Brief Genetics Report

Analysis of the Vitamin D Receptor Gene Sequence Variants in Type 1 Diabetes

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Vitamin D is known to modulate the immune system, and its administration has been associated with reduced risk of type 1 diabetes. Vitamin D acts via its receptor (VDR). Four single nucleotide polymorphisms (SNPs) of the VDR gene have been commonly studied, and evidence of association with type 1 diabetes has been reported previously. We sequenced the VDR gene region and developed its SNP map. Here we analyzed association of the 98 *VDR* SNPs in up to 3,763 type 1 diabetic families. First, we genotyped all 98 SNPs in a minimum of 458 U.K. families with two affected offspring. We further tested eight SNPs, including four SNPs associated with $P < 0.05$ in the first set and the four commonly studied SNPs, in up to 3,305 additional families from the U.K., Finland, Norway, Romania, and U.S. We only found weak evidence of association ($P = 0.02–0.05$) of the rs4303288, rs12721366, and rs2544043 SNPs. We then tested these three SNPs in an independent set of 1,587 patients and 1,827 control subjects from the U.K. and found no evidence of association. Overall, our results indicate that common sequence variation in the VDR gene has no major effect in type 1 diabetes in the populations tested. *Diabetes* 53:2709–2712, 2004

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IL, interleukin; SNP, single nucleotide polymorphism; VDR, vitamin D receptor.

The molecular mechanisms underlying type 1 diabetes are only partly understood. It develops as a result of a complex interaction of many genetic and environmental factors leading to the immune destruction of the insulin-producing β-cells (1). Three disease loci have been identified so far that contribute to the etiology of type 1 diabetes, the HLA complex, the variable number of tandem repeats locus located in the promoter region of the insulin (*INS*) gene, and the cytotoxic T-cell–associated antigen-4 gene (*CTLA4*) (1,2).

1α,25-dihydroxyvitamin D_3, the hormonally active form of vitamin D, has been successfully used to prevent autoimmune insulitis and reduce diabetes incidence in the mouse model of type 1 diabetes, as well as in animal models of other autoimmune diseases (3–6). In humans, population studies suggest that vitamin D supplementation in early childhood decreases type 1 diabetes incidence (7,8), raising hopes that it may be used as a type 1 diabetes preventive treatment. Vitamin D has been long known to play a central role in bone and mineral metabolism. Now it is widely recognized to regulate growth and differentiation in many target tissues and act as a modulator in the immune system (9). Its effects are mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcriptional regulators. VDR is found in >30 different tissues, including islet cells of the pancreas, circulating monocytes, dendritic cells, and activated T-cells (9). Upon binding 1α,25-dihydroxyvitamin D_3, VDR regulates gene expression by direct interaction with specific sequence elements in the promoter region of hormone-responsive target genes. In the immune system, 1α,25-dihydroxyvitamin D_3 was shown to suppress production of the interleukin (IL)-12, IL-2, tumor necrosis factor-α, and interferon-γ cytokines and activate expression of transforming growth factor-β1 and IL-4 cytokines, thereby inhibiting Th1-type responses and to induce regulatory T-cells (9). It can also regulate differentiation and maturation of dendritic cells critical in the induction of T-cell–mediated immune responses (10). These pathways may explain the beneficial effects of vitamin D in autoimmune diseases (6).

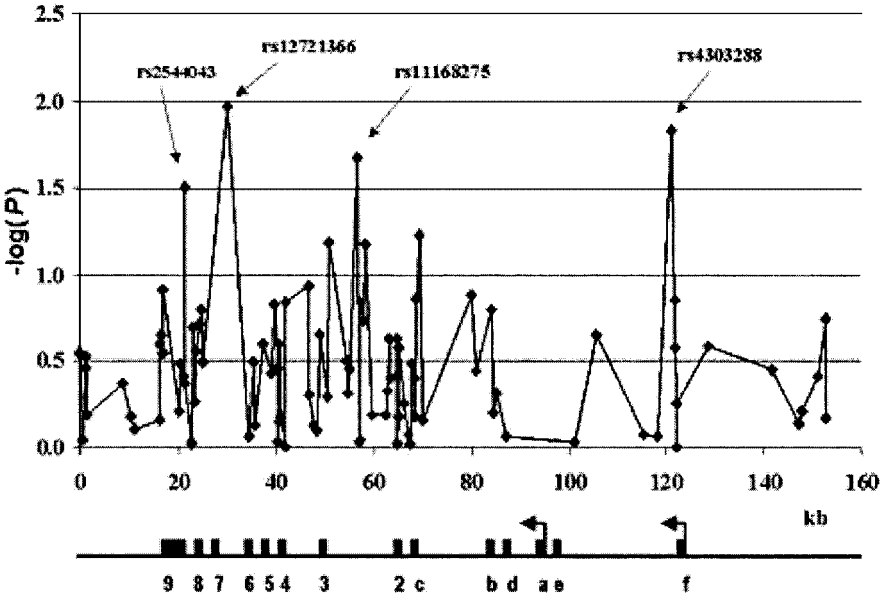

FIG. 1. Association analysis of the 98 SNPs in the VDR gene region. Exons of the VDR gene are shown as black boxes. Promoters are shown as arrows.

