Association between vitamin D receptor gene polymorphisms and Hypothyroidism in Saudi population.

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ABSTRACT  
Objective: To investigate three polymorphic sites Apal, BsmI and TaqI of vitamin D receptor (VDR) gene on chromosome 12q (12–12q14) as candidate for hypothyroidism (HT) in Saudi population.

Method: A sample of 100 Saudi classified as healthy (control, N= 50 (without HT)) or with HT (N= 50) was investigated the relationship of VDR gene polymorphisms at three restriction sites Apal, BsmI and TaqI to the risk of HT. Blood samples were taken and DNA was extracted from whole blood. The target part of VDR gene was isolated and amplified by the polymerase chain reaction (PCR). PCR products were digested by restriction enzymes: Apal, TaqI, and BsmI and electrophoresed on agarose gel.

Result: Thyroid-stimulating hormone (TSH) levels were statistically higher in HT than controls (P-value= 2.4E-10). The difference in Vitamin D Total (Vit.D) values between each two groups shows significant difference p-value =0.0207<0.05. However, we observed that there was no difference in the distribution of genotypes frequency of the BsmI TaqI and Apal VDR gene polymorphisms differed significant between HT and control groups (p= .0127<0.05) for BsmI (p= .0150<0.05) for TaqI and (p= .0376<0.05) for Apal.

Conclusion: The VDR gene polymorphisms analysis at all three restriction sites BsmI, TaqI and Apal were associated with the risk of hypothyroidism in Saudi population.

INTRODUCTION  
Hypothyroidism is a common endocrine disorder resulting from deficiency of thyroid hormone. It usually is a primary process in which the thyroid gland is unable to produce sufficient amounts of thyroid hormone. Several studies have shown that this disorder is quite common (10% in an elderly population). It is still a matter of controversy whether this disorder should be screened and treated.

Vitamin D is involved in biological processes such as bone metabolism, cell proliferation and differentiation. In the classical endocrine pathway, vitamin D enters the circulation attached to a D-binding protein, is first hydroxylated in the liver to 25(OH)D and then in the kidney to form the active metabolite, 1, 25 dihydroxy vitamin D (1,25-(OH)2D) or calcitriol.

Vitamin D exerts its genomic action via the nuclear vitamin D receptor (VDR), which shows an extensive polymorphism. The VDR gene is located on chromosome 12q (12–12q14) and is highly polymorphic. The VDR belongs to the steroid receptor super-family and is widely expressed in many cell types, including lymphocytes, macrophages, and pancreatic –cells. Four major polymorphic sites have been described with the VDR gene. A polymorphic FokI site in exon 2 results in an alternative transcription initiation site, leading to a protein variant with three additional amino acids at the amino terminus. Polymorphic BsmI and Apal sites are present in intron 8, and a silent T to C substitution creates a TaqI restriction site in exon 9.

Importantly, both vitamin D and thyroid hormone bind to similar receptors called steroid hormone receptors. A different gene in the Vitamin D receptor was shown to predispose people autoimmunity thyroid disease including Graves’ disease and Hashimoto’s thyroiditis. These reasons, it is important for patients with thyroid problems to understand how the vitamin D system works.

Recently, many studies have shown that low levels of vitamin D contribute to Graves’ disease (GD) and Hashimoto thyroiditis (HT) and that combining vitamin D with anti-thyroid drugs or thyroid hormone contributes to the treatment of autoimmune thyroid disease (AITD) by suppressing the autoimmune reaction and reducing serum levels of thyroid autoantibodies. However, other authors have proposed that vitamin D deficiency does not increase the risk of AITD and is not associated with early-stage AITD. Because the association between vitamin D levels and AITD is still controversial. Inconsistency was attributed to the environmental factors that potentially interfere with the VDR genotypes. At this time the research on its role in autoimmune and thyroid disease is not conclusive. The biological effect of vitamin D is thought to occur by binding to the vitamin D receptor (VDR) which belongs to the steroid receptor super-family. However, BsmI (rs1544410), Apal (rs7975232), and TaqI (rs731236) SNPs, located near the 3′ end of the VDR gene, are in strong linkage disequilibrium (LD) with each other. These three SNPs don't change the amino acid sequence of the encoded protein but have been shown to affect gene expression through regulation of mRNA stability. Therefore, we have investigated three polymorphic sites Apal, BsmI and TaqI of VDR gene on chromosome 12q (12–12q14) as candidate for HT susceptibility locus for the first time in Saudi population.

