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

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References

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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, Fok-I, Taq-I, Apa-I, 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 TaqI (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 TaqI 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: 1325–1332.

Abbreviations used in this article: BMI, body mass index; CI, confidence interval; CVD, cardiovascular disease; HC, healthy control subject; HDL, high-density lipoprotein- protein; KSA, Kingdom of Saudi Arabia; LD, linkage disequilibrium; OR, odds ratio; PC5, PE–Cyanin-5; PC7, PE–Cyanin-7; SNP, single nucleotide polymorphism; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; VDR, vitamin D receptor; VDRE, vitamin D response element.

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stability (5, 14). Another polymorphism, FokI, 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-I, Taq-I, Apa-I, and Bsm-I 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—rs731236 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 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—rs731236 AG, n = 28; AA, n = 30; GG, n = 30.

Biochemical analysis

Fasting serum samples were stored in a −20°C freezer before analysis. Fasting glucose (FG), lipid profile, albumin, phosphorus, and calcium were measured using a chemical 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, rs7975232, and rs10735810) were evaluated by allele 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 RNAeasy 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 eluted in nuclease-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

\[ E = \frac{10^{-\text{slope} / \text{E}_{\text{cycle}}}}{2} \]

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 cDNA, 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 internorm calibrator 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 FITC and PERM (FITC & PERM Cell Permeabilization kit; 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–Conjugated anti-CD4 (clone SFC112T4D11; mouse IgG1), FITC– or PE–Cy5–Conjugated anti-CD14 (clone 116; Beckman-Coulter, Fullerton, CA); anti–IFN-γ–PC5 (clone 45B3; mouse IgG1), eBioscience, San Diego, CA); anti-human IL-6–FITC (clone 1936, mouse IgG1 isotype); anti–human IL-12–FITC (clone 27537, mouse IgG1 isotype); and anti–human IL-17–PC5 (clone BL168, mouse IgG1 isotype; BioLegend).

Analyses were performed using a Beckman-Coulter Cytoflex CS-FC500 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...
bandpass filter, PC5 deep-red fluorescence was collected through a 670-nm bandpass filter, and PC7 blue fluorescence was collected through a 770-nm bandpass filter. Data were gathered using linear amplifiers for forward and side scatter and logarithmic amplifiers for FITC green, PE orange-red, PC5 deep-red, and PC7 blue fluorescence. Samples were first run using isotype control or single fluorochrome-stained preparations for color compensation. Rainbow Calibration Particles (Spherotech, Lake Forest, IL) were used to standardize results.

**HLA DRB1 genotyping**

HLA DRB1 typing was performed by PCR amplification with sequence-specific primers using commercial kits (BAG, Lich, Germany, or Astra Formedic Srl, Milan, Italy). Allele detection was done after amplification in a GeneAmp PCR 9700 thermocycler (Applied Biosystem) by gel electrophoresis on 2% agarose gel.

**Statistical analysis**

Data were analyzed using the Statistical Package for the Social Sciences for Windows (SPSS version 16.0; SPSS, Chicago, IL) and are expressed by mean ± SD. Independent sample test was used to test HC and T2DM groups. ANOVA was used to compare different genotypes in each SNP followed by Dunnett’s post hoc test. Analysis of covariance was used to compare the genotypes in rs1544410 (Bsm-I) adjusted for the covariant age. Significance was set at p < 0.05. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by multinomial logistic regression for the haplotype, allele, and genotype frequencies. The most common haplotype was used as the reference, and rare haplotypes were dropped from the analysis. Haplotype frequencies were estimated by the Expectation-Maximization algorithm implemented in PROC Haplotype in SAS Genetics statistical software package (SAS Institute, Cary, NC). The standardized measure of linkage disequilibrium (LD), termed r², was computed at pairs of SNP loci. Tests of departures from LD as used in PROC Allele of SAS Genetics. Finally, VDR polymorphisms and HLA DRB1 cosegregation in T2DM and HC were evaluated by 2X2 contingency table and Pearson’s χ² analysis. Bonferroni correction for multiple test (pB) 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/Products/REST2009Software.aspx?x=8042) 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 [H(1)] 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.

