THE ASSOCIATIONS BETWEEN COMMON POLYMORPHISMS IN INSULIN, IGF2, NAIP AND SELL GENES AND THE RISK FOR T1DM

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Abstract

T1DM patients without clinical proteinuria (n= 80) and healthy controls (n= 80) were included in this study. The Insulin -23Hph (T/A), Insulin +1127Pst1 (C/T), IGF2 Apa (820 G/A), SELL P213S polymorphisms and NAIP exon 5 deletion were genotyped in all samples. All genotypes were similarly distributed in subjects stratified according to gender. The exon 5 of NAIP gene was present in all samples and this polymorphism was excluded from further analysis. The distribution of Insulin -23Hph, Insulin +1127Pst, IGF2 Apa, SELL P213S genotypes in patients and controls are in agreement with Hardy-Weinberg equilibrium. The Insulin -23Hph AA genotype is significantly associated with T1DM (O.R. AA vs. non-AA  = 4.26; 95%CI: 2.06-8.8; p<0.0001). No other significant differences of genotypes, alleles or combined genotypes were detected between investigated markers and diabetes.

We found a strong association of polymorphisms from IDDM2 with T1DM in our population.

Key words: type 1 diabetes mellitus, insulin, IGF2, SELL

1. Introduction

Type 1 diabetes mellitus (T1DM) is a chronic disease with an increasing world-wide incidence. T1DM is most frequently characterized by autoimmune destruction of pancreatic β cells (type 1A), whereas in several percent of cases the disease is non-autoimmune (type 1B). Some environmental and genetic factors play important roles in the etiopathogenesis of this complex disease. A significant number of markers are tested for linkage or association with T1DM but only several genomic regions were reconfirmed in different populations (e.g. IDDM1, IDDM2, IDDM5, IDDM12) [1, 2]. Additive or interactive effects among different genomic regions can create distinct patterns of associations with diabetes [3, 4]. Overall, it is estimated that the IDDM1 and IDDM2 account for 50-70 % of the familial aggregation of diabetes [5].

Some IDDM2 polymorphisms (11p15.5) have been associated with the expression of insulin gene in relevant tissues for diabetogenesis [6-8] and with the presence of some autoantibodies against pancreatic islet beta cells (e.g. GAD [9], but not insulin autoantibodies [10, 11]). The polymorphisms from IDDM2 were considered markers for T1DM [12-14] in
Caucasians and Asians [15-18]. Statistically significant results were identified in different populations from Europe (e.g. Finnish [19], Swedish [20], French [21], Spanish [22], Polish [23] and Romanian [24]), excepting Czechs [25]. In other populations (e.g. Asians, Tanzanian blacks) this association is still under discussion [26, 27].

IGF2 encodes for a peptide with mitogenic properties (especially during intrauterine development) and with important autocrine/paracrine signaling effects on thymus, lymphocytes and pancreas. Insulin growth factors have important roles in the development of endocrine pancreas [28], suppression of apoptosis (e.g. developmental apoptosis or apoptosis induced by proinflammatory cytokines) in the pancreatic islets [29] and for induction of immune tolerance to proinsulin [30, 31]. The expression of this gene can be modulated by the IDDM2 polymorphisms [32-34]. Although IGF2 is a candidate locus for pathogenesis of T1DM [30, 35] no statistically significant evidence of association between IGF2 Apal and disease was found in our population [24].

Neuronal Apoptosis Inhibitory Protein (NAIP or BIRC1) has anti-apoptotic properties mediated by BIR domains which bind and inhibit caspase-3, -7, and -9 [36-39]. These domains of NAIP are encoded by exons 5 and 6. The deletion of these exons can affect NAIP functions. Its orthologous are involved in susceptibility for some infectious [40, 41] and bacterial-induced inflammation [42]. Destruction of pancreatic β cells, infections and inflammations are components of T1DM etiopathogenesis. Until now we have no data regarding the relations between NAIP mutations and T1DM.

SELL is a cell adhesion molecule which plays an important role in the recruitment of inflammatory cell (e.g. leukocyte migration to sites of local inflammation) and in the inflammatory response in different organs, including human pancreas [43-45]. The P213S polymorphism in exon 6 of the gene is responsible for an amino acid change (Proline213Serine) in the domain 1 of protein. This polymorphism can modify the interaction between leucocytes and endothelium. The high serum levels of L-selectin have been associated with different diseases including T1DM [44].

