetyczne pomiędzy HLA a 5′VNTR INS w szacowaniu ryzyka T1DM lub wieku zachorowania.

Materiał i metody: Efekt częstości alleli HLA i 5′VNTR przeanalizowano za pomocą modeli wieloczynnikowej regresji logistycznej uwzględniającej interakcje czynników. Liczebność grupy badanej zaplanowano na 590 dzieci. Wszystkich pacjentów planowano poddać genotypowaniu 5′VNTR INS i HLA.

Wyniki: Wśród 590 pacjentów włączonych do badania 302 chorowało na T1DM, a 288 stanowiło grupę kontrolną. Częstości haplotypów HLA ryzyka wynosiły: 117 DR-DQ2; 130 DR4-DQ8 (w tym 43 heterozygoty DR3-DQ2/DR4-DQ8). Stwierdzono silny związek genetyczny między tymi haplotypami a predyspozycją do cukrzycy ($p < 0,0001$). Najczęściej występującym allelem ochronnym był DQB1*0602, wykryty u 24 osób z grupy kontrolnej i u 2 z grupy T1DM ($p < 0,0001$). Homozygoty klasy I stanowiły 58% grupy kontrolnej (n = 174) oraz 78% grupy badanej (n = 224; OR: 2,63; 95% CI: 1,79–3,57). Interakcje pomiędzy 5′VNTR a wariantami DR3-DQ2 lub DR4-DQ8 nie były istotne statystycznie pod względem ryzyka T1DM (p = 0,54 i 0,24), ani też pod względem wieku zachorowania na T1DM (p = 0,14 i p = 0,64).

Wnioski: Interakcje pomiędzy HLA a genotypem 5′VNTR nie mają charakteru warunkowego. Oznacza to, że homozygotyczność klasy I 5′VNTR jest niezależną od HLA determinantą genetyczną T1DM u dzieci. **(Endokrynol Pol 2011; 62 (5): 436–442)**

Słowa kluczowe: *choroby autoimmunologiczne, układ HLA, gen proinsuliny*

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Introduction

Type 1 diabetes mellitus (T1DM) is a disease of undeniable autoimmunologic background, influenced primarily by genes of the HLA complex. However, other genomic loci also influence the overall risk for developing autoimmunity towards the beta cells [1]. One of these regions is the proinsulin-insulin-like growth factor 2 (*INS-IGF2*) promoter (5') site, which contains a variable number of tandem reapeats (VNTR) [2–5]. The number of repeats in white Caucasians is limited to two classes: class I (consisting of 26–63 repeats) or class III (consisting of 140–210 repeats) [6]. Both classes are associated with distinct functional capabilities of the *INS-IGF2* promoter region. Class I leads to reduced expression of proinsulin (*INS*) in the beta cells and the thymus — which in turn impairs tolerance towards own beta cells [7–10]. On the other hand, class III is protective against T1DM but, as it leads to overexpression of *INS*, it has been reported to increase the risk of type 2 diabetes, insulin resistance and obesity [6, 11, 12]. The relation between HLA and *INS* has been investigated in a Jewish population of various origins [13], patients of the Scandinavian Childhood Diabetes Study Group [14] and in adult Czech patients with late onset type 1 diabetes [15]. As the HLA haplotypes distributions in both studied populations deviated from those observed within the Polish population [16], we decided to investigate HLA and *INS* interaction and determine the type of relation between these two major factors determining autoimmunity in T1DM.

The aim of this study was to evaluate the potential for gene-gene interaction between the 5'VNTR region of the *INS-IGF2* promoter and HLA haplotypes in children with T1DM and healthy controls and to establish the risk of autoimmune diabetes associated independently with these loci.

Material and methods

The study received the approval of the Bioethics Committee of the Medical University of Lodz. Prior to inclusion, informed consent forms were obtained from parents and the patients themselves. The study group was intended to be composed of children with at least one sibling affected with T1DM. All patients enrolled in the study had to be positive at onset for at least one autoantibody specific for T1DM (ICA, anti-GAD, IAA and/or anti-IA2). Two aliquots of 2 mL of venous blood were obtained from both patients and healthy controls (non-diabetic blood donors aged 45 ± 5.1 years) into EDTA-containing test tubes and stored at –20°C.

Both study groups came from the same geographic region of central Poland and were of Slavic origin.

DNA extraction and 5'VNTR genotyping

DNA was isolated using genomic DNA extraction kit — Genomic Midi AX (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. The polymorphic site subjected to genotyping was dictated according to the HapMap database and preexisting information of complete linkage of the A allele at the –23HphI site (rs689) with class III allele [17].