The VDR gene is located on chromosome 12q12-q14 and includes eight protein-coding exons (exons 2–9) and six untranslated exons (exons 1a–1f), which are alternatively spliced, and two promoter regions (11). Four common single nucleotide polymorphisms (SNPs) in the VDR gene have been studied intensively (12): *Fok*I T>C (rs10735810), *Bsm*I A>G (rs1544410), *Apa*I G>T (rs7975232), and *Taq*I C>T (rs731236). Allele T of the *Fok*I SNP creates an alternative ATG codon leading to a three-amino-acid–longer VDR protein (13). SNPs *Bsm*I and *Apa*I are located in an intron, and *Taq*I is a silent SNP in exon 9. These four SNPs have been tested for association with various human traits and diseases and have been reported (12) to affect risk of cancers and bone density–related and immune-mediated disorders. Several studies reported association of type 1 diabetes with one of these four SNPs. However, the reported associations are inconsistent between studies (14–19). Since these SNPs, with potential exception of the *Fok*I variant, have no known functional role, these results may indicate that they are merely markers in linkage disequilibrium with a true causal variant(s), which remains unknown. Recently, we performed a comprehensive identification (20) of the sequence polymorphisms in the VDR gene region and developed its dense SNP map. Here we report a study of association between 98 SNPs in the VDR gene region and type 1 diabetes.

We genotyped a set of 458 U.K. families with two type 1 diabetic offspring in each for 98 *VDR* SNPs, including the four commonly studied SNPs (*Fok*I, *Bsm*I, *Apa*I, and *Taq*I). Additionally, we genotyped a set of 307 U.S. families with two type 1 diabetic offspring in each family for 40 out of these 98 SNPs. We found that four SNPs, rs4303288, rs11168275, rs12721366, and rs2544043, which were tested here for the first time, showed evidence of association with type 1 diabetes ($P = 0.01–0.03$) (Fig. 1 and supplementary table [available in an online appendix at http://diabetes.diabetesjournals.org]). We genotyped these four SNPs, as well as SNPs *Fok*I, *Bsm*I, *Apa*I, and *Taq*I, in an additional set of type 1 diabetic families with at least one affected offspring from the U.K., Finland, Norway, Romania, and U.S. In the analysis of the combined family set, none of the four commonly studied SNPs were associated ($P > 0.05$) (Table 1). Among SNPs first tested by us, rs12721366, rs2544043, and rs4303288 showed some evidence of association with type 1 diabetes in the combined family set ($P < 0.05$) (Table 1). These associations cannot be viewed as statistically significant on a genome-wide scale (21,22). Therefore, we analyzed these three SNPs in

TABLE 1
Association analysis of the eight *VDR* SNPs in the type 1 diabetic families

SNP ID no./name	Location*	Number of families	Allele frequency†	Transmissions (Percentage, allele)		P_{TDT}‡	P_{GTRR}§	P_{CO}‖	$P_{YB/CO}$¶
rs4303288	Intron (1f–1e)	2,667	T (0.60)/G (0.40)	1,559 (51.7, T)	1,454 (48.3, G)	0.06	0.02	0.60	0.24
rs10735810/*Fok*I	Exon 2	2,893	C (0.60)/T (0.40)	1,688 (51.2, C)	1,606 (48.8, T)	0.15	0.09	0.37	0.16
rs11168275	Intron (2–3)	3,239	G (0.21)/A (0.79)	1,298 (49.1, G)	1,343 (50.9, A)	0.38	0.19	0.14	0.77
rs12721366	Intron (6–7)	2,915	G (0.01)/A (0.99)	59 (60.8, G)	38 (39.2, A)	0.03	0.05	0.92	0.37
rs1544410/*Bsm*I	Intron (8–9)	2,916	A (0.42)/G (0.58)	1,558 (48.8, A)	1,637 (51.2, G)	0.16	0.35	0.49	0.41
rs7975232/*Apa*I	Intron (8–9)	3,763	G (0.47)/T (0.53)	2,097 (49.6, G)	2,132 (50.4, T)	0.59	0.63	0.39	0.15
rs731236/*Taq*I	Exon 9	2,594	C (0.39)/T (0.61)	1,385 (49.6, C)	1,406 (50.4, T)	0.69	0.11	0.86	0.69
rs2544043	Exon 9	3,358	C (0.07)/G (0.93)	452 (46.5, C)	521 (53.5, G)	0.03	0.06	0.35	0.56

