Vitamin D Receptor (VDR) mRNA and VDR Protein Levels in Relation to Vitamin D Status, Insulin Secretory Capacity, and VDR Genotype in Bangladeshi Asians

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Associations have been reported between vitamin D receptor (VDR) gene polymorphisms, type 1 diabetes, insulin secretion, and the insulin resistance syndrome. As VDR polymorphisms have no known functional significance, these findings may implicate a variant of the VDR gene or a locus in linkage disequilibrium with the VDR. We have examined VDR mRNA and VDR protein levels in relation to VDR polymorphisms (41 Bangladeshi subjects) and analyzed insulin secretory capacity (143 Bangladeshi subjects), allowing for other known determinants. Peripheral blood mononuclear cells (PBMCs) from subjects who had been genotyped for *Bsm*I, *Apa*I, *Taq*I, and *Fok*I VDR restriction fragment length polymorphisms were used for both total VDR mRNA quantitation (using TaqMan) and measurement of VDR protein levels (using a specific microimmunoassay). Stepwise multiple regression analyses were used (to $P < 0.05$) to analyze the data. For the insulin secretion index, the best-fit model ($n = 143$, $P < 0.0001$) gave age ($P = 0.002$), *Taq*I ($P < 0.0001$), and BMI ($P = 0.001$) as independent determinants; with the inclusion of VDR mRNA and VDR protein levels, VDR mRNA was the sole independent determinant ($n = 41$, $P = 0.024$). However, the best-fit model for VDR mRNA ($P = 0.004$) gave *Fok*I ($P = 0.044$) and *Taq*I ($P = 0.04$) genotypes and insulin secretory capacity ($P = 0.042$) as independent determinants. For VDR protein levels, the best-fit model ($P = 0.006$) gave *Taq*I genotype ($P = 0.005$) and circulating 1,25-dihydroxyvitamin-D levels ($P = 0.03$) as independent determinants. In conclusion, these studies confirm an association between VDR polymorphisms and insulin secretory capacity and demonstrate the VDR genotype to be a significant determinant of VDR mRNA and VDR protein levels in PBMCs, providing functional support to previously described genetic associations with the VDR gene. Furthermore, VDR expression has been shown to be a determinant of insulin secretory capacity. *Diabetes* 51:2294–2312, 2002

Classic actions of vitamin D include maintenance of mineral homeostasis by regulation of calcium absorption in the gut and reabsorption by the kidney, in addition to regulation of bone remodeling (1). Among many noncalcemic functions are the induction of differentiation in peripheral blood mononuclear cells (PBMCs) and antiproliferative effects in many cancers, as well as vitamin D's immunosuppressive properties; vitamin D also acts as a necessary adjunct for insulin secretion (2). The main sources of vitamin D are ergocalciferol (D2) and cholecalciferol (D3), found in dietary sources and supplements, and cholecalciferol produced in the skin by ultraviolet radiation of 7-dehydrocholesterol. Both these compounds are hydroxylated in the liver to form 25-hydroxyvitamin D [25(OH)D], which is the major circulating metabolite precursor to the hormonally active form, 1,25-dihdroxyvitamin D [1,25$(OH)_2$D]. Hydroxylation of 25(OH)D metabolite, stimulated by parathormone, produces the hormonally active metabolite 1,25$(OH)_2$D, mainly in the kidney. 1,25$(OH)_2$D is the main ligand for the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcriptional regulators (1,3). The 25(OH)D precursor is widely used in assessment of vitamin D repletion and has a slower rate of clearance from the circulation than 1,25$(OH)_2$D. Because the VDR is expressed in a large number of tissues, it is not surprising that ligand-activated VDR modulates the expression of many genes. The VDR gene, located on chromosome 12q, has 14 exons, 6 of which are in the 5′ untranslated region (1a–1f). At least 22 unique nonfunctional VDR variants have been described, most of which lead to rare syndromes associated with vitamin D–resistant rickets (4). A number of common chronic disorders of inflammatory, infective, and autoimmune etiologies, including both type 1 and type 2 diabetes and colorectal adenoma, have been shown to be associated with specific polymorphisms of the vitamin D receptor gene, although not all such associ-

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25(OH)D, 25-hydroxyvitamin D; 1,25$(OH)_2$D, 1,25-dihdroxyvitamin D; IHD, ischemic heart disease; MMP, matrix metalloproteinase; OGTT, oral glucose tolerance test; PBMC, peripheral blood mononuclear cell; RFLP, restriction fragment length polymorphism; VDR, vitamin D receptor.

