Original paper

Study on VDR Polymorphism Influence in Associating with Diabetes Mellitus

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Abstract

The aim of this study was to evaluate the association between vitamin D receptor, IL6 polymorphisms and DM. DNA was used to genotype the VDR FokI (rs2228570), TaqI (rs731236) and ApaI (rs7975232) polymorphisms by PCR RFLP and IL6 G-174C (rs1800795) by tetra-primer ARMS PCR. The presence of torque teno viruses DNA was assessed with heminested-PCR. For this study T1DM (n = 107) and T2DM (n = 124) patients and matched clinically healthy subjects (n = 200) were recruited. T1DM patients have a tendency to be more frequent carriers of the C allele and TTV infection than controls (OR = 1.9, p = 0.03). VDR tt genotype and VDR “FAt” haplotype are risk factors for T1DM. VDR “fAt” haplotype may increases the risk for T2DM. These associations were not changed after exclusion from statistical analysis of patients with hypertension, myocardial infarction, stroke or breast cancer.

Keywords

PCR – RFLP, diabetes, IL6, vitamin D receptor, haplotypes.

Introduction

Interleukin-6 (IL-6) can protect β-cell from cytokine-induced death and also may contribute to insulinitis. The presence of -174 C variant or of CC genotype of IL-6 -174 G>C (rs1800795) polymorphism were considered risk factors for type 1 diabetes mellitus (T1DM) in some populations. Also, the -174C variant was considered protective (HUTH & al [1], STEPHENS & al [2]), while the G variant or the GG genotype may represent risk factors for type 2 DM (T2DM) among Indians (MUHU-PADHYAYA & al [3]) and Europeans (WILLER & al [4]). However, these positive associations with DM has not been reconfirmed by meta-analyses (YIN & al [5], QI & al [6]).

Vitamin D and the nuclear receptor for vitamin D (VDR) seem to be involved in insulin secretion and sensitivity, glucose tolerance and metabolism, modulation of the immune system, secretion of several cytokines (e.g. IL1, IL2, IL6, IL12) and immune responses to viral infection, inflammation and adiposity. Thus VDR may contribute to predisposition for insulin resistance or DM, especially in some population (QIN & al [7], ZHU & al [8]).

**Torque teno virus (TTV), Torque teno mini virus (TTMV) and Torque teno mini virus (TTMV)** are human anelloviruses which can influence IL6 production and secretion (ROCHI & al [9], ZHENG & al [10]). In addition, viral genome may encode for miRNAs (KINCAID & al [11]) and for some short proteins with incompletely understood functions. Chronic viral infections may represent a stress factor for the host and thus an aggravating factor for disease onset or evolution.

The aim of the present case-control study was to test the association between four genetic polymorphisms and DM in the Romanian population.

Materials and Methods

**Subjects**

Data and biological samples from unrelated T1DM (n = 107) and T2DM (n = 124) patients were selected from National Institute of Diabetes, Nutrition and Metabolic Diseases “Prof. N. Paulescu”, Bucharest. The patients were selected based on the age (>18 years old), the duration of diabetes (>5 years) and the negative history for other autoimmune disorders, chronic pancreatitis, overt nephropathy or declared blood transfusions.

Clinically healthy subjects matched for age and gender with the T1DM and the T2DM patients were distributed into two control groups: HC1 (n = 100) and HC2 (n = 100).

**Genotyping methods**

DNA (Axyprep™ Blood Genomic DNA Miniprep Kit, Axxygen Biosciences, California) was used to genotype the VDR FokI (rs2228570), TaqI (rs731236) and Apal (rs7975232) polymorphisms by PCR RFLP and IL6 G-174C (rs1800795) by tetra-primer ARMS PCR (YE & al [12]). The presence of torque teno viruses DNA was assessed with heminested-PCR (NINOMIYA & al [13]).

**Statistical analysis**

The differences between the clinical parameters and the alleles and genotypes frequencies between lots were compared using the StatsDirect software. The SHEsis online platform and Plink software (version 1.07) have been used for haplotype analysis (SHI & al [14]). A Bonferroni corrected p value threshold was employed for correction of type I error.

**Results and Discussion**

The distributions of the four polymorphisms were similar in diabetic patients and in the control lot. Haplotype analysis revealed a significant association between T1DM and VDR F or Fat haplotypes (OR = 2.1, p <0.003). For T2DM, a trend of association with the VDR F at haplotype was observed (OR = 2, p < 0.05). Seven patients with T1DM (which had hypertension) and twenty-four patients from T2DM lot (which had, in different association, hypertension – 15, myocardial infarction – 4, stroke – 12, or breast cancer – 1) were excluded from statistical analysis to avoid potential confounding effects; the characteristics of the subjects which remained for further analysis are listed in Table 1.

