CLINICAL STUDY

Vitamin D and estrogen receptor gene polymorphisms in type 2 diabetes mellitus and in android type obesity

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Abstract

Objective: We studied the significance of BsmI restriction enzyme polymorphism of the vitamin D receptor (VDR) gene and the XbaI and PvuII polymorphisms of the estrogen receptor (ER) gene in patients with type 2 diabetes ($n = 49$), android type obesity with normal carbohydrate metabolism ($n = 29$) and healthy controls ($n = 138$).

Methods: The distribution of genotypes in the study groups, as well as their relationship to fasting and 1 h postprandial serum C-peptide levels were analyzed.

Results: Postprandial serum C-peptide levels of BB genotypes were significantly higher in the diabetes and obese groups (6.18 ± 5.09 ng/ml) compared with other genotypes (2.71 ± 2.45 vs. 1.72 ± 1.97 ng/ml, respectively, $P = 0.05$). Among patients with type 2 diabetes and obese subjects, the XX allelic variant of the ER gene was more frequent ($P = 0.00015$). Postprandial C-peptide levels of subjects exhibiting XX genotype were significantly lower compared with those with Xx genotype (1.67 ± 2.16 vs. 3.8 ± 3.72 ng/ml, $P = 0.021$). The BBXx allelic combination of the VDR/ER receptor genes was less frequent in diabetic patients than in healthy subjects or in obese patients. The BBXx genotype was associated with significantly elevated postprandial C-peptide levels in all subjects compared with other combinations (9.65 ± 3.14 vs. 1.35 ± 2.82 ng/ml, $P = 0.003$). No difference was found in the distribution of the PvuII polymorphism of the ER gene or in the association with the C-peptide levels among study groups.

Conclusion: Polymorphisms of the VDR/ER receptor genes might play a role in the pathogenesis of type 2 diabetes by influencing the secretory capacity of β -cells.

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Introduction

In the pathogenesis of type 2 diabetes, both insulin resistance and altered insulin secretion may be present. The prevalence of type 2 diabetes is increased in obesity, especially of the abdominal type. There is an ongoing effort to identify new factors, which may influence the secretory capacity of β -cells.

Vitamin D and its receptor complex – as a transcription factor – may play a regulatory role in β -cell insulin secretion (1). Vitamin D deficiency enhances the prevalence of type 2 diabetes, and the replacement of vitamin D may increase the secretion of insulin (1, 2). The vitamin D receptor (VDR) is a member of the steroid/thyroid hormone receptor family. The VDR is expressed in pancreatic β -cells (3), and the BsmI restriction enzyme polymorphism of the gene influences susceptibility to type 1 diabetes mellitus (4,

5). Another polymorphic site of the VDR gene (ApaI) influences the insulin secretory capacity of the β -cells in healthy Asians (6).

Estrogen also may influence insulin secretion in type 2 diabetes. The prevalence of type 2 diabetes is significantly lower among patients on estrogen replacement therapy (7). In ovariectomized mice, estrogen replacement has been shown to prevent the onset of diabetes (8). The estrogen receptor (ER) is also a member of the nuclear steroid/thyroid receptor family. The XbaI and PvuII restriction enzyme polymorphisms of the ER gene have been suggested to be involved in the pathogenesis of different diseases (9–12). Estrogen may increase the expression of the VDR gene (13). Certain allelic combinations of ER and VDR genes have been shown to influence bone mineral density more markedly than ER or VDR genotypes alone (14).

Table 1 Clinical data (means $\pm$ S.E.M.) from patients with type 2 diabetes (Group A), android type obesity with normal carbohydrate metabolism (Group B) and healthy controls (Group C).