The debatable results imply testing the effect of interactions between multiple markers on disease susceptibility and replication of any positive association in separate studies.

2. Objective

The aim of this case-control study was to test the association between T1DM and Insulin, IGF2, SELL and NAIP polymorphisms.

3. Materials and methods

Subjects

Unrelated volunteers of Caucasian ancestry have been recruited from N Paulescu Institute, Bucharest between 2012 and 2013. They were physical evaluated and data regarding anthropometrical parameters and medical history were recorded. We included in this study 160 subjects which were distributed into T1DM (T1DM patients without clinical proteinuria, n= 80) and HC (healthy controls, n= 80) lots. The patients with T1DM were selected based on: sudden-onset of diabetes (inaugural ketoacidotic status), the daily insulin treatment was started during the first 12 months from this episode, absolute insulin deficiency before 18 years and at least three years of daily insulin treatment. Patients were included in this study if renal albumin excretion was <300 mg at the selection time.

Clinically healthy controls were included in the study if they have reported no previous glucose metabolism abnormalities, renal diseases or different chronic infections. They were matched for age, sex and birth place with the patients.
Genotyping methods

Whole-blood samples were collected in EDTA–K3 tubes from each subject. Genomic DNA was extracted from 200 μl from each blood sample AxyPrep™ Blood Genomic DNA Miniprep Kit (Axygen Biosciences, California) and stored at -20°C until further molecular analysis. The DNA was quantified with Quant-iT™ PicoGreen® (Invitrogen Corp., Carlsbad CA) in a Rotor -Gene 6000 (Corbett Research) and its integrity has been evaluated by agarose gel electrophoresis.

The Insulin -23Hph (T/A, rs689), Insulin +1127Pst1 (C/T, rs3842752), IGF2 Apa (820 G/A,rs680), SELL P213S (rs2229569) [46] polymorphisms were genotyped in each sample according with previously published protocols. Briefly, these polymorphic regions were amplified by PCR (Rotor -Gene 6000 (Corbett Research) using 20 pmol of specific primers, 1 U Taq pol (Fermentas, Lithuania), 10mM dNTP (Fermentas, Lithuania). The PCR program was 1min at 94°C, 30 x 1 min at 94°C, annealing temperature, 1 min at 72°C, and 10 min at 72°C. The sequence of the primers (Sigma Genosys), the annealing temperature and the size of amplicons are present in Table 1.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primers sequence (Forward / Reverse)</th>
<th>Annealing conditions</th>
<th>Amplicon size (bp)</th>
<th>Endonuclease restriction</th>
</tr>
</thead>
<tbody>
<tr>
<td>INS -23Hph</td>
<td>5'TCCAGGACAGGCCTGCATCAG3'/5'AGCAATGGGCGGTGGCTCA3'</td>
<td>58°C /1 min</td>
<td>441</td>
<td>HphI</td>
</tr>
<tr>
<td>INS +1127Pst</td>
<td>5'CTCTACCAGCTGGAGAACTA3'/5'GGCTGGTTCAAGGGCTTTAT3'</td>
<td>60°C / 1 min</td>
<td>104</td>
<td>PstI</td>
</tr>
<tr>
<td>IGF2 Apa</td>
<td>F5’CTTGGACTTTGAGTCAAATTGG3'</td>
<td>R5’CCTCCTTTGTCTCTTACTGGG3’</td>
<td>55°C / 1 min</td>
<td>236</td>
</tr>
<tr>
<td>SELL P213S</td>
<td>5'TGATTCAGTGTAGCGCTTTG3'/5'CTTGACAGGGTTGGTCTG3'</td>
<td>60°C / 1 min</td>
<td>186</td>
<td>Hph I</td>
</tr>
<tr>
<td>NAIp exon 5</td>
<td>deletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’CTCTCAGCCTGCTCTTCAGAT3'/5’AAAGCCTCTGACGAGAGGAT3’</td>
<td>58°C / 1 min</td>
<td>436</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The sequence of primers, the annealing temperature and the size of amplicons.

Amplicons (5 μl) were digested with specific endonuclease (5U) for three hours, according to manufacturer instructions. Fragments resulted after enzymatic digestion were electrophoresed in agarose (2%) or polyacrylamide (8%) gels. After electrophoresis, the gels were stained with ethidium bromide (10μg/ml) and were visualized with an UVP Bioimaging System (Jencons PLS).

