Vitamin D and Calcium-Sensing Receptor Genotypes in Men and Premenopausal Women with Low Bone Mineral Density

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Abstract

Background: Genetic factors have been shown to play a major role in the development of peak bone mass, with hereditability accounting for about 50–85% of the variance in bone mass. Numerous candidate genes involved in osteoporosis have been proposed, but the precise genes and their relative contribution remain unknown.

Objectives: To gain insight into the genetic basis of idiopathic low bone mineral density in Israeli patients by analyzing the impact of two candidate genes: polymorphism of the vitamin D receptor gene and polymorphism A986S in the calcium-sensing receptor gene.

Methods: We analyzed 86 Jewish Israeli patients with LBMD: 38 premenopausal women and 48 men, and compared the allelic pattern distribution with that of the general population (126 men and 112 women). Genotyping of the VDR gene was performed in three polymorphic sites using restriction enzymes, and allelic analysis of A986S polymorphism in the CaSR gene was performed using the denaturing gradient gel electrophoresis technique.

Results: In LBMD women the distributions of VDR alleles in ApaI polymorphism were AA = 7/28, Aa = 16/28 and aa = 5/28; in TaqI polymorphism TT = 10/31, Tt = 16/31 and tt = 5/31; and in BsmI polymorphism BB = 7/32, Bb = 14/32 and 11/32. In LBMD men the distributions were AA = 17/39, Aa = 21/39 and aa = 1/39; in TaqI polymorphism TT = 12/42, Tt = 23/42 and tt = 7/42; and in BsmI polymorphism BB = 12/41 Bb = 18/41 and bb = 11/32. The distributions of all these polymorphisms in the control groups were not significantly different. Adjusting for the independent age and gender parameters confirmed that these three polymorphisms of the VDR gene did not have a significant effect on bone mineral density. Thirty percent (24/79) of LBMD patients of either sex displayed heterozygosity of the CaSR A986S polymorphism, compared with 40 of 203 controls (19.7%) ($P = 0.059$). Adjusting for age and gender in these patients revealed a significant difference in the femoral neck BMD between homozygotes and heterozygotes ($P = 0.002$). The age at menarche of the LBMD women was found to predict 61% of the variance of femoral neck BMD.

Conclusions: In Israeli Jewish men and premenopausal women VDR gene alleles do not seem to be associated with lower lumbar spine or femoral neck BMD. A trend towards heterozygosity for a CaSR polymorphism missense mutation was noted in the LBMD patients. Age at menarche in the LBMD women was found to be an important predictor of BMD. A significant difference was found between LBMD women and healthy control women towards heterozygosity for a CaSR polymorphism, as well as between homozygotes and heterozygotes for a CaSR polymorphism in BMD. The significance of these findings and their applicability to a larger population awaits further studies.

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Osteoporosis is characterized by low bone mineral density and micro-architectural changes in bone structure, leading to skeletal fragility and typical fractures in elderly men and women [1]. BMD in adults is the outcome of the peak bone mass achieved shortly after puberty and the process of lifelong bone loss, which is most prominent in postmenopausal women. The peak bone mass is largely determined by heredity, but the genes involved as well as their relative contribution are not yet fully understood. Most of the genetic studies in osteoporosis focused on female populations and rarely on males. Some genes involved in calcium homeostasis or cytokine secretion in bone were proposed as candidate genes in the pathogenesis of osteoporosis [2–4]. One major candidate is the gene for the vitamin D receptor. Morrison et al. [5] reported that VDR gene polymorphisms determine circulating osteocalcin levels and influence BMD. Two other independent studies found similar trends in British [6] and Japanese [7] populations. Allelic variation of the VDR gene did not seem to be related to BMD or osteoporosis in U.S. [8] or Swedish [9] cohorts. Inconsistent results were obtained in other ethnic groups, casting doubts on the importance of this particular candidate gene [10]. A meta-analysis of 16 studies in different populations and ethnic groups found a weak correlation between BMD and allelic variability of the VDR gene [11].

Another candidate for the genetic determination of osteoporosis is the gene for the calcium-sensing receptor. The CaSR belongs to the G-protein coupled receptor super-family and serves as a sensor of the extracellular calcium levels in different tissues. This sensor

LBMD = low bone mineral density
VDR = vitamin D receptor
CaSR = calcium-sensing receptor
BMD = bone mineral density

plays a key role in the regulation of secretion of calciotropic hormones, the parathyroid hormone and calcitonin [12]. More than 30 mutations in the encoding region for the CaSR gene have been described in humans so far. Inactivating mutations were found in familial hypocalciuric hypercalcemia and in neonatal severe hyperparathyroidism, while activating mutations are seen in various familial hypocalcemic syndromes [13]. A knockout mice model with complete lack of the CaSR is characterized by severe hyperparathyroidism, hypercalcemia and skeletal abnormalities [14]. An analysis of polymorphisms of the CaSR gene (A986S) in a Canadian population found a significant correlation with extracellular calcium levels [15]. CaSR is therefore an interesting candidate gene but its role in osteoporosis has not yet been studied in depth.