	Group A	Group B	Group C
Number of patients	49	29	138
Female/male	27/22	23/6	66/72
Mean age (age range) years	57 (29–77)	41 (18–67)	63 (23–83)
BMI (kg/m ²)	31.3 $\pm$ 7.5*	40.1 $\pm$ 7.2*†	24.8 $\pm$ 2.4
Basal C-peptide (ng/ml)	3.0 $\pm$ 2.2*	2.93 $\pm$ 1.5*	1.46 $\pm$ 0.25
pp C-peptide (ng/ml)	5.7 $\pm$ 4.7§	7.3 $\pm$ 2.3*	2.73 $\pm$ 0.6
HbA1c (%)	7.0 $\pm$ 1.2‡	5.5 $\pm$ 0.4	5.21 $\pm$ 0.5
Waist-to-hip ratio	0.89 $\pm$ 0.06*	0.98 $\pm$ 0.05*	0.78 $\pm$ 0.03

*vs Group C: $P = 0.00001$; †vs Group A: $P < 0.00005$; §vs Group C: $P = 0.00006$; ‡vs Group B and C: $P = 0.00005$.

In our present work, we studied the BsmI polymorphism of the VDR gene, as well as the XbaI and PvuII polymorphisms of the ER gene in patients with type 2 diabetes, android type obesity without disturbances of the carbohydrate metabolism, and in healthy controls. We also investigated the relationship between these genetic polymorphisms and serum fasting and 1 h postprandial (pp) C-peptide levels.

Subjects and methods

Forty-nine Caucasian patients with type 2 diabetes in acceptable (HbA1c < 7%) metabolic state (Group A), 29 patients with android type obesity (BMI > 30 kg/m², waist to hip ratio (WHR) > 0.9 in men, > 0.8 in women) with normal carbohydrate metabolism (Group B), and 138 healthy subjects (Group C, both groups had normal glucose tolerance on 75 g oral glucose tolerance testing, defined by the World Health Organization criteria 1985) were studied after giving their informed consent. Patients were selected from the Endocrine Clinic. In Group A, 26 patients were on insulin (2–4 times daily) and 23 subjects were controlled with diet and oral antidiabetics. The patients had been diabetic for at least 2 years. Clinical data from the patients and controls are summarized in Table 1. The study was approved by the Institutional Ethical Committee.

Analysis of the restriction site polymorphisms of the VDR and ER genes

Genomic DNA was isolated from peripheral blood. DNA was extracted using phenol–chloroform and precipitated with ethanol. The BsmI polymorphic site of the VDR gene and the XbaI and PvuII polymorphic regions of the ER gene were amplified using the polymerase chain-reaction (PCR) technique.

For the VDR gene, the following primers were used: primer A: 5' AAC CAG CGG GAA GAG GTC AAG GG 3' 23-mer; primer B: 5' CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA 3' 30-mer (10 μ mol/l final

concentration). The PCR reaction was carried out in 50 μ l of the final volume using the following materials: 5 μ l 10 $\times$ (Mg-free) PCR reaction buffer, 1 μ l dNTP (10 mmol/l), 5 μ l MgCl₂ (25 mmol/l), 10 μ l purified DNA, 1 μ l each of primers A and B (10 μ mol/l), 0.4 μ l Taq (Promega, Madison, USA) and 26.6 μ l 2D PCR water. The following reaction protocol was followed: 95 °C for 3 min, 35 $\times$ (72 °C for 90 s, 95 °C for 45 s) and 72 °C for 10 min. The amplified PCR product was digested using BsmI restriction enzyme (Hybaid-AGS, Teddington, Middlesex, UK, 10 U/ μ l) for 90 min at 65 °C. The BsmI restriction site is missing in the B allele and is present in the b allele.

For the ER gene, the following primers were used: primer A: 5' CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC 3' 33-mer; primer B: 5' TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA 3' 33-mer (10 μ mol/l final concentration). The PCR reaction conditions are the same as for VDR. The PCR product was subsequently digested using XbaI and PvuII restriction enzymes (Promega, Madison, USA, 10 U/ μ l) at 37 °C overnight. The XbaI/PvuII restriction sites are lacking in the X/P alleles whereas they are present in the x/p alleles.

For the PCR reactions, a Hybaid Touchdown thermocycler (Teddington, Middlesex, UK) was used. Electrophoretic separation was carried out in a 2% agarose gel containing 10 μ g/ml ethidium bromide.