TABLE 1
Determinants of insulin secretion index, VDR mRNA, and VDR protein

	n	Best fit model (*P*)	Determinant	Standardized coefficient B	*P*	Excluded variables (*P*)
Insulin secretion index	143	<0.001	Diabetic status*	–0.39	<0.001	Paan usage (0.4); cigarette usage (0.08); WHR (0.5); *Bsm*I (0.92); *Apa*I (0.24); *Fok*I (0.45); serum 25(OH)D (full range) (0.7)
			BMI	0.29	0.001	
			*Taq*I for VDR†	0.25	0.002	
Insulin secretion index (vitamin D insufficiency)	93	<0.001	Diabetic status*	–0.27	0.012	Paan usage (0.2); cigarette usage (0.5); WHR (0.8); *Bsm*I (0.98); *Apa*I (0.6); serum 25(OH)D <20 ng/ml (0.6)
			BMI	0.3	0.004	
			*Taq*I for VDR†	0.29	<0.001	
			*Fok*I for VDR‡	–0.22	0.022	
Insulin secretion index (extension study group)	41	0.024	VDR mRNA	0.397	0.024	VDR protein (0.7); VDR genotype ×4; (0.11–0.84); Paan usage (0.8); cigarette usage (0.84); BMI (0.4); WHR (0.12); diabetic status (0.74); age (0.7); sex (0.9); 25(OH)D (0.29)
VDR mRNA	41	0.004	*Fok*I for VDR‡	–0.32	0.044	*Apa*I (0.9); *Bsm*I (0.75); serum 25(OH)D (0.28) glycosylated hemoglobin (0.17)
			*Taq*I for VDR†	–0.32	0.04	
			Insulin secretion index	0.32	0.042	
VDR protein	38	0.006	*Taq*I for VDR†	–0.52	0.005	*Apa*I (0.28); *Bsm*I (0.07) *Fok*I (0.11); VDR mRNA (0.06); log insulin secretion index (0.34); serum 25(OH)D (0.6)
			$1,25(OH)_2D$	0.39	0.03	

Insulin secretion index was determined at OGTT (see RESEARCH DESIGN AND METHODS). *If diabetic status (normal, impaired, or diabetic on OGTT) is excluded, age also becomes a determinant in the total dataset ($P = 0.002$) and in vitamin D insufficiency defined by 25(OH)D <20ng/ml ($P = 0.003$). †*Taq*I polymorphism and ‡*Fok*I polymorphism of VDR. WHR, waist-to-hip ratio.

ations are found consistently in different populations (5–18).

We have initiated a study of risk factors for type 2 diabetes and ischemic heart disease (IHD) in relation to vitamin D status in healthy adult subjects from Bangladesh living in East London, U.K. This ethnic group is known to have a high prevalence of vitamin D deficiency. We have demonstrated a strong association between insulin secretion and *Apa*I, *Taq*I, and *Bsm*I polymorphisms of the VDR gene in this study group; these findings were independent of vitamin D status (19). The *Fok*I genotype was not examined in that study, and the reported findings were based on simple correlative tests and one-way ANOVA of the mean with VDR genotype (19). The results have recently been replicated in another ethnic group with an additional association between insulin secretory capacity and the estrogen receptor gene (20). A relationship between vitamin D status, VDR genotype, and the matrix metalloproteinase (MMP) system has also been found, with *Taq*I polymorphism of the VDR gene contributing to the determination of circulating tissue inhibitor of metalloproteinases 1 (TIMP1) concentration in Bangladeshi subjects (21).

The purpose of the present study was twofold. The first aim was to genotype the original cohort of Bangladeshi subjects for the *Fok*I polymorphism and reanalyze the combined data to see whether VDR genotype contributed to the determination of insulin secretion when other relevant factors (age, body build, and vitamin D status) were included in multiple regression analysis. The second aim was to quantitate both VDR mRNA and total VDR protein in freshly obtained PBMCs from a subgroup of subjects from the original cohort. This extension to the study was designed to include those with vitamin D insufficiency to determine whether either VDR mRNA or total VDR protein might vary with vitamin D status or with VDR genotype.

RESEARCH DESIGN AND METHODS

This study cohort consisted of 143 apparently healthy Bangladeshi adults (subjects with previously diagnosed diabetes were excluded), screened as having a fasting or a postprandial capillary glucose within the upper range of normoglycemia by previously defined criteria (19). These subjects have been extensively phenotyped for risk factors for type 2 diabetes including insulin secretion index (calculated from basal and 30-min glucose and insulin levels during an oral glucose tolerance test [OGTT] [22]). Glucose tolerance was normal in 75.4% of subjects and impaired in 16.4%; 8.2% of subjects were found to have diabetes by 1985 World Health Organization criteria current at the time of initial recruitment (23). DNA from the study subjects was digested with *Fok*I, and fragments were separated by standard agarose-gel electrophoresis; *Fok*I genotypes were designated by the lowercase letter f for

TABLE 2
Data by VDR genotype for the initial and extension study groups

	VDR polymorphism					
	*Apa*I			*Bsm*I		
	AA	Aa	aa	BB	Bb	bb
N (%) in group						
Extension	16 (39.02)	19 (46.3)	6 (14.6)	9 (21.9)	22 (70.9)	10 (22.4)
Initial	54 (31.6)	91 (53.2)	26 (15.2)	39 (22.8)	79 (46.2)	53 (30.9)
Age(SD) (years)						
Extension	45.7 ± 10.6	51.5 ± 9.1	45.3 ± 8.6	51.1 ± 10.0	47.5 ± 10.8	47.6 ± 7.9
Initial	44 ± 10.6	47 ± 9.1	45.6 ± 8.6	45.6 ± 9.4	46.9 ± 10.7	44.7 ± 10.3
Insulin secretion index						
Extension	225.4 ± 285.4	129.9 ± 110.3	101.8 ± 34.3	106 ± 51.7	204.5 ± 255.2	123 ± 93.8
Initial	222.6 ± 241.2	146.4 ± 196.3	90.5 ± 61.6	178.5 ± 207.5	190.3 ± 247.6	108.7 ± 85.5
VDR mRNA (copy number/μg total RNA)						
Extension	103,980 ± 38,372	120,040 ± 46043	121,784 ± 51,339	105,593 ± 33,964	108,160 ± 36,166	134,529 ± 61,272
VDR protein (μg/mg) total extract protein						
Extension	17.91 ± 10.1	16.97 ± 10.2	18.1 ± 8.2	21.2 ± 11.6	16.1 ± 9.6	16.18 ± 7.96
Serum 25(OH)D (ng/ml)						
Extension	10.3 ± 5.3	8.3 ± 3.8	9.9 ± 3.3	10.0 ± 2.6	9.3 ± 5.4	8.5 ± 3.4
Initial	16.8 ± 7.43	17.2 ± 7.8	20.52 ± 12.0	16.5 ± 7.0	18.1 ± 9.2	17.7 ± 8.5
Serum $1,25(OH)_2D$ (pm/l)						
Extension	84.3 ± 27.4	76.7 ± 27.9	80.4 ± 29.3	87.7 ± 30.1	78.3 ± 27.9	76.1 ± 23.9

