Association of Vitamin D Receptor Gene Polymorphisms and Type1 Diabetes in an Egyptian Population

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Abstract

Aim and background: Vitamin D is known for its immune modulatory functions and its role in normal insulin secretion. Vitamin D acts via vitamin D receptor gene (VDR). The study was conducted to assess the relationship between Apa I and Taq I restriction site polymorphisms of vitamin D receptor gene and type1 diabetes in an Egyptian population.

Subjects and Methods: One hundred Egyptian participants (1-20 years old) of both sexes were recruited in this study. Fifty of them were type 1 diabetes patients and the other fifty were control non diabetic subjects. Apa I and Taq I variations were tested using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism.

Results: Genetic analysis revealed that AA and Tt genotypes were predominant in the two study groups with higher frequency among patients group, whereas, aa and tt genotypes were predominant among controls (14% and 10%, respectively). Combined genotypes AA1T, AA1t and aA1T were significantly more frequent in controls (P value of odds ratio 0.032, 0.033, 0.002, respectively). The haplotype AT was more frequent among diabetics (41%), whereas, haplotypes At and aT were more frequent in control group (39%, 29%, respectively). Linkage analysis of Apa I and Taq I markers showed strong linkage disequilibrium between two markers (D=1).

Conclusion: Apa I and Taq I VDR gene polymorphisms have an association with T1D and may predispose to the risk of T1D affection. While, genotypes AA1T, AA1t and aA1T and haplotypes At and aT seem to have a possible protective effect against T1D.

Keywords: Genomic DNA; Single nucleotide polymorphism; Type 1 diabetes mellitus; Vitamin D receptor gene

Introduction

Type 1 diabetes (T1D) is an immune-mediated disorder where clinical disease develops as a result of interactions between genetic and environmental factors. T-lymphocytic infiltration occurs first in pancreatic islets causing insulin insufficiency and destruction of beta cells [1].

In 2013, The International Diabetes Federation stated that Egypt has the largest contribution to the total number of estimated childhood type 1 diabetes cases for Eastern Mediterranean and Middle East region which accounts for almost a quarter of the region’s total number. The incidence of type 1 diabetes is reported as 8 per 100,000 populations per year for those aged 14 years and under [2].

Type 1 diabetes is a multifactorial disease with strong genetic component. The major T1D susceptibility locus maps to the HLA class II genes and accounts for up to 30%–50% of genetic T1D risk. Other non-HLA T1D loci were known to produce smaller effects on disease risk compared to HLA genes. These loci include insulin gene (INS), cytokine T-lymphocyte associated protein 4 (CTLA4) genes and protein tyrosine phosphatase non receptor type 22 (PTPN22) gene [3].

The involvement of vitamin D in the etiology of both type 1 and type 2 diabetes has been suggested. Vitamin D compounds are known to suppress T-cell activation by binding to the vitamin D receptor (VDR). Polymorphisms of the VDR gene are likely to be related to T-cell mediated autoimmune disease [4].

Vitamin D is a fat-soluble vitamin that plays an important role in bone metabolism and seems to have some anti-inflammatory and immune-modulating properties [5]. Most of these biological actions of vitamin D are considered to be exerted through the nuclear vitamin D receptor (VDR)-mediated control of target genes [6].

The VDR gene, located on chromosome 12q13.11 encodes a polypeptide that binds 1, 25-dihydroxy-calciferol and interacts with target nuclei to produce a variety of effects [7]. Several major polymorphic sites have been described within the VDR gene. FokI in exon 2, Bsm I and Apa I both in intron 10 and Taq I in exon 11 are the four common single nucleotide polymorphisms (SNPs) for the VDR gene [8].

The Apa I (rs7975232) is located in intron 10. Apa I is a Restriction Fragment Length Polymorphism (RFLP) showing Guanine to Thymine nucleotide substitution. Chromosome position of the SNP is 47845054, and position of the SNP in the VDR gene is 64978, located on the forward strand [9].