*For the intronic SNPs, exons flanking the intron are shown in parentheses. †Allele frequency among parents of the type 1 diabetic patients from the U.K. is shown; ‡P_{TDT}: P value for the transmission-disequilibrium (1 df) test; §P_{GTRR}: P value for the genotype relative risk (2 df) test; ‖P_{CO}: P value for the effect of the country of origin on type 1 diabetes association; ¶$P_{YB/CO}$: P value for the combined effect of the patient's year of birth and country of origin on type 1 diabetes association.

TABLE 2
Association analysis of the three *VDR* SNPs in type 1 diabetic patients and control subjects from the U.K.

SNP	Case subjects	Control subjects	*P* value	OR (95% CI)
rs4303288				
n	1,548	1,739		
Allele G	38.6	40.2	0.19	0.94 (0.85–1.03)
Allele T	61.4	59.8	0.19	1.07 (0.97–1.18)
Genotype GG	14.9	16.4		
Genotype GT	47.4	47.5	0.39	
Genotype TT	37.7	36.1		
Genotype GG	14.9	16.5	0.21	0.89 (0.73–1.07)
Genotypes TT and TG	85.1	83.5	0.21	1.13 (0.93–1.36)
Genotypes GG and GT	62.3	63.9	0.34	0.93 (0.81–1.08)
Genotype TT	37.7	36.1	0.34	1.07 (0.93–1.23)
rs2544043				
n	1,575	1,785		
Allele C	6.3	6.7	0.50	0.94 (0.77–1.14)
Allele G	93.7	93.3	0.50	1.07 (0.88–1.30)
Genotype CC	0.1	0.5		
Genotype CG	12.4	12.5	0.23	
Genotype GG	87.5	87.0		
Genotype CC	0.1	0.5	0.88	0.28 (0.06–1.33)
Genotypes CG and GG	99.9	99.5	0.88	3.54 (0.75–16.7)
Genotypes CC and CG	12.5	13.0	0.67	0.96 (0.78–1.17)
Genotype GG	87.5	87.0	0.67	1.04 (0.85–1.28)
rs12721366				
n	1,587	1,827		
Allele A	99.2	99.1	0.62	1.14 (0.68–1.90)
Allele G	0.8	0.9	0.62	0.88 (0.53–1.47)
Genotype AA	98.4	98.1		
Genotype AG	1.6	1.9	0.62	
Genotype GG	0	0		
Genotype AA	98.4	98.1	0.62	1.14 (0.68–1.91)
Genotypes AG and GG	1.6	1.9	0.62	0.88 (0.52–1.47)

Data are percent, unless noted otherwise.

an additional independent set of up to 1,587 type 1 diabetic patients and 1,827 control subjects from the U.K. and did not find any evidence of association (Table 2). These results indicate that a major effect, such as odds ratio (OR) > 1.5 for a common allele, is unlikely to exist for the *VDR* polymorphisms in type 1 diabetes. Population substructure within case-control samples could affect association studies (23,24). However, given that we have found no convincing evidence of association between the *VDR* SNPs and type 1 diabetes in the large family collection, potential population substructure in the British case-control sample should not greatly alter our conclusion.

SNPs *Bsm*I, *Apa*I, and *Taq*I are in linkage disequilibrium, and their haplotypes were previously shown (15) to be associated with type 1 diabetes in the German population. Therefore, in 1,811 type 1 diabetic families who were genotyped for all three of these SNPs we tested association of the *Bsm*I-*Apa*I-*Taq*I, *Bsm*I-*Taq*I, and *Apa*I-*Taq*I haplotypes (15) and found no association (7 degrees of freedom [df], $P = 0.11$; 3 df, $P = 0.18$; and 3 df, $P = 0.16$, respectively).

Our study does not support previous reports (14–17) in much smaller samples, which showed some evidence of type 1 diabetes association for the *Fok*I, *Bsm*I, *Apa*I, or *Taq*I SNPs or their haplotypes. Spurious association and publication bias is a possible explanation of the previous positive results, particularly given that there is no consistency regarding the findings of the associated SNPs and their alleles between different studies.

