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Sibling Pair Linkage and Association Studies between Bone Mineral Density and the Insulin-Like Growth Factor I Gene Locus*

ISTVAN TAKACS, DANIEL L. KOLLER, MUNRO PEACOCK, JOE C. CHRISTIAN, SIU L. HUI, P. MICHAEL CONNEALLY, C. CONRAD JOHNSTON, JR., TATIANA FOROUD, AND MICHAEL J. ECONS

Departments of Medicine (I.T., M.P., S.L.H., C.C.J., M.J.E.) and Medical and Molecular Genetics (D.L.K., J.C.C., P.M.C., M.J.E.), Indiana University School of Medicine, Indianapolis, Indiana 46202

ABSTRACT

A major determinant of the risk for osteoporosis in later life is bone mineral density (BMD) attained during early adulthood. BMD is a complex trait that presumably is influenced by multiple genes. Insulin-like growth factor I (IGF-I) is an attractive candidate gene for osteoporosis susceptibility, because IGF-I has marked effects on bone cells and has been implicated in the pathogenesis of osteoporosis. The IGF-I gene contains a microsatellite repeat polymorphism approximately 1 kb upstream from the IGF-I gene transcription start site, and previous investigators have found a higher prevalence of the 192/192 genotype of this polymorphism among men with idiopathic osteoporosis compared to controls. In this study we used this IGF-I polymorphism to test for an association between this polymorphism and BMD in our large population of premenopausal women (1 sister randomly chosen from 292 Caucasian and 71 African-American families). We also used this polymorphism to detect linkage to BMD elsewhere in the IGF-I gene or in a nearby gene using sibling pair linkage analysis in healthy premenopausal sister pairs (542 sibling pairs: 418 Caucasian and 124 African-American). Neither test provided any evidence of linkage or association between the IGF-I gene locus and spine or femoral neck BMD in Caucasians or African-Americans. (*J Clin Endocrinol Metab* **84:** 4467–4471, 1999)

OSTEOPOROSIS is a common medical problem, with over 1.3 million fractures occurring annually (1). The major determinants of the risk for osteoporosis in later life are the peak bone mass achieved during adulthood and the subsequent rate of bone loss (2, 3). In the absence of a secondary cause of osteoporosis, peak bone mass attained may be the most important determinant of bone mineral density (BMD) in later life (4). Twin studies suggest that between 60–80% of the peak BMD of adult women is genetically determined (5–8). Peak BMD is a complex trait that is presumably is influenced by multiple genes (9). Identification of genes that affect peak bone mass will permit early detection of individuals who are at risk for osteoporosis and early institution of preventive measures. Insulin-like growth factor I (IGF-I) is an attractive candidate gene for osteoporosis susceptibility. It is an anabolic polypeptide known to stimulate skeletal growth (10, 11) and is important in osteoblast replication and bone matrix protein synthesis (12, 13) as well as osteoclast formation and activation (14). IGF-I is stored in large quantities within the skeletal matrix, and it is possible that IGF-I may serve to couple osteoblast and osteoclast function (15). IGF-I knockout mice exhibit delayed bone development, retarded growth, and growth deficiency (16). Furthermore, in one study (17) the serum IGF-I concentration accounts for more than 35% of the variance in femoral BMD in a cross between two inbred strains of mice. In humans, the serum IGF-I level is high in young women, when peak bone mass is attained, and it declines with age. (18) Serum IGF-I is positively correlated with BMD in a variety of clinical conditions (19–22).

In a recent association study Rosen *et al.* (23) reported an association between a microsatellite repeat polymorphism, located 1 kb upstream from the IGF-I gene transcription start site, and serum IGF-I concentrations. They found that the 192/192 genotype was more prevalent in male patients with osteoporosis than in controls and that there was a trend for normal males with the 192/192 genotype to have lower BMD than men with other genotypes (23). In the present study we used the same dinucleotide repeat polymorphism as that used by Rosen *et al.* (23, 24) to search for association and linkage between the IGF-I gene locus and peak BMD in a large population of healthy premenopausal sibling pairs.

