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Allelic variation in the vitamin D receptor influences susceptibility to IDDM in Indian Asians

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Summary Vitamin D has important immunomodulatory properties and prevents development of diabetes mellitus in an animal model of insulin-dependent diabetes (IDDM). We have studied the vitamin D receptor locus as a candidate for genetic susceptibility to IDDM in Southern Indian families. We found evidence for an association of one particular vitamin D receptor allele with IDDM susceptibility in this community. Ninety-three South Indian families consisting of available parents and an affected offspring were genotyped for three vitamin D receptor polymorphisms using the restriction enzymes *TaqI, ApaI* and *BsmI* as well as an adjacent microsatellite located to 12q14 (D12S85). Transmission disequilibrium testing analysis was used to assess preferential transmission of polymorphic markers and haplotypes with IDDM. There was significant excess transmission of vitamin D receptor alleles containing the *BsmI* restriction site to affected offspring in these families ($p = 0.016$). No association was found between D12S85 and IDDM. This study suggests that a polymorphism within or close to the vitamin D receptor gene may modify susceptibility to IDDM in this ethnic group. [Diabetologia (1997) 40: 971–975]

Keywords Vitamin D receptor, genotype, insulin-dependent diabetes, genetic susceptibility, South India.

Insulin-dependent diabetes mellitus (IDDM) is a multifactorial disease with a strong genetic component [1]. The main genetic contribution to IDDM susceptibility lies in the major histocompatibility complex (MHC) on the short arm of chromosome 6 and several non-MHC chromosomal regions are also involved [2]. Various initial approaches have been used to identify IDDM susceptibility regions including case-control studies of candidate genes [HLA, insulin gene regulatory region, interleukin-1 receptor type 1 gene (ILIR1)] [3–6], combined linkage and association-based studies of candidate genes (CTLA4 – a receptor on activated T cells) [7], and systematic total genome searches in addition to individual chromosomal regions [8–16].

There are clear differences in immunogenetic predisposition to IDDM between countries and these seem to vary with disease incidence [1]. IDDM in Southern India has a similar incidence (10.4/100 000 cases per year) to Asian children in the UK and white children of European extraction [17, 18]. While an MHC component to susceptibility is detectable [19, 20] in IDDM in Southern India no association with either the insulin gene [20] or interleukin-1 receptor type 1 [6] has been found using a case-control design. This finding suggests possible differences in the non-MHC IDDM component in this ethnic group compared to whites of European extraction. In the latter populations an association with the insulin gene has been universally reported [4, 5, 21], and an ILIR1

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Abbreviations: IDDM, Insulin-dependent (Type 1) diabetes mellitus; VDR, Vitamin D receptor; RFLP, restriction fragment length polymorphism; TDT, transmission disequilibrium test; MHC, major histocompatibility complex; ETDT, extended transmission disequilibrium test; mRNA, messenger RNA; ILIR1, interleukin-1 receptor type 1.

association with IDDM has been described in some studies of northern Europeans [6, 22].

IDDM develops as a result of autoimmune destruction of the insulin producing pancreatic beta cells; vitamin D has important immunomodulatory properties and influences insulin secretion [23]. The active form of vitamin D (1,25 dihydroxyvitamin D3) inhibits T cell proliferation and suppresses both tumour necrosis factor α (TNFα) and interleukin-1 production [24]. Furthermore, in the NOD mouse (an excellent animal model of IDDM) vitamin D3 administration prevents the development of IDDM as well as the associated autoimmune insulitis [25]. In a recent study in Bangladeshi subjects living in east London, vitamin D levels were found to be reduced in those most at risk for non-insulin-dependent diabetes [26]. Therefore, we have studied the vitamin D receptor (VDR) locus on chromosome 12q as a candidate gene for IDDM susceptibility in Southern Indian subjects using the transmission disequilibrium test (TDT) [27, 28] in family trios consisting of parents and affected offspring.

Subjects, materials and methods

IDDM families: A total of 93 Southern Indian families were recruited from patients attending the MV Hospital for Diabetes, Madras, India. All affected subjects were ketosis prone and had acute onset of disease which required immediate treatment with insulin. Cases of fibrocalculous pancreatic diabetes were excluded by radiology, ultrasonography and on clinical grounds. The mean age of onset in IDDM probands was 11.1 years (± 6.6 years; range 1–29 years), the male to female sex ratio was 0.45, mean body mass index at time of venesection was 18.2 ± 5.03 and 54% of 48 probands tested had antibodies to glutamic acid decarboxylase (GAD65; unpublished data). Blood samples were taken from 93 probands; in addition, both parents were studied in 83 of these families, and from 1 parent in the remaining 10 families. Overall NIDDM was present in 16% of parents and IDDM was diagnosed in 2 of the fathers. Informed consent was obtained from all families prior to blood sampling in Madras.

