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Cortical bone health shows significant linkage to chromosomes 2p, 3p, and 17q in 10-year-old children

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Introduction

Genetic studies of bone mass, bone mineral content (BMC), and/or bone mineral density (BMD) have demonstrated these measures to be highly heritable [1–11]. As substantial evidence for an additive genetic contribution to adult bone mass and risk for osteoporosis has now been well established, genetic research in this area has moved increasingly toward the identification of genomic regions or candidate genes influencing variation in such traits [12–14]. Recent genetic linkage and genome-wide association studies of bone mass in adults have identified regions on at least 11 separate chromosomes [7,15–18] potentially harboring genes that play a role in regional or total body BMD [12,18–25].

It is evident that researchers are closing in on specific genes influencing the maintenance or loss of adulthood bone mass. Research into childhood skeletal genetics, however, is less abundant and generally focuses on genes underlying specific bone disorders. This paucity of information regarding the genetics of pediatric skeletal growth is unfortunate given that the health of a child's skeleton, and the attainment of peak bone mass in young adulthood, is vital to the health of the adult skeleton. Further, the genetic underpinnings of bone accrual during childhood may differ from that of bone maintenance and/or loss during adulthood. This paper presents the results of genome-wide linkage analyses of measures of childhood cortical bone health.

Materials and methods

Study Sample

The study sample is comprised of a subset of 600 individuals participating in the Fels Longitudinal Study, the world's longest-running longitudinal study of human growth,

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development, and body composition change over the life span [26]. Subjects are primarily Caucasian and live (or lived at the time of evaluation) in southwest Ohio or immediately surrounding regions. Children represented in this sample are considered normal and healthy and were not selected for any disease or bone related trait. Specifically, we examined hand-wrist radiographs of these 600 individuals (317 boys, 283 girls) taken when they each were 10 years old. The majority of children were within 2 weeks of their 10th birthday at the time of radiography. It is important to emphasize that although individuals in this study span up to three generations (see below), all individuals were sampled at the same chronological age. This single age was chosen in order to minimize variation in bone measurements that could be attributed to age or growth status, and to minimize sex differences that normally arise with puberty. In doing so, we maximize the ability to detect genetic involvement in the study traits. Boys and girls were analyzed together in all genetic analyses (all bone measures were shown to have no significant differences between the sexes; Duren et al.[9]).

Because of the longevity of the Fels Longitudinal Study and the participation of individuals from several dozen large nuclear and extended families, the study sample of 600 predominantly white individuals from 144 families contains many relative pairings spanning up to three generations. Median family size for this sample is 4, the mean family size is 6, and the maximum number of individuals examined in a single family is 59. There are 2,925 relative pairings among the 600 individuals in the study sample. Pairings range from 1st degree relatives (e.g., sibling and parent-offspring pairs) to 8th degree relatives (e.g., 3rd cousins twice removed).

Bone Measurements

Radiographic measurements of a cylindrical bone such as the second metacarpal are generally more reliable than those taken from less cylindrical bones [27]. Because bone tissue is relatively evenly distributed around the circumference in the second metacarpal, slight variation in radiographic positioning does not have a large effect on metrics. Additionally, measures of cortical bone are less affected by short-term temporal fluctuation in bone mass caused by seasonal variation and weight changes than measures of BMD or BMC from dual energy x-ray absorptiometry (DEXA) [28].

Quantitative measurements of bone mass from radiographs were obtained from the second metacarpal in each individual according to standard methods [29]. Bone diameter, medial cortical thickness, lateral cortical thickness, and medullary diameter were each measured in millimeters on the second metacarpal (see Duren et al.[9]). All measurements were taken directly from original radiographs using digital Mitutoyo calipers with direct computer input. Two additional variables were derived from these measures: 1) total cortical thickness, calculated as the sum of medial and lateral cortex; and 2) cortical index (CI), calculated as total cortex/bone diameter [after 30]. Sample-wide coefficients of variation ranged from 11.8 to 15.5% [9].

