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RESEARCH ARTICLE

Vitamin D receptor and estrogen receptor gene polymorphisms in men with type 2 diabetes: Effects on Bone Metabolism

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Abstract

Purpose There is an increased fracture risk in type 2 diabetes mellitus [DM] patients independent of bone mineral density [BMD], both in men and women. Estrogen receptor [ER]-alpha and vitamin D receptor [VDR] gene polymorphisms may predispose patients to increased osteoporosis and fracture risk. This study aims to analyze the relationship of the ER-alpha gene and VDR gene polymorphisms with indicators of bone turnover and BMD in male type 2 diabetic patients.

Methods Type 2 diabetic men diagnosed with diabetes for at least one year and healthy controls were included in this cross-sectional study. BMD was measured by dual X ray absorptiometry. Gene polymorphisms were evaluated with polymerase chain reaction-restriction length polymorphism. Serum iPTH, calcium, beta-CrossLaps (cTx), osteocalcin, and free testosterone levels were also evaluated.

Results Participants were 141 type 2 diabetic men [55 ± 8 years] and 100 healthy controls [53 ± 7 years]. BMD measurements were not statistically different between the groups. While iPTH [$p < 0.05$] and serum calcium levels [$p = 0.03$] were higher in men with type 2 DM; beta-CrossLaps [$p = 0.0001$], osteocalcin [$p = 0.005$], and free testosterone [$p = 0.04$] were lower than controls. The differences in terms of the frequencies of VDR Apa, Taq, Bsm, Fok and ER-alpha polymorphisms were not statistically significant between the groups. No relationship was observed between polymorphisms and BMD in both groups.

Conclusions VDR and ER-alpha gene polymorphisms seem to have no effect on BMD and bone turnover in men with DM.

Keywords Bone density · Diabetes Mellitus, type 2 · Male · Polymorphism, genetic · Receptors, calcitriol · Receptors, Estrogen

Introduction

It is known that fracture risk is greatly increased in diabetic patients [1]. A relationship between the decrease in bone mineral density (BMD) and fracture risk in type 1 diabetics has been noted previously [2]. In type 2 diabetics, in spite of the increased fracture risk, a decrease in BMD has not been observed. Although the BMD in postmenopausal patients with type 2 diabetes mellitus (DM) is not different from controls, it has been shown that the risk of humerus, hip and vertebral fractures increases [3].

The pathogenesis of osteoporosis in Type 1 diabetics is not clear. Several studies have debated the existence of a relationship between blood glucose control and BMD [2, 4]. Changes in the bone turnover markers refer to osteoblast dysfunction [5]. The data associated with bone metabolism in type 2 diabetics are complex. It is shown that blood glucose dysregulation in older type 2 diabetics depends on levels of serum testosterone [6]. The difference between BMD in type 1 and type 2 diabetics is still not clarified even

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though it has been explained through the fact that the body mass index is high in the type 2 diabetics [7].

Genetic constitution, which is responsible for 60–75% of bone mass and metabolism, is also a focus of interest in diabetic patients [8]. The data relating to this subject are currently insufficient, and differ between communities.

In Chinese type 2 diabetic men, BMD is high in people with the "B" genotype of vitamin D receptor (VDR) Bsm1 polymorphism [9]. There are data on the fact that the collagen gene polymorphism is associated with femoral bone mass in premenopausal women [10]. The relationship of the VDR gene Taq polymorphism with lumbar bone mass is reported in a study carried out in our country [11].

It has been shown that ER gene polymorphisms are associated with BMD in postmenopausal women and osteoporotic men [12, 13].

The interest into research on bone metabolism in diabetic patients is new in the literature, and the existing data are insufficient. The measurement of BMD in diabetic patients seems to be insufficient in terms of evaluation of the risk of fracture. In this group of patients, more sensitive methods, such as quantitative tomography, are discussed in establishing the diagnosis of osteoporosis and in the treatment follow-up [14]. It is known that BMD, volumetric BMD, and bone turnover markers have a genetic transition in the general population [15]. There are insufficient data on the effects of genetic constitution to bone metabolism in the diabetic patient group.

The aim of this study is to analyze the relationship of the VDR gene and ER-alpha gene polymorphisms with bone turnover markers and BMD in type 2 diabetic patients.

