CLINICAL STUDY

Vitamin D receptor gene polymorphisms are associated with obesity in type 2 diabetic subjects with early age of onset

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Abstract

Objective: Allelic variations in the vitamin D receptor (VDR) gene were reported to modulate insulin secretion in response to glucose. VDR was investigated as a candidate gene for type 2 diabetes mellitus (T2DM).

Method: Four single nucleotide polymorphisms (SNPs) in intron 8 (BsmI, Tru9I, ApaI) and exon 9 (TaqI) of the VDR gene were examined in 309 unrelated French subjects with T2DM and 143 controls.

Results: The distribution of alleles and genotypes of the four SNPs was similar in patients and controls. However, in patients whose age at diagnosis of diabetes was ≤ 45 years, homozygous subjects for the T-allele of the TaqI SNP had a higher body mass index (BMI) (31.7 ± 6.7 kg/m², $P = 0.0058$) and an increased prevalence of obesity (81%, $P = 0.005$) with respect to heterozygous subjects (27.9 ± 5.0 kg/m²; 46%) or homozygous subjects for the t-allele (27.7 ± 5.0 kg/m²; 52%). Similar results were observed for homozygous subjects for the b-allele of the BsmI SNP. Logistic regression analysis demonstrated that TT homozygosity was independently associated with obesity in these subjects (odds ratio, 4.64; 95% confidence interval (CI), 1.64–14.76; $P = 0.0056$).

Conclusion: VDR is not a major gene for T2DM in French Caucasians. However, polymorphisms in the VDR gene are associated with the susceptibility to obesity in subjects with early-onset T2DM. The pathophysiological mechanisms of these associations remain unexplained, but they could be related to a direct effect of vitamin D in adipocyte differentiation and metabolism, or to an indirect effect by modulation of insulin secretion.

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Introduction

The vitamin D endocrine system regulates multiple aspects of calcium metabolism and of cellular differentiation and replication in many target tissues, including the endocrine pancreas (1–3). It is now clear that the vitamin D endocrine system plays an important role in the mechanism of insulin release and in the maintenance of glucose tolerance. Vitamin D deficiency in experimental animals and in humans is associated with impaired insulin secretion, which is normalised by 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) administration (4–6). The actions of the vitamin D endocrine system are mediated both by genomic and non-genomic pathways (7). The former are activated by the binding of 1,25-(OH)₂D₃ to a specific cytosolic/nuclear vitamin D receptor (VDR), a member of the steroid/thyroid hormone-receptor superfamily (8). It is well established

that pancreatic β -cells express VDR (3). Non-genomic pathways are activated via a putative membrane vitamin D receptor (mVDR), and might be responsible for rapid effects of vitamin D (9).

Allelic variations in the VDR gene were reported to be associated with a variety of phenotypes. These included variations in circulating levels of 1,25-(OH)₂D₃ (10), variations in bone mineral density (11), modulation of intrauterine and early postnatal growth (12), severity of coronary artery disease (13), and also susceptibility to type 1 diabetes (14, 15) and variations in insulin secretion in response to glucose (16). Indeed, an association of alleles of single nucleotide polymorphisms (SNPs) in the VDR gene with increased or decreased insulin secretion in response to an oral glucose tolerance test was observed in a cohort of Bangladeshi Asians (16) recruited from a population considered at risk for type 2 diabetes (5).

Table 1 Demographic and clinical profile of patients and controls. Data are expressed as means±s.d. (and range).

	Patients			<i>P</i> * value	Control group
	All subjects	Age at diagnosis ≤45 years (1st tercile)	Age at diagnosis >45 years (2nd–3rd terciles)		
Subjects (<i>n</i>)	309	106	203		143
Gender (M/F as %)	58/42	56/44	60/40	0.42	50/50
Age (years)	62±12	54±11	67±9	<0.0001	61±16
BMI (kg/m ²)	29.3±5.9	29.4±5.8	29.3±5.9	0.92	–
Age at diagnosis of diabetes (years)	50±12 (27–82)	38±5 (27–45)	56±8 (46–82)	<0.0001	–
Duration of diabetes (years)	13±9	18±11	11±8	<0.0001	–
Treatment (OHA/insulin as %) [†]	59/41	55/45	62/38	0.21	–
Total cholesterol >6 mmol/l (%)	34	27	37	0.08	–
Triglycerides >2 mmol/l (%)	51	46	53	0.31	–
Prevalence of obesity (BMI ≥ 27 kg/m ² ; %)	60	59	61	0.85	–
Prevalence of hypertension (%) [‡]	67	57	71	0.02	–

*Comparisons between the two subgroups of patients stratified by terciles of age of diagnosis of diabetes.

