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Vitamin D Receptor Gene Polymorphisms and HLA DRB1*04 Cosegregation in Saudi Type 2 Diabetes Patients

Nasser M. Al-Daghri,*†‡ Omar Al-Attas,*†‡ Majed S. Alokail,*†‡ Khalid M. Alkharfy,*†§ Hossam M. Draz,* Cristina Agliardi,¶ Abdul Khader Mohammed,* Franca R. Guerini,¶ and Mario Clerici*‖

The vitamin D receptor (VDR) gene has been involved in the modulation of susceptibility to inflammatory and autoimmune conditions, and could play a role in the pathogenesis of type 2 diabetes mellitus (T2DM). Susceptibility to T2DM was recently suggested to be associated with HLA alleles, and risk for development of T2DM by analyzing 627 individuals (368 T2DM patients and 259 healthy control subjects) part of a well-characterized cohort followed in Riyadh, Kingdom of Saudi Arabia. Genomic DNA was genotyped for the VDR gene single nucleotide polymorphisms of Fok-I, Taq-I, ApaI, and Bsm-I. Analyses were run by allelic discrimination real-time PCR. HLA genotyping was performed as well by PCR using sequence-specific primers, whereas cytokine production was evaluated by FACS. Results showed T2DM to be significantly associated with the VDR Taq1 (rs731236-AG) and Bsm-I (rs1544410-CT) genotypes, and the VDR rs1544410-T allele. Cosegregations resulting in significant increases of T2DM odds ratio were detected between Taq1 and Bsm-I VDR polymorphisms and HLA DRB1*04. Notably, the VDR polymorphisms observed to be more frequent in T2DM patients correlated with increased VDR expression and IL-12 production, as well as with metabolic parameters of susceptibility to T2DM, including serum cholesterol and high-density lipoprotein levels. VDR polymorphisms are present in T2DM, and correlate with HLA DRB1*04 and with immunologic and metabolic parameters; results from this study add T2DM to the list of diseases that are likely modulated by an HLA/VDR interaction. The Journal of Immunology, 2012, 188: 000–000.

The presence of a correlation between vitamin D receptor (VDR) polymorphisms and type 2 diabetes mellitus (T2DM)-associated metabolic parameters, including fasting glucose, glucose intolerance, insulin sensitivity, insulin secretion, and calcitriol levels, has been reported by observational studies (1–5). Among such VDR polymorphisms, Fok1, Taq1, Bsm-I, EcoRV, and ApaI are suspected to alter the activity of the VDR protein (6, 7) and to modulate susceptibility to T2DM. VDR binds vitamin D, a secosteroid hormone ingested in the diet or synthesized in the skin when 7-dehydrocholesterol reacts with UVB light. This hormone has important immunomodulatory properties, and its depletion could play a part in the pathogenesis of both type 1 diabetes mellitus (T1DM) and T2DM (8, 9). Thus, vitamin D deficiency results in reduced insulin secretion in rats and humans, and its replenishment improves the function of pancreatic cells and glucose tolerance (10–13).

Vitamin D modulates the expression of the insulin receptor gene, as well as insulin secretion, and exerts its actions on target tissues by binding to the cytosolic/nuclear VDR, which is a member of the steroid/thyroid hormone receptor family that functions as a transcriptional activator of many genes. The VDR gene is thus an interesting environmental candidate for T2DM pathogenesis and clinical manifestations (2). This gene is located on chromosome 12q13.1, consists of 14 exons, and has an extensive promoter region capable of generating multiple tissue-specific transcripts (4, 14, 15). In fact, VDR is expressed in a large number of tissues, including those involved in the regulation of glucose metabolism, such as muscle and pancreatic cells (16–18). Upon binding vitamin D, and after the subsequent specific phosphorylation by kinase cascades, the VDR undergoes a conformational change that facilitates its binding to the retinoid X receptor (19). The resulting heterodimer interacts with vitamin D-responsive elements in the promoter region of target genes, thereby modifying their expression (20).