Amplification of the studied fragment for subsequent digestion and genotyping was performed using polymerase chain reaction (PCR) with a pair of oligonucleotide primers spanning the region of the first exon of *INS* and its flanking sequence — the 5'UTR. The primers were expected to yield a product of 782 base pair length. The PCR reaction was conducted using the following reagents: Taq polymerase (TibMolbiol, Berlin, Germany) — 0.25 mL, MgCl2 containing buffer of 10 mM/mL concentration (Qiagen, Hilden, Germany) — 2.5 mL; genomic DNA sample of a concentration equal to 100 ng/mL — 5 mL; a deoxynucleotide mix of 4 mmol/mL concentration — 2 mL and forward and reverse primers — 0.5 mL each, with 14.5 mL of water added as reaction environment. The total volume in which the reaction was conducted equalled 25 mL. Cycling parameters were set as follows: 15 minutes at 95°C of initial denaturation, then 35 cycles at: 94°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec, followed by 10 minutes at 72°C of final elongation. The presence and quality of the amplified product was verified by agarose gel electrophoresis (Sigma, St. Louis, MO, USA). The PCR product was subjected to digestion using HphI restrictive enzyme, which was expected to cut the 782 bp product into three fragments of lengths of 321, 230 and 231 bp. If the polymorphic allele was present, and additional restriction site of a sequence GGTGA instead of GGTGT occured and was recognized by the applied restriction enzyme. The resulting cutting site generated an additional product of 40 bp length, derived from the longest band of 321 bp. The restriction enzyme reaction was run under the following conditions: PCR reaction product — 8 mL; distilled water — 9.5 mL; HphI enzyme (Fermentas, Burlington, Ontario, Canada) — 0.5 mL and 2 mL of appropriate reaction buffer (Fermentas). The reaction mix was incubated overnight at 37°C, and followed by 2% agarose gel electrophoresis.

HLA genotyping

Genotyping of the DRB1, DQA1 and DRB1 polymorphisms was performed by means of allele-specific probe hybridisation. The second exons of abovementioned genes were amplified using PCR reaction with specific oligonucleotide primers. Their sequences are listed in

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Table I. *Primer sequences for respective HLA genes*
Tabela I. *Sekwencje primerów dla poszczególnych genów HLA*

Gene	Primer sequence	
DRB1	DRB-GH4	5′-CCGGATCCTTCGTGTCCCCACAGCACG-3′
	2DRBAMP-B	5′-CCGCTGCACTGTGAAGCTCT-3′
DQA1	2DQAAMP-A	5′-ATGGTGTAAACTTGTACCAGT-3′
	2DQAAMP-B	5′-TTGGTAGCAGCGGTAGAGTT-3′
DQB1	2DQBAMP-A	5′-CATGTGCTACTTCACCAACGG-3′
	2DQBAMP-B	5′-CTGGTAGTTGTGTCTGCACAC-3′
INS	INS-F	5′-CCCACCCTCTGATGTATCTCG-3′
	INS-R	5′-CCCAGCCATCTGCCGAC-3′

Table I. PCR reaction was conducted in a volume of 100 mL containing 0.1–0.5 mg of genomic DNA, 8 mM DTP, 2–3 mM of MgCl2, 2 units of Taq DNA polymerase and adequate reaction buffer. Reaction conditions were set as: 10 minutes of initial denaturation at 95°C followed by 35 cycles of (1 min at 94°C — 1 min 56–58°C — 1 min at 72°C) and finally 10 minutes at 72°C of final elongation. Length and quality of amplified products was confirmed by 1.4% agarose gel electrophoresis. Following amplification, the DNA products were transferred onto a Hybond N+ nitrocellulose membrane (Amersham Biosciences, USA) using DotBlot Apparatus (Bio-Rad, USA). After transfer, the DNA was denaturated using 0.4 M NaOH and incubated for two hours at 80°C. Following that stage, the membranes were hybridised with site-specific oligonucleotide probes labelled with digoxygenin (Boerhinger–Mannheim, Germany). Probe detection was performed using terminal transferase activity which attached digoxygenin-11-2`,3`-dideoxiuridin-5`-triphosphate to the 3′ end of the probes. Overall, 25 probes for the *DRB1*, 18 for *DQA1* and 24 for *DQB1* loci were used. Hybridisation was performed at 54°C over 12 hours in 50 mL of total volume, containing a specific reaction buffer, 0.1% SDS; 0.1% Ficoll 400; 0.1% fraction V of BSA; 100 mg/mL of Salmon's sperm and distilled water.

