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Linkage of a Gene Causing High Bone Mass to Human Chromosome 11 (11q12-13)

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Summary

The purpose of this paper is to report the linkage of a genetic locus (designated "HBM") in the human genome to a phenotype of very high spinal bone density, using a single extended pedigree. We measured spinal bone-mineral density, spinal Z(BMD), and collected blood from 22 members of this kindred. DNA was genotyped on an Applied Biosystems model 377 (ABI PRISM Linkage Mapping Sets; Perkin Elmer Applied Biosystems), by use of fluorescence-based marker sets that included 345 markers. Both two-point and multipoint linkage analyses were performed, by use of affected/unaffected and quantitative-trait models. Spinal Z(BMD) for affected individuals (N = 12) of the kindred was 5.54 ± 1.40; and for unaffected individuals (N = 16) it was 0.41 ± 0.81. The trait was present in affected individuals 18–86 years of age, suggesting that HBM influences peak bone mass. The only region of linkage was to a series of markers on chromosome 11 (11q12-13). The highest LOD score (5.21) obtained in two-point analysis, when a quantitative-trait model was used, was at D11S987. Multipoint analysis using a quantitative-trait model confirmed the linkage, with a LOD score of 5.74 near marker D11S987. HBM demonstrates the utility of spinal Z(BMD) as a quantitative bone phenotype that can be used for linkage analysis. Osteoporosis pseudoglioma syndrome also has been mapped to this region of chromosome 11. Identification of the causal gene for both traits will be required for determination of whether a single gene with different alleles that determine a wide range of peak bone densities exists in this region.

Introduction

Peak mass of the adult skeleton is under genetic control. Twin studies show that the bone-mass variance between MZ co-twins is smaller than that between DZ co-twins (Smith et al. 1973; Moller et al. 1978; Dequeker et al. 1987; Pocock et al. 1991; Slemenda et al. 1991; Young et al. 1995). It has been estimated that as much as 60% of the variance in skeletal mass is inherited (Smith et al. 1973; Slemenda et al. 1991; Krall and Dawson-Hughes 1993). In addition, peak skeletal mass is the best measurable determinant of fracture risk in the elderly (Hui et al. 1989), although bone loss during later adult life is also a factor (Hui et al. 1990; Johnston and Longcope 1990; Chesnut 1991). Thus, study of the genetic basis of osteoporotic fracture can be approached through study of the molecular-genetic determination of peak bone mass.

Past research to identify specific genes that influence peak bone mass has focused mainly on candidate genes with identifiable polymorphisms. For example, the vitamin D–receptor (VDR) locus has been associated with spinal and femoral bone mass (Morrison et al. 1994; Howard et al. 1995; Riggs et al. 1995; Spector et al. 1995). However, studies of genetic control of bone mass are challenging, because bone mass is a polygenic, continuous trait, confounded by nongenetic (environmental) factors that include (among others) hormone status, nutrition, age, physical activity, and comorbid disease (Matkovic et al. 1979; Richelson et al. 1984; Dequeker and Guesens 1985; Kanders et al. 1987; Nilas and Christiansen 1987). Furthermore, each skeletal anatomic site may be influenced by several genes that themselves differ from site to site. Conclusions about inherited traits, reached from numerous reports on groups of unrelated persons, are likely to be confounded by problems such as genetic heterogeneity, variable allele frequencies, and variable gene penetrance. These problems may prove difficult to overcome in association studies, even with large sample sizes. It is thus not surprising that, when viewed as a group, the results of past VDR–bone-mass studies have been inconsistent, despite the inclusion of some studies of twins (Hustmyer et al. 1994; Morrison et al. 1994; Eisman 1995; Garnero et al. 1995; Peacock 1995; Spector et al. 1995). This inconsistency points to the need for additional approaches, such as the extended family–based design presented here.

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Family-based-study designs that use linkage analysis (e.g., sib-pair or pedigree studies) are generally more informative than association studies. The limited genetic heterogeneity within families, combined with detailed knowledge of the pedigree, is a key factor. Furthermore, genetic-linkage studies work best when the phenotype under study is discrete. The purpose of this paper is to report the linkage of a genetic locus in the human genome to very high bone mass (HBM), by use of a single extended pedigree. Follow-up studies may include an investigation of the genetic basis of a mechanism that regulates peak bone mass.

Material and Methods

The study was approved by the Creighton University Institutional Review Board. Each subject signed an informed-consent document prior to participating in the project.

