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Sibling Pair Linkage and Association Studies between Peak Bone Mineral Density and the Gene Locus for the Osteoclast-Specific Subunit (OC116) of the Vacuolar Proton Pump on Chromosome 11p12-13

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A major determinant of the risk of osteoporosis is peak bone mineral density (BMD), which has been shown to have substantial heritability. The genes for 3 BMD-related phenotypes (autosomal dominant high bone mass, autosomal recessive osteoporosis-pseudoglioma, and autosomal recessives osteopetrosis) are all in the chromosome 11q12-13 region. We reported linkage of peak BMD in a large sample of healthy premenopausal sister pairs to this same chromosomal region, suggesting that the genes underlying these 3 disorders may also play a role in determining peak BMD within the normal population. To test this hypothesis, we examined the gene responsible for 1 form of autosomal recessive osteopetrosis, TCIRG1, which encodes an osteoclast-specific subunit (OC116) of the vacuolar proton pump. We identified 3 variants in the sequence of TCIRG1, but only one, single nuclear polymorphism 906713, had sufficient heterozygosity for use in genetic analyses. Our findings were consistent with linkage to femoral neck BMD, but not to spine BMD, in a sample of 995 healthy premenopausal sister pairs. However, further analysis, using both population and family-based disequilibrium approaches, did not demonstrate any evidence of association between TCIRG1 and the spine or femoral neck BMD. Therefore, our linkage data suggest that the chromosomal region that contains OC116 harbors a gene that affects peak BMD, but our association results indicate that polymorphisms in the OC116 gene do not affect peak BMD. (*J Clin Endocrinol Metab* 87: 3819–3824, 2002)

OSTEOPOROSIS IS A complex disease with both environmental and genetic determinants. The major determinants of the risk for osteoporosis in later life are peak bone mass achieved during early adulthood and the subsequent rate of bone loss (1). 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 (2). As much as 80% of the variance in BMD can be attributed to heritable factors (3–5). BMD is a complex trait that presumably is influenced by multiple genes (6).

We previously reported linkage to chromosome 11q12-13 for peak BMD in a sample of premenopausal sister pairs (7, 8). This linkage was particularly interesting because three distinct Mendelian disorders (autosomal dominant high bone mass, autosomal recessive osteoporosis-pseudoglioma, and autosomal recessives osteopetrosis) involving changes in BMD had been linked to this same chromosomal region (9–11). Recently, TCIRG1 was identified as one of the genes responsible for autosomal recessive osteopetrosis (12), which had previously been mapped to chromosome 11q12-13 (11). Mutations in this gene may account for as many as 50% of the cases of recessive osteopetrosis (13). In mice, inactivation of TCIRG1 causes osteoclast-rich osteopetrosis (14). In addition, one of the murine models of recessive osteopetrosis, the *oc/oc* mouse, results from a deletion involving the 5′ portion of this gene (15).

TCIRG1 encodes the osteoclast-specific 116-kDa subunit (OC116) of the vacuolar proton pump [vacuolar-type adenosine triphosphatase (V-ATPase) complex]. The 116-kD protein is an integral component of the V-ATPase complex, whose action mediates H^+ transport into the resorption lacunae, where a low pH is a prerequisite for the dissolution of hydroxyapatite crystals (16). Thus, TCIRG1 plays a critical role in bone resorption. Although mutations in the TCIRG1 gene result in osteopetrosis, we hypothesized that minor changes in gene sequence, which would not entirely disrupt protein expression or function, could slightly alter protein expression or the ability of the protein complex to transport hydrogen ions. For example, changes in the amino acid sequence of the TCIRG1 gene could result in a protein with slightly increased or decreased ability to transport protons, whereas changes in noncoding regions of the gene could result in small changes in gene expression. Differences in proton transport of even a few percent between two polymorphisms could result in significant differences in BMD over the 2 decades that it takes to develop peak BMD. Therefore, the TCIRG1 gene is a strong candidate gene for influencing BMD in the normal population because of both its chromosomal location (11q13) and its function.