Almost 200 polymorphisms have been found in the VDR gene, but their influence on VDR protein function and signaling remains largely unknown (21). Most VDR gene polymorphisms, including the Bsm-I, ApaI, and Taq1 RFLPs, are located at the 3′ untranslated region of the gene (4). This region is involved in regulation of gene expression, particularly through the modulation of mRNA
stability (5, 14). Another polymorphism, Fok1, is localized within the 5’ end of the gene, near the promoter region. It consists of a T-to-C transition at exon 2 that eliminates the first potential ATG translation start site and allows a second one, 9 bp downstream, to be used (4).

T2DM is nevertheless a multifactorial disease in which HLA molecules are likely to play a role as well. This concept stems from results obtained in T1DM (22, 23) and was recently reinforced by the description of a possible protective role of HLA DRB1*02 in T2DM, probably by enhancement of self-tolerance, thereby protecting against the autoimmune-mediated reduction of insulin secretion (24).

An interaction between VDR and HLA alleles mediated by the vitamin D response element (VDRE) present in the promoter region of some HLA-DRB1 alleles was recently shown in T1DM (25). To verify whether associations could be established between VDR polymorphisms and HLA in T2DM, we analyzed the Fok-1, Taq-1, Apa-1, and Bsm-1 single nucleotide polymorphisms (SNPs) of the VDR gene and HLA DRB1 alleles in a Saudi Arabian population. The cosegregation of VDR SNPs and HLA-DRB1 alleles, as well as their possible influence on VDR expression and cytokine production, were also verified in the same individuals to evaluate possible interplays between these two genes, which are known to interact in autoimmune disease (24). Results indicate that the HLA DRB1*04:01 allele does cosegregate with specific VDR polymorphisms in patients affected by T2DM, and that this interaction is likely important in modulating disease susceptibility.

Materials and Methods

Patients and control subjects

A total of 627 Saudi individuals (368 T2DM patients and 259 healthy control subjects [HC]) were enrolled in the study. These individuals are part of the Biomarker Screening in Riyadh Project (RIYADH COHORT), a capital-wide epidemiologic study taken from more than ∼17,000 consenting Saudis coming from different Primary Health Care Centers. A generalized questionnaire aimed to seek demographic information and medical history was given to all participating subjects. Those with comorbidities that needed medical attention were excluded from the study. Written consent was obtained after orientation for the study. Ethical approval was granted by the Ethics Committee of the College of Science Research Center, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA). VDR mRNA expression and cytokine production were evaluated on a group of 88 additional individuals who were classified based on VDR genotypes as follows: VDR BsmI—rs51544410 CT, n = 33; CC, n = 29; TT, n = 26; VDR TaqI—rs7312326 AG, n = 28; AA, n = 30; GG, n = 30.

Anthropometry and blood collection

After an overnight fast (>10 h), participating subjects were requested to return to their respective Primary Health Care Centers for anthropometry and blood withdrawal. Anthropometry included height (to the nearest 0.5 cm), weight (to the nearest 0.1 kg), waist and hip circumference using a standardized measuring tape in centimeters, systolic and diastolic blood pressure measurements, and body mass index (BMI; calculated as kg/m2). Blood was transferred immediately to a nonheparinized tube for centrifugation. Serum was then transferred to a prelabeled plain tube, stored in −20°C freezer before analysis. Fasting glucose (FG), lipid profile, albumin, phosphorus, and calcium were measured using an automated analyzer (Konelab, Vantaa, Finland). Serum 25-hydroxy-vitamin D was measured by ELISA (IDS, Boldon Colliery, Tyne & Wear, U.K.).

VDR SNP analysis

Whole blood was collected in EDTA-containing tubes, and genomic DNA was isolated from whole blood using the blood genomic prep minispin kit (GE Healthcare) and stored at −20°C until analyzed. The four VDR SNPs (rs731236, rs1544410, rs7975322, and rs10735810) were evaluated by allelic discrimination real-time PCR using predesigned TaqMan probes (Applied Biosystems, Foster City, CA). The PCR consisted of a hot start at 95°C for 10 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min; fluorescence detection takes place at a temperature of 60°C. All assays were performed in 10-μl reactions, using TaqMan Genotyping Master Mix on 96-well plates using an ABI 7000 instrument (Applied Biosystems). Control samples representing all possible genotypes and a negative control were included in each reaction.

