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Association studies of *ALOX5* and bone mineral density in healthy adults

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Summary

Animal studies suggest that arachidonate 5-lipoxygenase (encoded by *ALOX5*) may be a genetic determinant of bone mineral density. We tested this hypothesis in a sample of healthy men and women and did not find consistent evidence for an association between variation in this gene and either lumbar spine or femoral neck BMD.

Introduction—Phenotypic variation in bone mineral density (BMD) among healthy adults is influenced by both genetic and environmental factors. A recent mouse study implicated *ALOX5*, which encodes arachidonate 5-lipoxygenase, as a contributing factor to areal BMD (aBMD).

Methods—Fifteen single nucleotide polymorphisms (SNPs) distributed throughout *ALOX5* were genotyped in three healthy groups: 1,688 European American, premenopausal sisters, 512 African American premenopausal sisters and 715 European American brothers. Statistical analyses were performed in the three groups to test for association between these SNPs and femoral neck and lumbar spine aBMD.

Results—Significant ($p \leq 0.05$) evidence of association was observed with three of the SNPs. However, despite the linkage disequilibrium between SNPs, adjacent SNPs did not provide statistical evidence of association in any of the three study groups.

Conclusions—These data do not provide consistent evidence of association between genomic variation in *ALOX5* and clinical variability in aBMD in healthy subjects.

Keywords

ALOX5; Association; Bone mineral density; Genetics; Race; Single nucleotide polymorphism

Introduction

Bone mineral density (BMD) is a complex phenotype and is influenced by both genetic and environmental factors [1]. Environmental factors, including nutrition and physical activity, influence the attainment of peak BMD; however, genetic factors also account for as much as 80% of the variability in peak BMD attained in early adulthood [2,3]. Racial differences occur in peak BMD; African Americans, on average, have higher peak BMD than European Americans [1].

To identify the genes contributing to the variation in BMD observed among healthy individuals, several ongoing studies have recruited multiple members within a family for detailed phenotypic evaluation which included BMD measurements at multiple skeletal sites. These phenotypic data combined with genotypic marker data have been used to perform linkage analysis to identify chromosomal regions linked to the phenotypic variation in BMD. Results from several studies suggest that multiple genes likely contribute to the observed variation in BMD [4]. Furthermore, there is growing evidence that different genes influence BMD in men and women [4–7] and that there may also be skeletal site-specific genetic effects, with different chromosomal regions linked to either spine or hip BMD [1–4,6–10].

It has been found that 5-lipoxygenase metabolites of arachidonic acid stimulate osteoclastic bone resorption [11–13] and inhibit osteoblastic bone formation [14]. Arachidonate 5-lipoxygenase (encoded by the *ALOX5* gene) belongs to a family of lipoxygenases that catalyze oxygenation of poly-unsaturated fatty acid substrates, including arachidonic and linoleic acids. In mice, *Alox5* has been implicated in the negative regulation of bone development. The disruption of *Alox5* in mice leads to increased bone as compared with normal mice [11,15]. Four independent mapping studies in mice have identified a quantitative trait locus (QTL) for bone related phenotypes in the distal portion of murine chromosome 6, a region that includes the *Alox5* gene [16–19]. A congenic mouse was created which is homozygous for B6 alleles throughout the entire genome except for the 25 cM region harboring *Alox5*, which is homozygous for the C3H donor alleles. These congenic mice had lower BMD than either the B6 background strain or the C3H donor strain [20]. Furthermore, integrative analysis of genotypic, expression and clinical trait data identified mouse *Alox5* as a susceptibility gene for BMD [15]. Therefore, its human homologue, *ALOX5*, is a strong candidate gene that may contribute to the BMD variation in healthy subjects.

Recently, another lipoxygenase, arachidonate 15-lipoxygenase (*Alox15*), was identified as a negative regulator of bone development in mice [21]. Association studies of human ortholog *ALOX15*, and its paralog, *ALOX12*, revealed that polymorphisms in *ALOX12* are correlated with changes in BMD [22,23]. In light of recent findings in the related lipoxygenase gene and evidence in mice, we sought to determine in a sample of healthy sisters and brothers whether polymorphisms in *ALOX5* are also associated with normal variation in peak BMD. To maximize the power of our familial sample to detect association we have performed our analysis using all genotyped members of each family. We have employed a correction for the relatedness of the subjects. This approach yields greater power than reducing the sample to only one individual per family.