We examined TCIRG1 as a candidate gene for peak BMD. Our strategy involved the identification of DNA polymor-

Abbreviations: BMD, Bone mineral density; LOD, log of odds; LRP5, low density lipoprotein receptor-related protein 5; QTDT, Quantitative Transmission Disequilibrium Test; V-ATPase, vacuolar-type adenosine triphosphatase; SNP, single nuclear polymorphism; TDT, Transmission Disequilbrium Test.

phisms in the TCIRG1 gene, estimating their allele frequencies in healthy unrelated individuals, and then testing the most informative polymorphisms for association with peak BMD in a large group of healthy premenopausal women. We investigated nine sequence variations, some involving more than a single base change. We validated an additional marker in the 11q13 region, between D11S1313 and D11S1314, which supports our previous findings of linkage for the femoral neck in this region. This finding led us to further investigate these data using both family and population-based methods to test for association between spine and/or femoral neck BMD and genotype for this marker in OC116.

Subjects and Methods

Subjects

Nine hundred ninety-five healthy, premenopausal, 20- to 45-yr-old sister pairs (797 Caucasian and 198 African-American pairs; all possible pairs, calculated as $n(n-1)/2$) were recruited in Indiana (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 determined for each sister along with history of oral contraceptive use and smoking. Informed written consent was obtained from all participants, and the study was approved by the institutional review board at Indiana University Medical Center. Blood samples were collected from all participating sisters and, whenever possible, from their parents. Genomic DNA was isolated using standard techniques (17).

For initial estimates of allele frequencies for the various polymorphisms, DNA from a group of previously studied (18) unrelated normal women from North Carolina was used. This group was not used in any other analysis in light of differences in phenotyping techniques between this group and the current sample.

BMD

BMD at the lumbar spine (L2–L4) and femoral neck was determined by dual energy x-ray absorptiometry, using either a DPXL or a Prodigy machine (Lunar Corp., Madison, WI). The short-term reliability of each measurement has a coefficient of variation of 1% or less. The values obtained from the two types of machines were adjusted using extensive cross-calibration data from subjects measured with both the DPXL and the Prodigy machines for this purpose. In all cases both members of a sister pair were measured on the same dual energy x-ray absorptiometry machine (Table 2).

Screening the TCIRG1 gene for sequence variants

A search was performed using the single nuclear polymorphism (SNP) database available at NCBI (http://www.ncbi.nlm.nih.gov/SNP), which identified three SNPs in the TCIRG1 gene. Six previously described (19) nucleotide changes were also examined (see Table 3). Some of these involved a 2- or 3-bp change in the sequence and, therefore, were not technically SNPs, but were investigated as polymorphisms.

TABLE 1. Demographics of sibling pair study participants

Variable	Caucasian	African-American
No. of participants	1185	316
No. of families	536	147
No. of sibling pairs		
2-Sibling families	447	129
3-Sibling families	72	16
4-Sibling families	13	1
5-Sibling families	2	0
6-Sibling families	1	1
7-Sibling families	1	0
No. of parents genotyped	277	29
Age of participants (yr, mean ± SD)	33.3 ± 7.0	32.9 ± 6.4
Weight of participant (kg, mean ± SD)	69.1 ± 15.4	80.8 ± 19.0

Genotyping of sequence variants

The restriction map of the sequence surrounding each polymorphism was determined using the program Webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html). Primers were selected using the program Primer3 from Whitehead Institute (http://www-genome.wi.mit.edu). All of the primers used in this study were synthesized by Invitrogen (Carlsbad, CA; Table 4). Restriction enzyme digestion assays for polymorphisms were designed. Products of 80–253 bp were amplified from genomic DNA, digested, and analyzed on horizontal agarose gel as restriction fragment length polymorphisms (see Table 4). GC42 was amplified by fluorescent PCR using a fluorescent-labeled forward primer and an unlabeled reverse primer (Table 4). SNP906713 and C226T were analyzed by fluorescent allele-specific PCR (20). The allele-specific primers were modified so that the 2 distinct products produced in a single PCR could be identified on a standard sequencer lane. The 2 forward primers differ on the 5′ end by both the number of nucleotides (2 extra bp for the HEX-labeled primer) and the fluorescent dye, and by the allele-specific nucleotide on the 3′ end. The PCR reactions were performed according to the following protocol: 40 ng DNA, 1.5 mM $MgCl_2$, 0.2 mM of each deoxy-NTP, 0.5 μM HEX-labeled primer and reverse primer, 0.125 μM FAM-labeled primer, and 0.5 U *Taq* polymerase (Perkin Elmer Corp., Branchburg, NJ) for a reaction volume of 10 μl. C226T was amplified at 64 C for 35 cycles, and SNP906713 at 66 C for 30 cycles. The corresponding PCR products were diluted, and an aliquot was added to the Genescan 400HD (ROX) size standard (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The final products were separated according to size on the capillary system of the 3100 Genetic Analyzer (PE Applied Biosystems), and the data were analyzed with Genotyper software and were overread independently by 2 investigators. The frequencies of the variants were established on a panel of 30 unrelated individuals. The most frequent SNP, SNP906713, located in intron 10 was genotyped in the 995 healthy sibling pairs using fluorescent allele-specific PCR.