SUBJECTS Subjects were consecutively recruited among patients who had been followed at the Outpatient Clinic of Marmara University Medical School Hospital, Endocrinology Section. A total of 141 male patients, between the ages of 40 and 60 years with a diagnosis of type 2 DM for at least 1 year, were included. Patients with another metabolic bone disease [hyperparathyroidism], chronic kidney disease, chronic liver disease, and chronic inflammatory diseases were excluded from the study. Age-matched healthy males were included as the control group.

The study protocol was approved by the Marmara University Faculty of Medicine Ethics Committee (Approval No: MAR-YÇ-2009-0106). Subjects were informed and included into the study after informed consent was obtained.

The study was planned and conducted as a cross-sectional study.

Materials and methods

Measurements

Bone Mineral Density

Dual-energy X-ray absorptiometry (DEXA) was used to determine bone mineral density (BMD) at the lumbar spine (L1–L4) in the anteroposterior (AP) projection and the three sites of the right hip (femoral neck,) using Lunar DEXA device, DPX-L.

Osteopenia was defined according to the classical WHO criteria, as a T-score for the lumbar AP or femoral neck between − 1 SD and − 2.5 SD. Osteoporosis was defined as the BMD T-score on DEXA at the lumbar spine or femoral neck that is less than or equal to -2.5 SD for postmenopausal women and men aged over 50 years. For premenopausal women and men aged below 50 years, it was defined as a BMD Z-score of less than − 2 SD or equal at the lumbar spine or femoral neck.

Vitamin D receptor gene (VDR) and estrogen receptor gene (ER) polymorphisms

DNA isolation DNA was isolated from peripheral blood samples using the Roche high pure PCR template preparation kit and stored at -20^{O}C.

VDR Bsm 1 PCR products were moved on 1% agarose gel and displayed with ethidium bromide. After 870-bp bands were determined, RFLP was applied.

RFLP: 20 ml PCR product was turned off at 37^{O}C in 50 ml reaction volume using 10 U BsmI enzyme over a 1 h period.

The BB genotype was determined/detected as 870 bp, the Bb genotype was determined as 870, 700 and 170 bp, and the bb genotype was determined as 700 and 170 bp.

VDR Fok 1 PCR products were moved on 1.5% of agarose gel and were displayed through ethidium bromide. After 265-bp, bands were determined, RFLP was applied.

RFLP: 30 ml PCR product was turned off at 55^{O}C in 50 ml reaction volume using 10 U FokI enzyme for a 1 h period. The products were moved on 2% agarose gel and were displayed with ethidium bromide.

The FF genotype was determined/detected as 265 bp, the Ff genotype was determined as 265, 196 and 69 bp, and the ff genotype was determined as 196 and 69 bp.

VDR apa 1 and taq 1 PCR products were moved on 1.5% of agarose gel and were displayed with ethidium bromide. After 740-bp bands were determined, RFLP was applied.

Apa I RFLP Conditions: 20 ml PCR product was turned off at 37^{O}C in 50 ml reaction volume through 10 U ApaI enzyme for a 1 h period. Taq I RFLP Conditions: 20 ml PCR product was turned off at 65^{O}C in 50 ml reaction volume through 10 U TaqI enzyme for 1 h period. The products were moved on 2% of agarose gel and were displayed with ethidium bromide.

The AA genotype was determined/detected as 740 bp, the Aa genotype was determined as 740, 530 and 210 bp, and the aa genotype was determined as 530 and 210 bp.

The TT genotype was determined/detected as 495 and 245 bp, the Tt genotype was determined/detected as 495, 290, 245 and 205 bp, the tt genotype was determined/detected as 290, 245 and 205 bp.

Estrogen receptor alpha gene polymorphisms

ER-alpha gene, was amplified using the F 5'-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC and 5'-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA primaries.

Xba RFLP: The PCR product was incubated through/with XbaI enzyme at 37^{O}C for 8 h. It was moved on 2% of agarose gel and was displayed under UV after it was painted with ethidium bromide. The presence of 1.3 kb [uncut] band was regarded/evaluated as "X" and the presence of the 910 and 390 bp band [cut] was regarded/evaluated as "x".

Pvu RFLP: The ER PCR product was incubated through/with the Pvu II enzyme at 37^{O}C for 12 h. The acquired product was displayed under UV after it was painted with ethidium bromide on 2%-agarose gel.