Taq I polymorphism (rs731236) is a Restriction Fragment Length Polymorphism (RFLP) in exon 11 of the VDR gene. Taq I is a thymine to cytosine polymorphism. SNP lies on the forward strand of the VDR gene on position 65058 [10].

The conflicting results and unclear associations between VDR gene polymorphisms and T1D in addition to unavailable data in Egypt have encouraged us to examine two of the restriction site polymorphisms of VDR gene which are Apa I and Taq I in an Egyptian population.

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Subjects and Methods

Subjects

The study was conducted on fifty T1D Egyptian patients with median age 8.5 (5-12.7) years. Disease onset ranged from 1 to 5 years old. Fifty healthy participants matching in age and gender were recruited to the study. T1D patients with chronic illnesses or syndromes were excluded. The study was carried out upon attendants of The Pediatrics Clinic, Insurance Hospital, Ismailia. Individuals enrolled in the study were subjected to a structured interview together with their parents. A questionnaire was given to each parent and filled in. A short account about the aim of study and the procedures done were explained for each participant. The study was conducted in accordance with the guidelines in the Declaration of Helsinki and was approved by the Ethical Committee of the Faculty of Medicine, Suez Canal University (research approval No. ID 1662014). An informed consent was obtained from all participants.

Genotyping

Molecular analysis of Apa I and Taq I restriction site polymorphisms of vitamin D receptor gene was done to every participant. Five ml of venous blood was collected from each individual in a heparinized tube. Blood samples were either freshly used for DNA extraction or stored at -20°C for later use. DNA was extracted using QIAamp® DNA Blood Mini Spin Column Kits (Germany), catalog no. 51104. Amplification of target DNA sequence of VDR gene was done using 96-well Thermal Cycler, (Applied Biosystems Veriti (USA) P/N: 43757860). Resultant amplicon is represented in Figure 1. Figure 2 shows a demonstration using bioinformatics tools of amplified DNA segment and site for primers binding to the VDR segment [11-13].

PCR was done using 12.5 μl of Master Mix, GoTaq® Green Master Mix, (Promega (USA) Catalog no. M7112). Then adding 1 μl each of forward primers and reverse primers together with 1-5 μl of extracted DNA samples at concentration 200 ng/μl. Apa I and Taq I were targeted by common Apa/ Taq forward and reverse primers [14], PCR conditions are shown in Table 1. For testing PCR amplification of the target DNA segment of VDR gene, gel electrophoresis was done using 2% agarose gel.

Amplified DNA sequences were digested separately by Apa I Restriction Enzyme, (FastDigestApa I, Fermentas (USA), catalog no. FD1414) and Taq I Restriction Enzyme, (FastDigest Taq I, Fermentas (USA), catalog no. FD0674). For each SNP, 17 μl of nuclease free water, 2 μl of buffer, 1 μl of enzyme and 10 μl of PCR product were added. Tubes digested by Apa I were incubated at 65ºC for 20 min, while tubes digested by Taq I were incubated at 65ºC for 5 min. Digested DNA fragments were electrophoresed on 2.5% agarose gel and finally viewed under ultraviolet transilluminator (Figures 3 and 4).

Depending on the presence or absence of Apa I restriction site, alleles found are AA (745 bp), aa (217 and 528 bp) and Aa (745, 217 and 528 bp). For Taq I, alleles are TT (494 and 251 bp), Tt of (294, 200 and 251 bp) and Tt (494, 294, 200 and 251 bp). Upper case letters denote the absence of restriction site and lower case letters denote presence of restriction site.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Initial Denaturation</th>
<th>Subsequent Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>95°C</td>
<td>95°C</td>
<td>67°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td>Duration</td>
<td>2 min</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
<td>5 min</td>
</tr>
</tbody>
</table>

Table 1: PCR conditions.

Statistical analysis

Data were managed using Microsoft® Excel 2010 and "Statistical Package for Social Sciences (SPSS) for windows", version 20.0. Descriptive statistics were represented as median and ranges from the 25th to the 75th percentile for non-parametric variables calculated using Minitab 17 version 17.2.1. Qualitative variables were described in percentage. Chi square and Kruskal Wallis tests were used.

