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First-stage autosomal genome screen in extended pedigrees suggests genes predisposing to low bone mineral density on chromosomes 1p, 2p and 4q

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Osteoporosis is characterized by low bone density, and osteopenia is responsible for 1.5 million fractures in the United States annually.[1] In order to identify regions of the genome which are likely to contain genes predisposing to osteopenia, we genotyped 149 members of seven large pedigrees having recurrence of low bone mineral density (BMD) with 330 DNA markers spread throughout the autosomal genome. Linkage analysis for this quantitative trait was carried out using spine and hip BMD values by the classical lod-score method using a genetic model with parameters estimated from the seven families. In addition, non-parametric analysis was performed using the traditional Haseman-Elston approach in 74 independent sib pairs from the same pedigrees. The maximum lod score obtained by parametric analysis in all families combined was +2.08 (θ = 0.05) for the marker CD3D on chromosome 11q. All other combined lod scores from the parametric analysis were less than +1.90, the threshold for suggestive linkage. Non-parametric analysis suggested linkage of low BMD to chromosomes 1p36 (Z_{max} = +3.51 for *D1S450*) and 2p23-24 (Z_{max} = +2.07 for *D2S149*). Maximum multi-point lod scores for these regions were +2.29 and +2.25, respectively. A third region with associated lod scores above the threshold of suggestive linkage in both single-point and multi-point non-parametric analysis was on chromosome 4qter (Z_{max} = +2.95 for *D4S1539* and Z_{max} = +2.48 for *D4S1554*). Our data suggest the existence of multiple genes involved in controlling spine and hip BMD, and indicate several candidate regions for further screening in this and other independent samples.

Keywords: quantitative trait; linkage; sib pair; non-parametric; bone density; osteopenia; osteoporosis; genome screen; family study; genes; skeleton

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Introduction

Skeletal quality is a composite of both the amount of mineral in bone and the microarchitecture of the bone structure. The individual contribution of genetics, lifestyle and environment to the overall quality of the skeleton is difficult to quantify because these three factors interact with one another. For example, genetic factors may strongly influence the biochemical and physiological responses to dietary and lifestyle factors that are known to have an effect on the quality of bone. Since bone mineral density (BMD) can be readily quantified, whilst microarchitecture cannot, most studies have focused on assessing bone quality by determining BMD. BMD measured at a single point in time reflects the peak bone density attained during growth as well as the rate at which mineral is lost from bone during aging.[2–5] The relative contribution of these two processes to an individual's BMD thus depends on age.

Studies of twins have suggested that as much as 75% of the variability in BMD is due to genetic factors.[6–9] Daughters of women with low bone density are more likely to also have lower than normal bone density.[10,11] Family history is a significant predictor of low bone density in both men and women.[12] However, identifying the genetic factors that might influence BMD is a daunting task because of the very large number of genes that could be involved. Numerous studies have demonstrated that mutations that alter glycine codons or otherwise disrupt the normal function of the *COL1A1* or *COL1A2* genes for type I collagen dramatically increase bone fragility and reduce BMD.[13–15] Milder mutations in *COL1A1* may also be associated with low BMD.[16,17] Linkage and association studies have suggested that some other genes that condition BMD are the vitamin D receptor (see[18] for review) and the estrogen receptor.[19] Linkage studies in two disorders, osteoporosis–pseudoglioma syndrome[20] and a high bone mass trait[21] indicate that a gene or genes on chromosome 11q can influence bone density.

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Recently we described seven families in which many individuals have low BMD.[22] Statistical analysis of the distribution of the spinal BMD values[22] and the femoral neck BMD values (Devoto M and Spotila LD, 1996) in these seven families indicated that a bimodal curve fitted the actual BMD distribution better than a unimodal curve ($p = 0.001$). This observation was consistent with the hypothesis that a single major gene predisposed individuals in these kindreds to low BMD. Simulations of linkage for both spinal and femoral neck BMD suggested that six of the families were sufficiently informative to give lod scores greater than +2.00. Preliminary linkage analysis indicated that neither *COL1A1*, *COL1A2*, nor the *VDR* genes were linked to the low BMD trait measured at the lumbar spine.[22] Therefore we performed a genomic wide screen with polymorphic microsatellite repeats in order to identify loci that might be linked to BMD. Our study is the first attempt to identify by linkage analysis loci involved in bone density in extended pedigrees with low BMD, utilizing BMD as a quantitative trait.