RNA extraction, DNase treatment, and cDNA synthesis

Total RNA was extracted using the RNeasy Mini extraction kit (Qiagen, Hilden, Germany) from 1.5 ml fresh peripheral blood. RNA was eluted in 35 μl RNase-Free water (Qiagen), and RNA concentrations were quantified by measuring the OD at 260-nm wavelengths using a spectrophotometer. Purity was determined as the 260/280 nm ratio with expected values between 1.8 and 2.0, indicating absence of protein contamination, and integrity was determined by electrophoresis on a formaldehyde agarose gel in the presence of ethidium bromide. Samples were treated with TURBO DNA-free DNase (Ambion, Austin, TX) following the manufacturer’s instructions. The reverse-transcription step was conducted on 1 μg RNA using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each 100-μl reaction mixture contained 10 μl of 10× RT buffer, 4 μl of 25× dNTP mix (100 mM), 10 μl of 10× RT random primers, 5 μl of MultiScribe Reverse Transcriptase, 21 μl nuclelease-free H2O, and 1 μg RNA isolated in nuclelease-free H2O in a volume of 50 μl. The thermal cycling conditions were as follows: one step at 25°C for 10 min followed by one step at 37°C for 2 h. cDNA samples were immediately stored at −20°C.

Quantitative real-time PCR

Experiments were performed in 96-well plates using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and a premade TaqMan probe (assay ID: Hs_01045840_m1). Three housekeeping genes (GAPDH, ACTB, YWHAZ) were used for normalization (assay IDs: Hs_99999905_m1, Hs_99999903_m1, and Hs_03044281_g1, respectively). The assessment of the amplification efficiencies of target and reference genes was carried out using the REST software (http://gene-quantification.com). Serial dilutions in triplets of a pool of 10 cDNAs were used for each transcript (VDR, YWHAZ, GAPDH, ACTB). The software determines the slope with a logarithmic algorithm, as well as an indication of the linearity of this logarithmic alignment using Pearson’s correlation coefficient. The efficiency (E) is in the 1 (minimum value) to 2 (theoretical maximum and optimum) range and is calculated from the slope, according to the equation $E = 10^{-1/slope}$.

Real-time relative expression experiments were performed according to the manufacturer’s instructions. In brief, 1 μl cDNA was used in a final PCR volume of 20 μl, containing 10 μl Gene Expression Master Mix, 8 μl H2O, and 1 μl TaqMan probe. PCR cycles were as follows: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Reactions were performed in triplicate, with nontemplate control for each gene and one intern calibration that removes the technical run-to-run variation between samples analyzed in different runs.

Cytometric analyses

PBMCs separated on lymphocyte separation medium (Organon Teknika, Durham, NC) were stained with CD4 and CD14 mAbs (Beckman Coulter, Brea, CA) before being treated with FIX and PERM (FIX & PERM Cell Permeabilization kits; eBioscience, San Diego, CA), fixed for 10 min in fixation medium (100 μl), washed, and finally resuspended in 100 μl permeabilization medium with FITC or PE-conjugated IFN-γ, IL-6, IL-12, or IL-17 specific mAb for 30 min at 4°C in the dark.

mAbs

The following mAbs were used: PE–Cy5–labeled anti-CD4 (clone SFCII12TD11; mouse IgG1), FITC– or PE–Cy5–labeled anti-CD14 (clone 16; Beckman-Coulter, Fullerton, CA); anti–IFN-γ–FITC (clone 4S.B3; mouse IgG1); eBioscience Cornerstone Court West, San Diego, CA); anti-human IL-6–FITC (clone 1936, mouse IgG1 isotype; R&D Systems, Minneapolis, MN), anti-human IL-12–FITC (clone 27537, mouse IgG1 isotype; R&D Systems), and anti-human IL-17–PC5 (clone BL168, mouse IgG1 isotype; BioLegend).