**Results**

Clinical and epidemiologic characteristics of T2DM patients and HC are presented in Table I. Significantly higher systolic and diastolic blood pressure, BMI, and waist circumference, as well as higher serum concentrations of cholesterol, high-density lipoprotein (HDL) cholesterol, glucose, calcium, and vitamin D were observed in T2DM patients compared with HC.

**Genotype and allele frequencies**

Genotype and allele frequencies of the examined VDR gene polymorphic sites were in Hardy–Weinberg equilibrium; the observed frequencies are shown in Table II. Bivariate and multivariate χ² analysis was performed comparing T2DM and HC. Results showed that some VDR SNPs are significantly more frequent in T2DM patients. Thus, the VDR rs731236-AG genotype (OR 1.43, 95% CI 1.03–2.00, p = 0.03) and the VDR rs1544410-T allele and -CT genotype were significantly associated with T2DM (OR 1.3, 95% CI 1.03–1.66, p = 0.024 and OR 2.08, 95% CI 1.48–2.92, p < 0.001, respectively). Conversely, the VDR rs1544410-CC genotype was more frequent in HC than in T2DM patients (OR 0.51, 95% CI 0.36–0.72; p < 0.001). No significant differences were detected in the other VDR SNPs (rs7975232, rs10735810).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HC</th>
<th>T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>259</td>
<td>368</td>
</tr>
<tr>
<td>Age (y)</td>
<td>44.1 ± 9.9</td>
<td>51.5 ± 8.6*</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>118.0 ± 13.6</td>
<td>127.7 ± 14.7*</td>
</tr>
<tr>
<td>Diastolic BP (mm Hg)</td>
<td>75.2 ± 8.9</td>
<td>79.4 ± 9.2*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.5 ± 6.2</td>
<td>32.0 ± 5.7*</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>104.9 ± 20.3</td>
<td>105.8 ± 20.7</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>89.9 ± 18.9</td>
<td>100.6 ± 19.4*</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>2.3 ± 0.42</td>
<td>2.6 ± 0.30*</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.1 ± 0.99</td>
<td>5.6 ± 1.0*</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.4 ± 1.2</td>
<td>10.4 ± 1.3*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>4.1 ± 0.97</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.70 ± 0.27</td>
<td>1.0 ± 0.31*</td>
</tr>
<tr>
<td>Phosphate ion (mmol/l)</td>
<td>1.1 ± 0.26</td>
<td>1.2 ± 0.27</td>
</tr>
<tr>
<td>Triglyceride (mmol/l)</td>
<td>1.4 ± 0.10</td>
<td>2.1 ± 0.11*</td>
</tr>
<tr>
<td>Corrected calcium (mmol/l)</td>
<td>2.2 ± 0.50</td>
<td>2.5 ± 0.23*</td>
</tr>
<tr>
<td>Vitamin D (nmol/l)</td>
<td>19.4 ± 6.9</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.

**LD and haplotype frequency**

A significant difference was detected in the haplotype rs731236_A + rs1544410_C + rs7975232_C + rs10735810_C, because this haplotype was more common in T2DM patients than in HC (OR 0.48, 95% CI 0.26–0.87; 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; 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 1.39-fold increase (p[H1] = 0.02; 95% CI 0.95–1.99) of VDR mRNA was detected in the VDR rs1554440 CT compared with CC genotype (Fig. 2A). In addition, a 1.36-fold increase (p[H1] < 0.01; 95% CI 0.95–1.453) 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 relationship 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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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 (Bsm-I) 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 χ² analysis showed that the HLA DRB1*04/VDR rs731236 (TaqI) and HLA DRB1*04/VDR rs1544410 (Bsm-I) 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

| Haplotype | HC | T2DM | OR (95% CI) | p Value
<table>
<thead>
<tr>
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</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.
Statistically 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).