Materials and Methods

Families, BMD Measurements, Collection of Material

The seven families in this study, composed of 149 individuals, were ascertained by identification of a proband with a spinal BMD Z-score of < -2.00 or radiographic evidence of osteopenia with pathological fractures and follow-up of those probands who indicated other family members might be similarly afflicted. All participants were examined and had BMD determinations by dual energy x-ray absorptiometry (DEXA) at both the lumbar spine (L2–L4) and the femoral neck.[22] The spinal and hip BMD measurements were adjusted for age, weight and gender by comparison with the normal population database supplied by the bone densitometer manufacturer (Lunar, Madison WI; Hologic, Waltham MA). Thirty-seven individuals had adjusted spinal Z scores < -2.00. A 5–15 ml blood sample was collected from each participating individual for either genomic DNA extraction or lymphocyte culture or both. All subjects gave written informed consent, and the study was approved by the Institutional Review Boards of the participating institutions.

Genotyping Analysis

DNA was extracted from blood directly or from Epstein-Barr virus-transformed lymphoblasts using the Genepure 341 (Applied Biosystems Inc., Foster City, CA, USA). Polymorphic short tandem repeat markers were analyzed by PCR amplification of approximately 100 ng of genomic DNA.[23,24] The 330 PCR primer pairs were purchased from Isogen Bioscience (Amsterdam, The Netherlands) or Research Genetics (Huntsville, AL, USA). PCR conditions were 2–5 pmole of each primer, one of which had been labeled at the 5' terminus with γ^{32}P-ATP, 1.5 mM $MgCl_2$, 250 μM of each nucleotide, and 0.5 units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA) in a reaction volume of 20 μl.

Thermal cycling was performed in a Perkin-Elmer model 9600 thermocycler under optimized annealing conditions and cycle number for each primer pair. The PCR products were resolved on 6% denaturing polyacrylamide gels either with or without formamide (32%) and visualized by autoradiography. Allele numbers were arbitrarily assigned for each marker, and autoradiographs were scored independently by two investigators.

Parametric Lod-Score Analysis

Parametric two-point lod-score analysis was carried out between each marker and spine and hip BMD separately using the MLINK program of the LINKAGE package version 5.1.[25] In this analysis, a single gene with two alleles (one normal and one predisposing to low values of spine or hip BMD) was assumed to contribute to the distribution of the quantitative traits. Parameter values for each quantitative trait used in the lod-score analyses were estimated from the distributions observed in our families as described[22] and were the same as used in the previous simulation analysis. A dominant transmission of each trait phenotype was assumed. The mean values for the age, weight, and gender adjusted measurements (Z-scores) for the normal and susceptible (i.e. predisposing) genotypes were –0.004 and –1.656, respectively (common variance 0.841) for spinal BMD, and +0.998 and –0.787, respectively (common variance 0.618) for hip BMD.

The frequency of the low BMD predisposing allele was assumed to be 0.01 for both hip and spine. This was probably an overestimate of the frequency of any single gene predisposing to low BMD, and as such took into account the possibility that more than one predisposing allele segregated in each family. To test the robustness of the linkage results with respect to disease allele frequency, lod scores were recalculated using a gene frequency of 0.001 for those markers that had lod scores greater than +1.00.

Allele frequencies for each marker were calculated from our family data using the ILINK program of the LINKAGE package. Correct estimation of allele frequencies was particularly important since not all founder individuals were available in our pedigrees. ILINK utilizes information on all available individuals and takes familial relationships into account. This procedure may lead to an overestimate of the frequency of any single marker allele segregating with the trait within each family, but it should not lead to an increase of false positive results. However, false positive results may be increased by an underestimation of the frequency of the trait-associated allele.

Sib-pair Analysis

The traditional Haseman-Elston test[26] based on regression of the squared difference of the sib-pair trait on the proportion of alleles shared identical by descent was carried out separately for spine and hip BMD using the MAPMAKER/SIBS program version 2.0.[27] For the purpose of carrying out sib-pair analysis, the seven pedigrees were subdivided into their nuclear components. There were 30 nuclear pedigrees with at least one sib pair typed (Table 1). Of these, 15 had both parents typed, ten had only one parent typed, and five had no parents available for typing. However, in many cases parental genotypes could be inferred unambiguously from the sibs' genotypes. In the 17 sibships with more than two sibs, only the independent pairs formed by matching the first sib with all the other sibs were included in the sib-pair analysis. For example, only seven of 28 possible pairs from a nuclear pedigree with eight sibs were considered. Together with the 13 pairs provided by the single-pair nuclear pedigrees, there was a total of 74 independent sib pairs.