Date are *n* (%) or means ± SD.

presence of the cutting site and by the uppercase F for its absence; *Apa*I, *Taq*I, and *Bsm*I genotyping was available from the original study (19).

Forty-one subjects, aged 31–65 years (mean age ± SD, 48.32 ± 9.9), from the original cohort gave informed consent to provision of a further blood sample for the present study, as approved by the District Ethical Committee. Of these, 39% were men and 61% were women. A total of 12.2% had developed diabetes and 4.9% proven IHD since the original study. The data on VDR genotype from the original and current studies was combined for analysis (see below). Serum 25(OH)D concentration was measured by radioimmunoassay (IDS Immunodiagnostics) (intra- and interassay coefficients of variation 8.8% and 10.8%, respectively); levels of 25(OH)D <20 ng/ml defined vitamin D insufficiency (24). 1,25-Dihdroxyvitamin D was measured by specific radioimmunoassay after immunoextraction [Gamma-B 1,25(OH)D RIA kit; IDS] and measured both bioactive forms of calcitriol (1,25-dihydroxyvitamin-D2 and -D3) equally well; intra- and interassay coefficients of variation were <8% and <10%, respectively. The reference range for normal adults is 48–110 pmol/l.

PBMCs were isolated from freshly heparinized venous blood using standard RPMI solutions and Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) separation. The cells were resuspended at 1×10^6 cells/ml in cold PBS and counted in Neubauer chambers. Aliquots containing 10^7 cells were used in both the VDR mRNA and VDR protein studies.

Total cellular RNA was extracted from 10^7 PBMCs using the RNeasy mini RNA isolation kit (Qiagen, Crawley, UK), according to the manufacturer's recommendations, and quantified using a GeneQuant RNA/DNA Calculator (Pharmacia Biotech) with storage at −70°C.

Forward (ATCTGCATCGTCTCCCCAGAT) and reverse (AGCGGATG TACGTCTGCAGTG) oligonucleotide primers and a TaqMan probe (TGATT GAGGCCATCCAGGACCGC) were designed for the VDR gene (accession no. AC004466) using Primer Express software (PE-Applied Biosystems, Warrington, U.K.). The RT-PCR assays were carried out with an ABI Prism 7700-sequence detector as described (25) and were analyzed using sequence detector software (version 1.6.3). Absolute mRNA copy numbers were determined from a VDR-specific standard curve obtained by serially diluting a single-stranded sense oligonucleotide specifying the 98-bp VDR amplicon. Absolute mRNA levels were expressed as VDR mRNA copy numbers per microgram of total RNA (25).

Total 1,25-dihydroxyvitamin-D3 receptor protein (VDR protein) concentrations in PBMC homogenates were measured by specific micro enzyme-linked immunosorbent assay against standard curves (correlation coefficient, 0.999) for purified human VDR protein; intra-assay coefficient of variation was <6.6% (26). Results from two samples with results below the limit of sensitivity were expressed as being at that limit (1 μg/mg of total extract protein).

Statistics. SPSS for Windows (version 10) was used to examine frequencies, calculate correlations, examine variation of means with genotype by one-way ANOVA, calculate odds ratios, perform χ^2 analyses, and carry out multiple regression analyses (stepwise to $P < 0.05$) for the determination of best-fit models. Where VDR genotype was used in multiple regression analysis, all four polymorphisms were included. Pairwise linkage disequilibrium between *Fok*I and the three previously studied polymorphisms of the VDR gene was assessed using the estimate haplotype program (27). The distribution of VDR genotypes was examined for compliance with Hardy-Weinberg equilibrium for the group as a whole, for the vitamin D–insufficient subgroup, and for the extension study subgroup, and the proportions in these groups were examined for the Wahlund effect.

RESULTS

Insulin secretion. The distribution of the insulin secretion index in the initial study ($n = 143$) was normalized by log transformation, as in our previous report (showing it to vary with *Apa*I, *Bsm*I, and *Taq*I polymorphisms of the VDR gene) (19). Multiple regression analysis showed the significant independent determinants of the insulin secretion index at initial OGTT to be diabetic status (normal, impaired, or diabetic glucose tolerance) ($P < 0.001$), BMI ($P < 0.001$), and *Taq*I genotype ($P < 0.001$) in the best-fit model obtained ($P < 0.001$) (Table 1). If diabetic status was excluded from analysis, on the basis that insulin secretion index is a determinant of diabetic status, then the best-fit model ($P = 0.002$) gave age ($P = 0.002$), *Taq*I genotype ($P = 0.001$), and BMI ($P = 0.011$) as independent determinants of the insulin secretion index, the constant remaining significant for each of these