Statistical analysis

Stepwise regression analysis was performed separately for each race using height, weight, history of oral contraceptive use, and smoking along with age to identify significant ($P < 0.10$) covariates for BMD.

TABLE 2. BMD of the sibling pair study participants

Site	Caucasian	African-American	*t* test (*P*)
Lumbar spine (L2–L4)	1.27 ± 0.14	1.34 ± 0.14	<0.0001
Femoral neck	1.01 ± 0.13	1.10 ± 0.14	<0.0001

Values (mean ± SD) are expressed as grams per centimeter squared.

TABLE 3. Description of the polymorphisms detected in the SNP database or in the genomic sequence from Heinemann *et al.* (19)

SNP	Position	Polymorphism	AA change
SNP906713	Intron 10	G/A	none
SNP1129633	Exon 11	G/A	K430K
SNP1047817	Exon 20	C/T	3′UTR
GCC1868	Exon 15	+ GCC/− GCC	-A603
GC1189CG	Exon 10	GC/CG	A377R
C1942T	Exon 15	C/T	R628W
G14	Exon 1	+ G/− G	5′UTR
GC42	Exon 1	+ GC/− GC	5′UTR
C226T	Exon 3	C/T	R56W

SNP identification number according to the SNP database or numbering of nucleotides according to Heinemann *et al.* (19).

TABLE 4. PCR primers and restriction enzymes for detection of OC116 polymorphisms

Name	Primers sequence (5′-3′)	Enzyme
SNP1129633	F: CATGGTCCTTGCGGAGAAC R: GGCAGACTTTCTTCAGGGGC	Hae III
SNP1047817	F: CAGATGACTAGGGCCCACTG R: GAGGAATAAAGACGGTCCGC	DdeI
GCC1868	F: ACCTTCCTGCTGGGACTCTT R: CAACAGGCTGCTCTACCCC	BglI
GC1189CG	F: ATGGAGGAGGGAGTGAGTGC R: GCTACCAGGAGGTCAACCC	Sau3AI
C1942T	F: TGCTGGGACTCTTCGGTTAC R: CAAGATCCTCGTCCGAGAAA	NciI
G14	F: AGTGAGCGGCGCTTAGTC R: ATTTCCTGCCCACCCTCTT	BsrBI
GC42	F: HEX-GGCAGCCAGCAGCGGAGG R: GGTGGGGCGCCAGGATCTC	No restriction
FAS-PCR		
SNP906713	SNP1/P212: HEX-CCGTCCTCATCTTCACACG SNP1/P210: FAM-GGCCGTCCTCATCTTCACAC SNP1/PR475: ATGGGAACAAGTCCTCATGG	
C226T	SNP8/P131: HEX-CGCTTTGTGGTTGATGTTC SNP8/P129: FAM-GACGCTTTGTGGTTGATGTT SNP8/PR270: CTGGCAAACCCAGACTCTTC	

Regression residuals, representing covariate-adjusted BMD values, were computed and used in all analyses.

To examine possible racial effects at the TCIRG1 gene locus for BMD, the sibling pairs were analyzed as two separate groups based on self-reported race (Caucasian and African-American). The linkage analysis was performed with the MAPMAKER/SIBS program (21), using the maximum likelihood variance option. An advantage of the quantitative linkage method, as employed in this analysis, is that no arbitrary threshold for high or low BMD values is necessary; therefore, all sibling pairs measured for BMD were included in the analysis.

Association between the polymorphism and peak BMD was examined using the Quantitative Transmission Disequilibrium Test (QTDT). As originally described, the Transmission Disequilbrium Test (TDT) (22) directly tests for linkage disequilibrium between the trait studied and the marker locus and is not susceptible to false positive results due to the presence of stratification in the population. The test procedure compares the number of times that a parent heterozygous for the marker allele associated with the trait transmits the associated marker allele to the offspring with the number of times that they transmit the alternate marker allele. The TDT detects a relationship between the marker locus and the trait only if association, due to linkage disequilibrium, is present. We used a variant of this method, the QTDT (23) (http://www.well.ox.ac.uk/asthma/QTDT) to detect an association between the OC116 marker and our quantitative measures of interest (spine or femoral neck BMD).