Genotyping and the Whole Genome Linkage Map

Whole genome marker genotyping—Of the 600 individuals sampled in this study, 440 were genotyped for ~400 highly polymorphic genetic markers on autosomes using the ABI PRISM Linkage Mapping Set Version 2 (Applied Biosystems, Foster City, CA). This mapping set consists of fluorescently labeled PCR primers that amplify dinucleotide repeat microsatellite loci (STRs) selected from the Genethon human linkage map[31–33]. The ABI PRISM Linkage Mapping Set-MD10 consists of 400 markers. The set is designed to create a map with markers spaced on average 10cM apart (range 2.4 to 24.1 cM).

Genotyping error checking—Genotypes assigned to individuals were checked for Mendelian consistency using the PEDSYS[34] program INFER. Discrepancies were initially referred to the lab for re-typing of markers in order to resolve them. If discrepancies could not be resolved by re-typing, the program SimWalk2 [35] was used to make decisions about the appropriate genotypes to exclude in that situation.

Estimation of allele frequencies—Estimates of allele frequencies for each genetic marker locus were obtained using Sequential Oligogenic Linkage Analysis Routines (SOLAR) [36]. The linkage map was based on the deCODE map with non-deCODE markers added by interpolation using physical location.

Statistical and Genetic Analyses

We used a maximum likelihood-based variance decomposition approach implemented in SOLAR [36] to estimate heritability for each skeletal variable and to conduct multipoint linkage analysis of each skeletal trait. Univariate linkage analyses were performed to locate areas of the genome that harbor genes (quantitative trait loci, QTL) that influence measures of second metacarpal morphology. To examine QTL linked to our skeletal traits, the covariance matrix (Ω) for a pedigree is:

$$\Omega=\Pi\sigma^2_q+2\Phi\sigma^2_a+I_n\sigma^2_e$$

where σ^2_q is the genetic variance in the trait due to the QTL, Π is a matrix whose elements provide the number of genes that a relative pair share IBD at that QTL that is linked to a genetic marker locus, σ^2_a is the genetic variance due to additive genetic factors, Φ is the kinship matrix, σ^2_e is the variance due to individual-specific environmental effects, and I is an identity matrix.

The hypothesis of linkage was tested by comparing the likelihood of a restricted model in which σ^2_q (the genetic variance due to the QTL) equals zero (no linkage) to a model in which σ^2_q is estimated. The difference between these two $\log_{10}$ likelihoods produces a LOD (log-odds) score that is the equivalent of the classical LOD score of linkage analysis [38].

To control for the genome-wide false positive rate, we calculated genome-wide p-values for each LOD score using a modification of a method suggested by Feingold et al. [39] that takes into account pedigree complexity and the finite marker density of the linkage map. Accordingly, our threshold for significant evidence of linkage (corresponding to genome-wide $\alpha = 0.05$) was LOD = 2.87, while suggestive evidence of linkage occurred at LOD=1.67. These values correspond to the expected false positive rates of once per 20 ("significant") and once per 10 ("suggestive") genome-wide linkage screens [40].

The power to detect genetic effects was improved by accounting for environmental contributions to the phenotypic variance. Prior to all analyses, we used likelihood ratio tests to screen each of the following covariates for significant mean effects on the second metacarpal cortical variables: age, sex, age^2, age × sex, age^2 × sex, and skeletal age.

Results

Basic quantitative genetic analysis using the complete data set of 600 phenotyped 10-year olds revealed significant ($p<0.001$) additive genetic components to the total variance for all skeletal variables examined. Heritability (h^2) estimates ranged from 0.45 ±0.11 (for lateral cortical thickness) to 0.71 ±0.10 (for cortical index). Details and discussion of these results have been published previously [9]. In addition to these variables measured at the narrowest

location on the metacarpal shaft, cortical index was also recorded and analyzed at the midshaft location of the metaphysis (heritability estimate for this trait was 0.61 ±0.11).

Whole genome linkage analyses in the sub-sample of 440 genotyped 10-year-olds revealed several chromosomal regions harboring genes influencing childhood skeletal variation. Significant QTL (LOD > 2.87) were identified for three of the bone traits (see Table 1).