Subjects and methods

Sample

Families consisting of healthy siblings were recruited from the state of Indiana to identify genes contributing to bone mass [6,8]. Recruitment focused on families with two or more healthy sisters (n=1,002) or families with two or more healthy brothers (n=336). A small number of families meeting both criteria (at least 2 healthy sisters and 2 healthy brothers) were also included (n=25). Studies were performed at the General Clinical Research Center of Indiana University School of Medicine. Both the sister and brother studies were approved by the Institutional Review Board of Indiana University-Purdue University Indianapolis. Informed written consent was obtained from all subjects prior to their participation in the study.

The sample of sisters ranged in age from 20 to 51 years and included 766 European American and 236 African American families. Sisters were required to be within 10 years of each other in age. Women who had irregular menses, or a history of pregnancy or lactation within three months prior to enrollment were excluded. Women taking oral contraceptives were not

excluded. The sample of brothers ranged in age from 18 to 61 years. There was no exclusion based on the difference in age between the brothers. A sample of African American brothers was also recruited but was not large enough to provide reliable association analysis results. Therefore, only European American brothers were included in the association analyses.

Exclusion criteria for both sisters and brothers included a history of chronic disease, use of medications known to affect bone mass or metabolism, or inability to have BMD measured because of obesity (weight >136 kg). Health and life style questionnaires were administered. A blood sample for the extraction of DNA was collected.

The presence of genetically distinct subgroups within a sample, termed population stratification, can increase the likelihood of a false positive association result. This is particularly true when a sample of unrelated individuals is analyzed. Since we had genotypes available from a previously completed 10 cM microsatellite genome screen [6], we were able to statistically test for the presence of genetically based subgroups in our two racially defined samples (European American and African American). The program Structure [24] was used to test for population stratification. A sample consisting of only one member from each of the genome screened European American (n=982) and African American (n=196) families was tested for stratification.

Using the genotypes, we obtained evidence for two groups; the two groups could clearly be categorized based on the self-reported race of the subject. The two racial groups were then each tested separately for stratification. No statistical evidence was found that there were any subgroups within the European American sample (mean probability of classification into the group was 0.99) or the African American sample (mean probability of classification into the group was 0.93).

BMD, height and weight measurements

Areal BMD (aBMD) at the lumbar spine (vertebrae L2-L4) and femoral neck were measured by dual energy X-ray absorptiometry (DXA), using two DPX-L and one Prodigy instrument (GE Lunar Corp., Madison, Wisconsin). All three DXA instruments were cross-calibrated weekly using a step-wedge phantom. There was no detectable systematic difference between the three machines over the course of the study. The coefficient of variation on duplicate measurements after repositioning was 1.0% for femoral neck and lumbar spine. Siblings were measured on the same instrument. Height and weight were measured using a Harpenden stadiometer and a Scale-Tronix weighing scale, respectively which were regularly calibrated.

SNP selection

Fifteen SNPs distributed throughout the 14 exons and extending approximately 16 kb upstream and 11 kb downstream of *ALOX5* were selected for genotyping. Thirteen SNPs were selected because they had a minor allele frequency of ≥ 0.1 in the European American (CEU) samples genotyped as part of HapMap. One additional SNP was genotyped to provide better coverage of *ALOX5*. A nonsynonymous SNP in exon 6 (rs2228065; Glu254Lys) was also genotyped. Genotyping of SNPs was performed using the iPLEX genotyping assays on a MassARRAY[®] platform (Sequenom Inc., San Diego, CA). The SNPs were also genotyped in the available parental DNA. These genotypes were used to identify Mendelian inconsistencies in the SNP genotypes [26]. Using one randomly selected individual in each family, we tested each SNP for a significant ($p < 0.001$) deviation from Hardy Weinberg equilibrium. The missing rate for SNP genotyping ranged from 2% to 5%.

The program HAPLOVIEW [27] was employed to examine the extent of linkage disequilibrium (LD) between the SNPs to ensure that the SNP density was sufficient to evaluate

evidence of association. LD (D' statistic) was evaluated separately in the European American and African American samples using one randomly selected individual in each family. SNP Tagger [25] (<http://www.broad.mit.edu/mpg/tagger/>) was used to estimate how well the selected SNPs represented the genetic information contained in non-genotyped SNPs in the *ALOX5* region. The extent to which the genotyped SNPs correlated with all HapMap SNPs in the two regions was evaluated for different levels of LD, with the r^2 statistic.

Statistical analyses

Analyses performed in this sample of men and women have found only age and weight to be significant covariates of aBMD [6,8]; therefore, regression residuals, representing age and weight-adjusted aBMD were computed separately in each of the three study groups and used in all subsequent analyses.

