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Relation of common allelic variation at vitamin D receptor locus to bone mineral density and postmenopausal bone loss: cross sectional and longitudinal population study

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Abstract

Objective—To determine whether common allelic variation at the vitamin D receptor locus is related to bone mineral density and postmenopausal bone loss.

Design—Cross sectional and longitudinal population study.

Setting—Outpatient clinic in research centre.

Subjects—599 healthy women aged 27 to 72 and 125 women with low bone mass aged 55-77 had bone mineral density measured once in the cross sectional study. 136 women aged 45-54 were followed up for 18 years in the longitudinal study.

Main outcome measures—Bone mineral density measured at the lumbar spine, hip, and forearm and rate of bone loss at different times over 18 years in relation to vitamin D receptor genotype as defined by the endonucleases ApaI, BsmI, and TaqI.

Results—Vitamin D receptor genotype was not related to bone mineral density at any site. The maximum difference between homozygotes was 1.3% (P = 0.33, n = 723). Women with low bone

mineral density had almost the same genotype frequencies as the women with normal bone mineral densities. Vitamin D receptor genotype was not related to early postmenopausal bone loss from age 51 to 53 (mean (SD) total loss at the lower forearm -3.6% (3.6%)), late postmenopausal bone loss from age 63 to 69 (at the hip -6.2% (8.7%)), or to long term postmenopausal loss from age 51 to 69 (at the lower forearm -24.5% (11.4%)).

Conclusion—Common allelic variation at the vitamin D receptor locus as defined by the endonucleases ApaI, BsmI, and TaqI is related neither to bone mineral density nor to the rate of bone loss in healthy postmenopausal Danish women.

Introduction

Osteoporosis is a systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue. This results in an increase in bone fragility and susceptibility to fractures,¹ predominantly of the femoral neck, vertebrae, and lower forearm.²

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The incidence of osteoporotic fractures is increasing, and hip fractures are expected to afflict more than 6 million people worldwide by 2050.³ Identifying people at risk is therefore of prime importance as prevention is possible—for example, with hormone replacement therapy.¹ Bone mineral density measured by bone densitometry is currently the most important predictor of osteoporotic fractures.¹

Twin studies and studies of daughters of women with osteoporosis indicate a strong genetic component in the development of osteoporosis.⁴⁻⁵ Recent twin studies in Australia and the United Kingdom suggest that common allelic variation of the vitamin D receptor gene is related to bone mineral density, accounting for up to 75% of the total genetic effect on bone density.⁶⁻⁷ Other studies in the United States have, however, failed to show this relation.⁸ There are also conflicting reports on the relation between postmenopausal bone loss and vitamin D receptor genotype.⁹⁻¹¹ We therefore examined a large group of healthy Danish women for the relation of bone mineral density and postmenopausal bone loss to receptor genotype as defined by the three endonucleases ApaI, BsmI, and TaqI.

Subjects and methods

LONGITUDINAL STUDY

In 1977, 315 women aged 45 to 54 living in the Copenhagen region were recruited from a questionnaire survey and a medical screening procedure for a two year follow up study on the prevention of postmenopausal bone loss. Women who were immobile, who had diseases known to affect bone metabolism, or who had at any time after the menopause received drugs known to affect bone metabolism (including hormone replacement therapy) were excluded from the study. Women who were aged 45-54 and had had a natural menopause six months to three years previously were included. Details of the selection process are given elsewhere.¹²

The 260 women who were alive and living in the area in 1995 were asked to participate in this study, and 149 agreed. Thirteen were excluded for the reasons described above, so 136 women were included in the longitudinal study. The mean (SD) number of years since the menopause in these 139 women at baseline was 2 (1). These women did not differ significantly from the 179 women who were not included in terms of weight, height, age, and years since the menopause at baseline. Total bone loss was calculated as a percentage for three periods. Early postmenopausal bone loss at the distal forearm over two years (1977-9) was calculated by simple linear regression of nine measurements of bone mineral content; late postmenopausal bone loss at the distal forearm, proximal femur, and lumbar spine was calculated over six years from one bone mineral density measurement in 1989 and one in 1995; long term postmenopausal bone loss over 18 years was calculated from the first measurement in 1977 and one

measurement in 1995. Participants receiving hormone replacement therapy in 1977-9 and 1989-95 were excluded from the calculations of the given time period. Participants who had taken hormone replacement therapy for more than three years were excluded from calculations for 1977-95.