TABLE 2
Continued

VDR polymorphism					
*Taq*I			*Fok*I		
TT	Tt	tt	FF	Ff	ff
16 (39)	20 (48.8)	5 (12.2)	16 (39)	19 (46.4)	6 (14.6)
78 (45.6)	74 (43.3)	19 (11.1)	52 (30.4)	84 (49.1)	26 (15.2)
48.3 ± 9.2	47.9 ± 10.6	49.8 ± 11.4	50.8 ± 10.3	43.5 ± 9.1	46 ± 8.7
44.9 ± 10.1	47.7 ± 10.5	43.1 ± 9.5	46. ± 9.9	44.4 ± 10.7	46.7 ± 9.9
161.7 ± 227.4	159.8 ± 188.6	180.8 ± 157.0	206.4 ± 259.1	105.1 ± 50.9	101 ± 46.5
126 ± 135.8	173 ± 230.2	269 ± 279.1	195 ± 262.4	143.8 ± 143.4	147.2 ± 132.4
132,480 ± 52,382	101,450 ± 35,454	105,295 ± 22,645	126,495 ± 43,763	90,097 ± 24,522	105,67 ± 53,284
20.14 ± 10.3	16.44 ± 9.1	11.65 ± 7.1	18.5 ± 11.5	16.1 ± 6.5	18.0 ± 9.1
8.3 ± 3.5	10.1 ± 5.1	9.4 ± 3.3	8.75 ± 3.9	11.1 ± 6.6	8.8 ± 2.8
17.9 ± 9.1	17.8 ± 8.5	15.4 ± 5.9	17.8 ± 9.2	16.9 ± 7.7	18.7 ± 8.2
76.1 ± 20.1	83.5 ± 32.8	78.1 ± 24.3	89.5 ± 29.3	78.9 ± 25.6	61.77 ± 11.0

models. Both models showed *Taq*I t homozygotes to be associated with the highest levels of insulin secretion (by ~12%) (Table 2). In vitamin D insufficiency, the *Fok*I polymorphism appeared as an additional independent determinant (Table 1).

Examination for linkage disequilibrium between VDR restriction fragment length polymorphisms (RFLPs) demonstrated that the *Apa*I, *Bsm*I, and *Taq*I polymorphisms were in linkage equilibrium with *Fok*I ($P = 0.07$, 1.0, and 0.15, respectively), whereas there was tight linkage disequilibrium between the *Apa*I, *Bsm*I, and *Taq*I genotypes ($P < 0.001$), as previously shown (19). There was no significant deviation, using χ^2 tests, from Hardy-Weinberg equilibrium in this group or in the subgroup whose PBMCs were studied; furthermore, there was no evidence of the Wahlund effect for any of the four VDR genotypes.

Extension study: vitamin D axis. The mean ± SD serum 25(OH)D, measured on 39 of 41 subjects at the time of reexamination for these VDR studies (insufficient serum, $n = 2$), was 9.3 ± 4.4 ng/ml, range 1.6–25.8; the serum $1,25(OH)_2D$, measured on 34 of 41 re-bled subjects (insufficient serum, $n = 7$) was 80.1 ± 27.2 pmol/l, range 38.8–150.1. The serum 25(OH)D concentration did not vary with VDR genotype, whether examined in the group as a whole ($n = 143$) or in just those with vitamin D insufficiency ($n = 93$). Neither vitamin D status nor serum $1,25(OH)_2D$ (activated vitamin D) levels, measured at the time of blood sampling for further studies on the VDR ($n = 41$), varied with VDR genotype, nor did serum 25(OH)D and $1,25(OH)_2D$ levels correlate with each other ($P = 0.98$).

Insulin secretion in the extension study. The best-fit model ($P = 0.024$) on multiple regression analysis ($n = 39$) showed VDR mRNA to be the only independent determinant of insulin secretion index (Table 1).

VDR mRNA copy number. The values used in the analyses were the means derived from two separate experiments, in which samples from all 41 subjects studied were measured together. However, the results did not differ significantly from the means derived from the three other measurements made for each sample. Determinants of VDR mRNA copy number were examined using stepwise multiple regression analysis (Table 1). The best-fit model obtained, $P = 0.004$, showed *Fok*I ($P = 0.044$) and *Taq*I ($P = 0.04$) genotypes and insulin secretion index ($P = 0.042$) to be independent determinants of VDR mRNA in the PBMCs; neither vitamin D status nor activated vitamin D were significant factors. The *Taq*I TT and *Fok*I FF genotypes were associated with the highest VDR mRNA copy numbers by ~25.8% and ~19.7% compared with tt and ff genotypes, respectively (Table 2), although this did not differ significantly with VDR genotype on simple one-way ANOVA.

VDR protein concentration. Cellular VDR protein concentrations were analyzed after we had excluded the results on three samples, where the protein content of the extracts was too low for accuracy to be ensured. Independent determinants of VDR protein in the PBMC extracts in the best-fit model using multiple regression analysis ($P = 0.006$), and including $1,25\text{-}(OH)_2D$ and vitamin D status in the analysis, were *Taq*I genotype ($P = 0.005$) and $1,25(OH)_2D$ ($P = 0.03$) (Table 1); these findings were not altered by reanalysis including the three discarded VDR protein values (see above). The *Taq*I genotype TT was

associated with the highest VDR protein levels (by ~72.9% compared with that for tt) (Table 2). Messenger RNA copy number approached significance as a determinant of VDR protein concentration in the PBMCs ($P = 0.06$). Insulin secretion index ($P = 0.34$), factors relating to either body build or diabetes, and the other VDR genotypes (*Apa*I, $P = 0.28$; *Fok*I, $P = 0.11$) did not affect these findings, although *Bsm*I did approach significance ($P = 0.07$).