Analyses were performed using a Beckman-Coulter Cytomics FC-500 flow cytometer equipped with a single 15-mW argon ion laser operating at 488 nm and interfaced with CXP Software 2.1. A total of 200,000 events were acquired and gated on CD4 or CD14 expression and side scatter properties. FITC green fluorescence was collected through a 525-nm bandpass filter, PE orange-red fluorescence was collected through a 575-nm
The software integrates randomization and bootstrapping methods that analyses were performed using the REST 2009 Software (http://www.qiagen.com) to compare VDR expression levels within them. Relative expression analysis was performed by using the Expectation-Maximization algorithm implemented in PROC Haplotype in SAS Genetics. Notably, VDR polymorphisms and HLA DRB1 cosegregation in T2DM and HC were evaluated by 2XN contingency table and Pearson’s χ² analysis. Bonferroni correction for multiple test (p<0.05) was applied.

For real-time PCR experiments data analysis, samples were subdivided into groups according to their VDR rs731236 and VDR rs1544410 genotypes to compare VDR expression levels within them. Relative expression analyses were performed using the REST 2009 Software (http://www.qiagen.com/) that permits use of multiple reference genes for normalization, improving the reliability of results (25). The software integrates randomization and bootstrapping methods that test the statistical significance of calculated expression ratios by the hypothesis test (p[H1]) representing the probability of the alternate hypothesis that the difference between groups is due only to chance. Relative quantification of the target transcript is based on the mean cycle threshold deviation of control and sample groups, normalized by reference transcripts.

LD and haplotype frequency
A significant difference was detected in the haplotype rs731236_A + rs1544410_C + rs7975232_G + rs10735810_C, because this haplotype was more common in T2DM patients than in HC (OR 2.08, 95% CI 1.48–2.92; p = 0.020). Such differences were not observed in any of the other examined haplotypes (Table III). Notably, VDR gene polymorphisms in rs731236 (TaqI) were in LD with both rs1544410 (Bsm-I) and rs7975232 (ApaI) SNPs were also in LD with each other (R² = 0.84 and 0.44, respectively). Finally, the rs1544410 (Bsm-I) and rs7975232 (ApaI) SNPs were also in LD with each other (R² = 0.41). These results are shown in Fig. 1.

Association of VDR variants with VDR gene expression
To verify whether the different VDR rs731236 (TaqI) and VDR rs1544410 (BsmI) genotypes modulate the expression of the VDR gene, we evaluated mRNA expression by quantitative real-time PCR in an additional group of 88 additional individuals who were classified based on VDR genotypes (BsmI: rs1554440 CT, CC, or TT; VDR TaqI: rs731236 AG, AA, or GG). Results indicated the presence of a slight but consistent and statistically significant increase in VDR expression that was associated with those genotypes that are more common in T2DM patients. Thus, a 1.393-fold increase (p[H1] = 0.02; 95% CI 0.959–1.49) of VDR mRNA was detected in the VDR rs15544410 CT compared with CC genotype (Fig. 2A). In addition, a 1.369-fold increase (p[H1] = 0.01; 95% CI 0.935–1.453) of VDR mRNA level was seen in the VDR rs731236 (TaqI) AG compared with AA genotype (Fig. 2B).

Association of VDR variants with cytokine production
Because it has been suggested that increased amount of VDR protein could modulate cytokines production and, in particular, could result in an increased generation of IL-12 (26), we evaluated the expression of IL-12 and other inflammatory cytokines in relationhip with the different VDR genotypes; differences were calculated by ANOVA.