[†]OHA, oral hypoglycaemic agents.

[‡]Hypertension was defined as: (1) systolic blood pressure (SBP) greater than 140 mmHg and/or a diastolic blood pressure (DBP) greater than 85 mmHg; (2) SBP and/or DBP below these values in the presence of antihypertensive medication and a history of hypertension.

These findings prompted us to investigate the role of VDR as a candidate gene for susceptibility to type 2 diabetes mellitus (T2DM).

Subjects and methods

Subjects

We studied a group of 309 unrelated Caucasian subjects with overt T2DM consecutively recruited at the Diabetes Department and outpatient clinics of Hôpital Necker, Paris, France. All subjects were tested for islet cell autoantibodies (ICAs) and none gave positive results. Because of the well-known clinical and genetic heterogeneity of T2DM, patients were stratified in more clinically homogeneous subgroups according to the age at diagnosis of diabetes (1st tercile versus 2nd–3rd terciles of the distribution). Analyses were performed in the whole population and in the more clinically homogeneous subgroup of patients. The control group consisted of 143 unrelated Caucasian subjects without known histories of diabetes, recruited among members of the CEPH (Centre d'Etudes du Polymorphisme Human) family reference panel (one per family) and among spouses of subjects with type 2 diabetes. The demographic and clinical characteristics of the patients and the control subjects are shown in Table 1. The study was approved by the ethical committee of Hôpital Necker (CCPPRB Paris Necker).

DNA studies

Genomic DNA was extracted from peripheral blood samples by standard procedures. Four SNPs in intron 8 (BsmI, Tru9I, ApaI) and exon 9 (ATT→ATC, Ile352Ile: TaqI) of the VDR gene (chromosome 12cen-q12) were examined by polymerase chain reaction/restriction

fragment-length polypeptide (PCR-RFLP)-based methods. Experimental conditions were as described previously for the detection of these ApaI and TaqI (17), and BsmI and Tru9I (18) restriction sites. It is noteworthy that the Tru9I SNP that we have recently identified (18) is located in the reverse primer binding site of primers that have been frequently used in the literature to genotype the BsmI SNP (19). We have observed that the presence of the Tru9I rarer allele may result in allele drop-out when the BsmI RFLP is genotyped with the original set of primers (18). In this situation, subjects that are heterozygous for the BsmI variant, and who also carry the Tru9I variant, can be mistaken for homozygous carriers of the sole BsmI amplified allele. Thus, a new set of primers was used to avoid mistyping. Genotypes were designated conventionally by the first letter of the name of the enzyme, except for the Tru9I genotypes, which were designated using 'U' or 'u' to avoid confusion with TaqI genotypes. A capital letter indicates the absence of the cut site, whereas a lower-case letter indicates its presence.

Statistical analyses

Results are expressed as means±s.d. Contingency table chi-squared tests were used to compare genotype and allele frequencies and other qualitative data. Fisher's exact test was used in calculations involving small samples. Odds ratios were computed when data were significantly different. ANOVA was used to compare quantitative data. When this test was significant, comparisons between pairs were made using the Tukey–Kramer Honestly Significant Difference test. Logistic regression analysis was performed to evaluate associations between clinical and genetic parameters. Statistical analyses were performed with JMP software (SAS Institute Inc., Carey, NC, USA).

Table 2 Vitamin D receptor genotypes in diabetic and control subjects. Genotypes are in Hardy–Weinberg equilibrium.

