

Genes Influencing Variation in Serum Osteocalcin Concentrations Are Linked to Markers on Chromosomes 16q and 20q*

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ABSTRACT

Osteocalcin (OC) is an important constituent of bone that is synthesized by osteoblasts. Serum levels of OC have been used as a biochemical marker of bone turnover. To identify the genes influencing variation in serum OC levels, we conducted a genome-wide scan in 429 individuals comprising 10 large multigenerational families. OC levels were measured by immunoassay, and genetic markers were typed at approximately 10-cM intervals across the genome. Quantitative trait linkage was tested using a multipoint analysis based on variance component methodology, adjusting for the effects of age, sex, and oral contraceptive use. Significance levels for linkage were obtained empirically, by Monte Carlo simulation.

The heritability of OC levels in this population was $62 \pm 8\%$. We detected significant evidence for linkage between a quantitative trait locus influencing serum OC levels and markers on chromosome 16q, and suggestive evidence for linkage of OC levels with markers on chromosome 20q. The multipoint lod scores peaked at 3.35 on chromosome 16 and 2.78 on chromosome 20, corresponding to *P* values of 0.00004 and 0.00017, respectively. A potential candidate gene for bone formation in the linked region on chromosome 20 is *CDMP1*, which encodes cartilage-derived morphogenetic protein 1. Future studies should evaluate whether variation in *CDMP1* or in other genes in the linked regions on chromosomes 16 and 20 influence the rate of bone turnover. (*J Clin Endocrinol Metab* 85: 1362–1366, 2000)

LOW BONE MASS in adult life may occur as a result of either achievement of a suboptimal peak bone mass or an accelerated rate of bone loss. Twin and family studies have established that genes explain a substantial proportion of the variation in peak bone mass (1–5). Because bone mass reflects the overall balance between bone formation and bone resorption, genetic effects on bone mass may be mediated through genes affecting bone remodeling. Identification of the specific genes involved may provide important insights into bone biology and the pathogenesis of osteoporosis.

Serum concentrations of osteocalcin (OC) have been used as a biochemical marker of bone turnover. Serum OC, also known as bone Gla protein, is a 49-amino acid protein synthesized by osteoblasts (6). The majority of secreted OC is incorporated into bone, where it becomes incorporated into the bone matrix (7). A fraction of newly synthesized OC is also released into circulation, where it can be detected by immunoassay. Additional evidence suggests a role for OC in the recruitment and differentiation of osteoclast precursors at the bone surface (8, 9). Serum OC levels are increased in children and young adults compared with older individuals

(7) and are also elevated in conditions associated with rapid bone loss (10) and excessive bone turnover (6, 7, 11, 12).

Several studies have evaluated the impact of genetic factors on serum OC levels. Kelly *et al.* (1) reported that variation in OC levels among female twin pairs was substantially smaller in monozygotic compared with dizygotic twins, and from these results they estimated that genetic factors accounted for 82% of the total variance in circulating OC levels. Somewhat lower estimates were obtained by Garnero *et al.* (13), who reported a heritability of 37% in their study of postmenopausal twin pairs. Few, if any, studies have estimated the heritability of OC levels from large family-based samples, especially ones including both men and women.

Despite the important role that genetic effects are believed to play in bone turnover, the individual genes that mediate these effects are not known. With the exception of one early study that reported an association between polymorphisms in the vitamin D receptor and serum OC levels (14), no other single gene effects on OC levels have been reported.

The aim of our study was to identify, using quantitative trait linkage analysis, chromosomal regions that might contain genes influencing variation in serum OC levels in a randomly ascertained set of families. To accomplish this aim, we conducted a genome-wide scan using markers spaced at a 10 cM average density throughout the genome.

Subjects and Methods

Families used in this analysis were identified through The San Antonio Family Heart Study (SAFHS), a population-based family study designed to identify the genetic determinants of atherosclerosis and its

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risk factors (15). Using a house-to-house recruitment procedure, we identified 40- to 60-yr-old residents with large families from low income neighborhoods in San Antonio, Texas, and invited them and their first-, second-, and third-degree relatives to participate. The invitation to participate was extended regardless of the probands' (or relatives') medical history. A total of 1431 nonpregnant individuals from 41 families were recruited into the baseline phase of the SAFHS. Extensive genotyping has now been completed on 471 of these individuals, comprising our initial set of 10 large families. The present study is based on these 471 subjects.