In order to maximize the amount of information available for each quantitative trait, multiple sibships were ranked within each nuclear pedigree starting from the sib with the lowest BMD Z-score value, according to increasing values of spine and hip BMD, separately. In this way, the sib pair with the lowest concordant values and the sib pair with the most discordant values were always included in the analysis. This has been shown to provide an effective way of pursuing linkage for quantitative traits.[28] Since this procedure was carried out independently for spine and hip BMD, the sib pairs included in the non-parametric analyses of the two traits were not necessarily the same. In particular, for spine there were 18 pairs with Z-scores for BMD less than –2 in both sibs; 32 with Z-scores for BMD less than –2 in one sib only; and 24 with Z-scores for BMD above –2 for both sibs. For hip, these numbers were 5, 22, and 47 respectively.

Two-point sib-pair analysis was carried out for all markers, and multi-point analysis was carried out including all markers from a given chromosome for which information regarding their map location and distance from adjacent markers could be obtained from independent sources such as GDB, CHLC, or Genéthon databases (http://gdbwww.gdb.org; http://www.chlc.org; http://www.genethon.fr/genethon_en.html, respectively). In some instances where no other information on marker location was available, maps provided by Isogen were also used (information supplied by manufacturer). The maps used for each chromosome in the multi-point analyses are available from the authors on request. Marker allele

Table 1 Description of nuclear pedigrees used in sib-pair analysis

	Number of pedigrees with:				
No. of sibs	*2 parents*	*1 parent*	*0 parents*	*Total pedigrees*	*No. of sib pairs*
2	7	4	2	13	13
3	3	4		7	14
4	3	1		4	12
5	1			1	4
6	1	1		2	10
8			3	3	21
Total	15	10	5	30	74

frequencies were the same as used in the lod-score analysis (also available upon request).

Results

Spine and hip BMD were treated as quantitative traits; no assessment was made whether an individual was affected or unaffected. Although the two traits are correlated, we treated them separately because the genetic contribution to BMD at each site varies[7] and the variability in anatomical site in each patient is considerable.[29] The genome screen was performed with 330 polymorphic markers, and the results were initially analyzed using standard two-point lod-score linkage methods for quantitative traits.[25] When results were summed over all the families, only one marker, *CD3D* on chromosome 11, gave a lod score above the threshold of +1.90 for suggestive linkage in parametric analysis ($Z_{max} = +2.08$) at a recombination fraction of 0.05 for the hip trait). In addition, one marker on chromosome 14 (*D14S54*) produced a lod score of +2.14 ($\theta = 0.00$ in a single family, also for the hip trait. All other lod scores, whether summed over all families or observed in individual families, were below the threshold of +1.90. In addition, heterogeneity tests carried out on the results for *CD3D* and *D14S54*, as well as for 27 additional markers for which at least one family had a lod score greater than +1.00, were non-significant.

The lod scores obtained by parametric analysis were not of the magnitude expected on the basis of the simulation study.[22] We decided, therefore, to perform non-parametric analysis using the classical Haseman-Elston test for quantitative traits as implemented in the MAPMAKER/SIBS[27] program. Single-point lod scores above the threshold of +2.20 ($p = 0.00074$) suggestive of linkage in sib-pair analysis were obtained for nine loci (Table 2). Of these nine markers, those located on chromosomes 1 and 4 were separated by less than 15 cM, and therefore can be considered as identifying the same chromosomal region. Different markers for the same region of chromosome 4 were positive in both spine and hip, whereas all other positive results were observed for the hip trait.

