Brief Genetics Report

Vitamin D Receptor Allele Combinations Influence Genetic Susceptibility to Type 1 Diabetes in Germans

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Vitamin D has been shown to exert manifold immunomodulatory effects. Because type 1 diabetes is regarded to be immune-mediated and vitamin D prevents the development of diabetes in the NOD mouse, we investigated the role of the vitamin D receptor (VDR) gene as a candidate for type 1 diabetes susceptibility. A total of 152 Caucasian families with at least one affected offspring were genotyped for four VDR restriction-site polymorphisms (*Fok*I, *Bsm*I, *Apa*I, and *Taq*I). Whereas the *Bsm*I, *Apa*I, and *Taq*I polymorphisms are in strong linkage disequilibrium with each other, no significant linkage disequilibrium with the *Fok*I site was observed. Extended transmission disequilibrium testing (ETDT) was used to detect preferential transmission of allelic combinations to affected offspring. We found significant haplotype-wise ETDT results for the *Bsm*I/*Apa*I/*Taq*I (χ^2 = 18.886, df = 7, P = 0.0086), the *Bsm*I/*Taq*I (χ^2 = 8.373, df = 3, P = 0.0389), and the *Apa*I/*Taq*I (χ^2 = 17.182, df = 3, P = 0.0006) haplotypes. The "*At*" and "*Bt*" alleles confer an increased risk, whereas "*AT*" and "*at*" are protective. The combination with the strongest susceptibility was the "*BAt*" haplotype (64% transmitted, P = 0.0106). Analysis of the *Fok*I site does not provide more information on susceptibility (*Fok*I/*Bsm*I/*Apa*I/*Taq*I [χ^2 = 24.702, df = 15, P = 0.0541]). These findings suggest a linkage of VDR itself or a nearby gene with type 1 diabetes susceptibility in Germans, confirming respective observations previously made in Indian Asians. *Diabetes* 49:504–507, 2000

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$1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CD, cluster of differentiation; ETDT, extended transmission disequilibrium testing; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RFLP, restriction fragment-length polymorphism; TDT, transmission disequilibrium testing; UTR, untranslated region; VDR, vitamin D receptor.

Type 1 diabetes results from an immune-mediated destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. The activation of autoreactive lymphocytes and the cytokine-induced apoptosis of pancreatic β-cells play a major role in the etiology of type 1 diabetes. 1,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] inhibits lymphocyte activation and affects other elements of the immune system, such as cytokine and immunoglobulin production, as well as major histocompatibility complex (MHC) class II and cluster of differentiation (CD)-4 expression (1). In NOD mice, the development of diabetes can be prevented by administration of $1,25(OH)_2D_3$ (2), which inhibits lymphocyte activation and restores the altered ratio of CD4/CD8 cells.

Vitamin D exerts its genomic action via the nuclear vitamin D receptor (VDR), which shows an extensive polymorphism. The VDR belongs to the steroid receptor super-family and is widely expressed in many cell types, including lymphocytes, macrophages, and pancreatic β-cells (3). Four major polymorphic sites have been described within the VDR gene. A polymorphic *Fok*I site in exon 2 results in an alternative transcription initiation site, leading to a protein variant with three additional amino acids at the amino terminus (4). Polymorphic *Bsm*I and *Apa*I sites are present in intron 8, and a silent T to C substitution creates a *Taq*I restriction site in exon 9. Recently, an association of VDR alleles with type 1 diabetes in Indian Asians has been reported (5). We therefore examined the VDR locus on chromosome 12q12-14 as a candidate gene for type 1 diabetes susceptibility in German families using extended transmission disequilibrium testing (ETDT).

Pair-wise transmission distortion testing revealed a strong linkage disequilibrium between "B" and "A" (0.1514 ± 0.0145), between "B" and "T" (–0.1953 ± 0.0148), and between "A" and "T" (–0.1322 ± 0.0144). No significant linkage disequilibrium between *Fok*I and any of the other sites was detectable. The allele combinations "*baT*" (35.3%), "*BAt*" (29.5%), and "*bAT*" (16.8%) were most frequent in the analyzed population (Table 1).