Ischemic heart disease, diabetes, or hypertension (or combinations of more than one of these conditions) had developed in eight extension study subjects during the years between the initial and the current studies. The distribution of the four VDR genotypes was compared (for *Apa*I, *Bsm*I, *Taq*I, and *Fok*I) between those with and without these problems. The prevalences of the VDR genotypes ff and tt were increased from 11.1% to 16.6% and from 6.6% to 25% in subjects with these disorders. The relative risk of these disorders (95% CI) for absence of the tt genotype was reduced to 0.15 (−0.4 to 1.1) and for the ff genotype to 0.29 (0.15 to 0.49).

DISCUSSION

We have demonstrated an association between VDR polymorphism and insulin secretory capacity and have shown that this gene is also a significant determinant of the amount of VDR mRNA and VDR protein expressed in PBMCs.

A feature of those subjects re-bled for the VDR mRNA and protein studies is that all but one of the subjects was 25(OH)D insufficient, probably reflecting the fact that these subjects were restudied in the autumn and confirming the inadequacy of the British summer and diet for maintenance of vitamin D repletion in this population (28). Although 25(OH)D levels were low, normal levels of calcitriol [hormonally activated $1,25(OH)_2D$] were maintained and there was, therefore, no simple correlation between 25(OH)D and $1,25(OH)_2D$ levels. We have reported insulin secretion to vary with vitamin D status and, independent of that status, to vary with VDR genotype (19,28). Several studies, including our work on MMP system activation (21), suggest that VDR genotype disease associations may be modulated by vitamin D status. Associations between VDR genotype and rickets, colorectal cancer, tuberculosis, hepatitis B, and leprosy are all more marked in subjects with vitamin D deficiency or in populations where vitamin D deficiency is common (5, 16, 19, 21, 29–31). Furthermore, phytohemagglutinin-stimulated growth of PBMCs varies with *Fok*I genotype when studied at half the maximal effective concentration of $1,25(OH)_2D$, whereas no such variation was found at the maximal concentration of activated vitamin D (32).

Further to our previous study (19), we have now used multiple regression analysis to allow for the effects of factors known to affect insulin secretion other than VDR genotype, age, body build, and vitamin D status. We have, in the same way, included factors that are known to influence VDR mRNA formation such as insulin secretion (33) and that influence VDR expression, such as activated vitamin D. The best determinants of insulin secretion were *Taq*I genotype, BMI, and age, although simple analysis of variation of the mean with genotype had suggested in our initial study (19) that the strongest association was with the *Apa*I genotype. Because *Apa*I, *Bsm*I, and *Taq*I polymorphisms are in tight linkage disequilibrium, the fact that our reanalysis suggests *Taq*I genotype to be a significant determinant of VDR mRNA formation, rather than *Apa*I, is not at variance with our original observations. The data are complicated by the fact that disease associations have been described with four different RFLPs, as defined by *Fok*I, *Bsm*I, *Apa*I, and *Taq*I, and with a mononucleotide repeat (A_n) in the 3′ untranslated region (5,34). The *Bsm*I and *Apa*I RFLPs are intronic (between exons 8 and 9), and the *Taq*I polymorphism is located in exon 9, but none of these changes lead to amino acid substitutions. Although it has been postulated that these RFLPs might affect VDR mRNA stability, this has not been confirmed, nor do they appear to affect ligand binding (35–37). These inconsistent results on the relationship between VDR genotype and VDR expression may have several explanations. In analyzing our data on VDR expression for possible effects of VDR polymorphisms, we have allowed for factors that influence VDR expression; indeed, had we relied simply on variation of our VDR data with VDR genotype, we might have concluded that associations did not exist, adding further confusion to this field. Furthermore, several, but not all, other studies have used less precise methods of mRNA analysis on a variety of tissues (PBMCs, parathyroid, fibroblasts, and cell lines) (37–44). Nonetheless, some positive results have been found in the latter studies, including variation in *Taq*I allele or associated haplotype-specific mRNA levels (37,39,41). In contrast to the other VDR polymorphisms, the *Fok*I polymorphism results in the incorporation of three extra amino acids in the NH_2 terminal of VDR protein, which influences transcriptional activity by modulating interaction with the transcription factor IIB (TFIIB) (43,44).

In those subjects with 25(OH)D insufficiency in the original study (serum levels <20 ng/ml), *Fok*I was a determinant of insulin secretion in addition to *Taq*I genotype and age. This is consistent with linkage equilibrium between *Fok*I and *Taq*I polymorphism (unlike the strong linkage disequilibrium that exists between the *Apa*I, *Bsm*I, and *Taq*I polymorphisms). It is therefore of interest that we observed significant variation of VDR mRNA with the *Fok*I polymorphism. Because PBMC production of activated vitamin D increases in vitamin D deficiency (45), *Fok*I genotype may contribute to feedback control of expression of the 25-hydroxyvitamin D-1-α-hydroxylase (CYP27B1) gene, located in the same chromosomal region as the VDR gene and active in PBMCs (46,47). Alternatively, the *Fok*I polymorphism may interfere with formation of VDR heterodimers with the retinoid X receptor to form effector complexes (48). This study does suggest that *Taq*I genotype contributes to the determination of both VDR mRNA and VDR protein levels independently, whereas *Fok*I genotype and insulin secretory capacity also contribute to the determination of VDR mRNA levels. Homozygosity for the VDR *Taq*I t genotype is associated with lower levels of PBMC VDR mRNA and protein but with higher levels of insulin secretion. This might reflect differences in the effects of ligand-activated receptors between different tissues, but the correlation of PBMC VDR mRNA with insulin secretory capacity indicates that this is unlikely.