CROSS SECTIONAL STUDY

From 1992 to 1994, 61 116 short questionnaires were posted to randomly selected women living in the Copenhagen region in order to form a database from which to draw participants for future studies. In all, 30 657 questionnaires were returned to the centre. From these completed questionnaires 1008 premenopausal, perimenopausal, and postmenopausal women were selected to take part in clinical and epidemiological studies after excluding those who were immobile, who had diseases affecting bone metabolism, who had received hormone replacement therapy within the previous six months or for more than three years, or who had taken any other drug known to affect bone metabolism. Most of the women included in the postmenopausal group of this study were aged between 49 and 61 or between 65 and 73 years. One baseline measurement of bone mineral density in the distal forearm, proximal femur, and lumbar spine was taken in all 1008 participants. These measurements were taken from 1992 to 1994. In 1995 these women were asked for a blood sample for determination of vitamin D receptor genotype. In all, we obtained 489 samples. To this number was added 110 participants from the longitudinal study who fulfilled the inclusion criteria for the cross sectional study; we used their bone mineral densities from 1995.

These 599 women were divided into three different groups according to the time since the menopause. Fifty were premenopausal, 153 were perimenopausal (time since the menopause six months to three years), and 396 were postmenopausal (time since the menopause being more than three years).

In 1993 we also included a further 180 women from the same database in a clinical trial. The inclusion criteria were (a) a bone mineral density in the forearm of less than -1.5 of the standard deviation of the mean value in premenopausal women (corresponding to a bone mineral density 9% lower than that of an age matched reference population) and (b) that more than 10 years had elapsed since the menopause. The exclusion criteria were the same as described above. In 1995 we asked the 180 women for a blood sample to determine vitamin D receptor genotype; 125 gave their consent and formed the group with low bone mineral density in our study. In 1993 bone mineral density of the lower forearm was measured in all 125 participants. In 1993 we also measured bone mineral density in the lumbar spine and proximal femur in 124 of them (one participant did not turn up for appointment).

Table 1—Frequency of vitamin D receptor genotypes according to menopausal state and bone mineral density in cross sectional study

Group	No	Mean (SD) age (years)	Mean (SD) years since menopause	Genotype frequencies (%)								
				BB	Bb	bb	AA	Aa	aa	TT	Tt	tt
Premenopausal	50	35(6)	NA	16	50	34	40	34	26	36	34	30
Perimenopausal	153	54(2)	2(1)	13	50	37	24	55	21	37	48	15
Postmenopausal	396	60(6)	11(6)	14	51	35	27	52	21	35	49	16
Above three groups combined	599	56(9)	NA	14	50	36	27	51	22	36	47	17
Low bone mineral density	125	65(5)	17(6)	13	53	34	30	50	20	34	52	14

NA = not applicable.

MEASUREMENTS

Vitamin D receptor genotype was determined by the polymerase chain reaction and analysis of restriction fragment length polymorphism. In brief, DNA was isolated from leucocytes from whole blood. A region of the receptor gene carrying the three reported polymorphic restriction sites BsmI, ApaI, and TaqI (situated in intron 8 and the 5' end of exon 9; 2100 base pairs)⁶ was amplified by the polymerase chain reaction. The specific primers for the vitamin D receptor were upstream primer positioned in exon 8 (5'-CAACCAAGACTACAAGTACCGCGTCAGTGA-3') and downstream primer positioned in exon 9 (5'-CGAGCACAAGGGGCGTTAGC-3'). DNA (50

ng) was amplified on a Perkin Elmer 9600 Thermocycler (Forster City, CA) for 33 cycles at 94°C for 25 s, 63°C for 30 s, and 72°C for 4 minutes, with an additional extension of 5 minutes at 72°C in the final step. The products of the reaction were digested with the restriction endonucleases BsmI, ApaI, and TaqI. Resulting restriction fragments were separated by gel electrophoresis in a 1% agarose gel and the genotype directly determined by looking at the DNA fragment pattern under ultraviolet light. The technique was validated against the standard Southern blot technique before implementation, and samples were tested in random order and blinded to clinical information.