Based on the linkage disequilibrium data, ETDT analysis of the 3′-haplotypes (*Bsm*I/*Apa*I/*Taq*I) showed a significant transmission distortion (χ^2 = 18.886, df = 7, P = 0.0086). These observations (Table 2) were confirmed when looking at the two-locus haplotypes that are part of the *Bsm*I/*Apa*I/*Taq*I

TABLE 1
Distribution of VDR haplotypes in the studied population

5′-3′-Haplotype	Percent of 5′-3′-haplotypes	3′-Haplotype code	Percent of 3′-haplotypes
F baT	18.1	1	35.3
f baT	17.2		
F BAt	19.0	2	29.5
f BAt	10.5		
F bAT	11.4	3	16.8
f bAT	5.4		
F BAT	3.9	4	6.0
f BAT	2.1		
F bAt	2.4	5	3.6
f bAt	1.2		
F Bat	1.8	6	2.4
f Bat	0.6		
F BaT	3.0	7	3.3
f BaT	0.3		
F bat	2.1	8	3.0
f bat	0.9		

The distribution of haplotypes (n = 332) in the studied population was derived from their frequency among (unrelated) parents that were considered in the ETDT analysis. The haplotypes are listed according to the previously suggested code for the 3′-haplotypes (6,7).

haplotypes: the ETDT for *Bsm*I/*Taq*I (χ^2 = 8.373, df = 3, P = 0.0389) was significant, as was the ETDT for the *Apa*I/*Taq*I haplotypes (χ^2 = 17.182, df = 3, P = 0.0006).

We failed to detect any transmission distortion of the "*baT*" allele (3′-code: 1) (45 of 82 times [55% transmitted, P = 0.3770]) or of its corresponding two-locus haplotypes "*bT*" (44 of 93 times [47% transmitted, P = 0.6041]) and "*aT*" (50 of 88 times [57% transmitted, P = 0.2008]). The "*BAt*" allele (3′-code: 2) was preferentially transmitted to affected offspring (52 of 81 times [64% transmitted, P = 0.0106]). Its two-locus haplotypes "*Bt*" (54 of 87 times [62% transmitted, P = 0.0244]) and "*At*" (52 of 84 times [62% transmitted, P = 0.0291]) also conferred an increased risk for type 1 diabetes. Transmission disequilibrium testing (TDT) of the "*bAT*" allele (3′-code: 3) was not significant, but the derived "*AT*" allele was preferentially not transmitted (20 of 63 times [32% transmitted, P = 0.0038]). The "*at*" allele—derived from the minor three-locus haplotypes "*Bat*" (3′-code: 6) and "*bat*" (3′-code: 8)—showed preferential nontransmission (4 of 17 [24% transmitted, P = 0.0290]). Adding the *Fok*I haplotype to the 3′-haplotypes in analysis of the transmission distortion diminished the statistical strength of the distortion observed for the 3′-haplotypes alone (ETDT for *Fok*I/*Bsm*I/*Apa*I/*Taq*I: P = 0.0541 [χ^2 = 24.702, df = 15]). Transmission of the single-locus alleles "*F*" (P = 0.3843), "*A*" (P = 0.6892), "*B*" (P = 0.2088), or "*T*" (P = 0.1573) alone did not differ significantly from the expected frequencies. All significant haplotype-wise results obtained by ETDT were confirmed by TRANSMIT analysis (Table 2).