The presence or homozygous deletion of NAIp exon 5 was identified by electrophoresis of amplicons in agarose gel and was confirmed by melting analysis and high resolution melting (HRM) analysis using SYBR® Green I and SensiMix® with EvaGreen® (Bioline, USA), as was previously described [47].

As a quality control, the genotypes were interpreted by two independent researchers and 10% of randomly selected samples were re-genotyped. The genotypes were concordant in all cases.

Statistical analysis

The chi squared test and Fisher exact test were used to examine the differences in the distribution of genotypes and alleles between groups and the Hardy-Weinberg equilibrium. The odd risk (OR) was calculated with StatsDirect statistics programs. Values of p<0.05 were considered statistically significant.
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**Ethics**

The study was conducted in accordance with the principle of the Helsinki Declaration and was approved by the ethical committee of the N Paulescu Institute (Bucharest) before initiation of the study. Informed written consent was obtained from each subject included in this study by the participating investigators.

**4. Results and discussions**

The clinical and laboratory characteristics of the groups are summarized in Table 2. The distribution of sex and age (p>0.05) in the DM and HC groups are similar. Subjects from the control lot were more frequent smokers (Pearson's p=0.04) and alcohol consumers (Fisher two side p= 0.04) compared to the patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Type 1 diabetes mellitus</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male/Female)</td>
<td>40/40</td>
<td>40/40</td>
</tr>
<tr>
<td>Age (year) *</td>
<td>25.88±4.12</td>
<td>26.44±3.1</td>
</tr>
<tr>
<td>(Range)</td>
<td>(19-33)</td>
<td>(19-33)</td>
</tr>
<tr>
<td>Age at the onset of T1DM* (Range)</td>
<td>13.23±2.52</td>
<td>-</td>
</tr>
<tr>
<td>(6-17)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (Kg/m²)*</td>
<td>23.71±1.59</td>
<td>23.41±1.34</td>
</tr>
<tr>
<td>(Range)</td>
<td>(20.62-26.45)</td>
<td>(20.96-25.56)</td>
</tr>
<tr>
<td>Smokers**</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Drinkers***</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Persons with tooth decay / missing tooth</td>
<td>19/8</td>
<td>13/6</td>
</tr>
</tbody>
</table>

*Data are presented as mean± SD; ** at least five cigarettes per day, for at least one year; *** at least 25 g of alcohol per day, for at least one year*}

The *Insulin -23Hph* (T/A, rs689), *Insulin +1127Pst1* (C/T, rs3842752), *IGF2* Apa (820 G/A, rs680), *SELL P213S* (rs2229569) polymorphisms and *NAIP* exon 5 deletion were genotyped in all samples (Table 3). In each lot all genotypes were similarly distributed in subjects stratified according to gender and, in consequence, the dichotomy males-females were not shown in the table.

The genotypic and allelic frequencies were calculated after direct counting genotypes in all samples. The *Insulin -23Hph* and *+1127Pst* polymorphisms were found to be identically distributed in investigated lots. The exon 5 of *NAIP* gene was present in all samples and this polymorphism was excluded from future analysis. The distribution of genotypes in patients and controls were in agreement with Hardy-Weinberg equilibrium (p=0.07-0.69).

Potential associations between diabetes environment and decayed or missing teeth were also investigated in different studies. These associations may be influenced by genetic and nongenetic factors. In our study the *Insulin*, *IGF2* and *SELL* genotypes considered individually or in two-by-two combinations were not associated with the presence of dental damage.

The AA genotype of *Insulin -23Hph* is more frequent in T1DM patients than in control group (66 vs. 42). In both groups genotypes were distributed similarly in men and women. The result of chi squared test shows that this genotype is significantly associated with T1DM
O.R. AA vs. non-AA = 4.26; 95%CI: 2.06-8.8, p<0.0001. Moreover, this genotype seems to confer greater risk in women (O.R. AA vs. non-AA = 5.21, p=0.002) compared to men (O.R. AA vs. non-AA = 3.48, p=0.03). No other significant differences of genotypes or alleles were detected between investigated markers and diabetes.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotypes\ Alleles</th>
<th>Lots</th>
<th>Type 1 diabetes mellitus</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><em><em>Insulin -23Hph</em> (T/A, rs689)</em>*</td>
<td>AA</td>
<td>66</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>13</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>1</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAF T</td>
<td>9.4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td><strong>HWE</strong></td>
<td></td>
<td>p=0.69</td>
<td>p=0.07</td>
<td></td>
</tr>
<tr>
<td><strong>IGF2 Apa (820 G/A, rs680)</strong></td>
<td>GG</td>
<td>49</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>29</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAF A</td>
<td>79.37</td>
<td>78.75</td>
<td></td>
</tr>
<tr>
<td><strong>HWE</strong></td>
<td></td>
<td>p=0.33</td>
<td>p=0.08</td>
<td></td>
</tr>
<tr>
<td><strong>SELL P213S (C/T, rs2229569)</strong></td>
<td>CC</td>
<td>56</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>19</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAF T</td>
<td>18.13</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td><strong>HWE</strong></td>
<td></td>
<td>p=0.07</td>
<td>p=0.28</td>
<td></td>
</tr>
<tr>
<td><strong>NAIP exon 5</strong></td>
<td>present</td>
<td>80</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>absent</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. The distribution of *Insulin, IGF2, SELL, NAIP* gene polymorphisms in T1DM patients and healthy control subjects (p value for Fisher exact test (two side); HWE = Hardy Weinberg equilibrium; MAF= minor allele frequency; NA = not applicable)