Experimental methods. DNA was extracted from whole blood (Puregene kits; Centra Systems Inc., Minneapolis, USA). All family members were initially genotyped for three restriction fragment length polymorphic (RFLP) sites (*BsmI* and *ApaI* in successive introns between exons 7 and 9 of the VDR gene, and *TaqI* within the 9th exon) [29]. Genotypes were determined by two separate polymerase chain (PCR) amplifications followed by restriction endonuclease digestion of the products (*ApaI* and *TaqI* for the first PCR; *BsmI* for the second). Fragments were separated on 1.5% agarose gels (in the case of *BsmI*), and 3% for *ApaI* and *TaqI;* the gels were visualised by ethidium bromide ultraviolet illumination. Genotypes were designated conventionally by a lower case letter for the presence of a cut site and a capital letter for the absence, e.g. the 'b' allele designates the presence of a *BsmI* site, and, likewise, 'B' the absence. The genotypes therefore are bb, BB (homozygous for the presence and absence of the cut site respectively) and Bb (heterozygous). All samples were analysed at least twice to ensure the validity of genotyping, and all results assessed blindly by an independent investigator. A fluorescently labelled microsatellite (D12S85) located 2cM from the VDR was also used to genotype all members of these families. PCR primers were chosen from published sequences [30] and a touchdown technique was employed. Amplified fragments were separated by polyacrylamide gel electrophoresis on an ABI 373 DNA sequencer and fragments analysed using GENESCANNER software. There were at least 10 alleles of this marker in the families with a size range of 104–130 base pairs (bp) [30].

Statistical analysis. The transmission disequilibrium test (TDT) was used to detect preferential transmission of the RFLP alleles to affected subjects [27]. For each heterozygous parent, the probability of transmitting either allele to an affected offspring is equal unless the polymorphism is linked and associated with the disease, deviation of transmission probabilities from 50:50 therefore provides good evidence for association due to linkage disequilibrium. In addition, haplotypes were constructed where possible from combinations of two RFLPs and also from a combination of all three RFLPs; these haplotypes were then treated as "pseudo-alleles" and analysed using the extended transmission disequilibrium test (ETDT) which deals with multiallelic polymorphisms [27]. The ETDT provides an overall test of transmission distortion for a multiallelic polymorphism either by considering all heterozygous parental genotypes separately (genotype-wise analysis), or by combining information across genotypes to detect effects due to particular alleles (allele-wise analysis). Where overall tests were positive for a haplotype system individual haplotypes were assessed to see which one accounted for the overall effect. Genotype distributions between subgroups of patients of IDDM patients were compared using the chi-square test.

Results

Restriction fragment length polymorphism (RFLP) data were available for all three enzymes in all 93 families. Since TDT relies on transmissions from heterozygous parents to affected offspring the numbers of informative families vary according to the enzyme used. ETDT analysis for individual RFLPs found that the "b" allele of the *BsmI* RFLP was preferentially transmitted to affected offspring [53 of 84 times (53/84); $p = 0.016$]; ETDT analysis for haplotypes based on two RFLPs also demonstrated significant preferential transmission of the 'bT' haplotype (41/59; $p = 0.003$) (Table 1). There was further evidence for preferential transmission of the *BsmI/ApaI* (allele-wise $p = 0.026$, genotype-wise; $p = 0.080$) and *BsmI/TaqI* haplotypes (allele-wise $p = 0.011$, genotype-wise; $p = 0.036$) (this latter effect is due mainly to the significant transmission of 'bT'). The disease associated alleles and haplotypes were equally transmitted by fathers and mothers. There was weak evidence for an extended haplotype effect (i.e. all three RFLPs) (allele-wise $p = 0.074$, genotype-wise; $p = 0.047$). Table 2 shows that the 'bAT' haplotype was preferentially transmitted to affected offspring on 35 out of 54 occasions ($p = 0.0295$) (seven of the eight possible haplotypes were observed in these families). The bAT

Table 1. *BsmI/Taq1* vitamin D receptor haplotypes in 93 South Indian families

	Haplotypes			
	BT	bT	Bt	bt
Transmitted	11	41	21	2
Not transmitted	27	18	29	1
p values	0.009	0.0028	0.26	–

Chi-square for allele-wise TDT = 11.12 (3 degrees freedom) $p = 0.011$
Chi-square for genotype-wise TDT = 11.88 (5 degrees freedom) $p = 0.036$

Table 2. *BsMI/ApaI/TaqI* vitamin D receptor haplotypes and IDDM

	Haplotypes						
	BAT	Bat	bAT	bAt	baT	BaT	bat
Transmitted	13	23	35	1	34	2	5
Non-transmitted	29	25	19	2	28	2	8
p values	0.014	0.77	0.0295	–	0.446	–	0.405

Chi-square for allele-wise TDT = 11.47 (6 degrees freedom) $p = 0.0747$
Chi-square for genotype-wise TDT = 23.91 (14 degrees freedom) $p = 0.0470$

haplotype was the third most common haplotype found in the parents of the IDDM probands (frequency 0.22 compared to 0.26 for BAt and 0.25 for baT).