Association analyses were performed using the program PedGenie [28], which tests population-based hypotheses regarding disease associations while properly accounting for relatedness of study subjects who are members of the same pedigree. This is achieved by computing a statistic equivalent to the traditional ANOVA F-statistic using the full sample, and then employing a permutation approach using the phenotype and genotype data observed to determine the empirical distribution of this statistic. P-values reported for association are empiric p-values obtained from the PedGenie method.

Results

A sample of 2,915 subjects completed detailed phenotypic assessment (Table 1). DNA was obtained from the parents in many of these families increasing the genotypic dataset to 3,851 individuals. Fifteen SNPs were genotyped throughout the 14 exons, as well as the promoter, upstream and downstream flanking regions of *ALOX5* (Fig. 1; Table 2). In both the European American and African American samples, there was no significant ($p < 0.001$) deviation from Hardy Weinberg equilibrium for any of the SNPs. LD between adjacent SNPs was high in the European American samples (average $D' = 0.95$; Fig. 2a) and the patterns of LD were consistent with those found in the European American HapMap database. The LD in the African American sample (average $D' = 0.91$) was also sufficient to evaluate the evidence of association throughout the gene, despite differences in the LD pattern as compared with the European American sample (Fig. 2b).

Thirteen of the 15 SNPs were in the HapMap database and had genotype data for European Americans. To further evaluate whether the SNPs genotyped in this study provided adequate representation of the sequence information in this chromosomal region, we compared the genotypic information in these 13 SNPs with the genotypic data available from all *ALOX5* SNPs genotyped in the European American HapMap database. From this comparison, we estimate that the 13 SNPs genotyped in this study captured the genotypic information (with an $r^2 \geq 0.5$) contained in 78% of the SNPs in the region that have a minor allele frequency ≥ 0.05 . Because two of the 15 genotyped SNPs were not present in the HapMap database, these estimates of our SNP coverage of *ALOX5* gene are an underestimate. HapMap only contains SNP data from Yorubas (Africans) rather than African Americans; therefore, a similar comparison of SNP coverage was not possible in the African American sample.

Among the 15 SNPs analyzed, one yielded nominal evidence for an association ($p < 0.05$) with femoral neck aBMD in African American women, and two with lumbar spine aBMD in European American men. In no case did the flanking SNPs provide any evidence for association, despite substantial LD. Only one of these SNPs, rs3780901, yielded a p-value less than 0.01; this was in the brother sample when tested for association with lumbar spine aBMD.

Notably, there was no evidence for association when the adjacent SNPs were evaluated, suggesting that these results are most likely to be a false positive association.

Discussion

Combining genotypic, gene expression and clinical data from various mouse models led researchers to postulate that arachidonate 5-lipoxygenase influences bone traits as well as other metabolic measures [15]. The *Alox5* allele carried by the DBA mouse, which has higher BMD than B6, contains a sequence variation that reduces the enzymatic activity of arachidonate 5-lipoxygenase. Furthermore, *Alox5* $-/-$ mice, which have decreased arachidonate 5-lipoxygenase expression, had higher BMD compared to normal mice [11,15]. These lines of evidence suggested that variation in *ALOX5* in humans might contribute to the observed variation in BMD.

We tested 15 SNPs throughout *ALOX5* to test for association with variation that might affect gene expression or protein function. These SNPs spanned a region from 16 kb upstream of the first exon to 11 kb downstream of the final exon, which is likely to cover most, if not all, regulatory elements of *ALOX5*.

In evaluating the evidence of association, we considered two important factors. First, we employed a more stringent statistical threshold for association ($p < 0.01$), that we felt would limit the likelihood of a false positive result. Second, we reviewed the pattern of the association results in the context of the LD between SNPs. In the event of a true association with aBMD, we expected to see significant association results across several SNPs which were in LD with each other.

Only one SNP met our nominal significance level ($p < 0.01$). Flanking SNPs did not show any evidence of association, despite high to moderate LD with adjacent SNPs. This SNP was not found in any apparent functional or conserved regions of the gene (nor were the other two that met a looser standard of $p < 0.05$). Thus, we interpret these data as false positives. Even if the association is true, we estimated by randomly selecting one brother from each of the families that the genotypes at SNP rs3780901 could account for no more than 1.9% of the variation in spine aBMD in men; there was no evidence for an effect in women.

It is interesting to speculate why variations in *ALOX5* are associated with BMD in mouse but are not associated in humans. It is possible that *ALOX5* is strongly influenced by variation in other genes in humans that are not present in inbred strains of mice. It is also possible that the effects of *ALOX5* on BMD are so small as to be undetectable in samples of the size of this study. Our largest sample is the group of 1,688 European American premenopausal women. When employing one randomly selected sister per family ($n = 766$), we have 80% power to detect an association accounting for 1.8% of the variation in aBMD with an alpha of 0.01. We saw no evidence in this sample.