Participants received a medical examination in our clinic in the morning following a 12-h fast. By interview, we obtained information about demographics, dietary habits (including history of smoking and alcohol consumption), current medication use, and for women, reproductive history. Women were classified as postmenopausal if they reported having had both ovaries removed or if at least 12 months had elapsed since their last menstrual period. To our knowledge, there was no history of metabolic bone disease or hyperthyroidism in these subjects since the subjects were members of "randomly" ascertained families, although our survey did not specifically inquire about these conditions. Blood samples were obtained using standard venipuncture technique for DNA processing and measurement of blood chemistries. Serum was separated from clotted blood by centrifugation and then stored at -80°C until assayed. Diabetes was defined according to the plasma glucose criteria of the World Health Organization (16).

Serum concentrations of intact OC were measured in duplicate with the NovoCalcin(r) assay (Metra Biosystems, Mountain View, CA), an enzyme immunoassay in a microtiter stripwell format using a murine monoclonal anti-OC antibody (17). In our laboratory, the interassay coefficient of variation for serum OC is 6.6%.

DNA was isolated from lymphocytes for PCR and automated genotyping. The DNA was amplified with fluorescently labeled primer pairs from MapPairs Human Screening Set Version 6 (Research Genetics, Inc., Huntsville, AL) that detect highly polymorphic microsatellite markers. PCR reactions were performed according to the manufacturer's protocol. Aliquots of the PCR reactions were pooled into multiplexed panels for genotyping with PE Applied Biosystems (Perkin-Elmer Corp., Foster City, CA) Model 377 DNA Sequencers and Genescan and Genotyper DNA Fragment Analysis software.

A total of 376 microsatellite markers from 22 autosomes were included in the analysis. The mean (\pm SD) heterozygosity of these markers in this population was 0.74 ± 0.08 . The distances between markers were computed from our data using CRI-MAP(18). The average spacing between markers was 10.0 cM, and the largest spacing was 27 cM (on chromosome 9).

Heritability of serum OC levels was defined in the "narrow sense," as the proportion of the total trait variance attributable to the additive effects of genes. Generally speaking, heritability is estimated from the degree of resemblance between relatives and corresponds to the observed correlation between relatives expressed as a proportion of the correlation that would be found if the trait were completely inherited (19). In pedigree structures, the covariances among different classes of relatives are generally not independent, and we, therefore, applied the method of maximum likelihood to obtain a pedigree-based estimate of heritability (20, 21). This method is more efficient than classical quantitative genetic methods that are based on pairs of relatives since all pedigree information is considered jointly. Briefly, estimates are obtained by modeling the observed covariances between two individuals within a pedigree as having an expected value given by the product of their coefficient of relationship (which is equal to two times their kinship coefficient), the heritability, and the phenotypic variance of the trait (conditional on covariate effects). Based on this simple model, the likelihood of the pedigree data is computed assuming multivariate normality as a working model. Parameter estimation is performed by finding those values of the parameters (including the heritability) that yield the maximum ln likelihood, which is summed over all pedigrees.

We tested for linkage with the genetic markers using a multipoint linkage analysis procedure based on variance component methodology (22). In this approach, the expected genetic covariances between relatives is modeled as a function of the probability that the relatives have inherited both alleles at the marker locus from a common ancestor. The total phenotypic variance is then partitioned into effects due to covariates, effects of a specific locus, and residual additive genetic effects. The

variance attributable to the quantitative trait locus (QTL) is parameterized by the probability of allele sharing (*i.e.* the identity-by-descent matrix) at a specific locus, whereas the variance attributable to the residual heritability is parameterized as a function of the relationship among individuals (*i.e.* the kinship matrix). The hypothesis of linkage is evaluated by testing whether the variance attributable to the QTL is significantly greater than zero. Model parameters are obtained using maximum likelihood estimates, and the likelihood of the pedigree data is compared for nested models using the likelihood ratio test. For multipoint analysis, the probability of allele sharing was estimated at arbitrary chromosomal locations (*e.g.* at 1-cM intervals across the chromosome) using the multipoint approximation approach of Fulker *et al.* (23), generalized for use in extended families. All analyses were conducted using the SOLAR software package (22).