The allele frequency within each group was determined as the number of occurrences of an individual allele divided by the total number of alleles. The carriage rate was calculated as the number of occurrences of a particular allele divided by the total number of alleles.
individuals carrying at least one of the investigated alleles divided by the total number of individuals in each group [15,16].

Hardy-Weinberg equilibrium (HWE) was used to calculate the expected genotype frequencies among patient and control groups [17].

Differences in genotypic and allelic distribution of VDR polymorphisms between patients and controls were determined by Pearson Chi-square ($\chi^2$). The $P$ value less than 0.05 was regarded as statistically significant. Haplotype analysis was performed by CHAPLIN 1.2.3 software. Pairwise linkage disequilibrium (LD) between the VDR gene polymorphisms was computed and LD plots were constructed using Haplovie software, version 4.2.

Results

Genotype frequencies of Apa I and Taq I among patients and controls are shown in Table 2. Regarding Apa I genotype, AA genotype is predominant as both diabetic (60%) and control groups (56%). Genotype aa was the least frequent in both groups, however, aa genotype was observed to have increased frequency in control group (14%) than patients group (4%). Differences in distribution are statistically insignificant. In Taq I genotype, the frequency of TT was the highest among the two groups, while genotype tt was the lowest. TT genotype showed the same distribution among patients and controls (36%). Tt genotype showed increased distribution among patients while tt showed increased distribution among controls. Chi square test and odds ratio (OR) test were done with no significant differences. P value for Hardy Weinberg Equilibrium is calculated for Apa I and Taq I genotypes (Table 2). All results are in equilibrium with Hardy Weinberg.

Distribution of Apa I and Taq I genotypes and alleles according to gender, family history or age at diagnosis in both patients and controls revealed no significant results. In addition, distribution of haplotypes and genotype combinations according to gender and family history in patient and control groups showed statistically insignificant differences.

The frequency of Apa I alleles $A$ versus $a$ represented 78% and 71% versus 22% and 29% in patients and controls respectively. Taq I alleles showed also no significant difference between diabetic and control groups ($P=0.25$), where $T$ versus $t$ represented 63% and 61% versus 37% and 39% patients and controls ($P=0.77$), respectively (Figure 5). Odds ratios didn’t show increased risk of any allele to acquire disease than the control. In Apa I alleles, OR of $a$ allele (CI 95%) was 0.69 (0.36-1.31) and OR of $t$ allele (CI 95%) of Taq I variant was 0.91 (0.51-1.62). P values of OR tests are greater than 0.05.

A allele showed higher rate of carriage in the patients group (78%) than in the control group (71%). However there was no significant difference between diabetic and control group regarding the carriage of A allele. $T$ allele also showed higher rate in the diabetic group that was not significant as well (Table 3).

Haplotype analysis showed that the three haplotypes $AT$, $At$ and $AT$ were found among the diabetic and control groups in frequencies shown in Table 4. Haplotype “at” was absent among patient and control groups. Haplotype $AT$ was found in 41% of patients whereas;

<table>
<thead>
<tr>
<th>Variables</th>
<th>Patients (n=50)</th>
<th>Controls (n=50)</th>
<th>$X^2$</th>
<th>P value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apa I Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>30 (60)</td>
<td>28 (56)</td>
<td>3.1</td>
<td>0.2</td>
<td>Reference</td>
</tr>
<tr>
<td>$Aa$</td>
<td>18 (36)</td>
<td>15 (30)</td>
<td>1.12 (0.47-2.63)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$aa$</td>
<td>2 (4)</td>
<td>7 (14)</td>
<td>0.26 (0.05-1.39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{tac}$</td>
<td>0.72</td>
<td>0.054</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq I Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>18 (36)</td>
<td>18 (36)</td>
<td></td>
<td></td>
<td>Reference</td>
</tr>
<tr>
<td>$Tt$</td>
<td>27 (54)</td>
<td>25 (50)</td>
<td>0.4</td>
<td>0.8</td>
<td>1.08 (0.46-2.42)</td>
</tr>
<tr>
<td>$tt$</td>
<td>5 (10)</td>
<td>7 (14)</td>
<td>0.71 (0.19-2.67)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$P_{tac}$</td>
<td>0.44</td>
<td>0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Confidence interval (CI), Odds ratio (OR). Two sided Chi square test and odds ratio 95% CI test were used