Subjects and Methods

Subjects

Five hundred and forty-four healthy, premenopausal, 20- to 45-yr-old sibling pairs were recruited from central Indiana. We were unable to amplify DNA from 1 individual from a 3-sister sibship, so the resulting study group contained 542 sibling pairs (418 Caucasian and124 African-American pairs; Table 1). Only full siblings were studied. Sister pairs were excluded if their ages differed by more than 10 yr. The women were healthy with no known disease of the skeleton and did not take medications known to affect BMD or skeletal turnover. Height and weight were obtained for each sister along with history of oral contraceptive use and smoking. Stepwise regression analysis was employed using these four variables along with age to identify significant covariates for BMD. Regression residuals, representing covariate-adjusted BMD values, were

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Address all correspondence and requests for reprints to: Michael J. Econs, M.D., F.A.C.P., F.A.C.E., Indiana University School of Medicine, 975 West Walnut Street, IB 445, Indianapolis, Indiana 46202. E-mail: mecons@iupui.edu.

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TABLE 1. Demographics of sibling pair study participants

Variable	Caucasian	African-American	*t* test (*P* value)
No. of participants	646	165	
No. of families	292	71	
No. of sibling pairs			
2-sibling families	246	54	
3-sibling families	36	12	
4-sibling families	9	4	
5-sibling families	1	1	
No. of parents genotyped	157	1	
Age of participant (yr, mean ± SD)	34.0 ± 6.7	32.4 ± 6.1	0.0071
Difference in sibling age (yr, mean ± SD)	3.57 ± 2.48	3.59 ± 2.60	0.97
Wt of participant (kg, mean ± SD)	69.1 ± 15.2	75.6 ± 17.7	<0.0001
Oral contraceptive use (yr, mean ± SD)	5.52 ± 5.11	4.27 ± 4.84	0.005

computed and used in all subsequent analyses. Written informed consent was obtained from all participants, and the study was approved by the institutional review board at Indiana University Medical Center (IRB8502–23).

BMD

BMDs at the lumbar spine (L2–L4) and femur neck were measured using dual energy x-ray absorptiometry with the DPXL machine (Lunar Corp., Madison, WI). Sisters were measured with the same densitometer.

Genotyping

Blood samples were obtained from all participating sisters and, whenever possible, from one of their parents. DNA was separated using standard techniques (25). The PCR was performed using oligonucleotide primers, as originally described by Rosen *et al.* (23), to amplify a polymorphic microsatellite composed of variable cytosine-adenosine repeats, situated 1 kb upstream from the transcription start site of the IGF-I gene. Thirty nanograms of template DNA, 100 ng of each primer, 200 μmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L $MgCl_2$, 0.5 U *Taq* polymerase (Perkin-Elmer Corp., Branchburg, NJ), and the manufacturer's recommended buffers were combined in 20-μL reactions. The forward primer was radiolabeled with ^{32}P using T4 polynucleotide kinase (Life Technologies, Inc., Gaithersburg, MD). PCR amplification was performed using a PTC-200 DNA Engine thermocycler (MJ Research, Inc., Watertown, MA) programmed for 94 C for 10 sec followed by 35 cycles of 94 C for 30 s, 68 C for 30 s, and 72 C for 45 s. The reaction was ended with final extension at 72 C for 5 min. Radiolabeled PCR products were screened for length variation by electrophoresis on a 6.5% polyacrylamide gel using a Kodak BioMax STS 45I (Eastman Kodak Co., Rochester, NY.) sequencing gel apparatus at 70 watts for 2 h and 45 min. Autoradiographs were exposed for 4–12 h in cassettes without intensifying screens. Both phenotypic and genotypic data were available for all recruited subjects. Two independent investigators scored all genotypes. To assure consistency of results, 36% of the genotypes were determined twice, and genotyping was consistent on replication. Allele size was determined by comparison with sequences of pUC18 bacterium DNA-s (Amersham Pharmacia Biotech, Cleveland, OH).

Statistical analysis

Genotype data from a minimum of 50 highly polymorphic microsatellite markers were used to verify the full sibling relationships among the subjects, incorporating parental genotype information where available, using the computer program RELATIVE (26). To examine possible racial effects at the IGF-I gene locus for BMD, the sibling pairs were analyzed as two separate groups based on race (Caucasian and African-American).