Table 1 Demographic characteristics and bone turnover markers of the study groups

	Type 2 DM	Control	p
Age (year)	55.6 ± 8.1	53.5 ± 7.2	> 0.05
Duration of Diabetes (year)	9.8 ± 6.4	-	
BMI (kg/m^2)	28.2 ± 4.3	28.0 ± 3.2	> 0.05
Fasting Blood Glucose (mg/dl)	157 ± 68	90 ± 9	0.001
Hb A1c (%)	7.23 ± 1.8	5.0 ± 0.5	0.001
Calcium (mg/dl)	9.8 ± 0.5	9.5 ± 0.5	0.03
Phosphorus (mg/dl)	3.5 ± 0.5	3.3 ± 0.5	0.007
iPTH (pg/ml)	50.8 ± 21	48.3 ± 20	0.30
Serum CTx (ug/L)	0.18 ± 0.1	0.43 ± 0.2	0.0001
Osteocalcin (ng/ml)	6.3 ± 3.1	7.9 ± 4.2	0.005
Estrogen (pg/ml)	28.3 ± 12	31.3 ± 10	0.05
f Testosterone (pg/ml)	12.6 ± 4.4	13.9 ± 4.3	0.04

DM = diabetes mellitus; BMI = body mass index; PTH = parathyroid hormone; CTx = carboxy-terminal collagen crosslinks; f = free

The presence of the 850 bp band [uncut] was evaluated as "P" and the presence of 450 bp band [cut] was evaluated as "p".

Biochemistry:

Serum calcium and phosphate concentrations were measured spectrophotometrically (Modular P, Roche Diagnostics, Mannheim, Germany). The measured calcium limits were 0.2–20 mg/dL and the measured limits for phosphorus were 0.3–20 mg/dL. In calcium measurements, within-run and between-run predictions were observed as 0.4 to 0.9% for the average serum concentrations 8.48–13.26 mg/dL. For phosphorus measurements, within-run and between-run predictions between average 4.3-6.0 mg/dL values were found as 0.8 to 0.9%. Serum osteocalcin and intact-parathyroid hormone (iPTH) concentrations were measured through solid-phase, two-site chemiluminescent immunometric assays (Immulite 2000, Siemens, LA, USA). The reported measuring range of osteocalcin is 2-100 ng/mL and the lowest limit is 0.55 ng/mL. The calibration upper limit for intact-PTH is reported as 2500 pg/mL. For osteocalcin, the within-run and between-run precision value was below 10% for the average serum concentrations 41.2 ng/mL. Within-run and between-run precisions values for intact PTH were found to be below 5% in normal serum concentrations. Serum beta-crosslaps (CTx) concentrations were measured through the electrochemiluminescence assay (Modular E, Roche Diagnostics, Mannheim, Germany). The lower determination limit was 2 mg/L, within-run and between-run precisions values were below 2%. Serum blood glucose was studied through spectrophotometric analysis; A1c was studied through the HPLC method.

Statistical evaluation:

SPSS program was used for the statistical analyses. The student-t or Mann-Whitney-U tests were used for in between-group comparisons. For allele frequency, comparisons between the groups were performed using the $\chi 2$ or Fisher exact tests where appropriate.

The Hardy – Weinberg equilibrium was calculated by the $\chi 2$ test (with one degree of freedom). Allele frequencies were in Hardy – Weinberg equilibrium. The level of statistical significance was set at $p < 0.05$. All results are expressed as mean ± standard deviation.

Results

The demographic data and biochemical parameters of type 2 diabetic male and control groups are summarized in

Table 1. Serum calcium and iPTH levels were higher in type 2 diabetic males compared to healthy controls. Serum phosphorus levels were higher in the diabetic males compared to healthy controls. Bone turnover markers, serum Ctx and osteocalcin were lower in the diabetic group. Serum testosterone levels were statistically lower in diabetic men compared to the healthy controls.

Femoral head and lumbar BMD, T- and Z-scores of the study groups are given in Table 2. Bone density values, T-score, and Z-scores were similar in both groups' femoral and lumbar areas.

The frequency of VDR and ER-alpha gene polymorphisms were identical in patients with DM and healthy controls. The most frequently observed polymorphisms were heterozygotes in both groups " Pp", "Xx", "Bb", "Ff","Aa"; "TT" in diabetics and "Tt" in controls were observed most frequently for Taq (Table 3).