We have found circulating levels of activated vitamin D [1,25$(OH)_2$D] to contribute to VDR protein levels, confirming earlier in vitro studies in PBMCs (45,49), whereas total VDR mRNA (the amplicon used in this study amplifies all mRNA species) was a borderline determinant of VDR protein levels in the PBMCs. In contrast, the insulin secretory capacity was a predictor of PBMC mRNA but not protein. These observations are not unexpected, as the relation between mRNA and its protein will depend on several factors including the half-life of the mRNA compared to the protein and on posttranscriptional processing. Indeed, tissue expression of VDR is directed by a number of promoters and transcription initiation from exons 1a or 1d, and alternative splicing can generate at least 10 different VDR transcripts, each of which was found in all of the tissues examined by Crofts et al. (50). Furthermore, we have previously studied mouse fibroblast and intestinal cell lines and showed that the addition of calcitriol [1,25$(OH)_2$D] increased VDR protein levels without changes in mRNA (51); similar changes have been reported in humans for healthy macrophages (49).

Many VDR disease associations reported are specifically with the *Taq*I polymorphism or associated haplotypes. It is with the *Taq*I polymorphism that we have found variation in insulin secretion and in TIMP1 responses to increases in circulating MMP9 (21). The apparent variation in risk for IHD and type 2 diabetes with *Taq*I and *Fok*I polymorphisms therefore warrants further investigation in larger populations. Further work is needed to define the mechanisms by which such variations in VDR synthesis lead to variation in cellular function and to determine whether such variation is abolished by adequate vitamin D repletion. The prescient suggestion, made 25 years ago, that requirements for vitamin D may vary between individuals (52) is now supported by clinical findings in tuberculosis, uremia, and colonic adenoma (16,30,53,54). It is clear that susceptibility to a wide range of disorders associated with VDR polymorphism, including type 1 and type 2 diabetes, could be reduced if adequacy of vitamin D repletion could be redefined to include those with the highest requirements (6–8,19,20,55). Furthermore, appropriate supplementation and maintenance in populations where vitamin D status is commonly inadequate is recommended (28,56–58).