Significant linkages were identified on chromosomes 2p, 3p, and 17q for the traits medial cortex, lateral cortex, and cortical index, respectively (see Figure 1). The location of the linkage peak for medial cortical thickness (LOD = 3.86) is near marker D2S319, which corresponds to chromosomal region 2p25.2. The 1-LOD interval of this linkage spans an area 6.6Mb wide starting at the pter end of the chromosome. This QTL was estimated to account for 58% of the detected residual phenotypic variance in second metacarpal medial cortical thickness.

The QTL for lateral cortical thickness (LOD = 3.67) is located between markers D3S1304 and D3S1263, overlapping chromosomal regions 3p26.1 and 3p25.3. This linkage has an 18.9Mb wide 1-LOD interval, and accounts for 45% of the detected residual phenotypic variance. A second significant linkage (LOD = 3.30) was found at the same region for total cortical thickness which is directly related to lateral cortical thickness. Suggestive linkage (LOD = 2.27) was also found to this region for medial cortical thickness.

The third significant QTL identified in this analysis is for cortical index at metaphyseal mid-shaft (LOD = 3.14). This QTL was found at chromosomal region 17q21.2 region, between markers D17S798 and D17S1868. This linkage has a 1-LOD interval spanning 18.6Mb and was estimated to account for 55% of the detected residual phenotypic variance. Additional suggestive linkage (LOD = 2.11) was found to this region for medial cortical thickness.

Discussion

The current investigation represents the first genetic linkage study focusing on skeletal mass/cortical thickness phenotypes in young children. The field of skeletal biology/bone health has been very successful, notably in recent years, in identifying genomic regions harboring genes responsible for bone maintenance and loss. Linkage studies of bone mass phenotypes have focused mainly on adults and groups at risk for osteoporosis. However, the genetic underpinnings of childhood bone health may have significant impact on adult bone health. Here, we found cortical bone thickness in 10-year-old children to be significantly linked to three genomic regions. Each of these regions contains plausible candidate genes, some of which have previously been identified by genetic studies of adult bone mass (see below). The skeletal phenotypes in our study are based on radiographic representations of bone. Although most other genetic studies measure bone mass by DEXA, we are encouraged that our analyses have confirmed areas initially identified in studies of adults using different methodologies. Further, our analyses have additionally identified new areas of the genome influencing the skeletal system during childhood.

It is generally accepted that genes regulating bone metabolism may influence regional bone mass in different ways, and may represent only a part of the genetic regulation of “whole body” systemic bone mass [16,41]. In fact, most investigations of regional bone density or morphological parameters (e.g., hip geometry) typically identify different genomic regions influencing the various bone traits under investigation. While the pleiotropic effects of some genes will influence global aspects of bone remodeling, it is clear that there are site-specific genetic effects on bone. This may have much to do with the functional environment of the skeletal element or trait, so that a bone or region of a bone under substantial mechanical stress (e.g., during locomotion) may be differentially influenced by genes than an element

free of that type of mechanical strain. In addition to regional differences in genetic control, it is reasonable that the genetic "landscape" changes over the course of one's life. For example, genes that influence bone accrual in childhood are likely different than those influencing bone resorption and remodeling in adulthood, which may also differ from those influencing mineral maintenance through bone metabolism [42].

Candidate Genes

The three significant QTL identified for bone health phenotypes in this sample of 10-year-old children correspond to genomic regions identified by other groups in samples of adult populations. Furthermore, the 1-LOD intervals surrounding each of our linkage peaks contain plausible positional candidate genes relevant to skeletal health, and importantly, many of these gene products are vital to the accrual of bone mass.

Genomic Region 2p25.2

Areas of the 2p region have been identified by other studies as harboring genomic "hot spots" for bone-related traits [e.g., 20,22,43,44,45,46,47,48,49]. Our linkage at 2p25.2 lies in close proximity to the region that has previously shown linkage to adult spinal BMD [7,22]. This region contains several genes previously recognized as candidate genes involved in adult skeletal health, including calmodulin 2 (CALM2), serine/theronine (STK), and pro-opiomelanocortin (POMC)[22]. The CALM2 gene has specific roles in bone development and is involved in control of osteoblast proliferation and differentiation, and thus may have its adulthood effects on bone rooted in early life bone development. The STK gene, also located in this region, is expressed by cancellous osteoblasts and has been reported by at least two other studies of skeletal genetics as a candidate gene for bone mass [20,22]. Additionally, this region of chromosome 2p houses the POMC gene, involved in calcium homeostasis, which has also been identified as a candidate gene for bone health in perimenopausal women and similarly aged men [46]. Our evidence of linkage to this region confirms the potential role of these candidate genes influencing not only bone metabolism throughout life, but also their influential role in childhood bone growth and apposition.