In the present study, we investigated a possible linkage of polymorphisms in the VDR gene—defined by *Fok*I, *Bsm*I, *Apa*I, and *Taq*I restriction endonucleases—with type 1 diabetes. The observed allele frequencies as well as the linkage

TABLE 2
Transmission of VDR haplotypes

	Heterozygous for respective haplotype	Transmitted	Percent transmitted	Not transmitted	Percent not transmitted	P	Haplotype-wise results χ^2	df	P
baT	82	45	55	37	45	0.3770			
BAt	81	52	64	29	36	0.0106			
bAT	59	23	39	36	61	0.0906			
BAT	23	7	30	16	70	0.0606			
bAt	14	6	43	8	57	0.5390			
Bat	8	2	25	6	75	0.1573			
BaT	12	7	58	5	42	0.5637			
bat	11	3	27	8	73	0.1317			
ETDT*							18.886	7	0.0086
TRANSMIT*							21.841	7	0.0027
bT	93	44	47	49	53	0.6041			
Bt	87	54	62	33	38	0.0244			
BT	36	14	39	22	61	0.1824			
bt	26	9	35	17	65	0.1167			
ETDT†							8.373	3	0.0389
TRANSMIT†							13.009	3	0.0046
at	88	50	57	38	43	0.2008			
At	84	52	62	32	38	0.0291			
AT	63	20	32	43	68	0.0038			
at	17	4	24	13	76	0.0290			
ETDT‡							17.886	3	0.0006
TRANSMIT‡							16.589	3	0.0009

TDT, ETDT, and TRANSMIT results for the *Bsm*I/*Apa*I/*Taq*I, the *Bsm*I/*Taq*I, and the *Apa*I/*Taq*I haplotypes are shown. Absolute and relative numbers of transmitted and not transmitted alleles are given. Significant TDT results are in bold type. Details about the TRANSMIT calculation can be found in RESEARCH DESIGNAND METHODS. *Haplotype-wise results for *Bsm*I/*Apa*I/*Taq*I; †haplotype-wise results for *Bsm*I/*Taq*I; ‡haplotype-wise results for *Apa*I/*Taq*I.

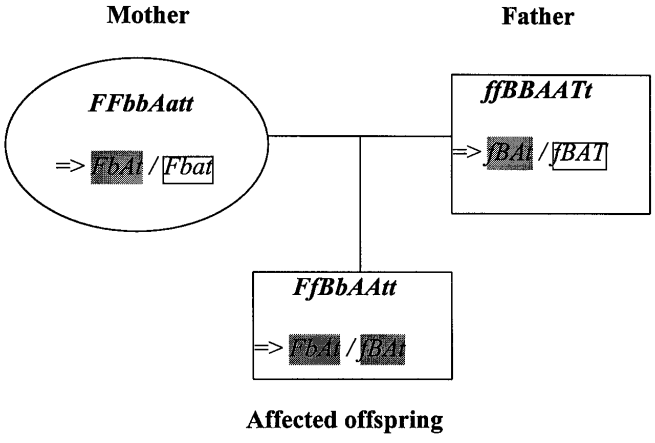

FIG. 1. Designation of haplotypes in the analyzed pedigrees. A schematic diagram representing the assignment of haplotypes based on the genotypes of the affected offspring, parents, and sibs is shown. Transmitted haplotypes are shown in shaded boxes and not transmitted haplotypes are shown in open boxes.

disequilibrium between the *Bsm*I, *Apa*I, and *Taq*I sites are in accordance with previously reported findings for Caucasian populations (6-8). Our report is the first to describe data for haplotypes comprising all four restriction sites.

We observed a significant transmission disequilibrium for several VDR haplotypes derived from the combined analysis of the *Bsm*I/*Apa*I/*Taq*I sites in the German population. Of those, the "*BAt*," "*At*," and "*Bt*" haplotypes conferred a significantly higher risk for type 1 diabetes, whereas the "*at*" and "*AT*" haplotypes showed significantly reduced transmission rates to the affected offspring, suggesting a protective effect of these haplotypes. Additional analysis of the *Fok*I site was not informative.

In a recent study, McDermott et al. (5) reported an association between VDR alleles and type 1 diabetes in Indian Asians. However, the "*bAT*" allele confers an increased risk in Indians, whereas the same allele is rather protective in Germans. The "*BAT*" haplotype is associated with a lower risk in both populations. The transmission of *Bsm*I/*Taq*I haplotypes also differs between the two ethnic groups. Overall, the "*b*" allele is associated with an increased risk for type 1 diabetes in Indian Asians and a decreased risk for type 1 diabetes in Germans. These findings suggest genetic heterogeneity within the VDR gene in the two populations, possibly due to different evolutionary lineages. Therefore, the studied VDR restriction fragment-length polymorphisms (RFLPs) appear to be markers rather than primary susceptibility loci in type 1 diabetes.