We derived the distribution of nominal logarithm of odds (lod) scores under the null hypothesis of no linkage empirically by Monte Carlo simulation. To generate this distribution, we simulated an unlinked marker locus with five equiprobable alleles, assigned genotypes to each founder, and then dropped genotypes down through the pedigree based on Mendelian expectations and the founder genotypes. The simulated unlinked marker had approximately the same information content (*i.e.* heterozygosity $\approx 80\%$) as the markers used in the genome scan. We then conducted linkage analysis of serum OC levels with the simulated unlinked marker. The unlinked marker locus was simulated using the PAP4 software (24), and the linkage analysis on each simulated data set was carried out using the SOLAR software program (22). We conducted 20,000 replicates and defined the probability of obtaining a false positive result as the proportion of replicates for which we obtained a specified lod score or higher. These probabilities were converted into lod scores, and it is these simulation-derived lod scores that are presented.

We initially performed a single locus analysis, estimating the QTL effect (*i.e.* lod score) at each 1-cM interval along the chromosome. We then performed an oligogenic (or multilocus) analysis, including in the analysis all loci for which the initial QTL effect achieved at least marginal levels of statistical significance (*e.g.* a lod score of 2.0 or greater). The locus with the highest lod score was included in the model first, and then the analysis for the second locus was repeated, conditional on the first locus (and covariates) being included in the model. The conditional lod score was calculated as the difference in likelihoods between the two-loci model and the one-locus model.

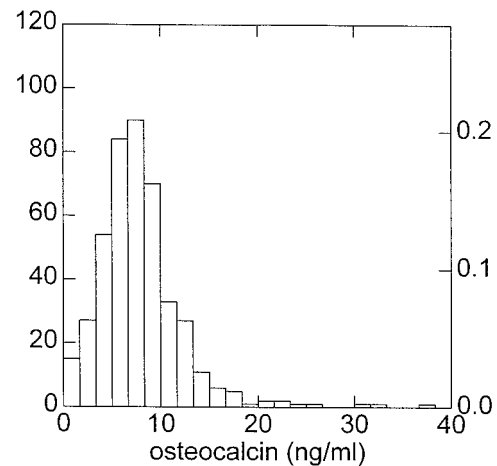


FIG. 1. Distribution of serum OC levels in the population.

TABLE 1. Mean (\pm SD) concentrations of serum osteocalcin according to age and sex

Age	Men	Women
16–30 yr	10.64 \pm 5.44 ng/mL (75) ^a	7.95 \pm 3.37 ng/mL (100)
31–44 yr	7.00 \pm 3.03 ng/mL (43)	5.76 \pm 2.94 ng/mL (69)
45–60 yr	6.97 \pm 4.52 ng/mL (47)	7.08 \pm 3.28 ng/mL (53)
≥ 61 yr	8.85 \pm 8.88 ng/mL (18)	8.89 \pm 3.45 ng/mL (27)

^a Number of subjects in parentheses.

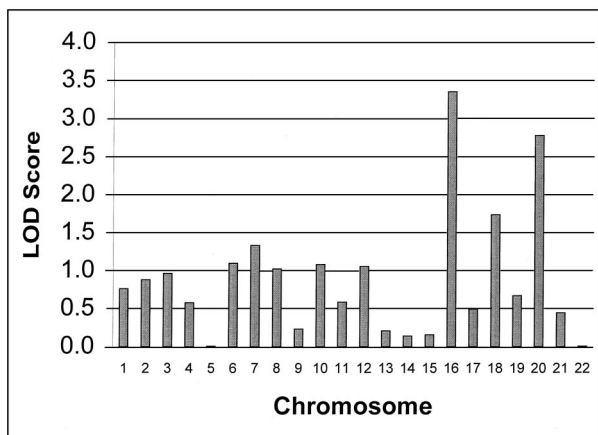


FIG. 2. Genome scan of OC concentrations: peak multipoint lod score by chromosome.