CD4⁺ and/or CD14⁺ cells that produce IFN-γ, IL-6, IL-12, and IL-17 were analyzed in basal conditions in the 88 additional individuals specified earlier. Results showed that IL-12–producing CD4⁺ cells were significantly increased (p < 0.05) in those VDR genotypes (VDR rs1544410 [BsmI] CT and VDR rs731236 [TaqI] AG) that are more common in T2DM patients. These results are shown in Fig. 2C and 2D. A tendency toward increased percent-

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Table I. Clinical characteristic of the T2DM patients and the HC enrolled in the study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HC</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>259</td>
<td>368</td>
</tr>
<tr>
<td><strong>Age (y)</strong></td>
<td>44.1 ± 9.9</td>
<td>51.5 ± 8.6*</td>
</tr>
<tr>
<td><strong>Systolic BP (mm Hg)</strong></td>
<td>118.0 ± 13.6</td>
<td>127.7 ± 14.7*</td>
</tr>
<tr>
<td><strong>Diastolic BP (mm Hg)</strong></td>
<td>75.2 ± 8.9</td>
<td>79.4 ± 9.2*</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>29.5 ± 6.2</td>
<td>32.0 ± 5.7*</td>
</tr>
<tr>
<td><strong>Hip circumference (cm)</strong></td>
<td>104.9 ± 20.3</td>
<td>105.8 ± 20.7</td>
</tr>
<tr>
<td><strong>Waist circumference (cm)</strong></td>
<td>89.9 ± 18.9</td>
<td>100.6 ± 19.4*</td>
</tr>
<tr>
<td><strong>Cholesterol (mmol/l)</strong></td>
<td>2.3 ± 0.42</td>
<td>2.6 ± 0.30*</td>
</tr>
<tr>
<td><strong>Glucose (mmol/l)</strong></td>
<td>5.1 ± 0.99</td>
<td>5.6 ± 1.0*</td>
</tr>
<tr>
<td><strong>LDL-cholesterol (mmol/l)</strong></td>
<td>5.4 ± 1.2</td>
<td>10.4 ± 1.5*</td>
</tr>
<tr>
<td><strong>HDL-cholesterol (mmol/l)</strong></td>
<td>4.1 ± 0.97</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td><strong>Phosphate ion (mmol/l)</strong></td>
<td>1.1 ± 0.26</td>
<td>1.2 ± 0.27</td>
</tr>
<tr>
<td><strong>Triglyceride (mmol/l)</strong></td>
<td>1.4 ± 0.10</td>
<td>2.1 ± 0.11*</td>
</tr>
<tr>
<td><strong>Corrected calcium (mmol/l)</strong></td>
<td>2.2 ± 0.50</td>
<td>2.5 ± 0.23*</td>
</tr>
<tr>
<td><strong>Vitamin D (nmol/l)</strong></td>
<td>19.4 ± 5.0</td>
<td>31.3 ± 1.8*</td>
</tr>
</tbody>
</table>

Median values ± SD are shown. *p < 0.05.
BP, blood pressure; LDL, low-density lipoprotein.
tages of CD14+CD16+ and CD4+IFN-γ cells, which nevertheless did not reach statistical significance, was detected as well in the same VDR genotypes that are more frequent in T2DM patients (data not shown).

**Association of VDR variants with metabolic parameters**

Metabolic parameters known to be associated with risk for development of T2DM were correlated next with the analyzed VDR SNPs. Results showed that higher cholesterol concentration was present in carriers of the AG rs731236 (TaqI) polymorphism compared with the AA genotypes (\( p = 0.022 \)). In addition, higher cholesterol (\( p = 0.014 \)) and lower HDL (\( p = 0.028 \)) concentrations, as well as lower calcium levels (\( p = 0.020 \)), were detected in the TT rs1544410 (BsmI) compared with the CC genotype (Supplemental Table I). Finally, lower cholesterol levels were observed in CC compared with the AA genotype of rs7975232 (Apa-I; \( p = 0.018 \); Supplemental Table II).

**Cosegregation of VDR genotypes and HLA alleles**

Analyses of HLA DRB1 distribution were performed in T2DM patients and HC; no significant differences were observed either when 4-digit resolution DRB1 (25 alleles) or when low-resolution DRB1 (13 alleles) distribution was analyzed (data not shown).

We next verified the presence of possible associations between VDR SNPs and HLA alleles; results of the cosegregation analysis of HLA DRB1 alleles together with different VDR genotypes evaluated by Pearson’s \( \chi^2 \) analysis showed that the HLA DRB1*04/VDR rs731236 (TaqI) and HLA DRB1*04/VDR rs1544410 (BsmI) complexes were significantly more frequent (\( p = 0.001 \) in both cases) in T2DM patients compared with HC (data not shown).