	Diabetic subjects	Controls	P
BsmI	(306)	(143)	
BB (%)	17.0	16.8	
Bb (%)	44.1	45.4	
bb (%)	38.9	37.8	0.96
Apal	(305)	(143)	
AA (%)	32.1	24.5	
Aa (%)	46.6	54.5	
aa (%)	21.3	21.0	0.20
TaqI	(305)	(143)	
TT (%)	39.3	37.8	
Tt (%)	44.6	46.1	
tt (%)	16.1	16.1	0.94
Tru9I	(306)	(142)	
UU (%)	73.2	80.3	
Uu (%)	25.8	18.3	
uu (%)	1.0	1.4	0.21

Results

There were no significant differences between diabetic and control subjects in the distribution of alleles or genotypes of the four SNPs (Table 2). Analyses were also performed in more homogeneous subgroups of patients with age at diagnosis in the first tercile or in the second and third terciles of the distribution. In these

subgroups, allele and genotype frequencies were similar to the frequencies in the whole group of patients and in the controls (data not shown).

Various parameters (age at diagnosis of diabetes, requirement for insulin therapy, body weight, BMI, prevalence of obesity ($\text{BMI} \geq 27.0 \text{ kg/m}^2$), hypertension, dyslipidemia) were compared among diabetic carriers of the different genotypes of the four SNPs. No significant differences in these traits were observed for the genotypes of any of the SNPs when patients were analysed as one single group, or when the subgroup of patients with age at diagnosis of diabetes in the second and third terciles of the distribution was considered (data not shown). However, in the group of patients with age at diagnosis of diabetes within the first tercile of the distribution (Table 3), homozygous subjects for the T-allele of the TaqI SNP presented a significantly higher BMI ($31.7 \pm 6.7 \text{ kg/m}^2$) than heterozygous subjects ($27.9 \pm 5.0 \text{ kg/m}^2$) or homozygous subjects for the t-allele ($27.7 \pm 5.0 \text{ kg/m}^2$; $P = 0.0058$). After correction for multiple testing due to stratification of age at diagnosis, this difference remained significant ($P = 0.017$). We have observed similar results for the bb genotype of the BsmI SNP ($P = 0.004$; corrected P value = 0.012), and a trend towards an association for the aa genotype of the Apal SNP. Data shown in Table 3 suggest a recessive model for these associations of the TT or bb genotypes with increased BMI. Moreover, the prevalence of obesity, defined as a $\text{BMI} \geq 27 \text{ kg/m}^2$,

Table 3 Vitamin D receptor genotypes and clinical profile of diabetic subjects in the first tercile of age at diagnosis (27–45) years).

Genotype	n	Age (years)	Percent of men	Age at diagnosis of diabetes (years)	Weight* (kg)	BMI* (kg/m^2)	Percent of obesity**
BsmI							
BB	20	58±12	50	38±6	79±18	27.9±5.0†	55
Bb	47	54±11	62	37±6	78±13†	27.8±4.9†	47
bb	39	52±10	51	38±5	87±17	31.8±6.7	80
P		0.16	0.53	0.80	0.025 (0.075)	0.004 (0.012)	0.0075 (0.023)
Apal							
AA	38	57±13	55	38±6	78±16	27.8±4.8	50
Aa	50	53±9	56	38±5	82±16	29.6±6.5	61
aa	18	52±11	56	38±5	88±16	31.4±5.8	81
P		0.10	0.99	0.95	0.095	0.089	0.09
TaqI							
TT	40	53±10	53	38±5	87±17	31.7±6.7	81
Tt	45	54±11	60	37±6	79±13‡	27.9±5.0‡	46
tt	21	58±12	53	38±5	78±18	27.7±5.0‡	52
P		0.17	0.74	0.68	0.039 (0.12)	0.0058 (0.017)	0.005 (0.015)
Tru9I							
UU	76	54±11	54	38±5	81±16	28.7±5.3	58
Uu	30	55±12	60	37±6	84±18	30.7±7.2	67
uu	—	—	—	—	—	—	—
P		0.78	0.57	0.62	0.38	0.15	0.49

*Age-adjusted comparisons.

†‡ANOVA P values (quantitative traits) are followed by Tukey–Kramer HSD test results when appropriate (see Methods): †statistically significant differences ($P < 0.05$) as compared with the BsmI bb genotype; ‡statistically significant differences ($P < 0.05$) as compared with the TaqI TT genotype.

P values after correction for multiple testing are given in parentheses.

**BMI $\geq 27 \text{ kg/m}^2$.

Table 4 Logistic regression analysis of traits associated with obesity in type 2 diabetic subjects.