Chromosome 16

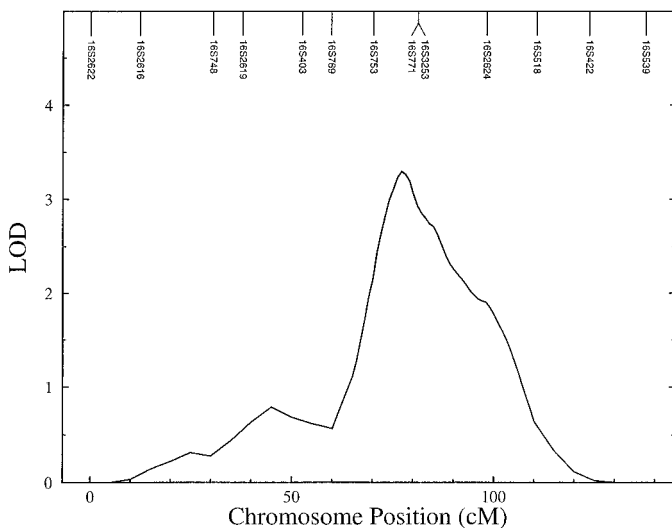


FIG. 3. Multipoint linkage analysis of OC levels with chromosome 16 markers.

Results

Serum OC concentrations were measured in 432 subjects, ranging in age from 16–94 yr. Values ranged from 0.45 ng/mL, the lower limit of detection of this assay, to 38.0 ng/mL. Mean values (\pm SD) were 8.67 ± 5.45 ng/mL and 7.26 ± 3.39 ng/mL in men and women, respectively. Eight individuals had serum OC measurements that were greater than 3 SD from the mean. To reduce the influence of these tail observations on the linkage analysis, we used a Huber-like transformation (25), in which we down-weighted these observations by replacing their values with the value corresponding to the 3-SD cutoff (*i.e.* 21.15 ng/mL). The distribution of measured OC values (*i.e.* before transforming the eight extreme values) in the population is shown in Fig. 1. The additive genetic heritability of serum OC in this population was $62 \pm 8\%$.

The distribution of serum OC according to age and sex is

Chromosome 20

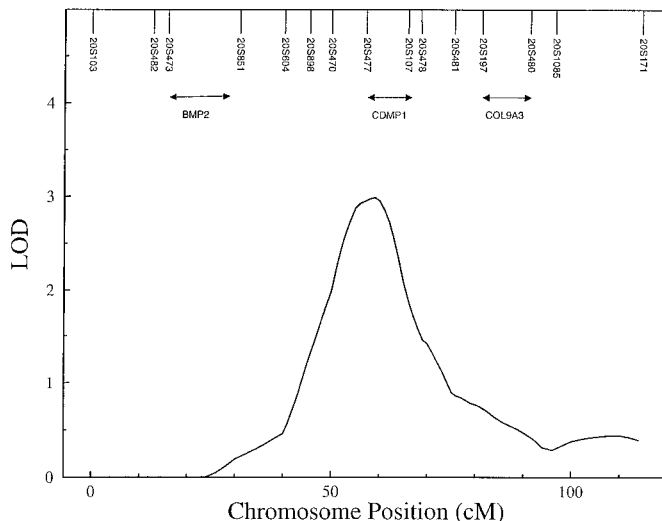


FIG. 4. Multipoint linkage analysis of OC levels with chromosome 20 markers. *BMP2*, Bone morphogenetic protein 2; *CDMP1*, cartilage-derived morphogenetic protein 1; *COL9A3*, collagen, type IX, α 3.

shown in Table 1. In both men and women, the distribution of serum OC was U-shaped with respect to age, that is OC levels were higher in subjects younger than 31 yr and older than 60 yr than in subjects between the ages of 30 and 60 yr. In general, women had lower OC levels than men before age 45, at which point the difference between the sexes disappeared.

Using likelihood methods, we evaluated the effects of diabetes, educational status, smoking and alcohol consumption habits, menopausal status, and oral contraceptive use on serum OC levels while adjusting for the effects of age, age squared, and sex. There were no significant effects of any of these variables, with the exception of oral contraceptive use. On average, OC levels were 1.58 ng/mL lower in women who currently used oral contraceptives ($n = 27$) than in those who did not ($P = 0.011$).

