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# Genome-Wide Haplotype Association Mapping in Mice Identifies a Genetic Variant in *CER1* Associated With BMD and Fracture in Southern Chinese Women

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**ABSTRACT:** BMD is a heritable trait and risk indicator for osteoporosis. In this study, we used a genome-wide haplotype association mapping (HAM) approach to identify a haplotype block within *Cer1* that partitions inbred mice strains into high and low BMD groups. A cohort of 1083 high and low BMD human subjects were studied, and a nonsynonymous SNP (rs3747532) in human *CER1* was identified to be associated with increased risk of both low BMD in premenopausal women (OR: 2.2; 95% CI: 1.0–4.6; $p < 0.05$) and increased risk of vertebral fractures (OR: 1.82, $p = 0.025$) in the postmenopausal cohort. We also showed that *Cer1* is expressed in mouse bone and growth plate by RT-PCR, immunohistochemistry, and in situ hybridization, consistent with polymorphisms potentially influencing BMD. Our successful identification of an association with *CER1* in humans together with our mouse study suggests that *CER1* may play a role in the development of bone or its metabolism. Our study highlights the use of publicly available databases for rapidly surveying the genome for quantitative trait loci.
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**Key words: CER1, BMD, association, fracture, southern Chinese women**

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## INTRODUCTION

BMD is a well-defined phenotype used for assessing the risk of osteoporosis, a common age-related disease characterized by the deterioration of bone microarchitecture, with consequent increased bone fragility and susceptibility to fracture. The WHO defines osteoporosis as a condition with reduced BMD of 2.5 SD below the mean for young adults.[1] This arbitrary cut-off is related to the cumulative lifetime risk of osteoporotic fractures in white women.[1] In particular, women are at a higher risk of developing osteoporosis and fractures in later life.

Bone mass in adult life is determined by peak bone mass acquired in young adulthood and rate of bone loss in late adulthood. Acquisition of a higher peak bone mass is believed to reduce the chance of having osteoporosis at a later age. Meanwhile, variation in BMD and the risk of having bone fractures are under strong genetic influence.[2] Several studies in mice suggest that genetic mechanisms can affect skeletal structure and remodeling, so that mice with high peak bone mass differ from those with low peak bone mass in terms of skeletal geometry, histomorphometry, and biomechanical competence.[3] Using the traditional mapping approach[4,5] to search for genetic factors affecting peak bone mass in mouse models, Klein et al.[6] successfully narrowed down the quantitative trait loci (QTLs) and identified *Alox15* as one gene that regulates BMD. The function of *Alox15* in bone cells was further elucidated by knockout mice models. The role of *Alox15* has also been identified in humans, and association studies by us and other groups have shown the association of single nucleotide polymorphisms (SNPs) in the 5′-flanking region and intron of this gene with BMD variation in women,[7,8] suggesting that the mouse is a good model organism for studying BMD variation.

To facilitate the identification of genetic factors associated with peak bone mass, we first took a genome-wide haplotype association mapping (HAM) approach using inbred mice strains that had been genotyped and phenotyped in the Mouse Phenome Project.[9] The idea of using

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multiple inbred strains for QTL mapping was first suggested by Grupe et al.[10] The method was questioned[11,12] until 2004, but when Liao et al.[13] used haplotype information to narrow down a QTL and identify a sequence variant associated with *H2-Ea* gene expression, the approach regained attention from the community. HAM was proposed to incorporate dense SNPs using a large inbred strain panel to analyze patterns of linkage equilibrium among chromosomal regions and search for QTLs genome-wide.[14,15] In HAM, a dense SNPs map was first partitioned into blocks of three SNPs with an average length of 1 Mb. Modified *F*-statistics were calculated for the whole genome to test whether blocks exist where the haplotypes can partition inbred strains into high and low BMD groups.[14] Multiple testing correction was accounted for using extensive permutations.[15] Although we[15] and other researchers[16] have recognized that a small panel of inbred strains may have low power in analyzing complex traits, HAM using dense markers is expected to have a resolution down to the single gene level.[13–16] In this study, the candidate gene *Cerberus 1* (*Cer1*) suggested from HAM analysis was eventually validated by a human case-control cohort of 1083 subjects.