A. Bone-Mass Measurements

Bone-mineral density (BMD; g/cm^2) measurements of the spine (L1–L4), hip, and total body, performed in Omaha, were done by dual energy x-ray absorptiometry (DXA; Hologic 2000). Measurement of BMD obtained at other centers used the available DXA equipment (Hologic or Lunar DPX). Absolute values for spinal BMD were normalized to that of age- and sex-matched individuals from the normal database obtained on each manufacturer's equipment, to enable calculation of spinal Z(BMD) for each person. Spinal Z(BMD) for an individual is the quantitative phenotype used in the linkage analysis. It is defined as the number of SDs above or below the mean of normal age- and sex-matched persons. Spinal Z(BMD) was obtained in every case; hip (N = 13) and total body (N = 7) measurements were made when possible. In addition, we obtained a complete clinical history and forearm radiograph in all cases. An extensive radiographic skeletal survey was performed on the proband and her mother.

B. Genotyping

Blood (20 ml) was drawn into lavender-cap (EDTA-containing) tubes by a certified phlebotomist and was stored refrigerated until DNA extraction. DNA extraction was done with a kit (Gentra Systems), according to the manufacturer's directions. The yield of DNA was routinely >40 μg of whole blood/ml, with a molecular weight of >50 kb. DNA was archived by storing coded 50-μg aliquots at −80°C as an ethanol precipitate.

DNA was genotyped by use of the fluorescence-based marker sets (Reed et al. 1994) commercially available through Perkin Elmer Applied Biosystems (ABI PRISM Linkage Mapping Sets) on an Applied Biosystems automated DNA sequencing system (model 377) running the GENESCAN™ 672 and GENOTYPER™ software for allele identification. These marker sets have been optimized for this hardware/software and are intended to be used in a multiplex format to facilitate rapid genotyping of large numbers of DNA samples. This linkage-marker set contains 345 markers that cover the human genome, excluding the X and Y chromosomes, at a spacing interval of 7–22 cM. The spacing of these markers is such that <2% of the genome lies >10 cM from a marker and ~50% lies <5 cM from a marker.

PCR reactions were performed in two fashions. All reagents were purchased from Perkin Elmer–Applied Biosystems. We performed individual PCR reactions with the markers from the Linkage Mapping Panels 12, 13, and 15–18, exactly as described by the supplier, using AmpliTaq DNA polymerase. All other panels were performed in a multiplex fashion. Markers from each panel were multiplexed in the PCR reaction, on the basis of color (dye label). Thus, for each DNA sample, only three reactions (one blue-, one green-, and one yellow-labeled group of markers) were performed with each panel. The multiplex PCR reactions were performed in a 50-μl reaction volume with the following final concentrations: 1 × PCR Buffer II, 2.5 mM $MgCl_2$, 10 pmol of each primer, 250 ng of DNA, and 3 units of AmpliTaq Gold DNA Polymerase. Thermal cycling was done by use of a Perkin Elmer 9600 thermal cycler. Conditions were 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and then followed by a 30-min extension at 60°C. After cycling, the reactions were cooled to 4°C until removal from the thermal cycler. The three separate multiplex reactions were pooled at the following ratios: 5 μl each of the blue (Fam-labeled) markers and green (Tet-labeled) markers, 10 μl of the yellow (Hex-labeled) markers, and 20 μl of deionized water. Pooled reactions were stored at −20°C until gel electrophoresis.

A portion (1.5 μl) of the pooled reaction was added to 3.5 μl of loading buffer containing deionized formamide, blue dextran, and TAMRA 350 size standards (Perkin Elmer–Applied Biosystems). After being heated at 95°C for 5 min to denature the DNA, the samples were loaded and electrophoresed as described in the operator's manual for the model 377 DNA sequencer.

After gel electrophoresis, the data were analyzed by use of GENESCAN and GENOTYPER software. Before the first analysis step, within the GENESCAN software, the lane tracking was optimized manually. After the gel-lane data were extracted, the standard curve profiles of each lane were examined and verified for linearity and size calling. Lanes with problems for either of these parameters were retracked and verified. Once all lanes had been tracked and the size standards had been identified correctly, the data were imported into GENOTYPER for allele identification. To expedite

allele identification, we obtained the program Linkage Designer from the Internet website of Dr. Guy Van Camp (http://alt-www.uia.ac.be/u/dnalab/ld.html; Van Camp et al., in press). This program facilitated importation of data generated by GENOTYPER, into the pedigree-drawing program Cyrillic (version 2.0; Cherwell Scientific Publishing).