Sex, sex-specific age terms (both age and age²), and oral contraceptive use were included as covariates in the linkage analysis. Information on oral contraceptive use was unavailable for three women, and so the linkage analyses were, therefore, based on 429 subjects. Fig. 2 shows the maximum lod scores observed on each chromosome. The highest lod score observed was 3.35, occurring on chromosome 16q, approximately 77 cM from pter. The second highest lod score was 2.78, occurring on chromosome 20q, approximately 58 cM from pter. The *P* values corresponding to these lod scores were 0.00004 (at chromosome 16q) and 0.00017 (at chromosome 20q). We detected only one other lod score as high as 1.50 elsewhere in the genome, with that occurring on chromosome 18p (lod = 1.74), approximately 24 cM from pter.

Figs. 3 and 4 show the multipoint lod scores for serum OC on chromosomes 16 and 20, respectively. On chromosome 16, peak evidence for linkage occurred in the interval flanked by markers D16S753 and D16S771 in cytogenetic band 16q11.1–16q13. The one lod unit support interval encompassed a 19-cM region extending from 70 cM from pter to 89 cM from

pter (16q11.1–16q22.1). The simulation-derived two-point lod scores for the markers in this region were: D16S769 (lod = 0.0), D16S753 (lod = 0.94), D16S771 (lod = 0.84), D16S3253 (lod = 0.44), D16S2624 (lod = 2.05), and D16S518 (lod = 0.13). On chromosome 20, peak evidence for linkage occurred between markers D20S477 and D20S107 in the region of 20p11.1–20q13.12. Fig. 4 also shows the locations of three bone formation candidate genes on chromosome 20 (see *Discussion*). The one lod unit support interval extended for 15 cM from 50–65 cM from pter (20p11.21–20q13.12). The two-point lod scores for the markers in this region were: D20S898 (lod = 0.32), D20S470 (lod = 1.12), D20S477 (lod = 2.30), D20S107 (lod = 0.70), D20S478 (lod = 0.48), and D20S481 (lod = 0.02).

We then performed a two-loci analysis to determine the effect of the chromosome 20 locus conditional on the linked chromosome 16 locus being in the model. Even after including the chromosome 16 locus in the model, adding the chromosome 20 locus significantly increased the likelihood of the data ($P = 0.0035$ by likelihood ratio test). Taken together, the loci on chromosomes 16 and 20 accounted for 41% and 29% of the residual variance in serum OC levels, respectively, after simultaneously adjusting for the effects of age, age², sex, and oral contraceptive use.

Finally, we conducted a second simulation study to evaluate the power to detect linkage in this set of families. For this study, we simulated a two-allele QTL that accounted for specific proportions of the total phenotypic variation in serum OC levels (ranging from 15–30%), and then dropped a tightly linked (recombination value = 0%) highly polymorphic marker down through the pedigrees. The total trait heritability was assumed to be 60%, and so the amount of the genetic variation not accounted for by the QTL was partitioned into a residual heritability. In our sample, the power to detect a QTL effect (*i.e.* lod score = 3.0) that accounted for 20% of the total trait variability was 33%. For a QTL accounting for 25% of phenotypic variation, the power was 59%, and the power to detect a QTL accounting for 30% of phenotypic variation was 85%.

Discussion

Serum OC levels are highly heritable in this Mexican American population, as they are in others (1, 13). What the genetic determinants of serum OC might be or in what pathways they might act is not clear. For example, at younger ages serum OC might predominantly reflect skeletal development events and serum OC levels might be strongly influenced by genes associated with bone formation. However, at older ages serum OC may predominantly reflect bone turnover rates and OC levels may be strongly influenced by genes affecting osteoblast or osteoclast function. In addition, at all ages there may be additional genetic influences on degradation of serum OC.