After hybridisation, the membranes were eluted using a solution containing antidigoxygenin antibodies labelled with alkaline phosphatase (Roche, Germany) and incubated for one hour. Afterwards, the membranes were transferred to a solution containing lumigen – a substrate for alkaline phosphatase (Boehringer-Mannheim, Germany), and incubated for 30 minutes at 37°C. Readouts were performed using autoradiographic cassettes with photographic film, which was labelled by activated photon-emitting lumigen. Final identification of HLA genotypes was performed by analysing the probe attachment patterns on the amplification product.

Statistical analysis

Continuous variables are expressed as means ± standard deviations (SDs). Univariate comparisons were performed by means of Chi2 test (Yates-corrected in case of group sizes below 15) or two-tailed Fisher's exact tests for group sizes below five. Odds ratio (OR) calculation with 95%CI was performed wherever possible. Gene-gene interactions were assessed using multivariate logistic regression model, which assumed multiplicative interaction between analysed variables. Variable selection was performed by using backward stepwise procedure for factors with a p value lower than 0.15. Sample size was estimated to achieve 80% of statistical power for detecting odds ratios lower than 0.75 or greater than 1.25 for univariate comparisons with a p level lower than 0.05. The required sample size calculated for such purpose equalled 273 individuals per group. Considering the population frequency of class III allele in white Caucasians (47.5 ± 12.8% according to HapMap project data) [17], the frequency of class III allele would have to be lower than 32.25% in the study group. Assuming a 10% safeguard against material or protocol errors, the planned sample size was increased to 608 individuals. Statistical analyses were performed using STATISTICA 8.0 (Statsoft, Tulsa, OK, USA).

Results

The planned number of 590 individuals was successfully genotyped. The cause of the different numbers of patients with known 5′VNTR and HLA data was lack of sufficient amounts of seven available materials for comprehensive HLA testing. Mean age at diagnosis was 9.47 ± 4.29 years. Gender distribution did not dif-

Table II. *Number of particular HLA alleles in the studied population*
Table II. *Liczba poszczególnych alleli HLA w badanej grupie*

DQA1	N	%	DQB1	N	%	DRB1	N	%
0101	102	13.4%	0201	310	30.8%	01	120	11.7%
0102	89	11.7%	0301	138	13.7%	03	224	22.3%
0103	22	2.9%	0302	220	21.9%	04	277	27.6%
0201	53	6.9%	0303	23	2.3%	07	91	9.1%
0301	241	31.6%	0401	1	0.1%	08	28	2.8%
0302	3	0.4%	0402	20	2.0%	09	2	0.2%
0401	18	2.4%	0501	116	11.5%	10	1	0.1%
0501	228	29.9%	0502	45	4.5%	11	68	6.8%
0502	5	0.7%	0503	3	0.3%	12	9	0.9%
0601	2	0.3%	0504	5	0.5%	13	73	7.3%
			0601	5	0.5%	14	12	1.2%
			0602	60	6.0%	15	72	7.2%
			0603	33	3.3%	16	48	4.8%
			0604	22	2.2%			
			0605	4	0.4%			

Table III. *Frequencies of HLA and 5'VNTR INS-IGF2 genetic variants within the study and control groups (OR — odds ratio; 95% CI — 95% confidence interval)*
Tabela III. *Częstości wariantów genetycznych HLA i polimorfizmu 5'VNTR INS-IGF2 w grupie badanej i kontrolnej (OR — iloraz szans; 95-procentowy CI — 95% przedział ufności)*

HLA haplotype	Patients with type 1 diabetes	Control group	p*	OR (95% CI)
DR3-DQ2 haplotype carriage	88 (75.21%)	29 (24.79%)	< 0.0001	3.69 (2.31–5.88)
DR4-DQ8 haplotype carriage	106 (81.54%)	24 (18.46%)	< 0.0001	6.33 (3.88–10.33)
DR3-DQ2/DR4-DQ8 heterozygosity	41 (95.25%)	2 (4.65%)	< 0.0001	21.39 (5.11–89.57)
DQB1*0602 carriage	2 (7.69%)	24 (92.31%)	< 0.0001	0.08 (0.03–0.28)
Other HLA haplotypes	118 (39.07%)	184 (60.93%)	< 0.0001	0.30 (0.21–0.42)
5'VNTR class I homozygosity carriage	224 (77.78%)	174 (57.60%)	< 0.0001	2.63 (1.79–3.70)