## MATERIALS AND METHODS

### *Genome-wide HAM of BMD in mice*

Thirty strains of mice including BTBR T+ f/J, BUB/BnJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/cdJ, C57L/J, C58/J, CAST/EiJ, CZECHII/EiJ, DBA/2J, FVB/NJ, I/LnJ, JF1/Ms, KK/HlJ, LP/J, MA/MyJ, MOLF/EiJ, MSM/Ms, NOD/LtJ, NZB/BlNJ, NZW/LacJ, PERA/EiJ, RIIIS/J, SJL/J, SM/J, SPRET/EiJ, SWR/J, WSB/EiJ, and 129S1/SvImJ with a dense set of genotyped SNPs[14] were used. Their whole body BMD, measured at 18 wk (Mouse Phenome Database: MPD103 [March 2005] http://www.jax.org/phenome;), was used for this study. We retrieved the mean of the female whole body BMD and SNPs for each strain and ran the HAM program used by Pletcher et al.[14] Basically, the program uses a sliding window with three SNPs and groups them into a block. A modified *F*-test was used to query for the existence of some haplotype structures that could partition mice with high BMD and low BMD. Given the dense number of markers of this study, traditional Bonferroni correction would be too stringent. Statistical significance was therefore assessed using a recent method of bootstrap estimation of common cut-offs based on the generalized family-wise error rate (gFWER), which is able to control for multiple testing while allowing for an acceptable false-positive rate.[17] Basically, we constructed a null reference distribution using a random bootstrap test to determine significance cut-offs by estimating the least upper bound of expected values reported at each locus. Ten thousand genome-wide simulations were performed by running $10 \times 10$ jobs of 100 genome-wide simulations for gFWER 1 = FWER, ..., gFWER 30, using the HPCPOWER System, which contained 128 compute nodes (http://www.hku.hk/cc/ccsystem/hpcpower/), at the University of Hong Kong. A distribution of the $k$th 10,000 values was plotted and the $(1 - \alpha)^{th}$ percentile, $\beta$, was used as our threshold.

### *Study population and selection of high and low BMD human subjects*

The study subjects came from an expanding database being created in the Osteoporosis Center at Queen Mary Hospital, the University of Hong Kong, to study the genetic and environmental risk factors for osteoporosis. Subjects of southern Chinese descent were recruited through advertisements in newspapers and also when they passed by road shows and health talks on osteoporosis held in various districts in Hong Kong between 1998 and 2003. Subjects would excluded from these studies if they had diseases known to affect bone metabolism, were premature to menopause (age < 40 yr), or had a history of bilateral oophorectomy or drug use that could affect bone turnover and BMD. Subjects included in the study all underwent a physical examination and height and weight measurements and were interviewed by a trained research assistant using a structured questionnaire to obtain information on ethnicity, social, medical, and reproductive histories, dietary and lifestyle factors, and family history of osteoporosis. A total of 5872 subjects were invited to the Osteoporosis Center at Queen Mary Hospital for BMD assessment. BMD ($g/cm^2$) was measured at the $L_1$–$L_4$ lumbar spine, femoral neck (FN), trochanter, and total hip using DXA (QDR 4500 plus; Hologic, Waltham, MA, USA). The hip and spine were chosen in the analysis because they are the most common osteoporotic fracture sites. Self-reported history of low-trauma fractures after the age of 45 yr was obtained using a structured questionnaire. Thoraco-lumbar spine X-rays were assessed for radiographic evidence of spine fracture at baseline using a visual semiquantitative method.[18] All low-trauma fractures at the spine, hip, and distal radius were included in the final analysis.