C. Linkage Analysis

Pedigree/marker data were exported from Cyrillic into the Makeped program and were converted to a file suitable for linkage analysis. Two-point and multipoint linkage analysis were performed by use of the MLINK and LINKMAP components of LINKAGE (Lathrop et al. 1985). Recombination fraction ($\hat{\theta}$) and maximum LOD score (Z_{max}) were calculated by use of the ILINK subroutine.

We used three different models to analyze for linkage: two affected-trait models, one with full penetrance and one with partial penetrance (.9); and a quantitative-trait model. For the affected-trait models, the following criteria were applied for selection of affected individuals: (1) spinal Z(BMD) > 3.0, (2) a clinical history without evidence of any known HBM syndrome, and (3) forearm radiographs showing a normal shape of the appendicular skeleton. For the quantitative-trait analysis, we defined NN as a mean spinal Z(BMD) of 0.41; ND as a mean spinal Z(BMD) of 5.54 and DD as a mean spinal Z(BMD) of 10.67, where N represents the normal allele and D represents the disease allele. The mean of the NN individuals was calculated from the spinal Z(BMD) of the unaffected individuals within the family (with individual 16 being excluded; see Discussion). Since the phenotype is very rare in the general population, we inferred that the allele frequency for the HBM-gene mutation conferring HBM is extremely low. We thus assumed that affected individuals were heterozygotes, and we calculated the mean for ND from the affected individuals in the family. We assumed an additive model to estimate the mean for DD individuals, on the basis of the difference, in means, between the NN group and the ND group. The variance for the unaffected individuals (excluding Individual 16) was 0.66, and that for the affected individuals was 1.96. In all three models, we assumed equal frequencies for each allele of a marker, since population-based frequencies were not available for all markers.

Results

Phenotype Inheritance and Description

The proband was referred, by her physicians, to the Creighton Osteoporosis Research Center, for evaluation of "unusually dense bones." She was 18 years old and had come to medical attention 2 years earlier because of back pain precipitated by an auto accident. She suffered soft-tissue injury to her lower back, manifested as pain and muscle tenderness. There was no radiographic evidence of fracture or subluxation. At the time of presentation, she had nearly recovered from the injury and, except for vigorous recreational and varsity sports, had resumed the usual activities of a high-school student. Physical exam revealed a normal healthy young woman. Radiographs of the entire skeleton revealed dense bones with thick cortices. All bones of the skeleton were dense and of normal shape. There were no skeletal radiographic lesions characteristic of any of the known conditions associated with HBM, in either the proband or her mother.

Table 1

Anthropomorphic and DXA Characteristics of Proband and Immediate Family

	Age (years)	Height (cm)	Weight (kg)	Spinal BMD (g/cm^2)	Spinal Z(BMD)
Proband	18	167.5	58.0	1.667	5.63
Mother	51	165.0	63.4	1.500	4.98
Father	52	183.0	84.7	1.118	.25
Brother	15	167.6	61.7	1.022	.45

The radiographic findings and the data in table 1 suggested that the proband had inherited from her mother a trait that resulted in very dense bones but an otherwise normal skeleton. Therefore, we focused further studies on the maternal kindred.

Studies of Maternal Kindred

The kindred was of mixed Caucasian (European) descent. The portion of the kindred used for the whole-genome screening and linkage analysis is shown in figure 1. The deceased maternal grandfather of the proband was phenotyped through medical records and antemortem skeletal radiographs. The phenotype of other deceased members was inferred from the pattern of inheritance, to expedite linkage analysis. The pattern of inheritance of the HBM trait in this family is autosomal dominant. X linkage is ruled out by the presence of male-to-male transmission from individual 12 to individuals 14 and 15. Twenty-two informative individuals completed both phenotyping and genotyping for the linkage analysis. In all, 28 individuals were phenotyped by DXA. Spinal Z(BMD) in the affected ($N = 12$) group was 5.54 ± 1.40; and that in the unaffected ($N = 16$) group was 0.41 ± 0.81, indistinguishable from that in normal members of the population.