*p values with Bonferroni's correction for multiple testing

fer between the study and control groups (48% *vs.* 45% males respectively; p = 0.89). Median duration of diabetes in the study group equalled 3.91 years (25–75%, 2.12–7.14). Metabolic control in the study group was satisfactory (HbA_{1c} = 7.57 ± 0.99). Mean daily insulin requirement equalled 0.52 ± 0.13 units. Distribution of HLA alleles at respective loci is presented in Table II. Distribution of DR3-DQ2, DR4-DQ8 and protective DQB1*602 haplotypes differed significantly between the study and control groups (Table III). Within the control group, 23 (7.62%) patients were homozygous for class III alleles, 105 (34.77%) were heterozygous for class I/class III and 174 (57.62%) were homozygous for class I. Allele frequencies in the control group did not show significant deviations from those expected from the Hardy-Weinberg equilibrium (p = 0.21). In the study group, genotype frequencies equalled three (1.04%); 61 (21.18%) and 224 (77.78%) respectively and differed

Table IV. *Multivariate results of 5′VNTR and HLA testing with gene-gene interaction evaluation for risk and protective effects of 5′VNTR classes (OR — odds ratio; 95% CI — 95% confidence interval)*

Tabela IV. *Wyniki analizy wieloczynnikowej uwzględniającej efekt polimorfizmu 5′VNTR i HLA oraz interakcje wariantów ryzyka lub ochronnych z genotypem 5′VNTR (OR — iloraz szans; 95-procentowy CI — 95% przedział ufności)*

	OR	95%CI		p
DR3-DQ2 carriage	1.72	1.37	2.16	< 0.00001
DR4-DQ8 carriage	1.86	1.43	2.41	< 0.00001
5′VNTR class I carriage	1.64	1.25	2.14	0.0003
DR3-DQ2 * 5′VNTR class I interaction	0.93	0.74	1.10	0.55
DR4-DQ8 * 5′VNTR class I interaction	0.85	0.66	1.05	0.23

Table V. *Predisposing effect of 5′VNTR class I homozygosity depending on HLA haplotype. Fractions denote the frequency of class III allele vs particular sample size*

Tabela V. *Efekt homozygotyczności klasy I 5′VNTR predysponujący do cukrzycy po uwzględnieniu haplotypu HLA. Ułamki oznaczają częstość allela klasy III względem liczebności danej podgrupy*

	Study group	Control group	p	OR (95% CI)
DR3-DQ2 carriers	15/88	10/29	0.08	2.56 (0.99–6.67)
DR4-DQ8 carriers	22/106	12/12	0.007	3.85 (1.52–10.00)
DR3-DQ2/DR4-DQ8 heterozygotes	7/41	2/2	0.04	–*
DQB1*0602 carriers	0/2	14/24	0.20	–*
Other HLA carriers	29/118	79/184	0.001	2.33 (1.39–3.85)

*OR impossible to estimate due to lack of patients in at least one subgroup

Table VI. *Multivariate regression model for age at diagnosis of diabetes with interaction assessment between HLA and 5′VNTR genotypes*

Tabela VI. *Model regresji wieloczynnikowej określającej wiek w chwili zachorowania na cukrzycę z uwzględnieniem interakcji między HLA a locus 5′VNTR*

Factor	F	p
DR3-DQ2 carriers	0.37	0.54
DR4-DQ8 carriers	1.99	0.16
5′VNTR class I carriage	1.41	0.24
DR3-DQ2 * 5′VNTR class I homozygosity interaction	2.20	0.14
DR4-DQ8 * 5′VNTR class I homozygosity interaction	0.18	0.67

significantly from the control group ($p < 0.0001$). Carriage of the class III allele was, as expected, protective against type 1 diabetes, while class I homozygosity conferred a strong predisposition for the disease (Table III).

Multivariate logistic regression analysis did not show any significant interactions between HLA and 5′VNTR haplotypes, but retained all variables tested within the model. This points to the presence of an additive effect to the risk of T1DM of class I allele rather than a multiplicative one (Table IV). This was confirmed by subgroup analyses, which showed that the protective effect of class III allele is retained regardless of HLA haplotype (Table V). Similarly, no evidence of any effect of either genetic variant in both 5′VNTR and HLA loci could be proven in terms of age at diagnosis of diabetes in multivariate model, despite the fact that DR4 haplotype carriage was a significant predictive factor for earlier diagnosis in univariate comparison (7.87 ± 0.67 in carriers vs. 8.05 ± 0.60 in non-carriers, $p = 0.03$). The final model assessing interactions between class I homozygosity and HLA risk haplotypes in determination of age at diagnosis of diabetes is shown in Table VI.