In 1977-89 bone mineral content and bone mineral density of the forearm were measured by single photon absorptiometry using iodine-125 (Bone Mineral Analyzer 1100; Mølsgaard Medical, Hørsholm, Denmark)¹³ and in 1995 by single energy x ray absorptiometry (DTX100; Osteometer, Rødovre, Denmark).¹⁴ The correlation between the two techniques in measuring bone mineral content at the distal forearm is good ($R = 0.99$), with measurements lying along the line of equality.¹⁵ Furthermore, the same external standard and calibration system has been used by this centre since 1977, thus making the results obtained at the different times comparable.

The bone mineral densities of the lumbar spine and of the proximal femur were measured by dual energy x ray absorptiometry. In the longitudinal study the machine used was a QDR 1000 and in the cross sectional study a QDR 2000 (both from Hologic, Waltham, MA).¹⁶

STATISTICAL ANALYSIS

Simple and multiple linear regression analyses were carried out to determine the relation between bone mineral density, age, weight, height, years since the menopause, and vitamin D receptor genotype. Analysis of variance using the general linear model of the SAS Institute¹⁷ was used for comparison between the genotypes, with a significance level of 5%. Differences in genotype frequencies between the four groups in the cross sectional study were tested by likelihood ratio χ^2 statistics.

Bone mineral density was expressed as a percentage of expected values; it was calculated for each group as the ratio of the observed bone mineral density to the value expected within the study group as a function (determined by linear regression) of years since the menopause. For the premenopausal women the percentage was calculated as the ratio of the observed bone mineral density to the mean value in the premenopausal group.

Results

There were no significant differences in genotype frequencies between the study groups (table 1). In particular there was no difference between the group selected for low bone mineral density and the other three groups combined. Genotype was not associated with age, years since the menopause, height, or weight (data not shown). Bone mineral density was related to age, years since the menopause, and weight, but adjustment for these factors did not change the results. Consequently, we present the crude unadjusted data except when bone mineral density was given as a percentage of expected values.

Figure 1 shows bone mineral density of the lower forearm, hip, and lumbar spine in relation to the BsmI genotypes. Analysis of the relation of the ApaI and TaqI genotypes at the same sites yielded similar results (data not shown).

We found a small difference between the AA and aa genotypes in bone mineral densities of the forearm

Fig 1—Bone mineral density (g/cm²) in forearm, hip, and lumbar spine in relation to BsmI genotypes (▲ BB, ● Bb, ■ bb). Mean (SD) values are shown for premenopausal and perimenopausal groups, P values relating to difference in mean values for the three alleles. Data for postmenopausal group show linear regression lines of bone mineral density on age for the three alleles, P values relating to slopes of regression lines

Table 2—Mean (SD) bone mineral densities as percentages of expected values in relation to vitamin D receptor genotypes in combined cross sectional study

Genotype	No of women	Forearm	Hip	Lumbar spine
BB	101	101.1 (12.8)	98.9 (13.6)	99.2 (16.3)
Bb	368	99.5 (13.2)	100.3 (13.8)	100.3 (15.0)
bb	254	100.2 (12.8)	99.2 (11.9)	99.9 (13.1)
P value		0.53	0.23	0.81
AA	201	99.6 (13.2)	100.0 (13.6)	99.9 (16.1)
Aa	368	100.3 (13.0)	100.5 (13.4)	100.7 (14.3)
aa	154	99.8 (13.1)	98.8 (11.9)	98.6 (12.8)
P value		0.84	0.42	0.33
TT	256	100.3 (12.7)	99.3 (11.8)	99.9 (12.8)
Tt	347	99.6 (13.6)	100.9 (13.8)	100.4 (15.2)
tt	120	100.5 (12.2)	99.0 (14.0)	99.2 (16.1)
P value		0.70	0.23	0.72

P values relate to difference in normalised bone mineral densities for the three different allelic types.