An additional positional candidate gene that lies at the peak of our QTL in this region is the thyroid peroxidase (TPO) gene. TPO encodes for a membrane-bound glycoprotein that plays an essential role in thyroid gland function. While it has not been identified as a candidate gene in previous linkage or association studies of bone health in adults, the TPO gene nevertheless is an attractive positional candidate gene for childhood bone accrual in the chromosomal region 2p25.2. Thyroid hormones are essential for bone growth, metabolism, and attainment of peak bone mass [50,51]. Mutations in TPO are associated with thyroid disorders (e.g., congenital goiter and hypothyroidism)[52], and thus likely influence bone metabolism. Further work is needed to confirm any role the TPO gene may have on pediatric skeletal health as measured in the current study.

Genomic Region 3p25.3

The 3p25.3 region identified by our linkage analysis is consistent with results of other genetic studies of adult skeletal phenotypes [53–56]. In each of these studies of bone mass, lumbar spine/spine BMD has shown linkage to the 3p25 region. Additionally, bone density of the ultradistal forearm showed linkage to the 3p25 region [55]. While none of the linkages identified in these studies reached genome-wide significance, they do identify this region as one of potential interest. The 1-LOD support interval on chromosome 3p (3p26.1 - 3p25.3) in our study contains genes of potential interest to child bone health including the calcium/calmodulin-dependent protein kinase I (CaMK1) gene. CaMK1 is expressed in many tissues, including osteoblasts of developing long bones [57]. Because calcium/calmodulin directly activates the CaMK1 by binding to the enzyme, and indirectly promotes

phosphorylation and synergistic activation of the enzyme, the CaMK1 gene, though ubiquitous throughout the body, may have an indirect impact on bone metabolism [57,58].

One candidate gene within the 1-LOD interval of our linkage results that has not been identified in other analyses is the oxytocin receptor gene (OXTR). OXTR is a member of the G protein-coupled receptor superfamily [59]. Oxytocin is classically known for its role in smooth muscle contraction in the uterus and mammary tissue [60], but evidence suggests OXTR is also an important bone mass regulator. In 1999 the receptor itself (OTR) was found to be functional in osteoblasts, mediating increases in intracellular calcium levels as well as prostaglandin E_2 synthesis [61]. In 2002, the OTR was discovered to be functional in osteoclasts as well, increasing proliferation of pre-osteoclasts upon ligand binding [62]. This finding was confirmed by Petersson [63] who also showed that oxytocin is important for bone metabolism, stimulating interleukin (IL)-6 production by osteoblasts. Oxytocin itself is also involved in skeletal health. A recent study of post-menopausal women showed an association between low levels of circulating oxytocin and severe osteoporosis [64]. Given these discoveries, and the delicate balance of osteoclastic and osteoblastic activity in bone remodeling, it would appear that oxytocin and its receptor may be important for bone mass regulation, especially during childhood.

To date, we are unaware of any other genetic study that has found significant linkage of bone mass traits to OXTR. Because of oxytocin receptor's role in bone regulation, the OXTR gene is an excellent candidate gene for bone mass, especially during childhood. While OXTR has not been identified by other genetic linkage studies, it is important to note that the OTR has binding affinity not only to oxytocin, but also to estrogen [61]. Estrogen has long been known to influence the regulation of bone mass, and several genetic studies have identified estrogen and estrogen-related genes responsible for bone mass variation [65].