Stratification of individuals based on the VDR genotypes and DRB1*04:01 presence (DRB1*04:01+/absence (DRB1*04:01−) showed significant differences in those subjects carrying at least one DRB1*04:01 allele together with VDR rs731236 and/or VDR rs1544410 (pc = 0.004). In particular, the copresence of the DRB1*04:01 allele and the VDR rs731236 (AA) genotype (DRB1*04:01+/AA) was statistically less frequent in T2DM patients (12.56 versus 61.1% in HC; OR 0.09, 95% CI 0.01–0.6).

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

<table>
<thead>
<tr>
<th>HLA DRB1*04</th>
<th>VDR rs731236 1 (TaqI) n (%)</th>
<th>T2DM, n (%)</th>
<th>HC, n (%)</th>
<th>p Value OR (95% CI)</th>
<th>VDR rs1544410 (BsmI) n (%)</th>
<th>T2DM, n (%)</th>
<th>HC, n (%)</th>
<th>p Value OR (95% CI)</th>
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<tr>
<td>DRB1*04:01</td>
<td>AA 3 (12.5) 11 (61.1) 0.003 0.09 (0.01–0.51)</td>
<td>CC 3 (12.5) 11 (61.1) 0.003 0.09 (0.01–0.6)</td>
<td>AG 17 (70.8) 5 (27.8) 0.14 6.31 (1.36–3.57)</td>
<td>CT 17 (70.8) 5 (27.8) 0.14 6.31 (1.36–3.6)</td>
<td></td>
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<tr>
<td></td>
<td>GG 4 (16.7) 2 (11.1) NS</td>
<td>TT 4 (16.7) 2 (11.1) NS</td>
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<td></td>
<td>24 18 pc = 0.004</td>
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<tr>
<td>DRB1*04:02</td>
<td>AA 0 0 NS</td>
<td>CC 0 0 NS</td>
<td>AG 2 (1.0) 1 (33.3) NS</td>
<td>CT 2 (1.0) 1 (33.3) NS</td>
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<td></td>
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<td>TT 0 0 NS</td>
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<tr>
<td>DRB1*04:03</td>
<td>AA 3 (27.3) 8 (40.0) NS</td>
<td>CC 3 9 (45.0) NS</td>
<td>AG 7 (63.6) 8 (40.0) NS</td>
<td>CT 7 9 (45.0) NS</td>
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<tr>
<td></td>
<td>GG 1 (9.1) 4 (20.0) NS</td>
<td>TT 1 4 (20.0) NS</td>
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<tr>
<td>DRB1*04:15</td>
<td>AA 0 0 NS</td>
<td>CC 0 0 NS</td>
<td>AG 1 (1.0) 0 NS</td>
<td>CT 1 (1.0) 0 NS</td>
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<td>GG 0 0 NS</td>
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<td>DR4+</td>
<td>AA 6 (17.6) 18 (48.6) 0.01 0.23 (0.07–0.75)</td>
<td>CC 6 (17.6) 20 (54.1) 0.004 0.19 (0.05–0.5)</td>
<td>AG 24 (70.6) 13 (35.1) 0.006 4.43 (1.46–13.8)</td>
<td>CT 24 (70.6) 11 (29.7) 0.001 5.67 (1.8–18.2)</td>
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<td></td>
<td>GG 4 (11.8) 6 (16.2)</td>
<td>TT 4 (11.8) 6 (16.2)</td>
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<td>34 37 pc = 0.008</td>
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Analyses 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 similarly modified in the presence of both HLA/VDR complexes. Finally, the distribution of VDR rs1544410 was different in HLA DRB1*04:01− subjects (p = 0.002). Thus, the VDR rs1544410 (CC) genotype (DRB1*04:01*/CC) was statistically less frequent in T2DM patients compared with HC (30.8 versus 44.6% in HC; OR 0.55, 95% CI, 0.36–0.84), whereas the DRB1*04:01*/CT genotype was more frequent in T2DM patients than in HC (53.0 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 DRB4*/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 as 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 (TaqI) 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-I 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-I, Bsm-I, and Taq-I 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 TaqI (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 (Bsm1) 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 autoimmune, 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 Bsm1 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 nonobese 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 IIb, a specific sequence (GGGTTGGAGGGTTCA) 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 overlapping 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*04 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 co-segregation of HLA-DRB*04 with the VDR SNPs that are des-