Because different DR4 subtypes (e.g., DRB1*04:01 versus DRB1*04:03) were suggested to have opposing effects on susceptibility to type 1 diabetes (23), cosegregation data of different VDR genotypes were analyzed next, together with high-resolution Table III. Haplotype frequency of examined VDR variants in T2DM versus HC

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>HC</th>
<th>T2DM</th>
<th>OR (95% CI)</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCC</td>
<td>30</td>
<td>27</td>
<td>0.48 (0.26–0.87)</td>
<td>0.020</td>
</tr>
<tr>
<td>GTAC</td>
<td>30</td>
<td>37</td>
<td>0.65 (0.37–1.15)</td>
<td>0.186</td>
</tr>
<tr>
<td>ACAT</td>
<td>32</td>
<td>49</td>
<td>0.81 (0.47–1.4)</td>
<td>0.490</td>
</tr>
<tr>
<td>ACCT</td>
<td>76</td>
<td>104</td>
<td>0.73 (0.47–1.1)</td>
<td>0.163</td>
</tr>
<tr>
<td>GTAT</td>
<td>65</td>
<td>122</td>
<td>1.0 (Reference)</td>
<td></td>
</tr>
</tbody>
</table>

Order of SNPs: rs731236, rs1544410, rs7975232, rs10735810. Pearson’s \( p \) value and OR (95% CI) were calculated by haplotype analysis.

Figure 1. LD analysis (\( \chi^2 \)) for the four analyzed SNPs. (1) rs731236 (TaqI) (chr12:46525024); (2) rs7975232 (ApaI) (chr12:46525104); (3) rs1544410 (BsmI) (chr12:46526102); (4) rs10735810 (FokI) (chr12:46559162). Genomic coordinates refer to the NCBI36/hg18 human genome assembly, \( r^2 \) represents the squared correlation coefficient between two SNP.
Distally significant associations were observed between the HLA DRB1*04:01 genotype and VDR rs731236 (TaqI), and between the HLA DRB1*04:01 genotype and rs1544410 (Bsm-I) complexes (Table IV).

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FIGURE 2. VDR is significantly upregulated in individuals expressing the BsmI rs1544410 CT (A) or the TaqI rs731236 AG (B) genotypes. IL-12–producing CD14+ cells are significantly increased in individuals expressing the BsmI rs1544410 CT (C) or the TaqI rs731236 AG (D) genotypes. Boxes represent the interquartile range, or the middle 50% of observations. Dotted line represents the median VDR expression (A, B) and the median percentage of CD14+IL-12+ cells (C, D); whiskers represent the minimum and maximum observations.

Table IV. Cosegregation of HLA DRB1*04 (high-resolution genotypes) with VDR rs731236 (TaqI) and VDR rs1544410 (BsmI) patterns in Saudi Arabian patients affected by T2DM and in HC

Answers were performed on DR4+ subjects. The number of DR4+ subjects is not equal to the sum of each genotype because four T2DM patients and four HC were heterozygous for DRB1*04:01:03.

n, Number of subjects positive for each genotype.
Similar results were observed for the DRB1*04:01 allele in association with the VDR rs1544410 (CC) genotype (DRB1*04:01/CC; 12.56 versus 61.1% in HC; OR 0.1, 95% CI 0.01–0.51). On the contrary, the DRB1*04:01/VDR rs731236 (AG) genotype (DRB1*04:01/AG) complex was significantly more frequent in T2DM patients (70.8 versus 27.8% in HC); notably, the presence of DRB1*04:01/AG complex was associated with a significantly increased OR (OR 6.31; 95% CI 1.36–3.57). Finally, the combination of the DRB1*04:01 allele and the VDR rs1544410 (CT) genotype (DRB1*04:01/CT), more frequently detected as well in T2DM patients (70.8 versus 27.8% in HC), was also associated with an increased OR (OR 6.31; 95% CI 1.36–3.57). Interestingly, OR were patients (70.8 versus 27.8% in HC), was also associated with an increased OR (OR 6.31; 95% CI 1.36–3.57). Notably, the presence of T2DM patients (70.8 versus 36.5% in HC; OR 1.97; 95% CI 1.32–2.96; Fig. 3).