Serum C-peptide measurement

Insulin secretion was evaluated by the determination of serum fasting and pp (1 h after the consumption of a 30 g carbohydrate-containing standard test-meal, corresponding to the carbohydrate content and composition of an average breakfast recommended for diabetic patients as previously described (15)) C-peptide values. C-peptide levels were measured post test feeding whilst on usual insulin treatment in those who required insulin. Blood samples were stored at –20 °C until determination. C-peptide was measured by a commercial RIA kit (BIODATA, Rome, Italy; normal fasting serum value: 0.6–2.2 ng/ml; intra-assay coefficient of variation (CV): 3.5%, inter-assay CV: 8.0%, sensitivity: 0.1 ng/ml).

Statistical analysis

The comparison of genotype distributions was carried out using the χ^2 test. Fischer's or randomization tests were applied where appropriate. Because of the skewed distribution of the C-peptide baseline values, the data were log-transformed and the mean values were compared using Student's *t*-test. The relation of genotypes to C-peptide changes (postprandial-fasting serum C-peptide levels) was evaluated using the Mann–Whitney test. Bonferroni corrections were performed where appropriate. Data have been adjusted for BMI.

Table 2 VDR gene BsmI and ER gene XbaI genotypes in patients with type 2 diabetes (Group A), in patients with android type obesity (Group B) and in healthy controls (Group C).

Distribution of genotypes	BB (%)	Bb (%)	bb (%)	XX (%)	Xx (%)	xx (%)
Group A (<i>n</i> = 49)	14	46	40	46†	51†	3†
Group B (<i>n</i> = 29)	27	46	27	31¶	69¶	0¶
Group C (<i>n</i> = 138)	19	48	33	14	64	22

†vs Group C: *P* = 0.00003, corrected *P* = 0.00015; ¶vs Group C: *P* = 0.0052, corrected *P* = 0.026.

Table 3 VDR gene BsmI and ER gene XbaI genotypes and mean ± s.d. basal/postprandial C-peptide levels in patients with type 2 diabetes and with android type obesity.

Genotypes	BB	Bb	bb	XX	Xx
Basal C-peptide (geometrical mean) (ng/ml)	0.97 ± 0.49 (2.64)	0.76 ± 0.72 (2.14)	0.98 ± 0.8 (2.66)	0.48 ± 0.87 (1.61)	1.05 ± 0.57 (2.85)†
Postprandial C-peptide (median) (ng/ml)	6.18 ± 5.09 (6.45)‡	1.72 ± 1.97 (1.2)	2.71 ± 2.45 (2.3)	1.67 ± 2.16 (1.0)	3.8 ± 3.72 (2.3)§

Basal C-peptide values were log-transformed. †vs XX: *P* = 0.03, corrected *P* = 0.09 (only in patients with type 2 diabetes); ‡vs Bb: *P* = 0.018, corrected *P* = 0.05; §vs XX: *P* = 0.007, corrected *P* = 0.02. (xx does not appear since its frequency was low, see Table 2).

Table 4 The distribution of the most frequent allele combinations of VDR and ER genes in patients with type 2 diabetes (Group A) and in patients with android type obesity (Group B) and in healthy controls (Group C).

Genotype	Group A (<i>n</i> = 49) (%)	Group B (<i>n</i> = 29) (%)	Group C (<i>n</i> = 138) (%)
BbXx	29	30	32
BbXX	17	22	8
BBXx	4	22	10

Table 5 Mean ± s.d. basal/postprandial C-peptide levels corresponding to the most frequent allele combinations of VDR and ER genes in patients with type 2 diabetes and android type obesity.

Genotypes	BbXX (<i>n</i> = 14, 18%)	BbXx (<i>n</i> = 23, 29%)	BBXx (<i>n</i> = 8, 10%)
Basal C-peptide (geometrical mean) (ng/ml)	0.5 ± 0.88 (1.65)	0.96 ± 0.51 (2.61)	0.92 ± 0.62 (2.51)
Postprandial C-peptide (median) (ng/ml)	1.35 ± 2.82 (0.35)†	2.02 ± 1.71 (1.7)‡	9.65 ± 3.14 (9.75)

Basal C-peptide values were log-transformed. †vs BBXx: *P* = 0.001, corrected *P* = 0.003; ‡vs BBXx: *P* = 0.038, corrected *P* = 0.1.