Discussion

Results shown by our group generally rule out the existence of an interaction effect between the INS

and INS-IGF2 5'VNTR site, despite the fact that the number of tandem repeats within that site did exert a strong effect on the risk of T1DM. This obviously does not negate the possibility that a relation between the two genotypes does exist. However, due to pre-specified sample size and ample statistical power, we can safely state that the potential effect would not be as strong as the influence of either of the analysed genes alone. The HLA haplotypes analysed within this study were, as expected, the strongest determinants of disease status, with ORs ranging from a supremely protective 0.08 to a strongly predisposing effect of 22. The 5'VNTR locus was, in comparison, a considerably weaker factor, with its protective effect associated with an approximately two-fold reduction of T1DM probability. The univariate effect of HLA haplotypes was very close to that shown by Cejkova and Benedek [13, 15], despite the fact that both mentioned studies enrolled considerably smaller groups of patients with autoimmune diabetes (n = 87 and 173 respectively) of different ethnic or geographic backgrounds. The effect of 5'VNTR polymorphism observed in the Polish population was divergent from that observed by Benedek et al., mostly due to a different class I/III allele distribution — class III allele was considerably less frequent in all four ethnically different groups of Jewish patients tested. Nevertheless, its protective effect was evident in both groups. Cejkova reported a similar result with the relative effect of the class III allele [15]. In both cited studies and this report, the effect of 5'VNTR and HLA genotypes was additive and no association suggesting mutual synergy or effect inhibition could be ascertained. This has led the authors to conclude that the effect of these genetic factors is separate and does not function in a conditional way and that 5'VNTR and HLA loci show independent effect. This conclusion is somewhat divergent from an earlier study by Julier et al., who stipulated that the effect of the 5'VNTR INS-IGF2 site is present only in HLA DR4 carriers [18]. This was probably caused by a smaller sample size in her study and underrepresentation of other HLA, but could also be potentially attributed to genetic differences resulting from the ethnic background of the analysed group. Results suggesting effects similar to our study were published by the Norwegian Childhood Diabetes Study Group [14] in 2008. Their study showed a lack of multiplicative interaction between HLA, 5'VNTR INS-IGF2 and *CTLA4* polymorphism, while confirming the existence of an interaction between *PTPN22* and HLA high-risk haplotypes (showing a significantly reduced effect than that expected) and in transmission disequilibrium testing of *PTPN22* and *CTLA4* polymorphic alleles. This led Bjornvold at al. to state that no evidence of interaction of 5'VNTR INS-IGF2 locus could be ascertained in the Scandinavian population with any of the top genetic risk factors of T1DM [14].

Based on results presented in this report, the same can be said for the Central European population of Slavic origin.

Therefore, assessment of a genetic risk profile of T1DM necessitates genotyping both the HLA and INS-IGF2 to fully estimate the genetic burden of autoimmune diabetes. Similarly, no evidence of interaction between HLA and 5'VNTR was detected in terms of age at diagnosis of diabetes. Despite the study being underpowered, the difference in age at diagnosis between respective 5'VNTR INS-IGF2 genotypes was non-existent.

This is somewhat discrepant from earlier reports [3], which showed, on a considerably smaller group, the existence of a three-way interaction between the 5'VNTR site and CTLA4 genotype. Additionally, Felner et al. showed that the detrimental effect of class I homozygosity was present in both the general population and in a subgroup of high-risk HLA positive patients, something which has been confirmed in our report [3]. No interdependency effect was noted between either HLA or 5'VNTR INS-IGF2 promoter region genotypes. In our study, the DR4 haplotype did in fact result in earlier onset of diabetes, but this association could not be confirmed in multivariate models. Similarly in the Scandinavian cohort [14], a lack of effect of genetic factors on age at onset was observed, which could suggest a more complex background of the rate of autoimmune process progression and manifestation of symptoms.

Conclusions

The protective effect of class III 5'VNTR INS-IGF2 modulates the risk conferred by HLA haplotype but does not show any evidence of multiplicative interaction or conditional effect, either on the risk of type 1 diabetes, or the age of its onset.

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