The present work is a pilot study that explores the contribution of polymorphisms of these two genes to BMD in an Israeli cohort of men and premenopausal women with idiopathic LBMD. We assumed that BMD in this cohort reflects mainly the peak bone mass since it is not affected by sex hormone deficiency.

Patients and Methods

Patients

We studied 86 consecutive LBMD patients: 38 premenopausal women and 48 men who were found to have low peak bone mass and were treated in the metabolic bone diseases clinics at the Sheba and Rambam Medical Centers. The study was approved by the institutional review board for research involving human subjects. Written informed consent was obtained from each participant. The eligibility criterion for the patients was BMD 2 SD below the mean of an age-matched reference population at the lumbar spine (L2–L4) and/or in the femoral neck. All patients had been questioned about previous and chronic illnesses, fractures, use of medications, and family history of bone diseases. Daily calcium intake was estimated by dietary history-taking. Exclusion criteria were: endocrinopathies, neoplastic diseases, vitamin D deficiency, known hereditary disorders, and other disorders (immobilization and chronic diseases such as liver/kidney failure, rheumatoid arthritis, hemoglobinopathies).

Detailed physical examinations were performed, and relevant laboratory tests were carried out in order to rule out secondary LBMD (i.e., liver and kidney function tests, serum calcium, phosphorus, alkaline phosphatase, parathyroid and thyroid hormones, 25 hydroxyvitamin D, sex hormones, complete blood count and protein electrophoresis, urinary calcium and Bence-Jones protein). Since no underlying disorder that could explain their low bone mass was found, all patients were diagnosed as having idiopathic LBMD. To serve as control groups for the genetic studies we chose at random 112 premenopausal healthy women and 126 men from the general population. The control subjects were questioned about their medical history but were not evaluated by laboratory testing.

BMD measurements

Bone density was measured at the lumbar spine (L2–L4) and the femoral neck by DXA (Lunar DPX-L, Lunar Corporation, Madison, WI, USA).

Polymerase chain reaction [16]

DNA was extracted from leukocytes isolated from anticoagulated peripheral venous blood using standard procedures. The PCR aliquots were of 50 L volume containing 0.2 mM of each nucleotide, 30 pmol of each of the primers, and 0.5 U Taq polymerase (Rhenium Bioprobe Systems, Moteil-Sous-Bios, France) in a buffer supplied by the manufacturer.

For the VDR gene, Ap fragment, the forward primer sequence was: 5′-CAGAGCATGGACAGGGAGCAA-3′ and 5′-GCAACTCC TCATGGCTGAGGTCT TC-3′ for the reverse primer, and for Bs fragment, the forward primer sequence was: 5′-CAACCAAG ACTA CAAGTACCGCGTCAGTGA-3′ and 5′-AACCAGCGG GAAGAGGTCA AGGG-3′ for the reverse primer. For CaSR, the forward primer sequence was: 5′-GCGGCCGCCCGTCCCGCCGCCCCCGCCCC GCCGCGGCCGCCAAGGACCTCTGGACCTCCCTTTGC-3′, and 5′-GAC CAAGCCCTGCACAGTG CCCA AG-3′ for the reverse primer. The PCR reaction was performed using 0.5–1 μg DNA as a template. Thermal cycling was performed in a PTC-100-60 (M.J. Research, Watertown, MA, USA) and was as follows: for Ap an initial denaturation step at 94°C for 4 minutes, followed by cycles of denaturation at 94°C (45 seconds), and extension at 72°C for 1 minute. Annealing temperature was 52°C for 24 cycles each of 2 minutes. For Bs and CaSR the same procedure was followed, except that the annealing temperature was 56°C and 60°C respectively. Following the reaction, all PCR products were analyzed in 1.5% agarose gels to confirm the success and specificity of the reaction.