To further test for association between the OC116 polymorphism and BMD, we randomly selected one sister from each family to ensure independence among the subjects. We then used ANOVA model fitting to test for significant differences in spine and/or femoral neck BMD between individuals with zero, one, or two copies of the A allele of SNP906713.

Results

We investigated 9 sequence variants localized in the TCIRG1 sequence, either previously reported (19) or present in the SNP database (Table 3). Only 3 of these variants were polymorphic in our test sample: GCC1868, C226T, and SNP906713 (Table 5). GCC1868 corresponds to the deletion of 1 amino acid, but its frequency was too low (1 in 120 alleles) to be further analyzed. C226T, which changes an arginine to a tryptophan in exon 3, appears to be a polymorphism specific for the Caucasian population, with a high prevalence of the C allele (95% of the alleles tested), whereas African-Americans all carried the C allele. SNP906713, present in intron 10, was further pursued; the minor allele (A) has a frequency of 0.28, providing sufficient power for statistical analysis. Therefore, we concentrated our efforts on this polymorphism.

TABLE 5. Frequencies of OC116 polymorphisms in our panel of unrelated individuals

SNP	No.	Allele 1	Allele 2
SNP906713	60	0.72	0.28
SNP1129633	60	1.00	0.00
SNP1047817	60	1.00	0.00
GCC1868	120	0.99	0.01
GC1189CG	60	1.00	0.00
C1942T	60	1.00	0.00
G14	60	1.00	0.00
GC42	60	1.00	0.00
C226T	60	0.95	0.05
	W: 384	0.94	0.06
	B: 344	1.00	0.00

No., Number of chromosomes screened. For polymorphism C226T, additional individuals were genotyped using DNA from our population of sisters. W, White; B, black.

The BMD values for our population were within the normal range, and as expected, the African-American population had higher BMD than the Caucasian population (Table 2). Only age and weight were identified as significant covariates ($P < 0.10$). Therefore, residuals from race-specific regression model fitting were used as age- and weight-adjusted BMD values in all subsequent statistical analyses.

Some 1539 DNA samples were genotyped for the marker SNP906713. No difference was observed between the allele frequencies for the SNP in the different populations ($P = 0.39$); the observed frequency of the A allele was 0.181 in Caucasians, 0.193 in African-Americans, and 0.183 in the combined sample. No deviation from Hardy-Weinberg equilibrium was observed in the Caucasian or African-American subgroup ($P = 0.75$ and $P = 0.97$, respectively) or in the combined sample ($P = 0.74$).

We found no evidence of linkage between SNP 906317 and BMD at the spine [linkage of difference (LOD) score =

0.00 for the combined sample of Caucasian and African-American sister pairs]. We did find evidence supporting linkage to femoral neck BMD in the combined sample (LOD = 1.30; $P = 0.007$). Simulations performed for a marker with heterozygosity equal to that of SNP 906317 showed that this LOD score was in the 98th percentile of the expected LOD score distribution for a gene, accounting for 10% of BMD variability, and in the 80th percentile of the expected LOD distribution for a gene, accounting for 25% of BMD variability.

We then used the same pedigree data to test for linkage disequilibrium between SNP906713 and a gene influencing BMD using the QTDT. We found no evidence of association between TCIRG1 and spine ($P = 0.99$) or femoral neck ($P = 0.21$) BMD. Although the TDT approach is robust to admixture, we analyzed the sample separately by race, and as expected, the QTDT results were similar in both the Caucasian (spine: $P = 0.89$; femoral neck: $P = 0.29$) and African-American samples (spine: $P = 0.70$; femoral neck: $P = 0.53$).

We subsequently tested for a population-based association between TCIRG1 genotype and BMD using a simple ANOVA to test whether the presence of zero, one, or two copies of the minor allele (A) affected peak BMD. We found no association between TCIRG1 genotype and BMD at either the femoral neck or lumbar spine in the Caucasian samples (femoral neck: $P = 0.48$; spine: $P = 0.84$; Fig. 1). Similarly, the African-American sample did not demonstrate any association with the TCIRG1 SNP (femoral neck: $P = 0.56$; spine: $P = 0.75$; Fig. 1).

