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CLCN7 Polymorphisms and Bone Mineral Density in Healthy Premenopausal White Women and In White Men

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Abstract

Introduction—Mutations in the chloride channel 7 gene (*CLCN7*) cause osteopetrosis, and polymorphisms of *CLCN7* in the non-disease allele are associated with penetrance of the autosomal dominant osteopetrosis (ADO) phenotype. Studies have also shown an association between *CLCN7* polymorphisms and bone mineral density (BMD) in women. However, there is no study to date that has examined whether *CLCN7* polymorphisms underlie normal variation of peak BMD in healthy premenopausal white women and in white men.

Methods—Six single nucleotide polymorphisms (SNPs) and one variable number tandem repeat (VNTR) polymorphism in the *CLCN7* gene were genotyped. Association was tested between *CLCN7* gene polymorphisms and both lumbar spine and femoral neck BMD. Healthy premenopausal white sisters (age 33.1 ± 7.2 , $n=1692$) and healthy white brothers (age 33.6 ± 10.9 , $n=715$) were studied.

Results—No significant association between *CLCN7* gene polymorphisms and BMD at the lumbar spine or femoral neck was found in white women or white men.

Conclusions—Genetic variation in the *CLCN7* gene is not a major contributor to the variability in peak BMD at the femoral neck and lumbar spine in healthy premenopausal white women and in white men.

Keywords

bone mineral density (BMD); association study; chloride channel 7 gene (*CLCN7*); single nucleotide polymorphism (SNP); variable number tandem repeat (VNTR)

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Introduction

Peak bone mineral density (BMD) is a major determinant of osteoporosis in later life and is affected by both genetic and environmental factors. Genetic factors are responsible for 60-80% of the variation in peak BMD in healthy subjects [1].

Mutations in the chloride channel 7 (*CLCN7*) gene result in both autosomal recessive (ARO) [2] and autosomal dominant (ADO) osteopetrosis in humans [3-5]. Both disorders result in decreased osteoclastic bone resorption with greatly increased BMD. Mutations in the *CLCN7* gene are fully penetrant in ARO, but the penetrance in ADO is incomplete. In ADO families, one third of the individuals with *CLCN7* mutations are unaffected gene carriers [3, 6, 7]. As we previously reported, two single nucleotide polymorphisms (SNPs), rs960467 and rs12926089 (V418M), of the *CLCN7* gene on the non-disease allele are associated with the penetrance of the ADO phenotype [6]. In 1077 Scottish women, aged 45 to 55 years, two *CLCN7* SNPs, rs12926089 (V418M) and rs12926669, which are 7 base pairs apart, were associated with variation in femoral neck BMD [8]. Additionally, in 425 French postmenopausal women, aged 50 to 80 years, a variable number tandem repeat (VNTR) polymorphism in intron 8 was associated with variation in femoral neck BMD [9].

The aim of this study was to determine whether polymorphisms in the *CLCN7* gene were associated with variation in peak BMD in healthy premenopausal women and in healthy men. In this sample, we genotyped the VNTR in intron 8 in *CLCN7* gene and the nonsynonymous SNP rs12926089 previously reported to be associated with BMD variation by other investigators, along with 5 other SNPs selected to span the gene. The association of these SNPs with lumbar spine and femoral neck BMD was tested in 1692 white sisters and 715 white brothers who took part in a sibling-pair study aimed at identifying the genes affecting peak BMD.

Subjects and Methods

Subjects

Healthy white premenopausal sisters and healthy white brothers living in Indiana were recruited as part of a sibling study to identify genes underlying peak bone mass. We studied 1692 premenopausal women and 715 men (Table 1). A detailed medical history was obtained through administration of health and lifestyle questionnaires. Those who had conditions known to affect BMD, including a history of chronic disease, use of medications known to affect bone mass or metabolism, or inability to have BMD measured because of obesity were excluded from the study. Women, who had irregular menses, or a history of pregnancy or lactation within 3 months before enrollment, were also excluded. However, women taking oral contraceptives were not excluded. Sisters, but not brothers, were required to be within 10 years of each other in age. Informed written consent was obtained from all subjects before their participation in the study. The study was approved by the Institutional Review Board of Indiana University-Purdue University Indianapolis. All studies were performed at the General Clinical Research Center of Indiana University School of Medicine. Blood samples were collected for the extraction of DNA.

BMD, Height, and Weight Measurements

Areal BMD (g/cm^2) at the lumbar spine (vertebrae L2-L4) and femoral neck were measured by DXA, using two DPX-L and one Prodigy instruments (GE Lunar Corp., Madison, WI, USA). All three DXA instruments were cross-calibrated weekly using a step-wedge phantom. There were no systematic differences among the three machines over the period of the study. The CV of duplicate measurements after repositioning was 1.0% for femoral neck and 0.52%

for lumbar spine. Sisters and brothers were measured on the same instrument as their sibling (s), usually at the same visit. Height and weight were measured using a Harpenden stadiometer and a Scale-Tronix weighing scale, respectively.