To increase the power of the study, a case-control association approach was adopted. Cases were arbitrarily defined as subjects with a low BMD (Z-scores $\leq -1.28$, equivalent to the lowest 10% of the population) at either the lumbar spine or FN; all cases had T-score $\leq -1$ at either the spine or hip, which was equivalent to osteopenia or osteoporosis according to WHO definition; control subjects were age- and sex-matched individuals with correspondingly high BMD (Z-scores > +1). A total of 1083 case-control subjects, with 613 spine case-control subjects and 900 FN case-control subjects, were included in the analysis, whereas 430 subjects were either case or control subjects in both sites (spine and FN). All participants gave informed consent, and the study was approved by the Ethics Committee of the University of Hong Kong and conducted according to the Declaration of Helsinki.

### *Sequencing and SNP genotyping*

All exons plus the exon–intron boundaries of *Cer1* were sequenced with four sets of primers (Supplementary Table S5).

SNPs were genotyped using the high-throughput Sequenom genotyping platform. Briefly, the genotypes

were determined with the Homogenous Mass EXTEND assay (Sequenom, San Diego, CA, USA). After PCR amplification, nonincorporated dNTPs were removed by shrimp alkaline phosphatase. A detecting primer immediately upstream from the polymorphic site was added together with a specific combination of deoxy dTTP and di-deoxy dATP, dCTP, dGTP, and thermosequenase (Amersham, Bioscience, Piscataway, NJ, USA). The extension products were analyzed by mass spectrometry (Sequenom Mass Array System). DNA from subjects with high and low BMD was randomly assigned to the 96-well plates, and genotyping was performed blind to the BMD status of the samples. Genotyping was repeated in 10% of the samples for verification and quality control. Genotype data were confirmed to have an error rate of <0.1%.

### *Statistical analysis of human data*

All association studies were analyzed separately for pre- and postmenopausal women. Genotype and allele frequencies for each SNP were determined by HAPLOVIEW.[19] Linkage disequilibrium (LD) for the SNP pair (rs3747532 and rs1494360) was estimated using HAPLOVIEW.[19] To assess the association of the SNPs and the BMD status (high versus low), ORs and 95% CIs were determined using binary logistic regression with BMD adjusted for age, height, and weight. Three genetic models were used to evaluate the association of BMD with *CER1* polymorphisms and BMD: (1) additive model of test allele *B*, where genotypes AA, AB, and BB were coded additively as 0, 1, and 2; (2) dominant model of test allele *B*: AA versus AB + BB; and (3) recessive model of test allele *B*: AA + AB versus BB. SPSS 14.0 for Windows was used for statistical calculations. The best genetic model was used to determine the association between the *CER1* SNP and vertebral fractures using a logistic regression model. We first calculated ORs with 95% CIs with adjustment of age, height, weight, and history of fall and then further adjusted for BMD at four skeletal sites (lumbar spine $L_1$–$L_4$, femoral neck, trochanter, and total hip). Bonferroni correction was used to correct for multiple testing.

### *Expression studies of Cer1 in mice*

For RT-PCR, total RNA was prepared from 10-day-old WT mice using Trizol reagent and reverse transcribed into cDNA with random hexamers. cDNA was used as a template in PCR for the amplification of *Cer1* using primers 5′-TGGCATCGGTTCATGTTCAGA-3′ and 5′-GTTCCGTCTTCACCATGCACTG-3′. In situ hybridization was performed using a DIG-labeled riboprobe protocol. The *Cer1* probe corresponded to positions 42–857 in mouse *Cer1* mRNA. *Cer1* cDNA was synthesized by PCR and cloned into pBluescript II. *Col10a1* positive and negative control probes were as previously described.[20] For immunohistochemistry, limbs were fixed in 4% paraformaldehyde before embedding in paraffin. Immunhistochemistry was performed using antibody for *Cer1* (R&D Systems, Minneapolis, MN, USA), and signals were detected using the secondary antibody—horseradish peroxidase (HRP)-conjugated polymer system (Envision+; Dako).