Our study had several strengths. All analyses were performed separately in the three study groups because of the sex and racial differences in BMD as well as the racial differences in SNP allele frequencies and patterns of LD. By performing the analyses in each group separately, we had the ability to detect race-specific or sex-specific effects. We also performed analyses that indicated there was no evidence of stratification in either our European American or African American samples. We genotyped fifteen SNPs distributed throughout the coding region of *ALOX5* and also extending upstream and downstream of the gene. This allowed us to evaluate the evidence of association anywhere within the gene. We also employed rigorous statistical criteria to limit the likelihood of a false positive result.

In summary, we have evaluated the evidence for association of SNPs in *ALOX5* with variation in BMD. We genotyped 15 SNPs throughout the gene, and did not find consistent evidence of association in any of our three study populations. We interpret these data to suggest that variation in *ALOX5* is unlikely to be a clinically significant risk factor contributing to peak BMD.

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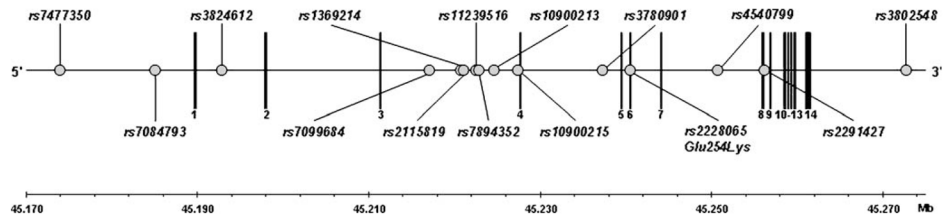


Fig. 1. Structure of *ALOX5* and location of the genotyped SNPs (NCBI Build 36.1). The circles indicate SNPs genotyped in this study. The vertical lines denote exons