SNP Genotyping

The *CLCN7* gene (RefSeq ID: NM_001287) consists of 25 exons spanning approximately 30 kb of chromosome 16p13. SNPs were chosen using the International HapMap Project Database, NCBI's LocusLink, and included many of the SNPs reported in other studies [6,8,9]. The genotyped polymorphisms were rs1033466, rs2235580, rs6600147, rs2235579 (Ala390Ala), rs12926089 (Val418Met), rs8767 and VNTR in intron 8 (Figure). Genotyping was performed using a modified single nucleotide extension reaction, with allele detection by mass spectrometry (Sequenom MassArray system; Sequenom, San Diego, CA). The multiplex assays were designed and run with the iPLEX™ format (Sequenom, San Diego, CA). PCR, SAP treatment and iPLEX reaction conditions are available on request.

To genotype the 50 bp-long VNTR in intron 8, PCR was performed using the Multiplex PCR Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with PCR primers (5'-CCGTGGGAAAGGTAACAAAG-3' and 5'-TGAACCTGAGTGGATCATCG-3'). Sizing of the PCR product was done by electrophoresis on 2% agarose gels. Gels were read independently by two individuals. The PCR product with 1 copy of the 50-bp VNTR was 194 bp; each copy of the repeat added 50 bp. The computer program PedCheck [10] was used to identify Mendelian inconsistencies in both SNP and VNTR genotypes.

Statistical Analysis

To identify significant covariates with BMD, stepwise regression analysis was used on lumbar spine and femoral neck BMD, using height, weight, oral contraceptive use (women only), pack-years of smoking, and age. A $p \leq 0.10$ was required for retention of a covariate in the regression model. Regression residuals, representing covariate-adjusted, sex-specific BMD values, were computed and used in all analyses.

A sample consisting of one sibling from each of the 1102 unrelated families was used to estimate the minor allele frequency. This sample was also used to test for significant deviations from Hardy-Weinberg equilibrium for each SNP using a χ^2 goodness-of-fit statistic. All subsequent analyses used genotype data from all available siblings. Linkage disequilibrium coefficients (Lewontin's D') for each pair of SNPs were computed using the Haploview software package [11].

To test for association between SNP genotypes and femoral neck and lumbar spine BMD variation, a population-based association test was performed using a linear mixed model framework. SNP genotype was modeled as a fixed effect (taking on three levels corresponding to the observed genotypes), and family (sibship) as a random effect in the mixed model. Correlation between subjects in the same family was modeled by assuming an error covariance matrix exhibiting compound symmetry. The model was fitted using the MIXED procedure in the SAS statistical software (version 9.1). The mixed model association test employs data from all siblings with both genotype and phenotype. A $p \leq 0.01$ was considered significant for all the analyses.

Results

The men were heavier and taller than the women but there was no difference in mean age (Table 1). Femoral neck BMD was 7% higher in men than women but there was no difference in spine BMD. Age and body weight were the only covariates that approached statistical significance

($p \leq 0.10$) in the regression model fitting of BMD for both women and men. Body weight and age together explained 10.3% and 18.9% of the variation in lumbar spine and femoral neck BMD, respectively, in women and 16.8% and 30.0%, respectively, in men.

The six genotyped SNPs (Figure) had minor allele frequency of at least 0.1 and were in Hardy Weinberg equilibrium ($p \geq 0.38$). The number of copies of the VNTR varied from 1 to 15 (Table 2). We found that 32% of the alleles were repeated 3 times and 53% of the alleles were repeated 5 times, which closely matches the allele frequencies observed by Pettersson et al (8). In women, there were 195 subjects with the 3/3 genotype, 490 with the 3/5 genotype, and 411 with the 5/5 genotype. In men, 64 subjects had the 3/3 genotype, 227 had the 3/5 genotype, and 200 the 5/5 genotype. Given the high frequency of the 3 and 5 alleles and the low frequency of all remaining alleles, the association results for the VNTR (Table 3) were based only on the data from individuals with the 3/3, 3/5, or 5/5 genotypes. We found no association between this VNTR and BMD at the spine or femoral neck in either of our two samples.

Since our intention was to extend the findings of the previous reports to men and premenopausal women, we tested rs12926089, which was found to be associated with femoral neck BMD by Pettersson et al [8]. We found no significant association between this SNP and lumbar spine or femoral neck BMD in either men or premenopausal women. Our sample size was nearly twice the size of Pettersson's, therefore our results are not likely to be due to reduced power. We then extended our analysis to include 5 additional SNPs that were distributed across the gene. We found no association between any of them and BMD at lumbar spine or femoral neck in either sample.