Two-point, nonparametric, quantitative linkage analysis was performed with the program SIBPAL, part of the S.A.G.E. (Statistical Analysis for Genetic Epidemiology) version 3.0 suite of programs. The linkage analysis was also performed with the Mapmaker/SIBS program (27), using both the Haseman-Elston and maximum likelihood variance options. These methods evaluate each marker individually for evidence of linkage to the BMD variables. An advantage of quantitative linkage methods as employed in these analyses is that no arbitrary threshold for high or low BMD values is necessary; therefore, all sibling pairs measured for BMD are included in the analysis.

To test for association between IGF-I genotype and BMD, we randomly selected one sister per family (n = 363). For each allele present in the overall population at 5% or more, we used ANOVA model fitting to test for differences in spine and/or femoral neck BMD between individuals with zero, one, or two copies of each allele.

Results

We found 10 IGF-I alleles in our population, with product sizes ranging between 176–202 bp (Table 2). Although we observed more alleles than previous investigators, the newly observed alleles are rare, and differences between our study and previous reports (23, 28) are probably due to our substantially larger sample size. As observed in other studies (23, 28), the 192 allele was the most common allele in Caucasians. There were substantial differences in allele frequencies between whites and blacks, with the 190-bp allele being significantly more common in blacks (32.6%) than whites (6.9%; $P < 0.001$; Table 2). To facilitate comparison for future studies, we genotyped three CEPH (Centre d'Etude du Polymorphisme Humain; www.cephb.fr) family members. The results are as follows: 1331–01 (194/192), 1331–02 (196/192), and 1347–02 (192/192).

Although there was a trend for spine BMD values to be slightly higher than the manufacturer's database, BMD values for our population were within the normal range, and, as expected, African-Americans had higher BMD than Caucasians (Table 3). Caucasian participants were slightly older than African-Americans, weighed less, and had more exposure to oral contraceptive agents (Table 1). On the average, sister pairs differed in age by 3.6 yr.

To test for association between IGF-I genotype and BMD,

TABLE 2. Bone mineral density of sibling pair study participants

Site	Caucasian	African-American	*t* test (*P* value)
Lumbar spine (L2–L4)	1.27 ± 0.14	1.33 ± 0.14	<0.0001
Femoral neck	1.00 ± 0.13	1.07 ± 0.14	<0.0001

Values (mean ± SD) are expressed as grams per cm^2.

TABLE 3. Distribution of IGF-I genotypes in the study population

Fragment size (bp)	Allele frequency (%)	
	Caucasian	African-American
176	0.4	1.5
186	0	3.2
188	2.1	6.2
190	6.9	32.6
192	63.5	29.4
194	19.6	16.5
196	5.7	7.6
198	1.9	1.2
200	0	0.9
202	0	0.9

we investigated whether the presence of zero, one, or two copies of the four most common alleles (frequencies >5%) affected BMD. We found no correlation between IGF-I genotype and BMD at either the lumbar spine or femoral neck in either the African-American or Caucasian samples (Fig. 1, A–D).

Although we found no association between the IGF-I gene microsatellite polymorphism and BMD, we also used this polymorphism as a genetic marker to detect linkage to BMD elsewhere in the IGF-I gene locus or in a nearby gene. Using the SIBPAL program, we found no evidence of linkage between BMD and the IGF-I gene polymorphism in either Caucasians or African-Americans for spine (Caucasian: $P = 0.24$; equivalent LOD score, 0.17; African-American: $P = 0.55$; LOD score = 0.00) or femoral neck BMD (Caucasian: $P = 0.43$; LOD score, 0.01; African-American: $P = 0.52$; LOD score, 0.00). Our results were similar using the Mapmaker/Sibs program in both races for spine (Caucasian LOD score, 0.44; African-American LOD score, 0.01) and femoral neck (Caucasian LOD score, 0.10; African-American LOD score, 0.00) with the maximum likelihood variance option. LOD scores computed with Mapmaker/Sibs using the Haseman-Elston method were the same or lower in all cases.