Genomic Region 17q21.2

The significant linkage found on chromosome 17 for cortical index corresponds to a region containing the sclerostin (SOST) gene. Sclerostin is a protein expressed nearly exclusively in osteocytes [66,67], which inhibits portions of the Wnt-signaling pathway, thereby inhibiting bone formation. Mutations in the gene that prevent sclerostin production result in sclerosteosis, a condition characterized by high bone mass throughout the body [68]. While those affected by this autosomal recessive trait exhibit marked increased bone mass, heterozygous carriers also exhibit higher than normal BMD values. Given the role of sclerostin in bone health, the SOST gene is likely to have a large role in bone acquisition and remodeling during the early and mid-childhood periods.

Conclusions

Replication of chromosomal regions has become an important criterion for genetic studies. Although we are unaware of any other study of child bone health that has reported linkage to any of these regions, we have provided evidence of significant genetic linkage to regions identified by adult studies as well as regions known to harbor genes influencing variation in bone morphology. We are also analyzing these bone traits at other childhood ages to determine if these regions are specific to bone health at a single time point or if the genes in these regions are influential on bone throughout childhood and/or adulthood.

The identification of three genomic regions influencing childhood bone health is a significant step for the field. Confirmation of the role of the specific genes contained in these regions may ultimately lead to better medical care for children with disorders of skeletal mass, and may shed light on gene action during childhood that has a lasting impact on skeletal health throughout the life span.

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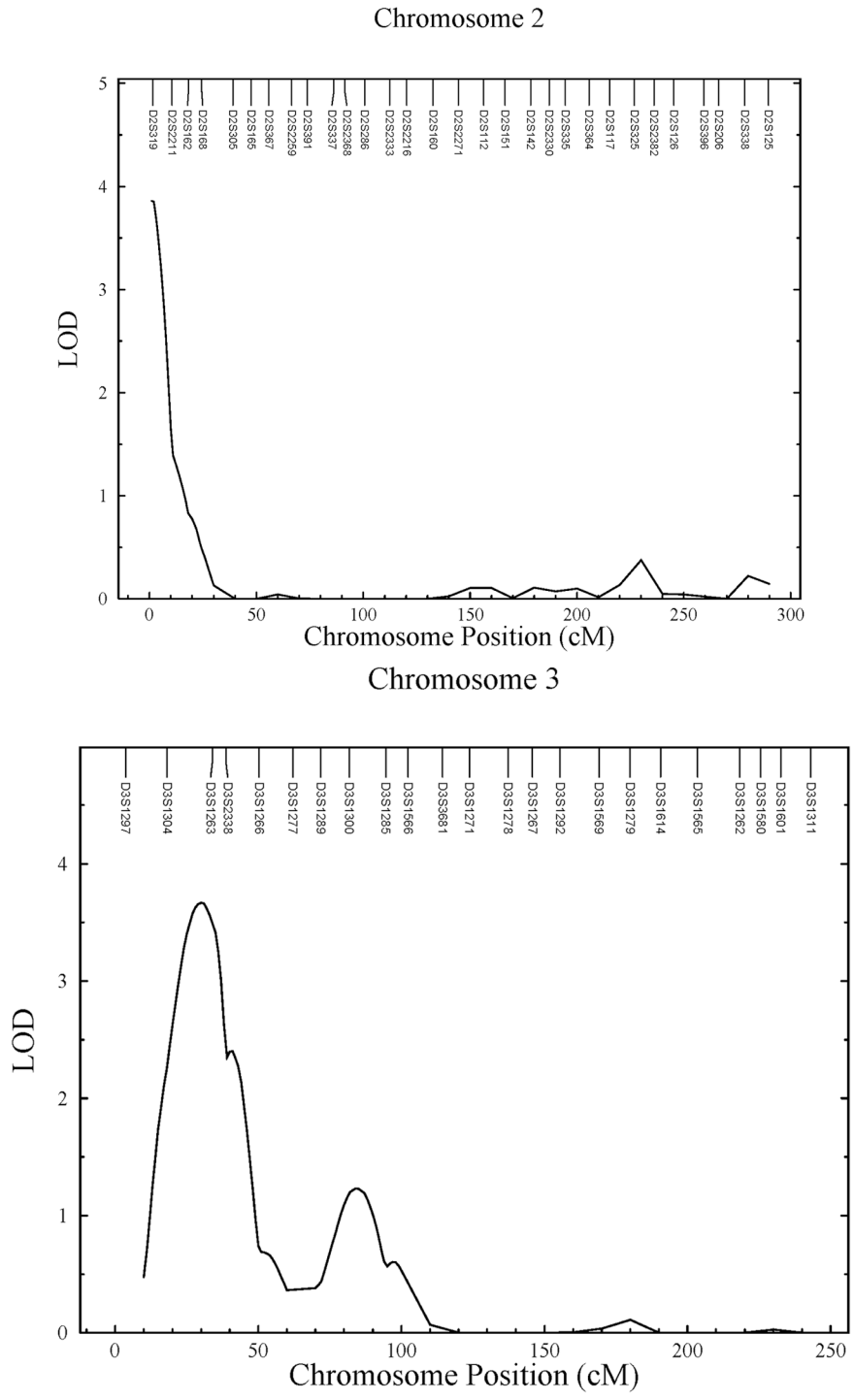
Highlights