Hemograms, standard serum clinical chemistry, and bone-marker measurements (alkaline phosphatase, bone-specific alkaline phosphatase, osteocalcin, pyridi-

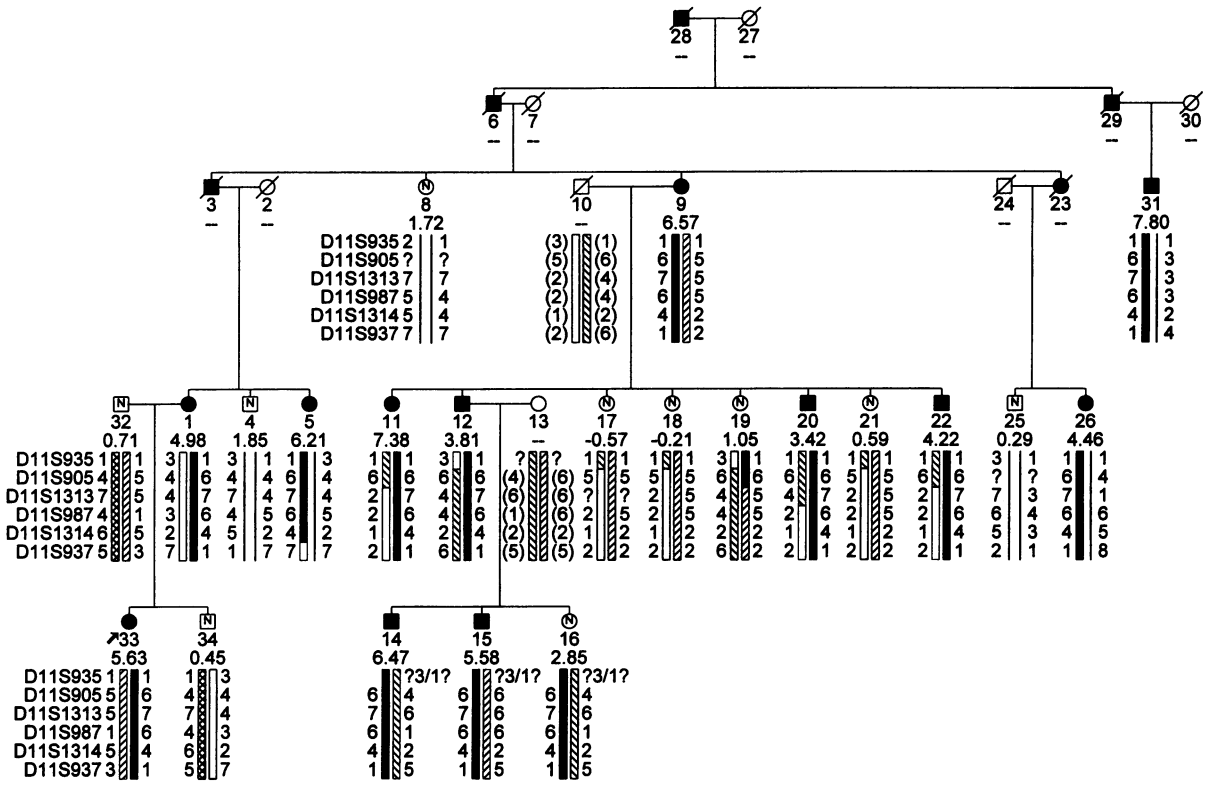

Figure 1 Pedigree of HBM family. Under each individual is an ID number, the spinal Z(BMD), and the allele identification for markers D11S935, D11S905, D11S1513, D11S987, D11S1314, and D11S937. Blackened symbols represent either affected individuals or, in cases where phenotype was not available, those presumed to be affected; and symbols containing "N" represent unaffected individuals. Genotypes within parentheses are inferred.

noline cross-links, and deoxypyridinoline cross-links) were performed on a subset of five affected individuals, revealing no abnormalities. DXA measurements for the hip and total body were congruent with the results from spinal Z(BMD). None of the affected individuals were aware of the presence of an unusual degree of HBM. None had clear-cut clinical, historical, radiographic, or laboratory findings that could be associated with HBM or that would point to one of the known syndromes of high bone density. No affected member of the kindred reported a history of any type of bone fracture. The HBM trait did not seem to affect the health or well-being of the affected individuals.

Genotyping

We performed the genome screening by using the 345 markers of the autosomal Perkin Elmer–Applied Biosystems Linkage Mapping Panels. The first-attempt success rate for markers used with individual PCR was 94% (73/78), whereas that for markers used with multiplex PCR was 92% (245/267). A listing of markers suffering from first-attempt failure is available on request. The advantage of the multiplex PCR is the estimated 10-fold increase in speed with which the panel sets can be analyzed.