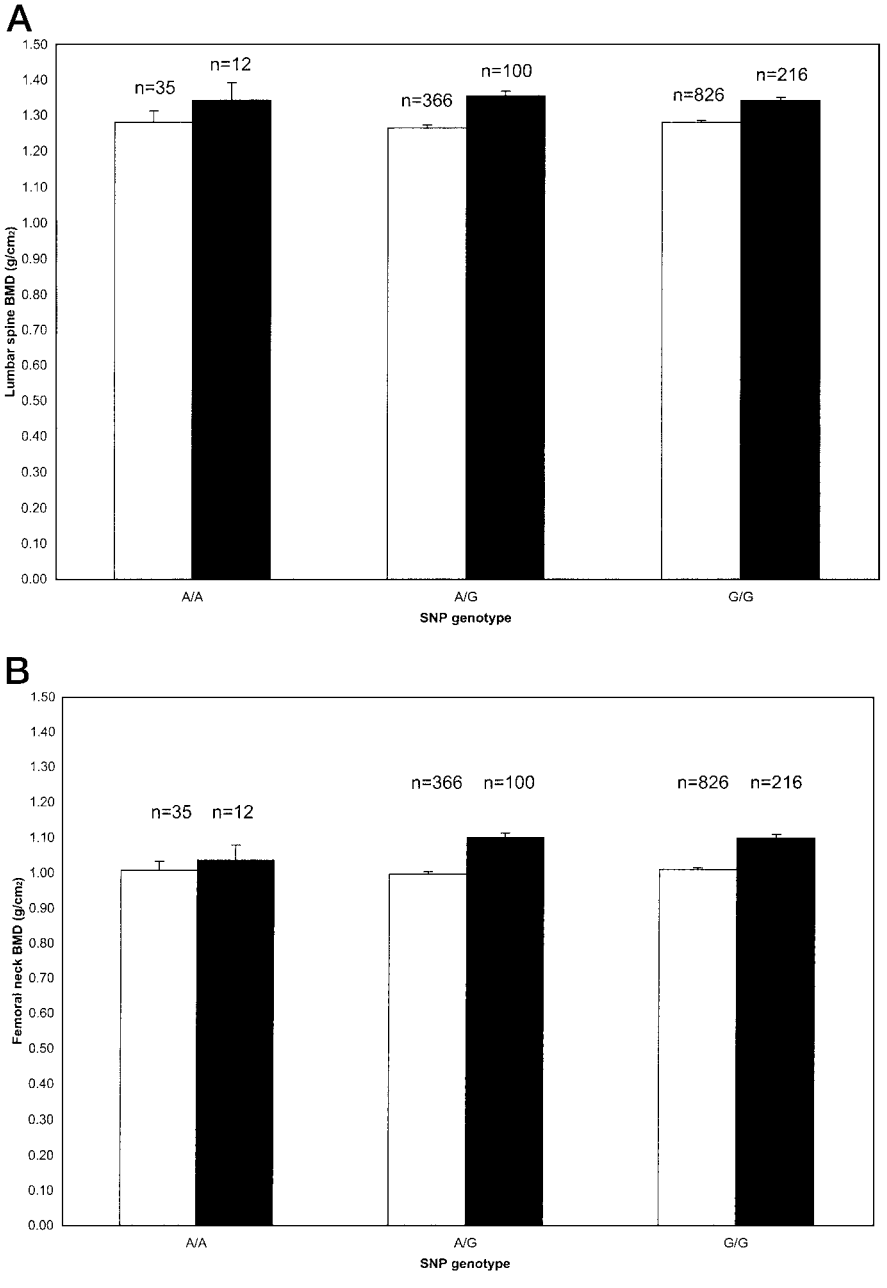

FIG. 1. The spine (A) and the femoral neck (B) BMD values (grams per centimeter squared, unadjusted) for each genotype in the Caucasian or African-American population are shown on the *vertical axis*. Data are expressed as the mean ± SD. The number of individuals is indicated *above the bars*. As described in the *Subjects and Methods*, we tested for differences between age- and weight-adjusted BMD values in individuals with zero, one, or two copies of each allele using ANOVA model fitting. Results did not differ using unadjusted values.