($P = 0.03$, premenopausal women) and hip ($P = 0.04$, perimenopausal women). This consisted of two positive test results out of 27, which at the level of 5% significance would be expected by chance. After Bonferroni's adjustment for multiple comparisons all the tests were clearly not significant.

In table 2 the results in the four groups in the cross sectional study have been combined using normalised values of bone mineral density (percentage of the expected value in the particular group) to show the overall relation of the vitamin D genotypes to bone mineral density. Again, no significant effect of genotype on bone density was discernible.

Table 3 shows the results from the longitudinal study. Early postmenopausal bone loss at the forearm was determined from nine measurements over two years in 110 subjects a mean of 2 (1) years after the menopause. The total mean change in bone mineral content was -3.6% (3.6%). Late postmenopausal bone loss was determined at the mean ages of 63(1) and 69(1) in 108 subjects over a period of six years. The mean total change in bone mineral density was -6.2% (8.7%) at the lower forearm, -3.7% (4.7%) at the hip, and -1.8% (6.0%) at the lumbar spine. Long term postmenopausal bone loss at the mean ages of 51 (1) and 69 (1) was determined at the forearm in 109 subjects and was -24.5% (11.4%). The number of subjects does not total 136 since not all participants showed up for every examination. Furthermore, some participants were excluded in some time periods because they had used hormone

replacement therapy. Bone loss at any time was not significantly related to the vitamin D receptor genotype.

Discussion

VITAMIN D RECEPTOR GENOTYPES AND BONE MINERAL DENSITY

Many studies have shown osteoporosis to be heritable.⁴⁻⁸ Given the many and complex factors controlling bone resorption and bone formation, the heritability of osteoporosis is likely to be multifactorial. However, Morrison *et al* found that common allelic variation in a single gene coding for the vitamin D receptor accounted for up to 75% of the total genetic effect.⁶ Others have since confirmed the relation of the vitamin D receptor gene with bone mineral density,^{7,11,18} although Hustmeyer *et al* found no relation between them.⁸ Preliminary data from a large population study in Rotterdam show an association of the baT haplotype with low bone mineral density,¹⁹ contrasting with the alleles reported by others.^{7,11,18}

Some groups have shown the relation between vitamin D receptor genotypes and bone mineral density to be more pronounced in premenopausal women¹⁸ and to decline with age.²⁰ We could not confirm this. Our premenopausal group was, however, too small to rule out a minor relation between genotype and peak bone mass, but the relation between genotypes and bone mineral density in our larger perimenopausal group was not significant. More importantly, the relation was clearly not significant in our postmenopausal group at the age when fractures begin to appear.

Like Looney *et al*,²¹ we did not find a higher prevalence of the BB genotype in women with a low bone mineral density. In the forearm bone mineral density was 9% below that of normal age matched women; these women with a low bone mineral density corresponded to the spinal wedge fracture group (mild osteoporosis) of Overgaard *et al*.²²

VITAMIN D RECEPTOR GENOTYPE AND RATE OF BONE LOSS

The first work on vitamin D receptor gene polymorphism showed a noticeable influence of receptor genotype on bone turnover, measured as circulating osteocalcin concentrations.²³ Subjects homozygous for BB had rates twice as high as those with Bb or bb genotypes.²³ As biochemical markers of bone turnover may predict the rate of bone loss,²⁴ we investigated whether receptor genotype affects the rate of bone loss. We followed up 136 women for 18 years with repeated bone mineral density measurements. We found no significant association of receptor genotype (BsmI, ApaI, or TaqI) with early postmenopausal bone loss over two years at the lower forearm, which is in contrast to the findings of Krall *et al*.¹¹ However, in their study of early postmenopausal bone loss Keen

Table 3—Early, late, and long term postmenopausal bone loss according to vitamin D receptor genotypes. Values are mean (SD) percentages