MATERIAL AND METHODS  
Subjects  
The VDR gene polymorphisms analysis study included a total of 100 Saudi volunteers. The diagnosis criteria of HT were according to the World Health Organization. Subjects were divided into two groups HT and non-HT. Total number of 50 Saudi patients (15 men and 35 women) with HT. Fifty healthy control subjects were studied (25 men and 25 women). They consisted of individuals with no history of thyroid or other autoimmune disease. This criterion depends upon two tests: Thyroid-stimulating hormone (TSH) and Vitamin D Total Test (Vit.D). We collected the clinical parameters for each HT volunteer upon diagnosis: TSH test, Vit.D test. These medical details were obtained from patient hospital files after obtaining the consent from the administration. The previous history of HT and family history of thyroid were taken from patient. The same...
parameters were recorded for the control volunteer. All subjects were selected from those who routinely attended diabetic clinic, Association of Diabetic Patient Friends Taif, King Faisal Hospital (KFH), Taif, Saudi Arabia. The study was approved by the ethical committee. All participants in the study filled a questionnaire and also signed a consent form.

Study Design
Genomic DNA was extracted from whole blood samples, in bio safety cabinet, using Thermo Scientific DNA extraction kit Blood (Thermo Scientific, USA. Cat.no. K0781). The extracted DNA was stored at -20°C for PCR amplification.

For Polymerase Chain Reaction (PCR), the reactions were prepared using Maxima® Hot Start Green PCR Master Mix (2x). The primers were from Macrogen. The forward primer was (5’-CAAGGACCACTCAGTGATGGAATG-3’) and the reverse one was (5’-CCAGGCTGCTAGAAGT-3’). For polymerase Chain Reaction (PCR), the master mix from Thermo Scientific was used. The mixture (50µl) contained 2X reaction buffer, 4mM Mg²⁺, 4µM deoxyribonucleoside triphosphates, 0.2µM of each primer, 0.45U Taq DNA polymerase and 10-30 ng of DNA template. The total reaction volume was made up to 50µl with nuclease free water.

The PCR tubes were transferred to thermal cycle. The amplification conditions were: an initial denaturation for 4 min at 95°C, 30 cycles each of which consisted of (denaturation at 95°C for 30 S, annealing at 60°C for 1 min and an extension at 68°C for 2 min), and final extension for 5 min at 72°C and ended at hold at 4°C. The PCR products were verified by 1% agarose gel electrophoresis at 100 V for 20˚C for PCR amplification.

VDR genotyping
Amplified PCR products (5 µl) were digested with 3000 U of each restriction enzyme from Thermo Scientific. These enzymes are Acetobacter pasteurianus ApaI, Bacillus stearothermophilus BsmI and Thermus aquaticus YTI TaqI. These enzymes were used to the supplier-recommended protocols by Thermo Scientific. By using the thermal cycler, the reaction was incubated in 37°C for 1 hour which is the activation temperature of BsmI and ApaI. Then the enzymes were inactivated by incubation at 65°C for 20 min. After that, the reaction was incubated 65°C for 1 hour which is activation temperature of TaqI, and then the enzymes were inactivated by incubation at 80°C for 20 min.

Statistical analysis
All statistical analyses were performed using the MegStat® version 9.0 computer program. Descriptive data were given as mean ± standard deviation (SD). Differences among groups were tested using the t-test. Differences in distribution of the genotypes between the females and males according to HT and Non-HT were examined with the chi-square analysis. Differences in genotype frequencies were considered statistically significant for p-value < 0.05.

Results
The Main Characteristic of the study group
The volunteers in this study were classified according to Thyroid-stimulating hormone (TSH) test as two groups HT (35 Females, 15 Males) and non-HT (25 Females, 25 Males) totally 50 subjects. The clinical and biochemical parameters for the two groups HT and control sample are shown in Table 1. The Thyroid-stimulating hormone (TSH) values between HT and non-HT patients shows significant difference p-value=2.44E-10<0.05, but non-significant difference between females and males in two groups. The vitamin D Total (Vit.D) values between each two groups females and males are significant difference p-value =0.0207<0.05 between HT and non-HT.

Table 1 Clinical and biochemical characteristics of non-HT and HT

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HT Mean ± SD</th>
<th>Non-HT Mean ± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D Total (Vit.D) (nmol/L)</td>
<td>17.3±14.50</td>
<td>20.2±15.33</td>
<td>0.0207</td>
</tr>
<tr>
<td>Thyroid-stimulating hormone (TSH) (nmol/L)</td>
<td>12.060±8.959 2.934±1.8273 2.44E-10*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Highly significant difference.

Data is represented as mean ± SD

P-Value for control groups and HT.