These results indicate that given HLA DRB1*04:01 allele and VDR genotypes cosegregate more frequently in T2DM; importantly, the detection of such HLA/VDR complexes significantly increases the OR for T2DM conferred by either the VDR polymorphism (Fig. 1) or the DR4*/VDR cosegregation alone. Taken together, these results suggest a synergistic effect of these two polymorphic sites toward T2DM development, a situation that was recently described to be present in multiple sclerosis patients.

**Discussion**

T2DM is a metabolic disorder characterized by hyperglycemia and insulin deficiency (5); this disease recognizes a multifactorial pathogenesis in which genetic factors play a complex and yet not clearly defined role. Several genes involved in the metabolic pathway of T2DM have been regarded as candidates for the onset of the disease (27), and among these, the VDR gene is considered as a particularly good candidate for disease susceptibility (28). Notably, as is the case with a number of inflammatory and autoimmune diseases, recent data have indicated a role for certain HLA alleles as well in the pathogenesis of T2DM (24, 29, 30).

To investigate possible relationships between VDR SNPs and HLA alleles, we analyzed these parameters in a group of Saudi T2DM patients, comparing results with those obtained in individuals of the same ethnicity. These subjects were chosen because they are part of a well-characterized cohort that is followed regularly in Riyadh, KSA. Results confirm that multiple SNPs in the VDR gene cluster in T2DM patients; these SNPs correlate with higher VDR expression, an increased production of IL-12, and metabolic parameters known to be associated with T2DM and, notably, synergize with HLA DRB1*04:01 in modulating the risk for development of T2DM.

Findings in this study indicate that polymorphisms in exon 9 (Taql) and intron 8 (Bsm-I) of the VDR gene were significantly associated with T2DM, whereas the genotype distribution and allele frequency of the polymorphisms in exon 2 (Fok-I) and intron 8 (Apa-I) of VDR did not differ significantly between patients and HC. These results confirm previous data indicating that Bsm-I and Taq-1 polymorphisms in the VDR gene are linked with the onset of T2DM (28, 31). These results are nevertheless not unequivocal because other researchers failed to demonstrate links between Fok-1, Bsm-1, and Taq-1 polymorphisms and T2DM in Indian (2), Turkish (32), Polish (33), and American populations (34). The reason of this discrepancy could be explained by the genetic differences in populations studied.

Polymorphisms in the VDR gene have been shown to influence VDR mRNA and protein levels (14), which, in turn, may influence the immunomodulatory function of VDR (26). Our results confirmed the presence of an increased VDR expression in those genotypes, Bsm-I (CT) and Taq-1 (AG), that are more commonly observed in T2DM patients. Because it was recently suggested that increased amounts of VDR protein might influence cytokine production, and in particular the generation of IL-12 (26), we next evaluated this parameter. Results indeed confirmed that IL-12–producing CD14+ cells were significantly increased in VDR rs1544410 (BsmI) CT and VDR rs731236 (TaqI) AG cells, that is, in those cells that are characterized by the VDR SNPs that are more frequent in T2DM patients. Augmented IL-12 production by APCs leads to differentiation of CD4+ T cells into IFN-γ-secreting Th1 cells; a strong Th1 response is often found in autoimmunity, resulting in the activation of downstream proinflammatory immune responses against autoantigens (reviewed in Ref. 35). These results thus seem to lend support to a possibly facilitating role played by the HLA/VDR circuit in the pathogenesis of autoimmune conditions.

The VDR SNPs that were seen to be more frequent in T2DM patients were also associated with metabolic parameters known to be altered in these patients. Thus, the typical pattern observed in T2DM is that of a dyslipidemia characterized by hypertriglyceridemia, higher concentrations of cholesterol, and lower concentrations of HDL. Interestingly, the TaqI and Bsm-I SNPs that were significantly more frequent in T2DM patients were associated with higher cholesterol levels and lower concentrations of...
HDL cholesterol. Notably, higher cholesterol levels and lower HDL concentrations are among the main risk factors for cardiovascular disease (CVD), and patients with T2DM have a significantly increased risk for all forms of CVD and a substantially increased risk for CVD-related death.