**Table 1. Clinical and biochemical data and genotype distributions of subjects selected for this study**

<table>
<thead>
<tr>
<th>Investigated characteristics</th>
<th>T1DM (Male)</th>
<th>T1DM (Female)</th>
<th>T2DM (Male)</th>
<th>T2DM (Female)</th>
<th>HC1 (Male)</th>
<th>HC1 (Female)</th>
<th>HC2 (Male)</th>
<th>HC2 (Female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>51</td>
<td>49</td>
<td>53</td>
<td>53</td>
<td>47</td>
<td>53</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>Age (years)</td>
<td>25.2 ± 3.5</td>
<td>25.67 ± 3.7</td>
<td>25.2 ± 3.4</td>
<td>25.7 ± 3.7</td>
<td>55.7 ± 5.0</td>
<td>54.4 ± 4.6</td>
<td>55.6 ± 4.9</td>
<td>54.5 ± 4.6</td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td>14.5 ± 4.2</td>
<td>14.5 ± 4.0</td>
<td>N.A.</td>
<td>N.A.</td>
<td>7.5 ± 1.8</td>
<td>7.1 ± 1.9</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Body Mass Index</td>
<td>23.1 ± 1.2</td>
<td>21.4 ± 1.8</td>
<td>23.3 ± 0.8</td>
<td>21.4 ± 1.1</td>
<td>27.4 ± 1.4a</td>
<td>26.5 ± 1.4a</td>
<td>24.4 ± 0.4a</td>
<td>22.9 ± 1.1a</td>
</tr>
<tr>
<td>Hemoglobin A1C (%)</td>
<td>8.5 ± 0.6</td>
<td>8.5 ± 0.7</td>
<td>N.A.</td>
<td>N.A.</td>
<td>7.9 ± 0.6</td>
<td>7.8 ± 0.5</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>&quot;A jeune&quot; blood glucose</td>
<td>N.A.</td>
<td>N.A.</td>
<td>87.4 ± 6.2</td>
<td>88.0 ± 5.7</td>
<td>119.0 ± 16.9</td>
<td>106.4 ± 15.6</td>
<td>91.0 ± 7.7</td>
<td>92.0 ± 7.3</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>188.1 ± 32.21</td>
<td>176.9 ± 29.6</td>
<td>149.3 ± 23.6</td>
<td>146.6 ± 21.3</td>
<td>211.6 ± 35.2</td>
<td>201.3 ± 27.7</td>
<td>171.8 ± 16.5</td>
<td>169.9 ± 17.1</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>139.8 ± 38.9</td>
<td>121.2 ± 31.3</td>
<td>96.2 ± 17.4</td>
<td>96.5 ± 16.9</td>
<td>183.6 ± 65.6</td>
<td>160 ± 56.4a</td>
<td>111 ± 24.3</td>
<td>111.6 ± 23.6</td>
</tr>
<tr>
<td>Smokers*</td>
<td>6</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Drinkers*</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>TVT infections</td>
<td>38</td>
<td>32</td>
<td>38</td>
<td>32</td>
<td>37</td>
<td>36</td>
<td>35</td>
<td>29</td>
</tr>
<tr>
<td>TVT</td>
<td>38</td>
<td>32</td>
<td>38</td>
<td>32</td>
<td>37</td>
<td>36</td>
<td>35</td>
<td>29</td>
</tr>
<tr>
<td>TVMDV</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>23</td>
<td>25</td>
<td>22</td>
<td>27</td>
<td>22</td>
</tr>
<tr>
<td>TVM</td>
<td>33</td>
<td>28</td>
<td>26</td>
<td>28</td>
<td>31</td>
<td>30</td>
<td>31</td>
<td>27</td>
</tr>
</tbody>
</table>

* a: the two-tailed P value for T test < 0.0001; b: the two-tailed P value for T test < 0.05;
N.A.: not available; #: more than five cigarettes per day, for at least one year; ##: more than 5 units of alcohol per day, for at least one year.
The distribution of the positive samples for torque teno viruses infection presented no significant differences between subjects. Some differences were found when data regarding other characteristics were tested in addition to the presence of viral DNA. First, healthy males without TTV (34.32 ± 14.15 vs. 42.58 ± 16.19, p = 0.014), T1DM females without TTMDV (24.35 ± 3.47 vs. 27.17 ± 3.55, p = 0.007) and T2DM females without TTMDV (53.39 ± 4.87 vs. 56.05 ± 3.92, p = 0.039) were younger compared with infected subjects from the same group. Second, healthy males infected with TTMDV had higher BMI (4.87 ± 0.76, p = 0.007) and T2DM females without TTMDV (22.56 vs. 18.90, p = 0.002) had higher total cholesterol levels compared to uninfected subject from the same group. The age of these sub-groups did not differ significantly.