Table 2 BMD parameters of study groups

	Type 2 DM	Control	p
L1-4 BMD (gr /cm2)	1.090 ± 0.2	1.080 ± 0.2	0.90
L1-4 T-score	-0.554 ± 1.5	-0.454 ± 1.5	0.60
Femoral neck BMD (gr/cm2)	0.93 ± 0.15	0.94 ± 0.13	0.70
Femoral neck T-score	-0.565 ± 1.1	-0.631 ± 0.9	0.50
Total femur (gr/cm2)	1.08 ± 0.1	1.00 ± 0.1	0.60
Total femur T-score	-0.208 ± 1.1	-0.406 ± 0.8	0.20

DM = diabetes mellitus; L1-4 = lomber 1–4; BMD = bone mineral density

Table 3 Frequencies of ER and VDR gene polymorphism in study groups

Polymorphisms		Type 2 DM (%)	Control (%)	p
Estrogen receptor alpha				
	PP	27	15	
PVU	Pp	45	54	p > 0.05
	pp	26	31	
	XX	20	20	
Xba	Xx	58	56	p > 0.05
	xx	22	24	
Vitamin D receptor gene				
	BB	18	16	
Bsm	Bb	46	56	P > 0.05
	bb	35	29	
	FF	52	52	
Fok	Ff	38	36	p > 0.05
	ff	10	33	
	AA	36	26	
Apa	Aa	42	54	p > 0.05
	aa	21	20	
	TT	45	36	
Taq	Tt	40	54	p > 0.05
	tt	15	11	

DM = diabetes mellitus

When the polymorphism frequencies were compared, the differences between the groups were not statisticallly significant. When the suitability of polymorphisms in terms of general community expected distribution with the Hardy-Weinberg equation is considered, it was observed that each polymorphism complied with the general expected distribution in each group. In other words, each polymorphism in each group was found to be appropriate for the Hardy-Weinberg equation.

The most frequently observed VDR haplotypes are given in Table 4. The most frequent haplotype in patients with DM and controls was "BbAaTt". The "bbaatt" haplotype was not observed in both groups. When VDR haplotypes were evaluated in terms of BMD and bone turnover parameters, no difference was observed in healthy controls and patients with DM.

There was no association between BMD and ER-alpha polymorphisms in diabetic men. In the healthy control group lumbar L1-4 BMD was higher in the ER-alpha "xx" genotype compared to the "Xx and XX" genotype (p = 0.01). A similar tendency was also observed for BMD measurements of the femur region (p = 0.07).

Discussion

In this study, although the bone turnover indicators were suppressed in type 2 diabetic male patients, BMD measurements were not statistically significantly different from healthy controls. This data is in accordance with the literature.

BMD measurement is the most effective method in predicting bone fractures in non-diabetics. However, studies

Table 4 Vitamin D receptor Bsm, Apa, Taq haplotype and ER-alpha PVU and Xba haplotype frequencies

	Type 2 DM	Control
BBAAtt	12%	1.4%
BbAATt	10.6%	8.1%
BbAaTT	5.6%	6.5%
BbAaTt	19.8%	27.8%
bbAATT	4.7%	1.6%
bbAaTT	15.4%	11.4%
bbaaTT	17%	9.8%
XXPP	24%	5%
XXPp	4%	5%
XXpp	-	1%
XxPP	13%	3%
XxPp	14%	6%
xxPP	-	2%
xxPp	7%	4%
xxpp	24%	13%

DM = diabetes mellitus

in diabetic women and men have shown that the BMD is similar to the control group despite the fact that fracture risk increases [16, 17]. In literature, bone metabolism has been studied on female diabetic patients. The studies on men are limited. Although, BMD has been reported to be normal, fracture risk increases in diabetic men, as well as diabetic women. Several meta-analyses report that the BMD values of diabetic patients are higher than values in non-diabetic controls; however, the risk of femur fracture is increased by 1.4 [7] or by 1.7 [18]. Our results reveal that BMD is found to be similar in the vertebral and femoral area when compared to the control group. Similar results are also available in the literature [19]. In a previous study, which we carried out on patients with type 1 DM, we reported that the difference of BMD measurements in the lumbar and femoral head of the patients and the controls was not statistically significant [20]. The reason for no change in BMD is not clear.

When compared to the control group, we determined that serum bone turnover markers are lower in patients with type 2 DM. A similar result is reported in another study, where in 133 patients with type 2 DM, bone formation markers were not found to be different, while bone resorption markers [CTx] were low [21]. In our study, both bone formation and bone resorption markers were found to be low, thus giving rise to the thought that bone metabolism is supressed in diabetic persons.

However, a new study, performed using a sensitive method evaluating the microarchitecture of bone, such as quantitative tomography, reports different results. Using the pQCT method in 1171 Type 2 diabetic men, bone density was lower in the cortical areas, compared to non-diabetics and was similar in the trabecular areas with regard to non-diabetics [22].