Multi-point non-parametric analysis was performed for 200 markers for which reliable information on map location was available. Among the regions that had given positive results with the single-point non-parametric analysis (Table 2), lod scores >+2.20 were observed on chromosome 1 for the hip trait in a region of 10 cM between markers *D1S450* and *D1S228*, with a maximum of +2.29 and on chromosome 4 for the hip trait in a region of 7 cM between *D4S1554* and *D4S1540*, with a maximum of +2.28 (Figure 1). An additional region on chromosome 2 reached a maximum of +2.25 for the spinal trait in a region of 4 cM between *D2S149* and *D2S387*. Marker *D2S149* had a maximum single-point lod score of +2.07, just below the threshold for suggestive linkage. A second broad region of chromosome 2 with a maximum multi-point lod score of 2.05 for the spine trait was identified by a single marker in the single-point non-parametric analysis, *D2S71* with a lod score of +1.72. All other multi-point analyses produced maximum lod scores <+2.00.

Discussion

In this study we have performed an autosomal genome screen for chromosomal regions that may contain genes involved in determining BMD at the spine and the femoral neck in seven pedigrees. The screen included over 300 polymorphic marker loci with an average spacing of 10 cM, although approximately 10% of the intervals were actually greater than 20 cM. In the computations of linkage analysis, BMD was considered as a quantitative trait and thus no designation of affected status or penetrance of the trait was required. Parametric linkage analysis was based on our initial model of a single major gene that was dominantly inherited in each of the seven families. Previous simulations of linkage under this model had indicated that five of the seven families could give lod scores greater than +2.00 for both of the traits.[22] However, actual linkage analysis gave only two lod scores greater

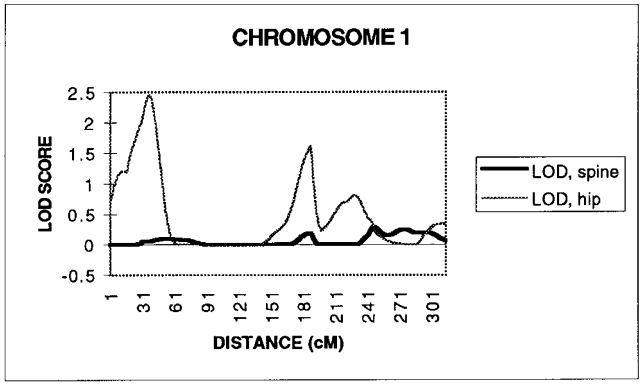
Table 2 Results of single-point Haseman-Elston sib-pair analysis. All markers with lod scores (Z_{max})>+2.20 are presented

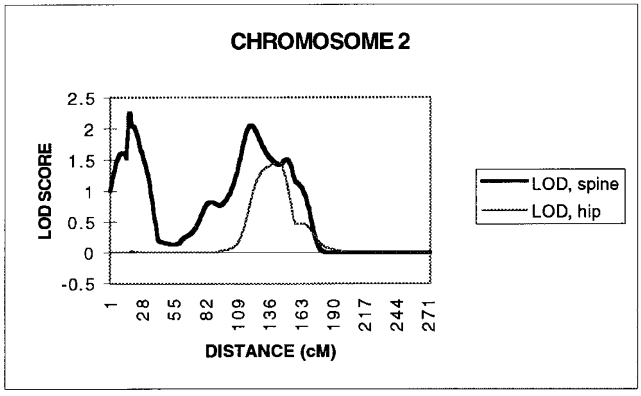
		Z_{max}	
Marker	*cM*[a]	*Spine*	*Hip*
D1S450	13.9	0.33	3.51
D1S214		0.00	2.62
D4S1539	14.4	2.95	0.14
D4S1554	2.9	0.32	2.48
D4S1535		0.09	2.74
D7S558		0.01	2.99
D17S261		0.21	2.34
D18S42	30	0.84	2.58
D18S70		0.17	2.14

[a]Distance from next marker in Table if less than 40 cM.

than +2.00 for the hip trait, suggesting that parametric linkage analysis had little power to identify BMD quantitative trait loci in these families.

We then performed non-parametric analysis on the 74 independent sib pairs that comprised the seven families. Three chromosomal regions were supported by lod scores above the threshold of suggestive linkage, > +2.20.[30] The first of these regions, 1p36, was identified with two marker loci separated by 13.9 cM (*D1S450* and *D1S214*) that gave lod scores in the single-point non-parametric analysis of +3.51 and +2.62, respectively, for the hip trait. The first value is close to the threshold for significant linkage of +3.60, corresponding to a genome-wide p-value of 0.00002.[30] The second region to receive support from non-parametric analysis was 2p23-p24. Here, markers *D2S149* and *D2S144* gave lod scores of 2.07 and 1.49, respectively, for the spinal trait. Multi-point non-parametric analysis based on all of the mapped markers resulted in an increase from +2.07 to +2.25 for this region of chromosome 2. The third chromosomal region (4q32-34) gave lod scores in the non-parametric analysis greater than +2.50 for several markers for both the spinal and hip traits.