*O.R. AA vs. non-AA = 4.26, 95% CI: 2.06-8.8, p<0.0001

T1DM is a complex disease which results from interactions between genetic and environmental factors. The genetic component of T1DM is polygenic and has a strong impact in disease etiopathogenesis. Analysis of markers from candidate genes, genome-wide association studies and meta-analyses of T1DM genome-wide association studies data sets provided new insights into genetic component of T1DM. However, only several percent of these positive results have been replicated in different studies. Although the results of replicated studies are not always fully concordant, they are useful for distinguishing true association by spurious association. The most common explanations for these discrepancies are differences in study design, disease and genetic heterogeneity, differences in genetic background of investigated populations.

IDDM2 has been involved in T1DM in different populations from Europe excepting Czechs [19, 21, 22, 24, 25]. It is difficult to identify which is the real marker(s) for T1DM susceptibility because polymorphisms from this region are in strong linkage disequilibrium, some of them (e.g. *INS VNTR, -23HphII*) can have functional consequences [48-50] and disease susceptibility can be influenced by interaction between neighboring markers [51]. The mechanism by which IDDM2 polymorphism(s) can modify the risk for T1DM was also a
subject of debate. Initially, it was considered that INS VNTR is responsible for this association [52, 53], because it can influence the efficiency of insulin genes transcription [54]. The contribution of other polymorphisms to the diabetes pathology was reconsidered in the next years [13, 55]. Thus, the SNP Insulin -23Hph (rs689) was mapped in a region predicted to influence the splicing efficiency, the length of mature RNA and the level of protein synthesis [49, 56].

We investigated two SNPs from the insulin gene region: 23Hph and +1127Pst. The genotypes of these markers were identically distributed in each investigated lot (Table 3). This particular distribution could be explained by the strong linkage between markers from insulin gene region previously reported [13, 57]. The results also showed that IDDM2 is very strongly associated with T1DM in Romanian population (O.R. AA = 4.26, p<0.0001). This result is in concordance with previous reports found in our population [24, 58] as well as in other European populations [20, 22].

A similar distribution of IGF2 and SELL polymorphisms was detected in patients and in control lots. Thus, analysis of individual polymorphism reveals no other association between investigated markers and T1DM.

5. Conclusions
The association of polymorphisms from IDDM2 with T1DM is very strong in our population.

Acknowledgements
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References
Increased and persistent circulating insulin-like growth factor II in neonatal transgenic mice suppresses developmental apoptosis in the pancreatic islets.


Two insulin gene single nucleotide polymorphisms associated with type 1 diabetes risk in the Finnish and Swedish populations. Dis Markers, 23(3), 139-145 (2007).


Two insulin gene single nucleotide polymorphisms associated with type 1 diabetes risk in the Finnish and Swedish populations. Dis Markers, 23(3), 139-145 (2007).


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THE ASSOCIATIONS BETWEEN COMMON POLYMORPHISMS IN INSULIN, IGF2, NAIP AND SELL GENES AND THE RISK FOR T1DM


[58] C. IONESCU-TIRGOVISTE, C. GUJA, M. HERR, E. CUCCA, K. WELSH, M. BUNCE, et al., Low frequency of HLA DRB1*03 - DQB1*02 and DQB1*0302 haplotypes in Romania is consistent with the country's low incidence of Type I diabetes. *Diabetologia.*, 44(Suppl 3), B60-B66 (2001).