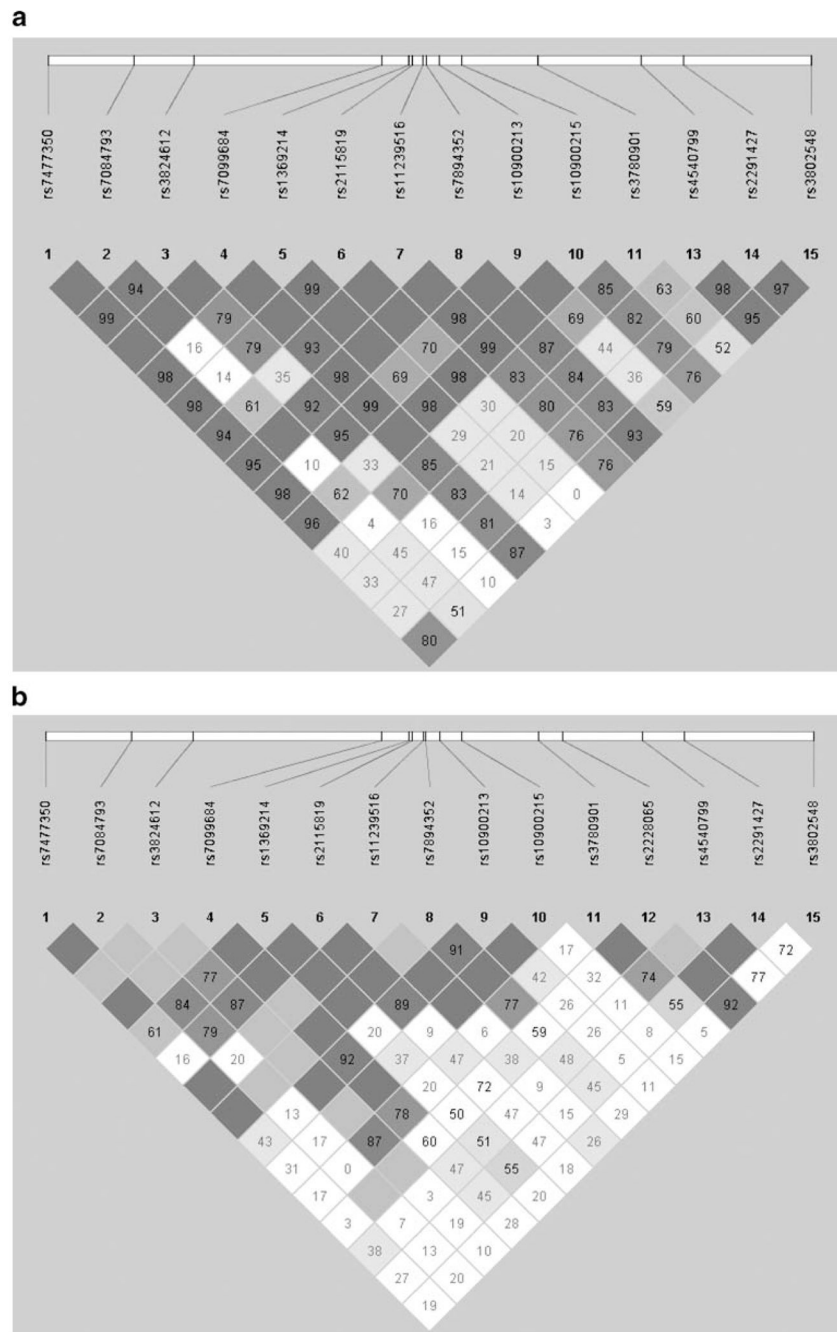


Fig. 2. Linkage disequilibrium (LD) computed between the genotyped SNPs in *ALOX5*. The pair-wise D' statistic is shown within each box (range 0–100; 0 indicating no LD; 100 indicating complete LD). The degree of shading represents the extent of LD. Darker shades indicate greater LD, lighter shades indicate less LD. When no number is shown within a box, and the box is dark gray, the corresponding D' is 100 (complete linkage disequilibrium). **a.** European American samples; **b.** African American samples

Table 1

Sample characteristics

	EA women ¹	AA women ²	EA men ¹
Number of families	766	236	336
Number of sibling subjects	1688	512	715
Number of genotyped parents	634	69	233
Age, years ³	33.1±7.2	33.0±6.6	33.7±10.9
Height, cm ³	165.4±6.0	164.4±6.2	178.1±6.9
Weight, kg ³	69.8±16.4	81.9±19.8	87.1±16.9
Spine BMD, g/cm ² ³	1.27±0.14	1.34±0.14	1.27±0.16
Femoral neck BMD, g/cm ² ³	1.02±0.13	1.11±0.15	1.09±0.17

¹ European American;

² African American;

³ mean ± SD

Table 2

SNP association results

SNP Id	Chr position ¹	SNP location ²	EA MAF ³	AA MAF ³	EA ⁴ women		AA ⁵ women		EA ⁴ men	
					Neck p-value	Spine p-value	Neck p-value	Spine p-value	Neck p-value	Spine p-value
rs7477350	45,173,955	5'	0.38	0.29	0.21	0.15	0.69	0.97	0.94	0.41
rs7084793	45,185,085	5'	0.16	0.20	0.47	0.73	0.04	0.19	0.15	0.02
rs3824612	45,192,870	Intron 1	0.38	0.09	0.45	0.26	0.95	0.77	0.64	1.00
rs7099684	45,217,161	Intron 3	0.19	0.11	0.44	0.53	0.68	0.11	0.59	0.25
rs1369214	45,220,735	Intron 3	0.47	0.50	0.80	0.35	0.72	0.36	0.96	0.69
rs2115819	45,221,095	Intron 3	0.47	0.25	0.67	0.36	0.28	0.72	0.89	0.57
rs11239516	45,222,543	Intron 3	0.14	0.14	0.39	0.45	0.49	0.87	0.43	0.70
rs7894352	45,222,918	Intron 3	0.19	0.11	0.62	0.71	0.58	0.14	0.84	0.41
rs10900213	45,224,720	Intron 3	0.44	0.41	0.34	0.44	0.20	0.72	0.78	0.94
rs10900215	45,227,460	Intron 3	0.14	0.26	0.63	0.46	0.31	0.58	0.43	0.70
rs3780901	45,237,382	Intron 4	0.31	0.39	0.83	0.99	0.85	0.17	0.30	0.006
rs2228065	45,240,512	Exon 6	0.00	0.09	N/A ⁶	N/A	0.67	0.95	N/A	N/A
rs4540799	45,250,791	Intron 7	0.30	0.12	0.29	0.22	0.58	0.30	0.13	0.08
rs2291427	45,256,230	Intron 8	0.32	0.16	0.18	0.24	0.33	0.08	0.17	0.20
rs3802548	45,272,751	3'	0.25	0.22	0.59	0.59	0.92	0.47	0.06	0.05

¹Chromosome positions are based on NCBI Human Genome Assembly v. 36.2.

²Position within or near gene.

³Minor allele frequency.

⁴European American.

⁵African American.

⁶N/A indicates result not available due to low minor allele frequency which invalidates the test of association.