	Odds ratio	95% Confidence interval	P
Gender	1.27	0.71–2.27	0.42
Age	17.83	2.98–116.59	0.002
Age at diagnosis of diabetes (1st versus 2nd–3rd terciles)	1.04	0.49–2.18	0.92
Duration of diabetes	0.13	0.01–1.16	0.07
Arterial hypertension	4.48	2.32–8.92	<0.0001
Total cholesterol >6 mmol/l	0.62	0.33–1.13	0.12
Triglycerides >2 mmol/l	1.71	0.97–3.04	0.07
Treatment by insulin	0.64	0.36–1.14	0.13
TT genotype* (1st tercile of age at diagnosis)	4.64	1.64–14.76	0.0056
TT genotype* (2nd–3rd terciles of age at diagnosis)	0.85	0.43–1.68	0.64

*TaqI single nucleotide polymorphism genotypes.

was increased in homozygous subjects for the T-allele of the TaqI SNP (81%) relative to the prevalence in carriers of other TaqI genotypes (46–52%; $P = 0.005$; corrected P value = 0.015). The odds ratio computed for this association of the TT genotype with obesity in a recessive model (TT versus Tt/tt) was 4.42 (95% CI, 1.76–12.34; $P = 0.0025$). The prevalence of obesity was also increased in homozygous subjects for the b-allele of the BsmI SNP relative to the prevalence in carriers of other BsmI genotypes ($P = 0.0075$; corrected P value = 0.023). The odds ratio for the association of the bb genotype with obesity in a recessive model (bb versus bB/BB) was 4.12 (95% CI, 1.64–11.53; $P = 0.004$). Other clinical parameters, such as the prevalence of hypertension or dyslipidaemia, were similar in carriers of the different genotypes. No gender-related differences were observed in any of these comparisons.

Homozygous subjects for the b-allele of the BsmI SNP and homozygous subjects for the T-allele of the TaqI SNP represent the same subset of patients, because of an almost complete linkage disequilibrium between the two SNPs (97.5% of TT homozygosity in bb homozygous subjects, $P < 0.0001$). These data are in agreement with the observation that baT and BA_T are the most frequent haplotypes in Caucasians (19, 20). Consequently, further analyses were performed with only one of these SNPs.

The multiple comparisons of clinical traits in carriers of the different genotypes plus the stratification of age at diagnosis are potential sources of type 1 error (false-positive results). To take these potential sources of bias into account, we performed logistic regression analyses with the ponderal status as the categorical dependent variable (obese versus non-obese) to assess correlations of this trait with clinical and genetic parameters, and to verify the independence of these correlations. Gender, age, age at diagnosis of diabetes (1st tercile versus 2nd–3rd terciles), duration of diabetes, the presence of arterial hypertension, increased total cholesterol levels (>6 mmol/l), increased triglyceride levels (>2 mmol/l), insulin requirement and TaqI SNP genotype (TT versus Tt/tt) were included as independent variables (Table 4).

As the effect of the TaqI SNP genotype on the ponderal status seems to occur only within a single level of the age at diagnosis variable (1st tercile), the model that was tested included the computation of the statistical effect of the TaqI SNP genotype for each level of the age-at-diagnosis variable. In statistics jargon, the TaqI SNP genotype variable is said to be 'nested' within the age-at-diagnosis variable. As expected, age and arterial hypertension were significantly associated with obesity. The odds ratio was 17.83 across the age range of the population (27–92 years) and 4.48 for hypertensive subjects as opposed to non-hypertensive subjects. These analyses also demonstrated that TT homozygosity was independently associated with obesity in diabetic subjects with age at diagnosis within the first tercile of the distribution (odds ratio, 4.64; 95% CI, 1.64–14.76; $P = 0.0056$, as compared with Tt/tt combined genotypes).

Discussion

We have observed no association between T2DM and four SNPs in intron 8 and exon 9 of the VDR gene. These data suggest that VDR is not a major gene for T2DM in French Caucasians. However, our study provides evidence that polymorphisms in the VDR gene might be associated with the susceptibility to obesity in subjects with early-onset T2DM. The presence of the TT genotype of TaqI SNP or of the bb genotype of the BsmI SNP accounted for a difference of about 9 kg of body weight or 4 kg/m² BMI, and for a ~30% increase in the prevalence of obesity in that group of subjects.