Table 2: Genotype frequency of Apa I and Taq I among patients and controls.
in controls it represents 32%. On the other hand at and Aa showed higher frequencies in controls (29% and 39%, respectively) than in patients (22% and 37% respectively). Chi square test ($\chi^2$) didn't reveal statistically significant differences.

Linkage disequilibrium (LD) analysis shows that Apa I and Taq I SNPs of the VDR gene are in a single haplotype block as shown in Figure (6). The normalized correlation coefficient (D') equals 1 which signifies high LD between the two SNPs.

Different genotype combinations of Apa I and Taq I variants studied are shown in Table 5. Chi square test showed significant difference among genotype combinations in both diabetic and control groups (P value=0.02). The Genotypes AAT, AATT and aatt were shown to be protective against T1D (P value of OR= 0.032, 0.033 and 0.002), respectively.

Discussion

In this study, the most frequent Apa I polymorphism in the control group was AA. This result was inconsistent with several studies who reported Aa genotype as the most frequent genotype among Egyptian control subjects in their studies [18-20]. However in another study, genotype frequency of Aa and AA were equal among an Egyptian control group [21].

The reported differences in genotype frequencies may be attributed to small sample sizes or ethnic differences. Iranian study found that genotype frequency of AA and Aa were equal among an Iranian control group [22]. Studies reported that Aa is the predominant genotype in a Saudi and a Syrian populations, respectively [23,24]. Throughout different populations Aa was found to be the most frequent genotype in Taiwan [25], Hungary [26], Finland [7], Croatia [26], Italy [27] and Spain [28]. Study on Japanese population revealed that genotype frequencies of Aa and aa were equal in the control group [29].

In the diabetic group, AA genotype of Apa I polymorphism was the most frequent genotype. This frequency was consistent with the findings of Egyptian study [19]. In addition, our results are in agreement with the findings in Saudi [23], Croatian [30] and Italian populations [27]. On the other hand, the studies conducted on Taiwanese [25], Hungarian [26], Finnish [7], Spanish [28] and Iranian [22] populations differed with our results where Aa genotype was the most frequent.

Regarding genotype frequency of Taq I polymorphism in control group, our study found that TT genotype was the most predominant. This result was consistent with two Egyptian studies [18,21]. However another two Egyptian studies reported that TT was the most common genotype among control group [19,20]. Again, small sample sizes may...
Table 5. Frequency of genotype combinations among patients and controls.

<table>
<thead>
<tr>
<th>Genotype Combination</th>
<th>Patients (%)</th>
<th>Controls (%)</th>
<th>X²</th>
<th>P Value</th>
<th>OR (95% CI)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>AATT</td>
<td>20</td>
<td>8</td>
<td>13.37</td>
<td>0.02*</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>AATt</td>
<td>30</td>
<td>34</td>
<td></td>
<td>0.35 (0.135-0.917)</td>
<td>0.032*</td>
<td></td>
</tr>
<tr>
<td>AAt</td>
<td>10</td>
<td>14</td>
<td></td>
<td>0.28 (0.090-0.905)</td>
<td>0.033*</td>
<td></td>
</tr>
<tr>
<td>AsTt</td>
<td>12</td>
<td>14</td>
<td></td>
<td>0.34 (0.111-0.95)</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>AtTt</td>
<td>24</td>
<td>16</td>
<td></td>
<td>0.60 (0.213-1.69)</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>atTt</td>
<td>4</td>
<td>14</td>
<td></td>
<td>0.11 (0.02-0.45)</td>
<td>0.002*</td>
<td></td>
</tr>
</tbody>
</table>