Linkage Analysis

On the basis of the bimodal distribution of spinal Z(BMD) (fig. 2), we first performed two-point linkage analysis using a simple affected (spinal Z[BMD] > 3.0), autosomal dominant model with full penetrance, to

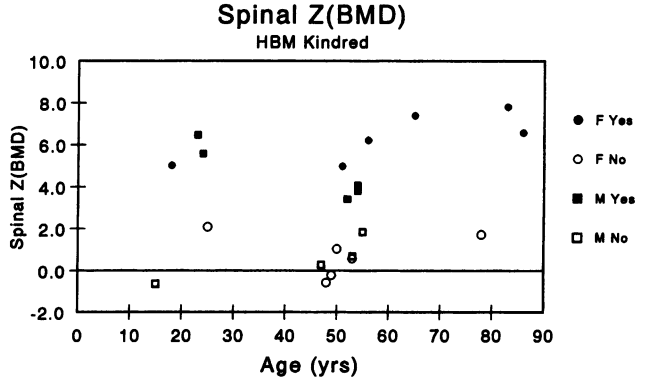

Figure 2 Spinal Z(BMD) of all kindred members contributing to this analysis. Affected females (F Yes), unaffected females (F No), affected males (M Yes), and unaffected males (M No) are represented as unblackened squares. The HBM trait appears at least by the time of skeletal maturity and persists throughout the remainder of the life span.

Table 2

Two-Point Z_{max}, by Model and Marker

Model and Marker	$\hat{\theta}$	Z_{max}
Affected with full penetrance:		
D11S905	.064	2.089
D11S1313	.107	1.572
D11S987	.044	4.055
D11S1314	.000	2.961
D11S937	.123	1.974
Affected with partial penetrance (.9):		
D11S905	.000	2.454
D11S1313	.000	1.932
D11S987	.000	4.474
D11S1314	.000	3.172
D11S937	.089	2.136
Quantitative trait:		
D11S905	.051	2.096
D11S1313	.000	2.679
D11S987	.000	5.207
D11S1314	.000	3.963
D11S937	.061	2.747

search for a region of linkage in the genome. The only region of linkage was to a series of markers on chromosome 11 (11q12-13). We next modeled the markers on chromosome 11, using an affected autosomal dominant model with partial penetrance. Finally, we used the quantitative-trait model. The results of all three models are shown in table 2. The multipoint map resulting from use of the quantitative-trait model is shown in figure 3. The Z_{max} (5.74) was near marker D11S987 (map position 55 in fig. 3). The 95% confidence interval places the HBM gene between D11S905 and D11S937, a region of ~30 cM (map position 41–71 in fig. 3). Haplotype analysis also places the HBM gene within this region (fig. 1). $\hat{\theta}$ and Z_{max} for the critical markers, derived from the two-point and multipoint linkage analyses, are shown in table 2.

Discussion

By analyzing DNA for linked markers in 22 members of an extended kindred, we have mapped to chromosome 11q12-13 a genetic locus (the HBM locus) that determines very high bone density. The 5.74 Z_{max} obtained in multipoint analysis was near D11S987. The 5.21 Z_{max} obtained in two-point analysis was at D11S987. The 95% confidence interval places the HBM locus in an ~30-cM region between markers D11S905 and D11S937. In this analysis, the haplotype of affected individuals, for markers D11S1313, D11S987, and D11S1314, appears as 7-6-4 (fig. 1).

Individual 16 (see fig. 1), with spinal Z(BMD) = 2.85, fits our a priori definition of unaffected (see Material and Methods). However, genotyping revealed that her haplotype was the same as that of the "affected" individuals. Several explanations could account for individual 16's status. The most likely possibility is that our arbitrary threshhold for determining affected status, spinal Z(BMD) > 3.0, is too high. Among members of a normal population, only 1/740 have spinal Z(BMD) > 3.0, and only 1/458 have spinal Z(BMD) > 2.85. This is a small difference. Simply reducing the threshhold to include Individual 16 as affected will increase the LOD score to that of the quantitative-trait analysis, but it compromises the rigor of our study in an unacceptable manner, because of the post hoc redefinition of affected status. A second possibility is that individual 16 has the marker haplotype but has a double crossover in the region between markers D11S987 and D11S1314, resulting in deletion of the HBM-gene mutation that is responsible for conferring HBM. Saturation mapping of the region by additional markers ultimately will address this possibility. Inaccurate phenotyping is a third posiblity, but it is excluded when a second spinal Z(BMD) measurement was indistinguishable from the first measurement.