Genotype	Early (2 years, ages 51-53)		Late (6 years, ages 63-69)						Long term (18 years, ages 51-69)	
	Forearm	No of women	Forearm	No of women	Hip	No of women	Spine	No of women	Forearm	No of women
BB	-1.8 (2.9)	13	-6.5 (6.4)	10	-5.3 (3.2)	10	-3.9 (6.1)	10	-26.7 (9.0)	10
Bb	-3.8 (3.4)	58	-6.8 (7.4)	55	-3.1 (4.9)	56	-1.9 (6.4)	56	-24.5 (11.1)	55
bb	-3.8 (3.9)	39	-5.2 (10.6)	43	-4.0 (4.6)	42	-1.2 (5.5)	43	-24.0 (12.5)	44
P value	0.18		0.66		0.33		0.43		0.79	
AA	-2.9 (3.9)	29	-6.1 (6.4)	26	-2.9 (5.1)	26	-2.9 (6.2)	26	-25.0 (10.7)	26
Aa	-3.5 (3.0)	58	-6.9 (9.6)	55	-3.8 (4.6)	55	-1.0 (6.4)	56	-23.7 (12.2)	55
aa	-4.7 (4.3)	23	-4.8 (8.8)	27	-4.2 (4.6)	27	-2.3 (4.7)	27	-25.5 (10.7)	28
P value	0.20		0.61		0.59		0.36		0.77	
TT	-4.2 (3.9)	39	-5.4 (10.3)	43	-4.1 (4.7)	42	-1.1 (5.6)	43	-23.9 (12.3)	44
Tt	-3.7 (3.3)	54	-7.3 (7.3)	52	-3.4 (4.6)	53	-1.9 (6.2)	53	-25.3 (10.8)	52
tt	-1.8 (3.3)	17	-4.4 (8.0)	13	-3.3 (5.6)	13	-3.7 (6.7)	13	-23.3 (11.2)	13
P value	0.07		0.44		0.75		0.38		0.77	

Key messages

- Osteoporotic fractures are a major health care problem, and their incidence is expected to increase considerably in the next decades
- Twin studies show a strong genetic component in the development of osteoporosis
- Finding a genetic marker for this disease would be important in identifying patients who could benefit from preventive treatment
- This study found that the vitamin D receptor genotypes previously suggested as accounting for up to 75% of the genetic effect on bone density did not affect bone mineral density in postmenopausal women
- Vitamin D receptor genotype is not related to postmenopausal bone loss

et al found no significant relation of vitamin D receptor genotype (TaqI) with rate of bone loss at the lumbar spine or femoral neck,¹⁰ thus supporting our findings.

Late postmenopausal bone loss was determined over six years from measurements at the lower forearm, hip and lumbar spine. Again there was no significant relation between receptor genotype and rate of bone loss. Finally, when long term postmenopausal bone loss at the lower forearm was considered the differences between the genotypes were clearly not significant. Our cross sectional study also supports the lack of association between vitamin D receptor genotype and rate of bone loss (fig 1).

Our selection process for the cross sectional study has not led to any bias in bone mineral density or vitamin D receptor genotype. The genotype frequencies in our study are similar to the frequencies found in white women.^{8 19 23} The cross sectional study does, however, have a slightly unequal age distribution in the postmenopausal group, with a relative lack of women between the ages of 61 and 65, but this is unlikely to have any bearing on the conclusions of this study.

CONCLUSION

If the vitamin D receptor genotype is related to bone mineral density in premenopausal women it is probably not clinically relevant since this relation seems to be lost at the menopause, whereas fractures occur mainly after the menopause. We therefore conclude, that receptor genotype is not related to bone density in postmenopausal women. Vitamin D receptor genotype was also not related to early (age 51-53), late (age 63 to 69), or long term postmenopausal bone loss (age 51 to 69).

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ONE HUNDRED YEARS AGO

BOXING THE EARS.

We are glad to see that a medical magistrate (Dr. Hetley) at Penge has drawn attention to the evil effects of boxing the ears in school children. Every trainer of youth should be impressed with the imperative duty of avoiding this form of punishment, not only from the harm which results to the ear itself, such as rupture of the membrana tympani and concussion of the labyrinth, but from the

even more serious consequences which may be caused by the resulting suppuration in the middle ear. It should also be recognised that the inattention of children is, as a rule, due to impaired hearing—a condition which in itself often renders the ears more liable to injury from blows.

(*BMJ* 1896;ii:35.)