The *Taq*I polymorphism results in a silent mutation in the VDR gene (9), which would therefore not be expected to alter VDR function. The *Bsm*I and *Apa*I sites are located within an intron of the VDR gene. Alterations in intronic sequences may influence protein expression, but the *Bsm*I polymorphism has been reported not to influence VDR mRNA levels in peripheral blood mononuclear cells (10).

The *Fok*I polymorphism results in a VDR protein that is three amino acids longer (4). Early in vitro studies demonstrated an increased transcription rate (1.7-fold) of the VDR gene in cells with the "*FF*" genotype (11). However, recent studies have failed to show any relevant alteration in either the affinity of binding of the VDR protein or in the steady-state transcription rate of VDR gene (12). Although various other immunologically and regulatory genes are located close to the VDR gene, candidate sites for type 1 diabetes susceptibility include 5′ untranslated region (UTR) variants (13) and a 3′UTR polymorphism within the VDR gene itself (8). Given the proven influence of vitamin D on the immune system, the existence of a "true" susceptibility locus for type 1 diabetes within the VDR gene appears more likely. The observation that, for example, the VDR 3′UTR polymorphism is linked with other RFLPs in Germans than it is linked with in Indian Asians (8) offers a possible explanation for the allelic heterogeneity between Caucasians and Asians that has also been reported for other diseases, such as osteoporosis (4,6). VDR genotypes have been implicated in osteoarthritis (14) and primary hyperparathyroidism (15). These different associations may reflect the pleiotropic nature of the VDR molecule in various tissues.

The identification of a single locus responsible for a functional difference in VDR expression or affinity for either its ligand, DNA binding sites, or the retinoid receptor (RXR)—with which it forms heterodimers—could explain how an impairment in vitamin D action might contribute to the pathogenesis of type 1 diabetes. In this respect, it is of interest that a recent epidemiological study from several European countries revealed vitamin D supplementation in early childhood to be associated with a reduced type 1 diabetes incidence (16).

How VDR variants exert their distinct actions in various tissues, including pancreatic islets, needs to be addressed in functional studies. This would also have to be related to different populations where these effects may depend on additional genetic (acting in *cis* or in *trans*) or environmental factors. Nevertheless, VDR represents a gene locus that marks susceptibility to type 1 diabetes.

RESEARCH DESIGN AND METHODS

A total of 152 families with at least 1 offspring affected with type 1 diabetes, encompassing 527 subjects, were genotyped. Type 1 diabetes was diagnosed according to World Health Organization criteria. Informed consent was obtained prior to blood sampling. Age of onset ranged from 1 to 35 years, with a mean of 11.2 years and a male:female ratio of 1.0. In nine families, two children were affected; in four families, one parent and one offspring had type 1 diabetes.

Genomic DNA was amplified using polymerase chain reaction (PCR). Amplified DNA was digested with restriction enzymes (purchased from New England Biolabs, Beverly, MA, and AGS, Heidelberg, Germany) according to manufacturer's instructions for 3 h. Digestion products were separated by PAGE on 8% gels and silver stained. Standard PCR conditions were as follows: initial denaturation for 4 min at 94°C, 30 cycles of 94°C, annealing temperature (see below) and 72°C for each 1 min respectively, and final extension for 5 min at 72°C. The *Fok*I polymorphism (annealing temperature: 58°C) in exon 2 was studied using primers Fok-for (5′-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′) and Fok-rev (5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′). The resulting 265-bp fragment was digested with *Fok*I, generating two fragments of 196 and 69 bp after digestion in presence of the *Fok*I site. For examining the *Bsm*I polymorphism (annealing temperature: 60°C), the first part of intron 8 was amplified using primers Bsm-for (5′-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3′) and Bsm-rev (5′-AAC CAG CGG GAA GAG GTC AAG GG -3′). The resulting 825-bp fragment was digested with *Bsm*I, generating two fragments of 175 and 650 bp in presence of the *Bsm*I site. The *Taq*I and *Apa*I polymorphisms (annealing temperature: 60°C) were examined in a 740-bp fragment of intron 8/exon 9 using the primers Taq/Apa-for (5′-CAG AGC ATG GAC AGG GAG CAA-3′) and Taq/Apa-rev (5′-GCA ACT CCT CAT GGC TGA GGT CTC-3′). Digestion with *Apa*I revealed two fragments of 210 and 530 bp when the restriction site was present. Digestion with *Taq*I resulted in three fragments of 290, 245, and 205 bp in the presence of the polymorphic site and two fragments of 245 and 495 bp in its absence, due to an additional monomorphic *Taq*I site. In samples heterozygous for the respective restriction sites, both digested and undigested DNA fragments were visible. Genotypes were assessed blindly and were designated by a lowercase letter for the presence of a restriction site and by a capital letter for its absence.