Of particular interest is the mapping of osteoporosis pseudoglioma syndrome (OPS) to this region of chromosome 11 (Gong et al. 1996). OPS is an autosomal recessive trait of juvenile osteoporosis and other complications, the opposite phenotype of the HBM trait. The localization of the HBM and OPS traits to the same region of chromosome 11 raises several possibilities.

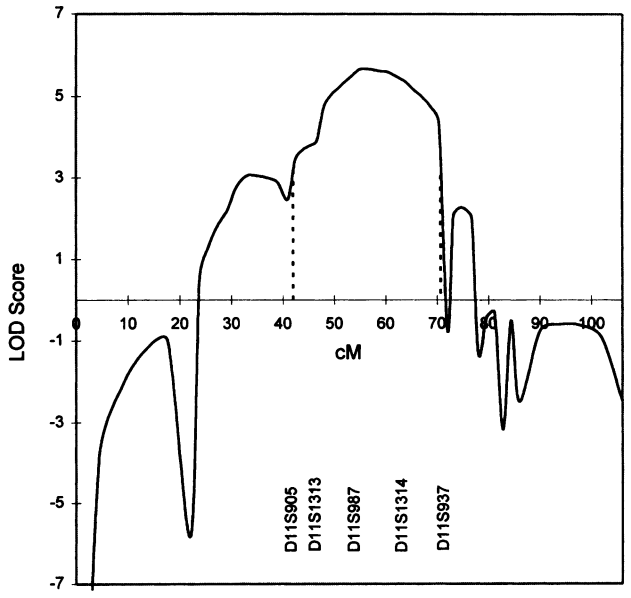

Figure 3 Multipoint map of chromosome 11. A region near marker D11S987 gave the Z_{max} (i.e., 5.74). The 95% confidence interval for the location of the HBM gene is an ~30-cM region demarcated by the dashed lines, that is bounded by markers D11S905 and D11S937.

One is that the traits are caused by allelic variation of the same gene. The mutation for OPS would behave as a recessive (loss-of-function) mutation, causing low bone mass. That for HBM would behave as a dominant (gain-of-function) mutation, causing HBM. A second possibility is that OPS and HBM are caused by separate genes. This would require the existence of two genes regulating BMD in a region representing <1% of the human genome. Identification of the causal gene for both traits will be required in order to answer this question. Of additional interest is whether allelic variation at genetic loci in this region is frequent enough to account for a significant portion of the variance in bone mass in the general population.

Aside from high bone density, this kindred has several additional features of interest. The HBM trait determines *peak* bone mass, because it is manifest in young adulthood (18 years of age), and its influence persists into the 9th decade. The bones of affected members, while appearing very dense radiographically, have normal external shape and outer dimensions. Medullary cavities appear subnormal in size without interference with hematopoiesis. Affected members appear resistant to fracture and live to advanced age without undue illness or disability. None of the members examined so far have neurological symptoms—or symptoms of impairment of any organ or system function—that can be related to the HBM trait. This phenotype matches no other disorder of bone, such as progressive diaphyseal dysplasia, pycnodystosis, or melorheostosis.

Our results, like those of others (Spotila et al. 1996), demonstrate that spinal BMD, a commonly applied, precise, continuous skeletal phenotype used routinely in osteoporosis research, can be used successfully as a quantitative trait for genetic-linkage studies. This kindred presented an unusual opportunity for linkage analysis, because of the extreme nature of the bone-density phenotype in some of its members. Their extreme values for spinal Z(BMD) caused a bimodal distribution that permitted linkage analysis using either a simple affected model or a quantitative-trait model. On the other hand, genetic mapping of *low*-bone-mass traits associated with increased risk of osteoporotic fracture will be more difficult. Low-bone-mass values associated with increased osteoporotic-fracture risk in the normal population do not present as a bimodal distribution. Furthermore, the range for low bone mass is considerably smaller than the range for the HBM trait analyzed here. The bimodal distribution of low BMD seen in seven families (Spotila et al. 1996) does not exist in population-based studies (Looker et al. 1995) that include many persons with osteoporotic fracture.

In conclusion, linkage analysis in this kindred places the HBM gene on chromosome 11, in the region 11q12-13. The identification of the HBM gene may represent a major advance in both the understanding of pathways that regulate bone density and the understanding of the pathogenesis of diseases such as osteoporosis.

Acknowledgments

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