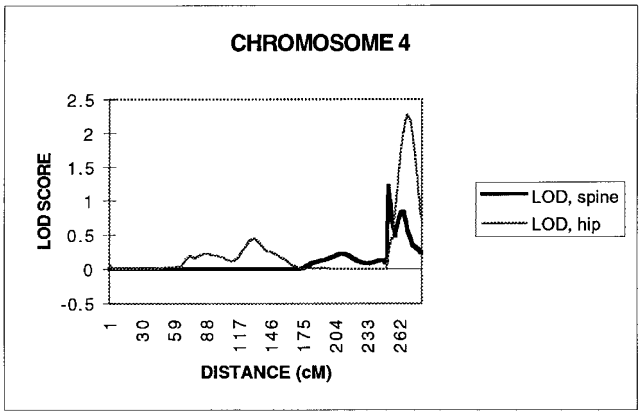


Figure 1 *Multi-point analysis for spinal and hip BMD for chromosomes 1, 2 and 4. Chromosome 1:* Z_{max} *for hip trait = +2.29 at 36 cM from 1pter; chromosome 2:* Z_{max} *for spine trait = +2.25 at 17 cM from 2pter; chromosome 4:* Z_{max} *for hip trait = +2.28 at 4qter*

Are there any candidate genes in the region that we have identified? Located within 1p36 is the gene for lysyl hydroxylase (*PLOD*), an enzyme required for the hydroxylation of specific lysines in type I collagen and subsequent glycosylation and cross-linking of those lysines in formation of the collagen fiber.[31,32] Also located in this interval is the gene for tumor necrosis factor α receptor 2 (*TNFR2*), that may have a role in osteoclast physiology.[33] However, there are 14 additional genes and 22 cDNA sequences (ESTs) within this interval (Human Transcript Map http://www.ncbi.nlm-.nih.gov/SCIENCE 96/). The interval 2p23-24 most likely contains a serine-threonine kinase that is expressed by cancellous bone osteoblasts in culture (Genbank Accession number: D87119), but again there are over 100 other cDNAs mapping to this region.

Two groups have previously reported lod scores suggestive of linkage of bone density to chromosome 11q[20,21] with a maximum at the locus *D11S987*. This marker is located near the centromere, probably within 11q12. We also analyzed this marker, but obtained lod scores less than +1.00 by both parametric and non-parametric methods. However, for *CD3D*, a marker located about 50 cM distal to *D11S987*, we obtained a lodscore of +2.08 ($\theta = 0.05$) over all seven families, suggesting that a broad region of 11q may be involved in BMD.

It is also worth noting that the marker *D7S558*, for which we obtained a lod score of +2.99, is within 3 cM of *COL1A2*, but that *D17S261*, for which we obtained a lod score of +2.34, is located on 17p and is therefore not close to *COL1A1*.

Proving linkage to a quantitative trait locus (QTL) in humans is difficult. In a simulation study, a locus explaining 33% of the total variance of a quantitative

trait produced only weak evidence of linkage (lod score 2.50) in a sample of 1000 unselected sib pairs.[27] An actual study of four quantitative variables associated with asthma carried out using the same methodological approach as the present study, identified five potential loci with associated p values of less than 0.0005 (corresponding to a lod score of 2.6 or more) using a sample of 172 sib pairs.[34] Another study of serum leptin levels (a quantitative indicator of obesity) using a variance component approach identified a single locus with a lod score of 4.95 in a sample of 458 individuals from ten Mexican–American families.[35] In contrast to these two studies, our sample size was small. In addition, our sib pairs were not necessarily selected for having extreme low or high BMD values compared to the general population, although the most discordant sib pair was always included in the non-parametric analysis. Nonetheless, we have identified three regions with suggestive linkage as indicated by results of non-parametric multi-point linkage analysis (chromosomes 1, 2, and 4). We are extending this study by performing a genome-wide screen on an independent sample of sib pairs, and are using additional marker loci in the original sample to further delimit selected chromosomal intervals.

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