Genotyping detects the presence of an allele in the diploid genome but does not provide any information regarding how these alleles are distributed between the two homologous chromosomes if the individual is heterozygous for more than one site. Thus, we reconstructed the VDR haplotypes as follows. An affected offspring has been genotyped "*FfBbAAtt*" and can possess either haplotype *FBAt/fbAt* or haplotype *fBAt/FbAt*. If the mother's genotype is "*FfbbAAtt*," her haplotype must be *FbAt/FbAt*. Because she is only able to transmit the "*FbAt*" haplotype to her child, the offspring's haplotype is unequivocally defined as *fBAt/FbAt* (Fig.1).

TDT was used to detect preferential transmission of the RFLP alleles to the affected subjects (17). The probability of a heterozygous parent transmitting either allele to the affected offspring is equal if there is no linkage between a certain allele and the disease in question. Deviation from the 50:50 transmission pattern suggests an association in the presence of linkage between a gene locus and this disease. The TDT is calculated as follows: $\chi^2 = (x_t - y_t)^2/(x_t + y_t)$, with x_t = number of transmission of the allele of interest x and y_t = number of transmissions of all other alleles. To assess the overall transmission distortion for the multilocus polymorphisms, haplotype-wise ETDT (18) was performed by logistic regression analysis using PROC LOGISTIC (SAS Institute, Cary, NC). TDT results are given uncorrected, whereas ETDT results have been corrected according to their degree of freedom. A probability of $P < 0.05$ was regarded as significant.

Only pedigrees in which all four parental haplotypes could be determined were included in the respective TDT/ETDT analysis. In other words, a family with only heterozygous members for the *Fok*I site could not be haplotyped for the *Fok*I alleles. This pedigree, therefore, would have been excluded for ETDT analysis of the *Fok*I/*Bsm*I/*Apa*I/*Taq*I haplotypes, but it would have been included for the ETDT analysis of the *Bsm*I/*Apa*I/*Taq*I haplotypes. Therefore, some of the pedigrees could not be considered in TDT/ETDT testing: *Bsm*I/*Taq*I (12 pedigrees), *Apa*I/*Taq*I (18 pedigrees), and *Bsm*I/*Apa*I/*Taq*I (19 pedigrees). Because a selection bias might result from the exclusion of pedigrees with uncertain haplotype phases, we applied the newly developed TRANSMIT test to control for a possible bias in our ETDT analysis. Because those pedigrees with uncertain haplotype phases are also considered in the TRANSMIT analysis, no systematic bias would be introduced in this test. The TRANSMIT test is based on a score vector that is averaged over all possible configurations of parental haplotypes and transmissions consistent with the observed data. Data from available siblings are used to narrow down the range of possible parental haplotypes that need to be considered (19). We found the TRANSMIT data to be highly consistent with our ETDT findings and can therefore exclude a significant bias in the presented ETDT results. The respective TRANSMIT and ETDT data are shown in Table 2. The pairwise linkage disequilibrium was calculated as described previously (20) and is given as disequilibrium ± SE.

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