There was no evidence for preferential transmission of either *TaqI* (allele-wise $p = 0.33$) or *ApaI* (allele-wise $p = 0.46$) alleles, and no association between IDDM and *ApaI/TaqI* haplotypes was found (allele-wise $p = 0.17$). TDT analysis of D12S85 revealed no evidence of association between this marker and IDDM (allele-wise $p = 0.13$).

Probands with an age of onset of IDDM of 15 years or below ($n = 72$) and those greater than 15 years ($n = 21$) were compared for genotype distribution of RFLPs; no differences were observed for *BsmI* ($p = 0.87$), *TaqI* ($p = 0.84$) or *ApaI* ($p = 0.9$). In the smaller number of probands tested for GAD65 antibodies ($n = 48$), no differences were observed according to the presence or absence of GAD65 antibodies (*BsmI*, $p = 0.82$; *TaqI*, $p = 0.96$ and *ApaI*, $p = 0.89$).

Discussion

The results of this study demonstrate preferential transmission of the "b" allele of the *Bsm1* RFLP to affected subjects. Extended haplotypes containing this allele, denoted bT and bAT, also demonstrate preferential transmission but we cannot determine whether this is due to a primary association between IDDM and these haplotypes, or whether the association is strictly with the "b" allele of the *Bsm1* RFLP itself.

The VDR locus has been extensively studied for association with susceptibility to osteoporosis, and the *Bsm1* 'B' allele has been associated with reduced bone mineral density in separate studies [31, 32]. However, these findings are not consistent, and several groups have failed to reproduce these initial observations [29, 33]; indeed at least one study has found the alternative "b" allele associated with particular subgroups of osteoporosis [34]. The 'bb' genotype has also been associated with primary hyperparathyroidism in Swedish patients [35]. Although the nucleotide sequence of the VDR gene has not been reported for the IDDM associated bAT haplotype, sequences from the two most common haplotypes in Caucasoids (Bat and baT) are available and only neutral coding region variants are described. Some polymorphic differences between the haplotypes were found in the 3' untranslated region between the baT and Bat haplotypes which were associated with reduced gene transcription [31, 36] and mRNA stability.

The VDR gene located on chromosome 12q12–12q14 is not in a region linked to IDDM in a genome scan of European and North American Caucasoid affected sibling pairs [8]. This does not exclude VDR gene involvement in IDDM as true linkage is easily missed in complex diseases, and for particular candidate loci association-based approaches such as TDT may be more powerful than linkage. Hence both the insulin gene and CTLA4 gene have been implicated in IDDM primarily by association studies, despite weak evidence in favour of linkage to these genes [4, 5, 7]. Because linkage disequilibrium only occurs over very short genetic distances in out-bred populations, association studies are not suitable for studying random markers in a genome scan but are the method of choice for candidate gene study. The case-control design is prone to false positives if population stratification is present (i.e. cases and controls are sampled from genetically different sub-populations), so results obtained by this approach must always be regarded with caution. By contrast, transmission disequilibrium testing uses those alleles not transmitted by parents to affected offspring to form the control group; positive results thus obtained are therefore likely to be due to a true genetic association between marker and disease loci. Our results are formally significant and seem unlikely to have occurred by chance, especially given the biological plausibility of the candidate gene studied. D12S85 is 2 cM from the VDR locus on 12q12–12q14. Since no association was found between this latter marker and IDDM, it would indicate susceptibility is located close to the VDR locus, and that D12S85 is in linkage equilibrium with the studied polymorphisms of the VDR locus. Recently, a locus on 12q24 (NIDDM1) has been found to be linked to insulin secretion in NIDDM [37]; furthermore, this region also contains the

MODY3 locus [38]. In maturity onset diabetes of the young, the MODY3 locus has recently been cloned and found to be due to a variant of hepatic nuclear factor-1α [39]. The distance between 12q12–12q14 (VDR location) and 12q24 (NIDDM1/MODY3) rules out our observations in IDDM being explained by associations between IDDM and either the NIDDM1 or MODY3 gene.

The immunogenetic predisposition to IDDM in South India has differences from that observed in whites of European extraction. While we have previously described associations between IDDM and DQB1*0302 and DQB1*0201 in South Indian subjects there is a decreased frequency of heterozygotes for DQB1*0302/DQB1*0201 in this ethnic group compared to whites [19, 20 and unpublished data]. Furthermore, there are also differences at non MHC-loci [6, 20]. Nonetheless, this is typical IDDM on clinical grounds, as indicated by the clinical presentation, the high frequency of GAD65 antibody positivity and an incidence of IDDM similar to many European populations [18]. Though the numbers are small, the VDR genotype distribution in the probands was not different according to GAD65 antibody status or age of onset of IDDM. Therefore, the VDR gene association we describe in this ethnic group is unlikely to be explained by an atypical form of IDDM or insulin-treated NIDDM in the guise of IDDM.

In conclusion, this study suggests that a polymorphism within or close to the VDR gene may modify susceptibility to IDDM in subjects living in South India. Replication of this result is necessary in other samples, preferably drawn from the same ethnic group and by identification of the particular polymorphisms within or close to VDR which directly affect susceptibility to IDDM.

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