Results

The xx genotype of the XbaI polymorphism of the ER gene hardly occurred in Groups A and B in contrast to the controls (Table 2). However, the XX genotype was significantly more frequent in Groups A and B compared with Group C (Table 2). The prevalence of the X allele was more frequent in both Group A and B than in Group C (Group A: 71%; Group B: 64%; Group C: 47%; *P* < 0.0002/0.024 vs control). The frequency of the x allele in these groups was 29, 36 and 53% respectively (*P* < 0.0002/0.024 vs control). No difference was found in the distribution of the PvuII polymorphism of the ER gene among study groups.

In Groups A and B, pp C-peptide levels were significantly higher in patients with the BB genotype

compared with those with the Bb genotypes (Table 3), however, we cannot find any differences in the BsmI genotype distribution between Groups A, B and C. The frequency of the different BsmI genotypes of the VDR gene in controls was similar to that reported in healthy Caucasian populations (16).

In both Group A and B, pp C-peptide levels were significantly lower in subjects with the XX genotype compared with those with the Xx genotype (Table 3). No association was found between the PvuII polymorphism and C-peptide levels (data not shown). C peptide levels and genotypes in Group C showed no correlation.

The BBXx allelic combination was less frequent in Group A than in Group C (Table 4). BBXx genotypes were associated with significantly higher pp C-peptide

concentrations in both patient groups while BbXX combinations were accompanied by significantly lower pp C-peptide levels (Table 5). The BbXX allelic combination was found more frequently in Group A than in Group C.

Discussion

We have demonstrated that the BB genotype of the VDR gene is associated with higher pp C-peptide levels. This observation is in accordance with the previously described finding that in an Indian ethnic group the susceptibility to type 1 diabetes is an association with this genotype (4). In NOD mice, a genetic model for type 1 diabetes, vitamin D and its analogues delayed the onset of diabetes (17). In humans, vitamin D supplementation may increase the insulin secretion in elderly patients (1, 2). Our findings suggest that the VDR gene BsmI polymorphism may influence the secretory capacity of β -cells.

We have also found an ER genotype, XX, which is associated with lower basal and pp C-peptide secretion in contrast to those with Xx. The genotype xx was extremely rare among diabetic and android type obese patients. At present, there is no evidence about the possible influence of the xx genotype on the long-term survival of these subjects. The XX genotype with the lower insulin secretory capacity was more prevalent in diabetic patients compared with healthy controls. The importance of the estrogen-ER system in carbohydrate metabolism might be further corroborated by evidence indicating that the prevalence of type 2 diabetes is significantly lower among women on estrogen replacement therapy (6).

The ER genotype distribution and C-peptide secretion of obese subjects were similar to those of type 2 diabetic patients. Type 2 diabetes is more prevalent among obese people, so our genetic findings are consistent with this observation. VDR and ER genotypes could be predisposing factors that might contribute to the pathogenetic processes leading to type 2 diabetes in android type obesity.

Our data suggests an allelic combination of the VDR and ER genes (BBXx genotype) in type 2 diabetic patients which is associated with higher pp C-peptide levels compared with other combinations. The frequency of this genotype was less prevalent among diabetic patients. A potential interaction between VDR and ER genes in bone metabolism has already been suggested by other investigators (14). A possible site for the interaction between estrogen and vitamin D system could be the aromatase gene possessing a vitamin D response element in its promoter region (18). The aromatase enzyme directs the peripheral conversions of androgens to estrogens. In type 2 diabetes, aromatase activity is decreased (19). Other genes, such as p21-ras

and jun that may be involved in the pathogenesis of diabetes, also have response elements for ER (20, 21).

Both the BsmI polymorphism of the VDR gene and XbaI polymorphisms of the ER gene are found in intronic regions. Intronic changes in gene sequence may have an impact on the expression of other genes by influencing the transcription and/or stability of mRNA of those genes (22, 23). The relationship between the intronic polymorphisms of ER/VDR genes and insulin secretion remains to be established.

Our observations in diabetic and obese people raise the possibility that certain VDR and ER genotypes associated with altered insulin secretion might contribute to the development of type 2 diabetes.

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