Confidence interval (CI), Odds ratio (OR). Two sided Chi square test and odds ratio 95% CI test were used. *: statistically significant (p ≤ 0.05)

Reference

To summarize, populations from different ethnic backgrounds were reported to have an association between Apa I-Taq I VDR gene variations and T1D. However, other populations from different ethnic backgrounds were reported to have no association: Southern Indians [34], Japanese [29], Hungarian [26], German [31], Croatian [32], Italian [27] and Spanish [28] populations. The T allele was predominant.

In our study, comparing patients to controls revealed that A and T alleles were more prevalent in patients than in controls on expense of a and t alleles which were observed to be higher in control group. This result may propose the possibility that a and t alleles have a protective role against T1D. The apparent discrepancies between our results and other studies could be due to ethnic differences and sample heterogeneity related to the distribution of VDR polymorphisms in these populations, as well as to interactions with other genetic or environmental factors involved in the pathogenesis of type 1 diabetes.

In this study, linkage disequilibrium (LD) analysis of VDR gene markers (Apa I and Taq I) showed that Apa I and Taq I are in strong linkage disequilibrium where D'=1. This result is in concordance with studies conducted on German [14], British [33] and Egyptian [19] populations who reported a strong LD between Apa I, Bsm I and Taq I.

Haplotyping analysis of Apa I and Taq I markers in T1D patient and control groups in our study, revealed that the most frequent haplotype in T1D patients was AT4 (41%) while in control group. AT haplotype predominates (39%). The affinity of A to T alleles increases in patients group resulted in increase of haplotype AT on expense of aT that decreases in patients. No significant difference was observed in haplotype distribution among patients and controls. This result is in disagreement with an Egyptian study that suggested an association between Apa I and Taq I haplotype and T1D in an Egyptian population [19].

A study conducted on 152 Caucasian families with at least one affected offspring with T1D revealed that haplotype At was preferentially transmitted to affected offspring which signified increased risk for T1D affection. In addition, haplotypes AT and at were considered to be protective against T1D as they were preferentially not transmitted [14]. A study tested Apa I–Taq I haplotypes on 1811 British diabetic families and found no significant association between this haplotype and T1D [33].

Genotype combinations in our study showed that AATT, AATT and aTt had statistically significant protective effect against T1D. This result was consistent with studies conducted on German [14], British [33] and Egyptian [19] populations.

In summary, populations from different ethnic backgrounds were reported to have an association between Apa I-Taq I VDR gene variations and T1D: Germans [14], Taiwanese [25], South Croatian [32] and Spanish [28]. However, other populations from different ethnic backgrounds were reported to have no association: Southern Indians [34], Japanese [29], Hungarian [26], Finnish [7], Chinese [35], European [33], Portuguese [36] and South Croatian [37]. Conflicts in associations between VDR gene markers and T1D may be explained by the relatively small sample sizes, laboratory techniques, and different ethnic backgrounds. However, environmental factors disposing to body weight, and growth should be considered as likely candidates to affect the association between VDR polymorphism and development of T1D in different populations.
Conclusion

1. The A and T alleles of the Apa I and Taq I VDR gene may predispose to the risk of T1D affection, whereas, a and t alleles may have a protective role against T1D.

2. Genotypes AA and Au of Apa I variants and TT for Taq I may have a role in T1D pathogenesis, whereas, aa and tt genotypes may have a possible protective effect against T1D.

3. The combined genotypes AATt, AAtt and aATT offer protection against T1D.

4. The haplotype AT may be implicated in T1D risk, whereas, haplotypes At and aT could have a possible protective effect against T1D.

Conflicts of Interest

Authors declare they have no conflicts of interest.

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10. http://www.ensembl.org/Homo_sapiens/Variation/Explore?db=core&g=ENSG00000111424; r=133273539-133273539;v=rs7975232;vdb=variation;vf=515885