VDR gene polymorphisms

Two fragments of the VDR gene, Ap (740 bp) and Bs (825 bp), were amplified. These PCR products represented two regions of the receptor gene. The first, Bs fragment, contains the BsmI restriction site with one primer originating in exon 7 and the other in intron 8. The second, Ap fragment, contains the ApaI and TaqI restriction sites with one primer in intron 8 and the other in exon 9. PCR products were then subjected to digestion of restriction enzymes: ApaI, TaqI and BsmI. The products were electrophoresed on horizontal agarose gel (1.5%), stained with ethidium bromide, visualized and photographed under ultraviolet light trans-illumination [7]. The letters A,B,T define the absence of the restriction enzymes ApaI, TaqI and BsmI respectively, and the letters a, b, t define their presence.

Denaturing gradient gel electrophoresis

Parallel denaturing gradient gels were cast with denaturing gradient of 20–80% as previously described [17]. After the gels were polymerized, PCR samples were mixed with gel-loading dye and were loaded on gels and electrophoresed overnight at 40 mA and 58°C, using the D-GENE apparatus (Bio-Rad). After completion of the running of samples in gels, the gels were stained with ethidium bromide and were photographed with an ultraviolet camera. The denaturing gradient gel electrophoresis technique was used to analyze the polymorphic locus in exon 7 of the CaSR gene. The PCR product of CaSR was 328 bp. Three forms of sample immigrations in

PCR = polymerase chain reaction

the denaturing gradient gel were observed – two types of homoduplexes and a heteroduplex.

Statistical analysis

Genotype frequency comparisons between LBMD patients and control groups in VDR and CaSR genes were performed by chi-square test for independence. Differences in BMD were tested with *t*-test for independent samples. Multivariable regression analysis was used to assess the independent factors that influence BMD. A value of $P<0.05$ was considered as significant.

Results

Characteristics of LBDM patients

Table 1 presents the demographic data, bone density results and estimated calcium intakes of the 38 premenopausal LBMD women and 48 Israeli LBMD men comprising the study group. The control groups consisted of 112 women aged 21–37 and 126 men aged 21–45 years, randomly selected, healthy, by self-reporting and by a medical history questionnaire.

Laboratory analysis

VDR gene polymorphism: Genotype frequency comparisons of LBMD women and LBMD men with their healthy counterparts (controls) revealed no significant differences [Table 2]. The age and gender-independent variables had a significant effect on BMD: at the lumbar vertebrae P=0.002 and at the femoral neck P=0.005. After adjusting for these variables in order to isolate the effect of the VDR gene polymorphisms, we found that these three sites do not have a significant effect on BMD. Age at menarche predicted 18% of the variance in lumbar spine BMD and 61% of the variance in the femoral neck BMD in LBMD women.

CaSR gene polymorphism: Distribution of the polymorphism patterns within LBMD patients displayed that 69.4% of the LBMD women and 69.8% of the LBMD men were homozygous in this locus. In the control groups 86.2% of women (P=0.030) and 75.9% (P=0.435%) of men were homozygous [Table 3]. In the LBMD group (women and men) 69.9% were homozygous versus 80.3% in the control group (P=0.059).

Neutralization of age and gender variables in order to isolate

Table 1. Characteristics of the Israeli LBMD patients in the study

	Women			Men		
	Mean	**Range**	**SD**	**Mean**	**Range**	**SD**
Sample size	38	–	–	48	–	–
Age (yr)	40.8	19–55	9.09	51.9	17–69	12.22
Age at menarche (yr)	12.8	11–15	1.00	–	–	–
Height (cm)	159.3	142–172	7.14	170.6	153–183	6.39
Weight (kg)	56.9	39–75	8.51	76.3	55–108	11.25
Calcium intake (mg/day)	443.3	200–1,200	315.60	527	200–1,000	280.98
BMD femur (g/cm^3)	0.726	0.551–0.910	0.07	0.796	0.571–1.086	0.11
Z score	-2.1	-3.4– -0.6	0.82	-2.0	-3.8– -0.58	0.98
BMD spine (g/cm^3)	0.936	0.625–1.103	0.10	0.902	0.677–1.209	0.11
Z score	-1.9	-4.1– -0.7	0.84	-2.5	-4.7– -0.26	0.9

Table 2. VDR gene genotype frequency comparisons between LBMD women and controls, and LBMD men and controls

		Women						Men					
		LBMD patients		Control				LBMD patients		Control			
Genotype		**No.**	**%**	**No.**	**%**	χ^2	P=	**No.**	**%**	**No.**	**%**	χ^2	P=
ApaI	AA	7	25.0	30	38.5	3.162	0.206	17	43.6	50	41.7	3.309	0.191
	Aa	16	57.1	42	53.8			21	53.8	55	45.8		
	aa	5	17.9	6	7.7			1	2.6	15	12.5		
TaqI	TT	10	32.3	32	43.2	1.271	0.530	12	28.6	56	44.8	3.524	0.172
	Tt	16	51.6	30	40.5			23	54.8	51	40.8		
	tt	5	29.4	12	16.2			7	16.7	18	14.4		
BsmI	BB	7	21.9	23	39.7	3.064	0.216	12	29.3	35	35.4	0.636	0.727
	Bb	14	43.8	18	31.0			18	43.9	37	37.4		
	bb	11	34.4	17	29.3			11	26.8	27	27.3		