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REFERENCES

1. DeLuca HF: The metabolism, physiology and function of vitamin D. In *Vitamin D, Basic and Clinical Aspects*. Kumar R, Ed. Boston, MA, Nijhoff Publishing, 1984, p. 259–302
2. Holick MF: Noncalcemic actions of 1,25-dihydroxyvitamin D3 and clinical applications. *Bone* 17:107S–111S, 1995
3. MacDonald PN: Molecular biology of the vitamin D receptor. In *Vitamin D. Physiology, Molecular Biology, and Clinical Applications*. Holick MF, Ed. Boston, MA, Humana Press, 1999, p. 109–128
4. Pike JW: The vitamin D receptor and its gene. In *Vitamin D*. Feldman D, Glorieux FH, Pike JW, Eds. San Diego, CA, Academic Press, 1997, p. 105–125
5. Zmuda JM, Cauley JA, PH, Ferrell RE: Molecular epidemiology of vitamin D receptor gene variants. *Epidemiol Rev* 22:203–217, 2000
6. McDermott MF, Ramachandran A, Ogunkolade BW, Aganna E, Curtis D, Boucher BJ, Snehalatha C, Hitman GA: Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians. *Diabetologia* 40:971–975, 1997
7. Pani MA, Knapp M, Donner H, Braun J, Baur MP, Usadel KH, Badenhoop K: Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. *Diabetes* 49:504–507, 2000
8. Chang TJ, Lei HH, Yeh JI, Chiu KC, Lee KC, Chen MC, Tai TY, Chuang LM: Vitamin D receptor gene polymorphisms influence susceptibility to type 1 diabetes mellitus in the Taiwanese population. *Clin Endocrinol* 52:575–580, 2000
9. Hauache OM, Lazaretti-Castro M, Andreoni S, Gimeno SG, Brandao C, Ramalho AC, Kasamatsu TS, Kunii I, Hayashi LF, Dib SA, Vieira JG: Vitamin D receptor gene polymorphism: correlation with bone mineral density in a Brazilian population with insulin-dependent diabetes mellitus. *Osteoporosis Int* 8:204–210, 1998
10. Ban Y, Taniyama M, Ban Y: Vitamin D receptor polymorphism is associated with Graves' disease in the Japanese population. *J Clin Endocrinol Metab* 85:4639–4643, 2000
11. Simmons JD, Mullighan C, Welsh KI, Jewell DP: Vitamin D receptor gene polymorphism: association with Crohn's disease susceptibility. *Gut* 47: 211–214, 2000
12. Ozaki Y, Nomura S, Nagahama M, Yoshimura C, Kagawa H, Fukuhara S: Vitamin-D receptor genotype and renal disorder in Japanese patients with systemic lupus erythematosus. *Nephron* 85:86–91, 2000
13. Garcia-Lozano JR, Gonzalez-Escribano MF, Valenzuela A, Garcia A, Nunez-Roldan A: Association of vitamin D receptor genotypes with early onset rheumatoid arthritis. *Eur J Immunogenet* 28:89–93, 2001
14. Keen RW, Hart DJ, Lanchbury JS, Spector TD: Association of early osteoarthritis of the knee with a Taq 1 polymorphism of the vitamin D receptor gene. *Arthritis Rheum* 40:1444–1449, 1997
15. Hennig BJ, Parkhill JM, Chapple IL, Heasman PA, Taylor JJ: Association of a vitamin D receptor gene polymorphism with localized early-onset periodontal diseases. *J Periodontol* 70:1032–1038, 1999
16. Kim HS, Newcomb PA, Ulrich CM, Keener CL, Bigler J, Farin FM, Bostick RM, Potter JD: Vitamin D receptor polymorphism and the risk of colorectal adenomas: evidence of interaction with dietary vitamin D and calcium. *Cancer Epidemiol Biomarkers Prev* 10:869–874, 2001
17. Van Shooten FJ, Hirvonen A, Maas LM, De Mol BA, Kleinjans JC, Bell DA, Durrer JD: Putative susceptibility markers of coronary artery disease: association between VDR genotype, smoking, and aromatic DNA adduct levels in human right atrial tissue. *FASEB J* 12:1409–1417, 1998
18. Uitterlinden AG, Burger H, Witteman JCM, van Leeuwen JPTM, Pois HAP: Genetic relation between osteoporosis and cardiovascular disease: vitamin D receptor polymorphism predicts myocardial infarction. *Osteoporosis Int* 8:8, 1998
19. Hitman GA, Mannan N, McDermott MF, Aganna E, Ogunkolade W, Hales CN, Boucher BJ: Vitamin D receptor gene polymorphisms influence insulin secretion in Bangladeshi Asians. *Diabetes* 47:688–690, 1998
20. Speer G, Cseh K, Winkler G, Vargha P, Braun E, Takacs I, Lakatos P: Vitamin D and estrogen receptor gene polymorphisms in type 2 diabetes mellitus and in android type obesity. *Eur J Endocrinol* 144:385–389, 2001
21. Timms PM, Noonan K, Mannan N, Hitman GA, Aganna E, Noonan K, Mills PM, Syndercombe-Court D, Patel B, Price C, Boucher BJ: Relationship between metalloproteinase-9, tissue inhibitor of metalloproteinase-1 and vitamin D status: its relevance to ischaemic heart disease in Asians (Abstract). *Am J Hyperten* 13:S100, 2000
22. Phillips DI, Clark PM, Hales CN, Osmond C: Understanding oral glucose-tolerance: comparison of glucose or insulin measurements during the oral glucose tolerance test with specific measurements of insulin resistance and insulin secretion. *Diabet Med* 11:286–292, 1994
23. WHO Study Group on Diabetes: Diabetes Mellitus: report of a WHO study group on diabetes mellitus. *WHO Technical Report Series* 727 1985
24. Malabanan A, Veronikis IE, Holick MF: Redefining vitamin D insufficiency. *Lancet* 351:805–806, 1998
25. Bustin SA: Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169–193, 2000
26. Uhland-Smith A, Prahl JM, DeLuca HF: An enzyme-linked immunoassay for the 1,25-dihydroxyvitamin D3 receptor protein. *J Bone Miner Res* 11:1921–1925, 1996
27. Xie X, Ott J: Testing linkage disequilibrium between a disease gene and marker loci. *Am J Hum Genet* 53:1107, 1993
28. Boucher BJ, Mannan N, Noonan K, Hales CN, Evans SJW: Glucose intolerance and impairment of insulin secretion in relation to vitamin D deficiency in East London Asians. *Diabetologia* 38:1239–1245, 1995
29. Slevaraj P, Kurian SM, Uma H, Reetha AM, Narayanan PR: Influence of