- Cortical bone accrual in childhood is under significant genetic control.
- Childhood cortical bone is linked to genes on chromosomes 2p, 3p and 17q.
- Candidate genes for childhood bone health include the sclerostin and oxytocin receptor genes.



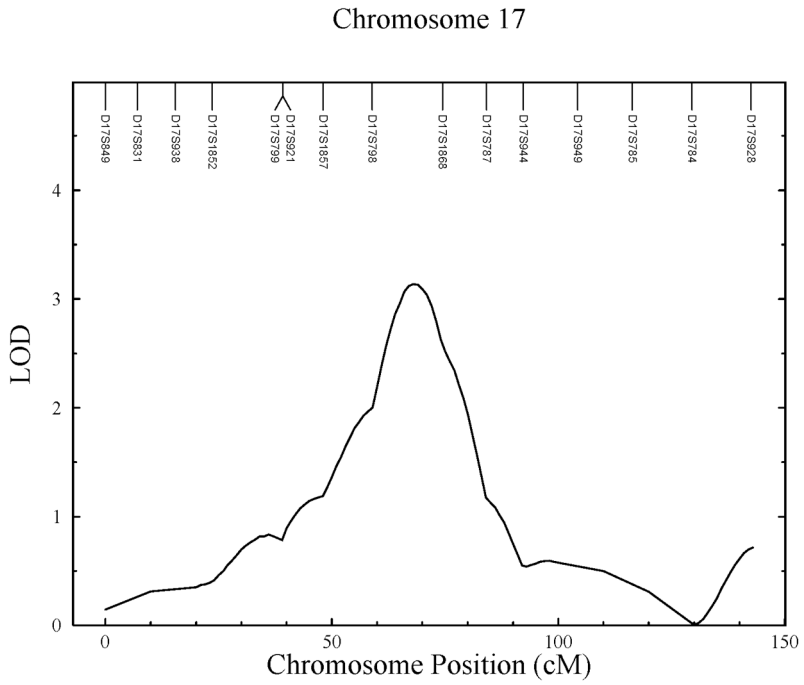


Figure 1.

Linkage results for childhood cortical bone traits. A) Significant linkage found on chromosome 2 for medial cortical thickness; B) significant linkage found on chromosome 3 for lateral cortical thickness; and C) significant linkage found on chromosome 17 for cortical index.

Table 1

Quantitative Trait Loci for childhood bone traits in Fels Longitudinal Study participants[a].

Trait	Cytogenic location	cM[b]	LOD	h_q^2	#BP in 1-LOD interval	# Genes in 1-LOD interval
Medial Cortical Thickness	2p25.2	1	3.86	0.58	6,607,526	18
Lateral Cortical Thickness	3p25.3	30	3.67	0.54	18,944,086	99
Cortical Index	17q21.2	68	3.14	0.55	18,629,864	392

[a] Maximum likelihood heritability estimates from linkage models: h_q^2 is the QTL-specific heritability, i.e., the proportion of the residual phenotypic variance due to the effect of the QTL.

[b] Location in centimorgans (cM) from pter-most marker locus in the human genetic linkage map for that chromosome.