To evaluate whether the genotyped SNPs adequately represented the variations in *CLCN7*, the linkage disequilibrium coefficients (D') estimated from our data (based on the reduced sample used in the VNTR analysis) were examined (Table 4). Overall there was good linkage disequilibrium between markers with the exception of rs6600147, which is not in linkage disequilibrium with the others. These findings are in excellent agreement with HapMap data, which also indicates poor linkage disequilibrium between rs6600147 and any other known marker.

In summary, we found no association between any of the tested SNPs or the VNTR and peak BMD in premenopausal women and in men.

Discussion

In this study, we found no genetic variant in *CLCN7* that accounted for a significant proportion of the variation in peak BMD in healthy premenopausal women and in healthy men. In particular, we could not confirm that rs12926089 was associated with variance in femoral neck BMD [8]. A possible cause for the difference in our findings and those of Pettersson and colleagues [8] is that we studied premenopausal women whereas they studied women around the time of the menopause. However, our results are in agreement with the study of Kornak and colleagues [9] on postmenopausal women, who also failed to find an association between rs12926089 and BMD. In contrast to Kornak et al [9], but in agreement with Pettersson and colleagues [8], we found no evidence for an association between the VNTR in intron 8 and BMD in women. It is possible that peak BMD may be more influenced by factors that influence bone anabolism while genes that affect bone loss, such as *CLCN7*, may be more important in the genetics of bone loss. However, this concept remains speculative without further data. Our negative results in men support our findings in women that genetic variation in *CLCN7* does not play a significant role in determining the variation in peak BMD at lumbar spine or femoral neck in healthy individuals.

This study had several strengths. First, we used two large samples, one of 1692 women and the other of 715 men. These samples had 99% and 91% power, respectively, to detect an association accounting for 2% of the variation in BMD at a significance threshold of 0.01. Second, the SNPs in this study have high heterozygosity, which increased our power to detect associations with peak BMD. Finally, we previously determined that our sample of whites is genetically homogeneous and thus is not susceptible to false positive association results due to population stratification [14].

In conclusion, we did not find an association between seven *CLCN7* polymorphisms and normal BMD variation in large samples of healthy white American women or men. These results suggest that genetic variations in *CLCN7* are not major contributors to the observed variability in peak BMD at either the femoral neck or lumbar spine.

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*MAF: Minor Allele Frequency;

Figure.

Diagram of the physical map and intron/exon boundaries of *CLCN7*. Vertical bars represent exons. The base pair coordinates on chromosome 16 (reference build 36.2) are shown at the top of the figure. One tick mark stands for 1 kb and the whole region is 40 kb. The SNP position (from NCBI), location, variation, minor allele frequency and missing data rates are also shown.

Table 1**Sample Characteristics**

	Men	Women
Number of Families	336	766
Number of Sibling Subjects	715	1692
Age, years [^]	33.6 ± 10.9	33.1 ± 7.2
Height, cm [^]	178.1 ± 6.9	165.5 ± 6.0
Weight, kg [^]	87.2 ± 16.9	69.9 ± 16.5
Lumbar Spine BMD, g/cm² [^]	1.27 ± 0.16	1.27 ± 0.14
Femoral Neck BMD, g/cm² [^]	1.09 ± 0.17	1.02 ± 0.13

[^] Mean ± SD

Table 2
Allele Frequency of VNTR from One Sample per Family

VNTR Repeats	Counts	Frequency
3	646	0.32
4	72	0.04
5	1065	0.53
6	96	0.05
9	79	0.04
1,2,7,8,10,11,15	52	0.02
Total	2010	1

Table 3
Association Results (*p* Value) for Femoral Neck and Lumbar Spine BMD

SNPs (<i>p</i> value)	White Women		White Men	
	Lumbar Spine	Femoral Neck	Lumbar Spine	Femoral Neck
rs1033466	0.10	0.46	0.08	0.16
rs2235580	0.30	0.32	0.70	1.00
rs6600147	0.85	0.74	0.58	0.96
VNTR	0.84	0.26	0.35	0.39
rs2235579	0.11	0.15	0.21	0.30
rs12926089	0.46	1.00	0.70	0.54
rs8767	0.08	0.81	0.56	0.38

Table 4
Linkage Disequilibrium (D') among Polymorphisms in the *CLCN7* Genes

White Sibpair Data	rs2235580	rs6600147	VNTR	rs2235579	rs12926089	rs8767
rs1033466			0.72	0.65	0.89	0.66
rs2235580	0.75	0.05	0.66	0.79	0.87	0.57
rs6600147		0.00	0.19	0.18	0.38	0.15
VNTR				0.56	0.10	0.32
rs2235579					0.98	0.70
rs12926089						0.72