non-MHC genes on lymphocyte response to *Mycobacterium tuberculosis* antigens and tuberculin reactive status in pulmonary tuberculosis. *Indian J Med Res* 112:86–92, 2000
30. Wilkinson RJ, Llewelyn, Toossi Z, Patel P, Pasvol G, Lalvani A, Wright D, Latif M, Davidson RN: Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. *Lancet* 355:618–621, 2000
31. Bellamy RJ, Hill AV: Host susceptibility to human tuberculosis. *Novartis Found Symp* 217:3–13, 1998
32. Colin EM, Weel AE, Uitterlinden AG, Buurman CJ, Birkenhager JC, Pols HA, van Leeuwen JP: Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D3. *Clin Endocrinol* 52:211–216, 2000
33. Krishnan AV, Feldman D: Stimulation of 1,25-dihydroxyvitamin D3 receptor gene expression in cultured cells by serum and growth factors. *J Bone Miner Res* 6:1099–1107, 1991
34. Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN, Eisman JA: Prediction of bone density from vitamin D receptor alleles. *Nature* 367:284–287, 1994
35. Gross C, Musiol IM, Eccleshall TR, Malloy PJ, Feldman D: Vitamin D receptor gene polymorphisms: analysis of ligand binding and hormone responsiveness in cultured skin fibroblasts. *Biochem Biophys Res Com* 242:467–473, 1998
36. Durrin LK, Haile RW, Ingles SA, Coetzee GA: Vitamin D receptor 3′-untranslated region polymorphisms: lack of effect on mRNA stability. *Biochim Biophys Acta* 1453:311–320, 1999
37. Verbeek W, Gombart AF, Shiohara M, Campbell M, Koeffler HP: Vitamin D receptor: no evidence for allele-specific mRNA stability in cells which are heterozygous for the Taq 1 restriction enzyme polymorphism. *Biochem Biophy Res Com* 238:77–80, 1997
38. Mocharla H, Butch AW, Pappas AA, Flick JT, Weinstein RS, De Togni P, Jilka RL, Roberson PK, Parfitt AM, Manolagas SC: Quantification of vitamin D receptor mRNA by competitive polymerase chain reaction in PBMC: lack of correspondence with common allelic variants. *J Bone Miner Res* 12:726–733, 1997
39. Carling T, Rastad J, Akerstrom G, Westin G: Vitamin D receptor (VDR) and parathyroid hormone messenger ribonucleic acid levels correspond to polymorphic VDR alleles in human parathyroid tumours. *J Clin Endocrinol Metab* 83:2255–2259, 1998
40. Ohtera K, Ishii S, Matsuyama T: Influence of the vitamin D receptor alleles on human osteoblast-like cells. *J Bone Joint Surg Br* 83:134–138, 2001
41. Yamagata M, Nakajima S, Tokita A, Sakai N, Yanagihara I, Yabuta K, Ozono K: Analysis of the stable levels of messenger RNA derived from different polymorphic alleles in the vitamin D receptor gene. *J Bone Miner Res* 17:164–170, 1999
42. Correa P, Rastad J, Schwarz P, Westin G, Kindmark A, Lundgren E, Akerstrom G, Carling T: The vitamin D receptor (VDR) start codon polymorphism in primary hyperparathyroidism and parathyroid VDR messenger ribonucleic acid levels. *J Clin Endocrinol Metab* 84:1690–1694, 1999
43. Arai H, Miyamoto K, Taketani Y, Yanamoto H, Iemori Y, Morita K, Tonai T, Nishisho T, Mori S, Takeda E: A vitamin D receptor gene polymorphism in the translation initiation codon: effect on protein activity and relation to bone mineral density in Japanese women. *J Bone Miner Res* 12:915–921, 1997
44. Jurutka PW, Remus LS, Whitfield GK, Thompson PD, Hsieh JC, Zitzer H, Tavakkoli P, Galligan MA, Dang HT, Haussler CA, Haussler MR: The polymorphic N terminus in the human vitamin D receptor isoforms influences transcriptional activity by modulating interaction with transcription factor IIb. *Mol Endocrinol* 14:401–420, 2000
45. Dusso AS, Finch J, Brown A, Ritter C, Delmez J, Schreiner G, Slatopolsky E: Extrarenal production of calcitriol in normal and uremic humans. *J Clin Endocrinol Metab* 72:157–164, 1991
46. Smith SJ, Rucka AK, Berry JL, Davies M, Mylchreest S, Paterson CR, Heath DA, Tassabehj M, Read AP, Mee AP, Mawer EB: Novel mutations in the 1 alpha-hydroxylase (P450cl) gene in three families with pseudovitamin D-deficiency rickets resulting in loss of functional enzyme activity in blood-derived macrophages. *J Bone Mineral Res* 14:730–739, 1999
47. Bell NH: Renal and non-renal 25-hydroxyvitamin D-1alpha-hydroxylases and their clinical significance. *J Bone Min Res* 13:350–353, 1998
48. Haussler MR, Whitfield GK, Haussler CA, Hsieh J-G, Thompson PD, Selznick SH, Dominguez CE, Jurutka PW: The nuclear vitamin D receptor: biological and molecular properties revealed. *J Bone Min Metab* 13:325–349, 1998
49. Kreutz M, Andreesen R, Krause SW, Szabo A, Ritz E, Reichel H: 1,25-dihydroxyvitamin D3 production and vitamin D3 receptor expression are developmentally regulated during differentiation of human monocytes into macrophages. *Blood* 82:1300–1307, 1993
50. Crofts LA, Hancock MS, Morrison NA, Eisman JA: Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. *Proc Natl Acad Sci U S A* 95:10529–10534, 1998
51. Wiese RJ, Uhland-Smith A, Ross TK, Prahl JM, DeLuca HF: Up-regulation of the vitamin D receptor in response to 1,25-dihydroxyvitamin D3 results from ligand-induced stabilization. *J Biol Chem* 267:20082–20086, 1992
52. O'Hara-May J, Widdowson EM: Diets and living conditions of Asian boys in Coventry with and without signs of rickets. *Br J Nutr* 36:23–36, 1976
53. Marco MP, Martinez I, Betriu A, Craver L, Fibla MJ, Fernandez E: Influence of *BsmI* vitamin D receptor gene polymorphism on the response to a single bolus of calcitriol in hemodialysis patients. *Clin Nephrol* 56:111–116, 2001
54. Dauncey MJ, White P, Burton KA, Katsumata M: Nutrition-hormone receptor-gene interactions: implications for development and disease. *Proc Nutr Soc* 60:63–72, 2001
55. Ortlepp JR, Lauscher J, Hoffman R, Hanrath P, Joost HG: The vitamin D receptor gene variant is associated with the prevalence of type 2 diabetes mellitus and coronary artery disease. *Diabet Med* 18:842–845, 2001
56. Boucher BJ: Inadequate vitamin D status: does it contribute to the disorders comprising syndrome 'X'? *Br J Nutr* 79:315–327, 1998
57. Hypponen E, Laara E, Reunanen A, Jarvelin M-R, Virtanen SM: Intake of vitamin D and risk of type 1 diabetes: a birth cohort study. *Lancet* 358:1500–1503, 2001
58. Utiger RD: The need for more vitamin D. *N Engl J Med* 338:828–829, 1998