The VDR gene and ER-alpha gene are two of the candidate genes for osteoporosis. The relationship between VDR gene polymorphisms and osteoporosis has been studied in various communities, and contradictory results have been obtained [23]. In a meta-analysis, consisting of studies perfomed mostly in potmenapausal women, more than half of the studies demonstrated an association between Fok1, Bsm1 and osteoporosis [24]. In a study evaluating VDR Apa and Taq polymorphisms in a Turkish type 2 diabetic population, it was observed that the Taq "tt" polymorphism was higher in diabetics than in the control group; despite the fact that the allele distribution was similar [25]. TaqI and ApaI VDR gene polymorphisms were not associated with type 2 DM. The TT genotype of TaqI VDR gene polymorphism was correlated with low levels of osteocalcin in overweight and obese subjects [26]. There was no correlation between VDR polymorphisms and BMD measurements in Turkish type 1 diabetic patients [27]. A nonsignificant influence of VDR gene polymorphism was evident in another study, where the BMD of the Bb genotype was higher than that of BB genotype and lower than that of bb genotype in Chinese diabetic patients [9]. In a Brazilian type 1 diabetic population, the distribution of VDR genotypes were not statistically significantly different from the controls. BB genotype was associated with a lower mean BMD at lumbar spine and femoral neck than in patients with Bb and bb genotypes [28]. In our study, the distribution of VDR Bsm, Fok, Apa, Taq polymorphisms were found to be similar in Type 2 diabetic men and in the control group.

The effects of ER-alpha gene polymorphisms on glucose metabolism in Type 2 diabetics have also been studied. In non-diabetic men, the presence of PP genotype was found to be associated with high levels of blood glucose. In multivariate analysis, ER-alpha Pvu polymorphisms were reported to be an independent risk factor in Type 2 diabetic men, while Xba polymorphisms were not found to be associated with the development of diabetes [29]. A study that investigated the peak bone mass in young men revealed that there were no significant differences in BMD among PvuII and XbaI genotypes of ER-alpha. However, an association between leptin receptor gene polymorphism and BMD was noted in the subjects carrying the PP homozygotes of PvuII or the X alleles of XbaI, but this was not significant in those without these genotypes [30]. Similarly the distribution of BstUI, Pvu II, and Xba I RFLPs was similar in the osteoporotic patients and the controls and the difference in terms of bone mass or bone turnover were not statistically significant between the patients having different genotypes [31]. The combination of Pvu II and Xba I polymorphisms of ER-alpha gene was significantly associated with both low lumbar L2-4 and trochanteric BMD in Chinese postmenopausal women [32].

The increase in serum calcium levels in the diabetic patients compared to the controls is statistically significant, but calcium levels are within normal limits. The increase in PTH is not statistically significant, and again the levels in the diabetics are within normal limits. Thus the changes in calcium and PTH seem to be subtle and do not have any clinical significance. Normally decreased calcium and increased PTH could be expected due to mechanisms like decreased intestinal absorbtion of calcium and increased calcium loss from the urine [33].

Our study is the first to study bone and VDR, ER-alpha gene polymorphisms in type 2 diabetic men. In type 2 diabetic men, PPXX and ppxx polymorphisms were found to be higher in diabetics than in the control group. Considering the effects of ER alpha polymorphisms on BMD, lumbar L1-4 BMD measurement was found to be higher in the "xx" genotype than in the "Xx and XX" genotype in the control group. A similar tendency was also observed for BMD measurements of the femur region.

The limitations of our study is that the size of our population was somehow limited for a genetic study. Moreover the nature of the study being a cross-sectional one precluded us from making direct associative conclusions.

In conclusion, the data we obtained reveal that the bone turnover is suppressed in type 2 diabetic men and this situation is not reflected in BMD measurements. In type 2 diabetic men, BMD measurement is not considered a sufficient method to determine bone fracture risk. The use of other methods, such as quantitative CT, indicating more developed microarchitecture must be studied as an option.

In type 2 diabetic men, the effect of VDR gene polymorphisms upon bone metabolism has not been observed. There is a minimal effect of ER alpha gene polymorphism on BMD in type 2 diabetic men. Studying the effects of osteoporosis candidate genes on bone metabolism in type 2 diabetic men with a wider patient group and more sensitive methods can shed more light on the subject.

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Declarations

Conflict of interest Authors have no conflict of interest.

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