Environmental factor(s), specific to some groups of type 1 diabetic patients, may alter the risk associated with particular SNPs in the VDR gene. For instance, VDR functions together with 1α,25-dihydroxyvitamin D_3, the level of which is dependent on various environmental factors (25). These factors include vitamin D intake in the diet or as a supplement and its synthesis from precursors in skin under ultraviolet light exposure. Such environmental factors may modulate risk associated with the sequence variation in the VDR gene, e.g., certain variants may only be functionally important among a subpopulation of subjects with vitamin D insufficiency. While it is difficult to design a study to evaluate such potential interaction directly, it may manifest itself as regional or temporal heterogeneity in association between patients, who would have developed type 1 diabetes in different environments. We therefore analyzed the effect of the country of origin of a patient and a combined effect of the patient's year of birth and a country of origin on association between type 1 diabetes and eight *VDR* SNPs that we studied in five different populations. We did not find any evidence for significant heterogeneity of the type 1 diabetes association (Table 1). However, environmental factors that influence levels of active vitamin D forms in humans are complex, and their effect may not be excluded by our analysis.

RESEARCH DESIGN AND METHODS

We obtained permission from relevant ethics committees and informed consent from all participating subjects. SNP detection is described in detail elsewhere (20). In brief, the VDR gene region was sequenced in eight healthy Caucasian subjects from the U.K. Coding exons were sequenced using an additional panel of 40 individuals from the same population to find rare variants. We sequenced 94 kb in a 164-kb region around the VDR gene and identified 245 SNPs.

We genotyped 98 SNPs in a minimum set of 458 U.K. families of Caucasian ethnicity, each with two children affected with type 1 diabetes (the Diabetes U.K. Warren repository). Additionally, a set of 307 U.S. families with two type 1 diabetic offspring in each (the Human Biological Data Interchange) was genotyped for 40 of 98 SNPs. The 458 U.K. family dataset provides at least 82% statistical power to detect association at $P = 0.05$, with OR 1.35 for alleles of >10% frequency. Four commonly studied *VDR* SNPs (*Fok*I, *Bsm*I, *Apa*I, or *Taq*I) and four SNPs that showed association with type 1 diabetes with $P < 0.05$ in the 458 U.K. families were then genotyped in an additional set of families with at least one child affected with type 1 diabetes. These families were of European origin and were collected in the U.K. ($n = 1,302$), Finland ($n = 1,543$), Norway ($n = 359$), Romania ($n = 335$), and U.S. ($n = 365$, including 307 Human Biological Data Interchange families). Therefore, in total we studied 4,362 type 1 diabetic families (the exact number of families genotyped for each SNP is shown in Table 1). Additionally we tested SNPs rs12721366, rs2544043, and rs4303288 in an independent sample of up to 1,587 type 1 diabetic patients collected across the U.K. and 1,827 control subjects who were selected from the 1958 cohort, which includes people born on 3–9 March 1958 in England, Scotland, and Wales (http://www.cls.ioe.ac.uk/cohort/ncds/mainncds.htm). This case-control sample provided 80% statistical power to detect association at $P = 0.05$ for ORs 1.15, 1.30, and 1.84 for SNPs rs4303288, rs2544043, and rs12721366, respectively, assuming a multiplicative model.

Genotyping was carried out using Invader (Third Wave Technologies, Madison, WI), TaqMan (Perkin Elmer Applied Biosystems, Foster City, CA), or BeadArray (Illumina, San Diego, CA). In the 458–U.K. family dataset, we assessed genotyping quality. All 98 markers tested had <10 families with misinheritances in the 458–U.K. family dataset. We tested genotype frequency

among parents for each SNP using Arlequin version 2.000 (http://lgb.unige.ch/arlequin) and found no unexpected deviation from the Hardy-Weinberg equilibrium ($P > 0.02$).

Statistical analysis was carried out with STATA version 8.1 (http://www.stata.com). *P* value calculations are based on robust variance estimates, used to correct for clustering of affected subjects within families. To assess an effect of the country of origin on type 1 diabetes association of the eight *VDR* polymorphisms we constructed contingency tables of transmitted and nontransmitted alleles in families from each country. Information on the year of birth was available for the type 1 diabetic patients from the U.K. (1924–1998), Finland (1941–1998), and Romania (1941–1998). To assess a combined effect of the patient's year of birth and country of origin we did a case-only permutation trend test. A null hypothesis that allele frequency does not change within a population with calendar years was tested. We used the UNPHASED program (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased) to calculate transmission of the haplotypes. We did not apply multiple testing corrections in this study, and all reported *P* values are uncorrected.

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