The pathophysiological mechanisms of these associations remain unexplained. A direct effect of vitamin D on adipocyte differentiation and metabolism is a possible mechanism, as VDR is expressed in preadipocytes (21). It has been shown *in vitro* that vitamin D inhibits the differentiation of preadipocytes (22, 23), and stimulates the terminal differentiation of adipocytes (24) and the synthesis and secretion of lipoprotein lipase (25). It is noteworthy that negative associations between BMI and circulating vitamin D₃ levels were

observed in Caucasian subjects (26, 27), and that VDR polymorphisms were found to influence circulating levels of vitamin D (10). It is tempting to speculate that the association of VDR polymorphisms with obesity in type 2 diabetic subjects could also be related to a modulation of insulin secretion. Hyperinsulinaemia is commonly found in obese subjects and may result from a β -cell compensatory response to insulin resistance. Alternatively, it might result from a primary over-secretion of insulin due to β -cell dysfunction (28, 29), and may contribute to the excess of fat deposition and to the development of insulin resistance.

The relationship between these genotypes and insulin levels could not be assessed in the population we studied, as all subjects were treated with oral hypoglycaemic agents and/or insulin. Their insulin levels, if measured, would mainly reflect the treatment of diabetes and/or the deleterious effects of chronic hyperglycaemia on β -cell function (glucotoxicity). It was reported that the ApaI, BsmI and TaqI SNPs influence insulin secretion in response to an oral glucose tolerance test in a cohort of non-diabetic Bangladeshi Asians (16). However, in that study, the aa, bb or TT genotypes were associated with reduced insulin levels (half those of AA, BB or tt genotypes, intermediate levels being observed in heterozygous subjects). Moreover, no differences in the BMI were noted in the carriers of the different genotypes. These observations appear at odds with our results, but they do not exclude the insulin hypothesis. Indeed, other studies have shown that variable genotypes in these SNPs may be associated with a particular phenotype in different populations or even in different subsets of the same population. For instance, a study of a cohort of healthy two-year-old French children suggested that VDR genotypes influence intrauterine and early post-natal growth via interactions with gender-related growth regulators (12). In that study, BB girls had higher length, body weight and body surface area, and, inversely, boys with the BB genotype had lower body weight, BMI and body surface area than their respective bb counterparts. Unfortunately, insulin levels were not measured in those children. Another example of variable-risk alleles in different populations is found in the associations of these SNPs with type 1 diabetes (14, 15).

Associations between VDR genotypes and bone mineral density in women have been reported (17, 19, 30), but the findings were refuted in several other studies (31–33). The results of a meta-analysis of 29 of such studies suggest a minor effect, there being a reduction in bone mineral density of about 2% in the BB-genotype groups relative to the bb-genotype groups (11). Could the higher BMI that we have observed in bb subjects be related to differences in bone mineral density? Indirect evidence suggests that this is not the case. A 2% variation in bone mineral density could not explain the variation in body weight and BMI that we

observed in our cohort, and, moreover, no differences in body weight or BMI were observed in most studies reporting VDR genotype-related differences in bone mineral density (17, 30). A study of healthy, non-obese (BMI < 30 kg/m²), premenopausal Caucasian women showed an association of bb genotypes with increased bone mineral density and with increased body weight (34). However, in that study, the association of the bb genotype with body weight was shown to be independent of variations in bone mineral density.

The genetic mechanisms behind the genotype/phenotype associations observed for these intronic or silent SNPs are still unclear. Linkage disequilibrium with a functional variant elsewhere in the VDR gene is a possible explanation. Some polymorphic differences in the 3' untranslated region have been observed between the BAt and the baT haplotypes (the most common haplotypes in Caucasians) (19, 20). These polymorphic differences were associated with variable gene transcription and mRNA stability. Alternatively, linkage disequilibrium with a variant in a nearby locus on chromosome 12cen-q12 could be responsible for the associations described.

In conclusion, our study suggests that polymorphisms in the VDR gene are associated with the susceptibility to obesity in subjects with early-onset T2DM. The vitamin D endocrine system is involved in adipocyte differentiation and metabolism, but also in the regulation of insulin secretion and in the maintenance of glucose tolerance. Studies in larger populations with more detailed phenotypes for insulin secretion, insulin sensitivity and anthropometric data are required to clarify the interactions of VDR variants with these traits.

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