The distribution of IL-6 genotypes has not deviated from Hardy-Weinberg equilibrium. The -174C allele was more common in patients diagnosed with T1DM in the first 10 years of life than in those with later onset (41/13 vs. 26/20, OR = 2.43, 95% CI: 1.03 - 5.69, p = 0.04). T1DM patients were also more frequent carriers of the G allele and TTV infection than controls (46 vs. 31, p = 0.03, OR = 1.9).

The IL-6 G-174C was not associated with T2DM, even if the gender, BMI, the age of diabetes onset or the presence of TTV infection were included in the analysis.

Single locus analysis revealed that VDR TaqI tt genotype (OR tt = 2.45; 95% CI = 1.29 - 4.62; p = 0.005) and VDR t variant (OR t = 1.99; 95% CI = 1.06 - 3.72; p = 0.02) increased the risk for T1DM whereas the TT genotype (OR TT = 0.50; 95% CI = 0.26 - 0.93; p = 0.03) and the T variant (OR T = 0.40, 95% CI = 0.21 - 0.76, p = 0.005) appeared as protective factors. A similar result was detected in the sub-group of women (OR tt = 2.72; 95% CI = 1.15 - 6.44; p = 0.02; OR T = 0.36, 95% CI= 0.15 - 0.86, p = 0.02), yet not in the case of male. Single locus analysis revealed no other significant associations with T1DM or T2DM (Table 2).

### Table 2. The distribution of VDR and IL6 polymorphisms in investigated lots

<table>
<thead>
<tr>
<th>Polymorphisms</th>
<th>T1DM</th>
<th>HC1</th>
<th>T2DM</th>
<th>HC2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>total</td>
<td>Male</td>
</tr>
<tr>
<td>IL6 GG</td>
<td>17</td>
<td>16</td>
<td>33</td>
<td>24</td>
</tr>
<tr>
<td>IL6 GC</td>
<td>26</td>
<td>23</td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td>IL6 CC</td>
<td>8</td>
<td>9</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>VDR FF</td>
<td>26</td>
<td>23</td>
<td>49</td>
<td>18</td>
</tr>
<tr>
<td>VDR Ft</td>
<td>19</td>
<td>20</td>
<td>39</td>
<td>23</td>
</tr>
<tr>
<td>VDR f</td>
<td>6</td>
<td>6</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>VDR AA</td>
<td>21</td>
<td>17</td>
<td>38</td>
<td>14</td>
</tr>
<tr>
<td>VDR Aa</td>
<td>21</td>
<td>21</td>
<td>42</td>
<td>24</td>
</tr>
<tr>
<td>VDR a</td>
<td>9</td>
<td>11</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>VDR t</td>
<td>15</td>
<td>23</td>
<td>38</td>
<td>8</td>
</tr>
<tr>
<td>VDR T</td>
<td>23</td>
<td>17</td>
<td>40</td>
<td>23</td>
</tr>
<tr>
<td>VDR T</td>
<td>13</td>
<td>9</td>
<td>22</td>
<td>20</td>
</tr>
</tbody>
</table>

a: OR t vs. T = 1.75, 95% CI = 1.00 - 3.04; p = 0.048; b: OR tt = 2.72; 95% CI = 1.15 - 6.44; p = 0.02; c: OR T = 0.36; 95% CI = 0.15 - 0.86; p = 0.02; d: OR tt = 2.45; 95% CI = 1.29 - 4.62; p = 0.005; e: OR C = 1.99; 95% CI = 1.06 - 3.72; p = 0.02; f: OR TT = 0.50; 95% CI = 0.26 - 0.93; p = 0.03; g: OR T = 0.40; 95% CI = 0.21 - 0.76, p = 0.005.