Our results suggest that among the genes influencing variation in serum OC levels may be two located on chromosomes 16 and 20. On chromosome 16, the peak lod score was 3.35, occurring in the interval flanked by markers D16S753 and D16S771 in a region that maps to 16q11.1–16q22.1. The peak lod score on chromosome 20 was 2.78, occurring in the

interval flanked by markers D20S477 and D20S107 in a region that maps to 20p11.21–20q13.12. Our statistical approach for lod score estimation was robust not only to outliers, since we down-weighted the influence of extreme observations, but also to violation of the multivariate normality assumption, since P values and lod scores were based on an empirical null distribution generated by Monte Carlo simulation. For only one of the 20,000 unlinked markers that we simulated did we obtain evidence for linkage as high as that which we observed on chromosome 16q.

The human OC gene has been mapped to chromosome 1q21–q23 (26, 27). One recent case-control study has reported an association between allelic variation at an anonymous marker tightly linked to the OC gene and bone mineral density, although the relationship between this polymorphism and OC levels was not reported (27). In our data, there was no evidence for linkage of OC levels to any markers in this region (multipoint lod = 0.30).

There are no obvious candidate genes that might potentially influence bone turnover in our region of linkage on chromosome 16. In contrast, there is one potential candidate gene for bone formation whose location coincides very closely with our region of peak linkage on chromosome 20. This gene (*CDMP1*) encodes cartilage-derived morphogenetic protein 1, a protein that is closely related to bone morphogenetic proteins 5–7 (28). Like the bone morphogenetic proteins, *CDMP1* is also a member of the transforming growth factor β superfamily, a large group of signaling molecules that mediate a variety of activities, including the induction of bone and cartilage formation (28, 29). Allelic mutations in *CDMP1* that result in reduced or absent activity of the protein are associated with inherited syndromes of acromesomelic dysplasia, characterized by skeletal abnormalities restricted to the limbs and limb joints (30, 31). *CDMP1* falls within the interval flanked by markers D20S477 and D20S107 on our map, making its likely position within 5 cM of our peak signal for linkage. There are, in addition, at least several other bone turnover candidate genes that have been mapped to chromosome 20, including bone morphogenetic proteins 2 and 7 (*BMP2* and *BMP7*), and collagen, type IX, α 3 (*COL9A3*). However, *BMP2* maps to the region of 20p12, approximately 30–40 cM to the pter side of our region of peak linkage, whereas *COL9A3* maps to 20q13.3, approximately 20–30 cM to the qter side of our region of peak linkage. Although *BMP7* has been assigned cytogenetically to chromosome 20, to our knowledge it has not yet been localized.

To our knowledge, our study represents the first genome-wide scan undertaken to identify regions containing genes influencing variation in serum OC levels. However, several previous studies have been carried out to investigate whether polymorphisms in selected bone formation candidate genes are associated with variation in serum OC levels. Morrison *et al.* (14) reported a significant association between OC levels and variants in the Vitamin D receptor gene (on chromosome 11), although later studies have failed to replicate this finding (32–35). Association studies of serum OC levels and gene variants have been performed for several other candidate genes, including type I collagen α 1 gene (chromosome 17q) (36), the estrogen receptor α gene (chromosome 6q) (37, 38), and the transforming growth factor β

I gene (chromosome 19q) (39), although few, if any, associations have been reported. We detected no evidence for linkage to any of these gene regions in our analysis. It is possible that the relative effects of different genes on phenotypic variation differ among populations, and results (including linkage) obtained from our Mexican American population may not be generalizable to others.

In our analysis, we parameterized the genetic effects on OC as additive genetic effects. However, the basic variance components model for linkage can be extended to incorporate parameters that account for additional sources of genetic variation, such as dominance effects, where the genotypic mean of heterozygotes does not fall halfway between that of the two homozygotes (22). In practice, however, estimation of dominance effects requires a large number of sib-pairs because this is the only type of relative pair that has the potential for sharing both zero and two alleles identical-by-descent. We did not incorporate dominance QTL effects in our model because of the low power to detect such effects in our sample. However, if dominance effects do exist at the QTL, then failure to account for them should underestimate the evidence for linkage.

In summary, our study provides evidence that genes influencing serum OC levels may be located on chromosomes 16 and 20. *CDMP1*, which has been mapped to the region of 20q11.2, is believed to play a role in bone formation, and its location corresponds to our region of peak linkage on chromosome 20. Further investigation of *CDMP1* and other genes in these regions may enhance our understanding of the factors influencing bone remodeling.

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