Discussion

IGF-I is an anabolic polypeptide produced by many tissues that functions as an autocrine and paracrine signal (29). IGF-I stimulates osteoblast replication (13), bone matrix protein synthesis (12), and osteoclast formation and activation (14). In rats, IGF-I infusion enhances osteoblast activity and decreases the number of osteoclasts (30). In humans, a short course of recombinant human GH treatment stimulates osteoblasts and activates bone remodeling (31). Clinical studies suggest that serum IGF-I may have an effect on BMD in males with idiopathic osteoporosis (32, 33) as well as in elderly women (22), and previous studies suggest that IGF-I genotype affects the serum concentration of IGF-I (23). Serum IGF-I concentrations are inversely correlated with age (18). Based on these data, we hypothesized that the IGF-I genotype could play a role in determining peak bone density.

We used the previously described IGF-I microsatellite polymorphism to detect linkage of BMD to either the IGF-I gene or a nearby gene. None of the tests performed provided any evidence of linkage or association between the IGF-I gene and spine or femoral neck BMD in whites or blacks. Our large sample of white premenopausal women has 80% power to detect association to a locus that accounts for at least 3% of the variability in BMD.

Our data do not address the possibility that the IGF-I genotype affects serum IGF-I concentrations, as we did not measure serum IGF-I concentrations or concentrations of the IGF-I-binding proteins in our study. Additionally, our study included only premenopausal woman. Therefore, we cannot exclude the possibility that IGF-I genotypes play a role in