Figure 1 Digestion results of the 3 polymorphism sites. Lane 1: 1kb DNA ladder. Lane 2 negative control, lane 3: PCR Products (2229 bp). ApaI digestion: control(2229bp,1900bp,500bp) patient(2229bp,1900bp,500bp). BsmI digestion: control (2229bp), patient(2229bp,1900bp,700bp). TaqI digestion: control (2229bp).patient(2229bp,250bp).

The PCR products in our samples from Saudi volunteers, ~ 2229 bp as shown in Figure 1, were digested with the BsmI, TaqI and ApaI restriction enzymes.

Statistical analysis of Genotypes Frequencies of BsmI,TaqI and Apal Polymorphisms in study Groups at HT and Control samples

Frequencies of VDR alleles in the two groups, irrespective of whether it was in females and males, shown in Table 2. Accordingly, the distribution of alleles frequency of the BsmI, TaqI and Apal VDR polymorphisms differed significantly between hypothyroidism and controls. The frequency of B allele was more frequent in control group compared with hypothyroidism patients and b more in hypothyroidism. Furthermore, the frequency of T allele was more frequent in hypothyroidism patients compared with control group and t more in control and frequency of A allele was more frequent in control and frequency of a allele was more frequent in hypothyroidism. Therefore was observed significant difference between them (p<0.0127<0.05) for BsmI (p=0.0150<0.05) for TaqI and (p=0.0376<0.05) for Apal.

Table 2 Distribution of VDR gene polymorphisms in patients with HT and non-HT

<table>
<thead>
<tr>
<th>VDR Polymorphisms</th>
<th>Case (n=50)</th>
<th>Controls (n=50)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsmI Genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BB</td>
<td>17</td>
<td>34</td>
<td>48</td>
</tr>
<tr>
<td>Bb</td>
<td>10</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>bb</td>
<td>23</td>
<td>46</td>
<td>5</td>
</tr>
<tr>
<td>Apal Genotypes</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>11</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td>Aa</td>
<td>6</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Bb</td>
<td>33</td>
<td>66</td>
<td>19</td>
</tr>
<tr>
<td>TaqI Genotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>21</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td>Tt</td>
<td>18</td>
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</tr>
<tr>
<td>tt</td>
<td>11</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>Bsm I Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>44</td>
<td>44</td>
<td>69</td>
</tr>
<tr>
<td>b</td>
<td>56</td>
<td>56</td>
<td>31</td>
</tr>
<tr>
<td>Apal Alleles</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>28</td>
<td>28</td>
<td>45</td>
</tr>
<tr>
<td>a</td>
<td>72</td>
<td>72</td>
<td>55</td>
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</table>

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We examined the association of VDR gene polymorphisms at three positions (Apal, BsmI and TaqI) with hyperthyroidism. Our results demonstrated alleles of BsmI gene polymorphisms was more frequent in healthy controls compared with HT, alleles of TaqI and Apal were more frequent in HT and there were significant difference in alleles frequency between cases and controls (p = 0.0127<0.05) for BsmI (p=0.9150<0.05) for TaqI and (p=0.0376>0.05) for Apal. Therefore, allele b of BsmI, allele T in TaqI and allele a in Apal region are risk alleles in Saudi population.

This outcome matches the outcome of the largest meta-analysis which found relationship between BsmI and TaqI polymorphisms in VDR and HT, whereas the Apal polymorphisms do not appear to have a significant association with overall HT risk. Therefore, three studies indicated TaqI polymorphism was associated with risk of HT in Croatian and Turkish populations (27); (28); (29). but four other studies from China, Japan, Italy and Serbia showed no association (30); (31); (32); (33).

Furthermore, the apparent discrepancies between this study and other could be a result of the effect of ethnic differences related to the distribution of VDR polymorphisms in these populations as well as interactions with other environmental factors (34). Therefore, the environmental factor influence levels of active vitamin D in humans are complex and a significant difference exists between vitamin D functions and VDR polymorphisms (35).

Some study found a higher frequency of variant allele of Apal and TaqI, but not significantly different between patients with HT and healthy controls (P > 0.05). In most epidemiological studies, however, associations were examined between SNPs, or between the BsmI,Apal and TaqI linkage group and the physiological parameter of interest ( (36); (37) ), but there is a lack of analysis of the direct influence of allelic variation on VDR protein expression or activity. In fact, BsmI, Apal or TaqI alleles have no effect on either the expression levels or the activity of the translated VDR protein ( (38); (39) ).

Conclusion

our case control study indicated that the VDR polymorphism in BsmI , Apal and TaqI regions are associated with hyperthyroidism in the Saudi population. We recommend using the same methodology with larger sample size in the same population. In addition, future studies on the correlation between environmental factor such as UV immune response, VDRSNPs and HT may also be considered.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

References:

21. Variation in associations between allelic variants of the vitamin D receptor gene and