Table 3. CaSR gene genotypes frequencies comparisons between LBMD women and controls, and LBMD men and controls

	Women						Men					
	LBMD patients		Control				LBMD patients		Control			
Genotype	**No.**	**%**	**No.**	**%**	χ^2	P=	**No.**	**%**	**No.**	**%**	χ^2	P=
Heterozygote	11	30.6	12	13.8	4.706	0.030	13	30.2	28	24.1	0.609	0.435
Homozygote	25	69.4	75	86.2			30	69.8	88	75.9		

only the effect of the analyzed polymorphism in the CaSR gene within the LBMD patients demonstrated a significant difference in the femoral neck BMD in the homozygous compared to heterozygous subjects (P=0.002).

Discussion

We studied allelic variations of two candidate genes in this pilot study of LBMD Jewish Israeli men and premenopausal women who did not have any clinical or laboratory evidence for secondary osteoporosis. The average calcium intake in our cohort was lower than the recommended daily allowance, although it is quite common in the Israeli population aged 25–40 (D. Nitzan-Kaluski et al., unpublished data of the National Health and Nutrition Survey – MABAT). It could not have caused the uniformly low BMD that was observed in our cohort. It was previously reported that BMD measured in post-menarchal Israeli girls with a calcium intake below 800 mg/day was distributed normally around the average when compared to age-matched controls despite their low calcium intake [18]. This cohort was chosen because its bone mineral density is presumably close to the peak bone mass.

It seems that allelic variations of the VDR gene do not contribute significantly to bone mass. Our results are similar to those obtained in other ethnic groups worldwide [2,3,7,8]. The initial expectation, following the study of Morrison et al. [5], that VDR alleles may predict BMD and fractures was replaced by the realization that this predictive value is limited to certain populations. The different and sometimes conflicting results regarding prediction of BMD by VDR genotypes may be due to unique interactions of genetic background and environmental factors. Another possibility is that the VDR alleles that were studied, and were presumed markers of the VDR functional activity, do not contribute much to peak bone mass but may affect the rate of bone loss in osteoporotic subjects. The VDR is expressed in many tissues including the reproductive tract, and allelic variations may have a tissue-specific effect [19]. Mice lacking the VDR signal display ovarian abnormalities [20]. We found that the age at menarche determines BMD, mainly at the femoral neck. One may speculate that the ovarian VDR plays a role in the onset of menses, but evidence for this is lacking. A correlation between age at menarche and BMD was also found in Japanese women [21].

The CaSR gene seems to be an attractive candidate gene for determining BMD and osteoporosis due to its profound involvement in calcium homeostasis. We found significant differences in the CaSR genotype distribution (P=0.03) among LBMD and healthy women. We also found a trend for heterozygocity at the studied locus in LBMD subjects (P=0.059) regardless of gender. Most significantly, in LBMD subjects, femoral neck BMD differed between homozygous and heterozygous CaSR genotype carriers (P=0.002). The link between functional activity of the CaSR and the polymorphic site we studied is speculative. It may indicate a change in the set point for extracellular calcium levels, which is a potential mechanism for the development of osteoporosis. Presumably, mutations or polymorphism of this gene may change the set point of the parathyroid cell for secretion of parathyroid hormone, leading to inappropriate parathyroid hormone secretion and increased bone resorption. Other polymorphic loci of the CaSR gene will be analyzed in a large number of subjects from different ethnic origins (humane gene mutation database http://www.uwcm.ac.uk/uwcm/mg/ns/1/134196.html).

In conclusion, in Israeli Jewish men and premenopausal women, VDR gene alleles do not seem to be associated with lower lumbar spine or femoral neck BMD. A trend towards heterozygosity for a CaSR polymorphism missense mutation was noted, as was a significant difference between LBMD women and healthy control women towards heterozygosity for a CaSR polymorphism. Moreover, a significant difference was found in BMD between homozygotes and heterozygotes. Menarchal age of the LBMD women was found to be an important predictor of BMD. Our results need to be corroborated in larger numbers of subjects, or by linkage analysis in families with several osteoporotic members.

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