Discussion

OC116 is the human α_3-subunit of V-type H^+-ATPase, involved in bone resorption mediated by osteoclasts. The gene coding for this protein, TCIRG1, is mutated in a subset of patients with recessive osteopetrosis. Most of these mutations are deletions, insertions, splice site mutations, and nonsense substitutions that are predicted to abolish protein function (12, 13, 24). These mutations result in osteopetrosis, because the osteoclasts are unable to resorb bone. We hypothesized that functional polymorphisms of the TCIRG1 gene could result in subtle differences in gene expression or activity between allelic variants. These differences could result in minor differences in osteoclast activity that would influence the development of peak BMD in the normal population. The TCIRG1 gene is localized in the region between D11S1313 and D11S1314, where linkage for BMD has been previously reported. Based on the function and chromosomal location of TCIRG1, we postulated that polymorphisms in TCIRG1 could influence normal BMD.

SNPs occur, on the average, every 1000 bp and have a low mutation rate, both of which are characteristics that may have particular advantages for association analyses. However, as biallelic markers, SNPs are generally less informative than microsatellite markers, which may be a disadvantage for both association and linkage studies. To generate markers for the study, we searched for SNPs and other polymorphisms. We investigated all of the polymorphisms previously described in TCIRG1. Two of the polymorphisms that we validated led to a change in the protein sequence. Due to the low frequency of these two polymorphisms, we were unable to detect any individual homozygous for the minor allele. Even though they were not useful for our study, these polymorphisms might affect the biochemical properties of the proton pump. GCC1868 involves the deletion of an entire amino acid (alanine) in the fourth transmembrane domain of the protein and therefore may affect the activity of the protein. C226T also appears interesting, because it involves the replacement of a basic amino acid by a neutral amino acid. To date, the detailed structure of OC116 is unknown, making it difficult to predict with precision the effect of such a deletion or substitution.

Unless a functional SNP directly influencing the disease susceptibility is included in the battery of markers tested, there is no clear advantage to testing SNPs in exons rather than in introns (25). We thus used an SNP in intron 10 of TCIRG1 to detect linkage or association between the TCIRG1 gene and the lumbar spine or femoral neck BMD in Caucasians or African-Americans. The linkage observed between TCIRG1 and femoral neck BMD is not totally unexpected because the gene is localized between D11S1313 and D11S1314 where we previously reported linkage for BMD. These data support our previous linkage findings for femoral neck BMD.

We did not detect an association between TCIRG1 and BMD using the ANOVA model fitting or the QTDT, indicating that this polymorphism is not responsible for a significant portion of the variance in peak BMD in our populations of Caucasians and African-Americans from Indiana. Although we cannot completely rule out TCIRG1 as a candidate gene because we have only investigated one marker, which was not fully informative (25), we did examine all known polymorphisms in the gene, and the polymorphism tested was the only known polymorphism that occurred with sufficient frequency to potentially have a role in determining peak BMD within the normal population. Furthermore, this polymorphism is not in linkage disequilibrium with another polymorphism that affects peak BMD. However, it should be noted that linkage disequilibrium generally exists over fairly small distances, usually less than 1 cM and often much shorter distances (26–28). Therefore, it is likely that linkage would be detected over a large chromosomal segment, perhaps 10–20 megabases, whereas linkage disequilibrium would only be observed with a marker very near or within the putative QTL, typically less than 0.1 megabase. Therefore, we cannot exclude the possibility that another nearby gene may influence peak BMD. Indeed, despite our lack of evidence of association with the TCIRG1 locus, our linkage data with SNP906713 supports the hypothesis that another gene in the region potentially influences femoral neck BMD. In this regard, investigators have reported that an amino acid change in the low density lipoprotein receptor-related protein 5 (LRP5) results in an autosomal dominant high bone mass phenotype (29), which appears to produce no clinical features or sequelae (9). The G to T transversion in exon 3 of the *LRP5* gene results in a glycine to valine amino acid change (G171V) (29). This amino acid is evolutionarily conserved and appears to alter the local hydrophobic environment at the surface of the protein. Inactivating mutations in this same gene have also been found to produce the autosomal recessive disorder osteoporosis pseudoglioma (30). A wide variety of mutations leading to LRP5 inactivation produce this phenotype, which is characterized by very low bone mass and eye abnormalities. Our data do not exclude, and are indeed consistent with, the concept that functional polymorphisms in the LRP5 gene could play a role in peak BMD variation. Therefore, LRP5 is an attractive candidate in which to test polymorphisms for a possible effect on normal variation in BMD.

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