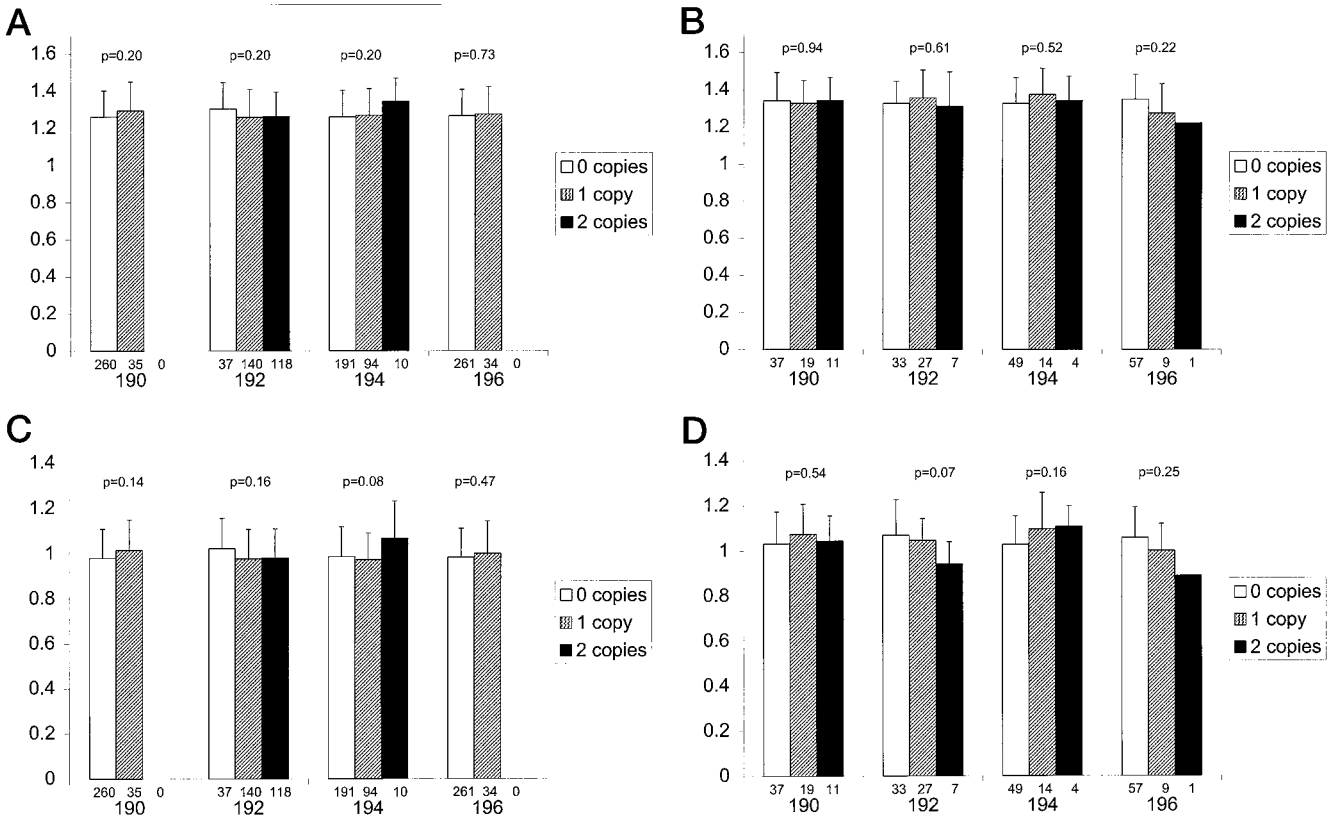

FIG. 1. The spine (A, Caucasians; B, African-Americans) and femoral neck (C, Caucasians; D, African-American) BMD (grams per cm^2) for each genotype are shown on the *vertical axis*. One sister was selected randomly from each family. Data are expressed as the mean ± SD. The number of individuals per group is indicated *below the bars*. We used ANOVA model fitting to test for differences between individuals with zero, one, or two copies of each allele that was present in overall populations at 5% or more.

determining peak BMD in men or in determining the rate of postmenopausal bone loss. In this regard, a recent study in a large population of elderly individuals (72–94 yr old) found an association between the serum IGF-I concentration and BMD in women, but not in men (22). However, another study (28) of 314 healthy postmenopausal Japanese women did not find an association between BMD and IGF-I genotype.

As noted in previous studies of Caucasians (23, 24), the 192-bp allele was the most common allele. However, there were differences between allele frequencies in blacks and whites. The difference in allele frequencies between these two groups do not explain the difference in BMD found in the two populations, as we found no linkage between BMD and the IGF-I gene in either racial group, but these differences could be connected to the previously reported racial differences in GH secretion between blacks and whites (35).

Our data do not support the results of a previous association study by Rosen *et al.* (23), who found a higher frequency of the 192/192 genotype in 30 osteoporotic men than in nonosteoporotic controls and a nonstatistically significant association between BMD and IGF-I genotype in healthy men. It is possible that the difference between our results and those of Rosen *et al.* resulted from gender differences between our two populations. It is also possible, although unlikely, that there were environmental and/or nutritional factors that differed between our two populations that interacted with the IGF-I genotype, leading to the disparate results seen in the two studies. Alternatively, association studies are susceptible to many factors that lead to association without a causal relationship (36). One possibility is that there is linkage disequilibrium between this IGF-I polymorphism and another polymorphism (either in the IGF-I gene or a nearby gene) that affects peak BMD. This is unlikely, because we found no evidence of linkage between IGF-I and peak BMD. On the other hand, population stratification (the presence of two subpopulations within a sample) could be responsible for the observed association between genotype and BMD in the study by Rosen *et al.* (23). In this regard, when we purposely combined our black and white subjects (each of which provided no evidence for association) in a single analysis, we found an association between femoral neck BMD and both the 190 and 192 alleles. Femoral neck BMD for zero, one, or two copies of the 190 allele were 0.99, 1.04, and 1.04, respectively ($P = 0.02$), whereas femoral neck BMD for individuals with zero, one, or two copies of the 192 allele were 1.04, 0.99, and 0.98, respectively ($P = 0.002$). These spurious results are due to the differences in allele frequencies between blacks and whites, and the well-known difference in BMD between these two populations rather than a causal relationship between IGF-I and BMD. In other words, the 190 allele is associated with higher BMD, as it is the common allele in blacks, and blacks have higher BMD than whites. The 192 allele is associated with low BMD, as it is the most common allele in whites. Although investigators usually avoid obvious stratification, such as between races, it is often difficult to detect subtle ethnic differences that result in stratification. Inability to detect deviations from Hardy-Weinberg equilibrium do not guarantee a nonstratified population (37).

In summary, we found no evidence to support a relationship between IGF-I genotype and peak bone mass at the spine or femoral neck in either Caucasians or African-Americans. Future studies of genetic contribution to peak bone mass should focus on other loci.

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