Significantly lower levels of calcium were detected as well in individuals showing the TT genotype of the Bsm-I polymorphism, a SNP significantly more frequent in T2DM patients. This result supports a report by Kiel and colleagues, who found an association between dietary calcium intake and Bsm-I polymorphism (36) and concluded that Bsm-I polymorphism might play a role in the absorption of dietary calcium. Notably, calcium together with vitamin D are strongly suspected to play a role in the pathogenesis of T2DM. Thus: 1) low calcium levels are known to impair insulin release (37), 2) calcium is essential for insulin-mediated processes in insulin-responsive tissues (38–41), 3) calcium repletion normalizes glucose tolerance and insulin secretion in vitamin D-depleted rats (42), and 4) hypocalcemia results in reduced insulin secretion in non diabetic individuals (43, 44). Higher serum 25-hydroxyvitamin D levels were also recently shown to be associated with a significantly reduced risk for T2DM in Australian adult men and women (45).

As shown by the results of statistical analyses, particular VDR gene SNPs associate with HLA DRB1*04:01 alleles in T2DM; this results in a modulation of the risk for development of T2DM. Which are the possible mechanisms explaining the interaction between VDR and HLA molecules? The biologically inert vitamin D3 is hydroxylated in the liver and in the kidney into [1,25(OH)2D]; this form becomes active upon binding to the VDR (46). In the presence of [1,25(OH)2D], VDR translocates to the nucleus (47) and generates heterodimers with the retinoid X receptor. The heterodimers can then bind, together with the transcription factor (47) and generates heterodimers with the retinoid X receptor. The heterodimers can then bind, together with the transcription factor IIB, a specific sequence (GGTTGGAGGGTTCA) called VDRE on DNA, resulting in the activation of a plethora of genes (48, 49). Recent reports demonstrated the presence of a VDRE element in the proximal promoter region of the HLA DRB1 gene (26, 50). VDREs present a multitude of sequence variations and a spectrum of binding affinities for VDR; this variability enables VDREs to respond optimally to different concentrations of the VDR/(1,25 [OH]2D) complex (51). Notably, a region almost perfectly matching that of VDRE is present in the promoter region of HLA DRB1*04 (52).

Data in this study also show that the VDR SNPs that are more frequent in T2DM patients correlate with metabolic parameters known to be associated with this disease, as well as with increased production of IL-12, a proinflammatory cytokine that has been involved in the pathogenesis of different autoimmune conditions. Based on these results, it is tempting to hypothesize that these VDR SNPs could be used to screen T2DM patients and identify those subjects that, because they are at greater risk for development of CVD and are more likely to secrete reduced quantities of insulin, would be treated in a more aggressive way and would be more closely monitored.

T1DM has been long known to be associated with HLA (22, 23); correlations between T2DM and HLA alleles have only been shown recently (24, 29, 30). Thus, data obtained in the seminal Pima Indian cohort study have indicated that the presence of HLA-DRB1*02 confers a lower risk for development of T2DM, possibly as a secondary effect on self-tolerance. Williams and colleagues (24) did not observe correlations between T2DM and other HLA alleles. Such correlation was not observed in the Saudi cohort either. Nevertheless, analysis of possible associations between VDR SNPs and HLA alleles showed the presence of a cosegregation of HLA-DRB*04 with the VDR SNPs that are described to preferentially be expressed in T2DM individuals. Notably, the presence of HLA-DRB*04 with such VDR SNPs resulted in an increased risk for development of T2DM.

Our findings, albeit needing to be confirmed in bigger cohorts, are novel in that they show a cosegregation between VDR and HLA alleles in T2DM patients, and add this disease to the list of those pathologies (53), including multiple sclerosis (54), that are likely to be modulated by the HLA/VDR axis.

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Disclosures

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