No significant results for T1DM and T2DM were obtained when allelic by allelic epistasis between the four SNPs were performed for case – control samples (p > 0.08). However, VDR haplotype-based association analyses revealed some significant results for T1DM. The most significant results were estimated for Ft haplotype (OR = 2.36, p = 0.0003). Overall, these results indicated that VDR Taq polymorphism considered independent or in association with VDR FokI polymorphisms increased the risk for T1DM in Romanian population (Table 3).

### Table 3. The logistic regression for VDR haplotype-based association analysis

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>SNPs</th>
<th>Haplotype</th>
<th>Freq</th>
<th>OR</th>
<th>T from Wald test</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1DM</td>
<td>Apol-TaqI</td>
<td>aT</td>
<td>0.342</td>
<td>0.59</td>
<td>6.13</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Apol-TaqI</td>
<td>At</td>
<td>0.387</td>
<td>1.65</td>
<td>6.13</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Fokl-TaqI</td>
<td>FT</td>
<td>0.328</td>
<td>0.64</td>
<td>4.19</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Fokl-Apol</td>
<td>FA</td>
<td>0.345</td>
<td>1.65</td>
<td>4.83</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Fokl-Taql</td>
<td>Ft</td>
<td>0.319</td>
<td>2.36</td>
<td>13.1</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td>Fokl-Apol-Taql</td>
<td>Fat</td>
<td>0.235</td>
<td>2.21</td>
<td>8.69</td>
<td>0.003</td>
</tr>
<tr>
<td>T2DM</td>
<td>Fokl-Apol-TaqI</td>
<td>fAt</td>
<td>0.161</td>
<td>2.05</td>
<td>4.57</td>
<td>0.03</td>
</tr>
</tbody>
</table>

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The TaqI and Apal VDR polymorphisms displayed linkage disequilibrium (D' = 51) (Figure 1). Genomic coordinates of polymorphisms were established according to human genome assembly GRCh38.p2. A significant LD was identified only between rs731236 (TaqI) and rs7975232 (Apal) (D' = 51). The estimation of linkage disequilibrium (LD) was computed for pairs of SNPs using Haploview 4.2 software.

![Figure 1. The Linkage Disequilibrium (r2) between the VDR TaqI (rs731236; chr12:47844974), VDR Apal (rs7975232 chr12: 47845054) and VDR FokI (rs2228570, chr12: 47879112).](image)

Although carriers of risk genotypes in both VDR TaqI and Apal or FokI polymorphisms seemed to be associated with T1DM, the results were not significant, if correction for multiple tests have been applied. In addition the conclusions regarding these associations may be interpreted with caution because only a low number of carriers of these combinations have been detected in this study.

The investigation of the relation between IL6 -174 polymorphism and DM provided contradictory results. The -174 C allele and the -174 CC genotype were considered risk factors for T1DM in some studies. These markers seemed to increase the disease risk in both gender (COOPER & al [15]) and, may predispose to the onset of disease at younger age in women (GILLESPIE & al [16]). In this study the IL6 -174C allele was found to be more common in patients diagnosed with T1DM in the first 10 years of life than in those with later onset (41/13 vs 26/20, OR = 2.43, 95% CI: 1.033 - 5.698, p = 0.04). This observation was not confirmed when each gender was tested independently.

The prevalence of torque teno viruses depends on the prevalence of linkage disequilibrium (D' = 51) (Figure 1). Genomic coordinates of polymorphisms were established according to human genome assembly GRCh38.p2. A significant LD was identified only between rs731236 (TaqI) and rs7975232 (Apal) (D' = 51). The estimation of linkage disequilibrium (LD) was computed for pairs of SNPs using Haploview 4.2 software.

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VDR was associated with T2DM in Chinese (XU & al [35]), Saudi Arabian (AL-DAGHR & al [36]) and Indian population (BID & al [37], MUKHOPADHYAYA & al [3]), but not in Polish (MALECKI & al [38]), Turkish (DILMEC & al [39]), North Indian populations (BID & al [37]) and Tunisians (MAHJOUBI & al [40]). Our results are in agreement with studies which detected non-significant association between VDR polymorphisms and T2DM.

The size and stratification of the population, exposure to risk factors, differences in alleles frequency within the population and age range may partially explain differences between results of this study compared to those previously published.

Conclusion

Subjects carriers of -174C allele and infected with TTV were more common in T1DM patients than in controls. This allele has a modest association with T2DM in the Romanian population.

Our data suggest that VDR Taq1 genotype and VDR haplotype “F*1” represents a risk factor for T1DM. The presence of VDR F-A1 haplotype presented a trend of association with T2DM.

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References