## RESULTS

### *Genome-wide HAM of BMD in mice*

Genome-wide HAM was performed using 30 inbred strains with their whole body BMD variations among strains.[21] The significance threshold for multiple testing correction was estimated to be $-\log(p)=4.114$ ($\alpha < 0.01$, $k = 10$) by gFWER. The results indicated 22 blocks in the genome that could contain genes for BMD in female mice (Table 1).

### *Identification of Cer1 as a potential candidate regulating BMD in mice*

Among 22 blocks identified in the HAM analysis, there were two regions on different chromosomes where there were two peaks in close proximity: chromosome 4, 82.2–87.9 Mb, and chromosome 12, 26.9–28.6 Mb (NCBI mouse build 36.1). The existence of peaks in close proximity may result from two scenarios: (1) two distinct QTLs or (2) a single peak split into two peaks because of a high number of missing SNPs or low frequency of the allele in or near the candidate gene, leading to reduced information near the candidate gene. When we examined the gene list in these two regions, 8 and 52 genes could be found in the flanking region in chromosome 12, 26.9–28.6 Mb, and chromosome 4, 82.2–87.9 Mb, respectively. However, of these 60 genes in these two regions, we excluded 50 of them because of their hypothetical status, leaving 10 well-characterized (known) genes: *Sox11*, *Zdh21*, *Cer1*, *Frem1*, *Snpc3*, *Psip1*, *Bnc2*, *Sh3g2*, *Atl1,* and *Adfp* (USCS mouse genome browser; assembly: February 2006). From these 10 RefSeq known genes in the two candidate regions, we focused on the gene *Cer1*, which bears two exons (3398 bp) residing on chromosome 4 (42.6 cM). It was chosen because it is in close proximity to the peak, and the gene product of *Cer1* is a putative cysteine knot protein that might be directly involved in bone-related development or metabolism. We did not exclude the possibility of having other candidates in this region.

Because no SNP in *Cer1* was included in the initial HAM analysis, no direct association could be studied. To supplement our claim that *Cer1* is a gene affecting BMD variation, all exons of *Cer1* were sequenced, and all five nonsynonymous SNPs on *Cer1* were genotyped in 38 mouse strains (Table 2). We found that the minor allele of rs32341805, which changes a Met to Ile at position 232, was strongly associated with lower whole body BMD of female mice [$r^2 = 0.27$; $F(1,26) = 14.3879$; $p = 0.0005$]. Haplotypes inferred from all these five nonsynonymous SNPs were also found to have a significant association with BMD [$r^2 = 0.30$; $F(6,31) = 3.68$; $p = 0.0071$; Table 2).

To estimate how much QTL variance could be explained by the *Cer1* polymorphism in chromosome 4 QTLs, we performed the HAM analysis again with BMD data adjusted with the rs32341805 genotype information (multiplying the BMD value of mouse strains in the Ile group by the ratio of the mean BMD of the Met group to the mean BMD of the Ile group), and the significant peaks in the region disappeared after reanalysis [the $-\log(p)$ of the peak near *Cer1* and the flanking region dropped from 4.22 to 1,

**TABLE 1.** Significant Peaks Found From Genome-Wide HAM for Female Mouse 18-wk BMD

| *Chr* | *Physical region (NCBI build 36.1)* | *5′ flanking SNP* | *3′ flanking SNP* | *Peak log(p)* |
|---|---|---|---|---|
| 1 | Chromosome 1, 74.8–75.4Mb | rs13475927 | rs13475928 | 6.00 |
| | Chromosome 1, 93.5–94.8Mb | rs13475980 | rs13475985 | 4.52 |
| 2 | Chromosome 2, 25.7–26.3Mb | rs13476390 | rs6227113 | 6.00 |
| | Chromosome 2, 126.8–127.1Mb | rs13476760 | rs3022899 | 4.21 |
| | Chromosome 2, 168.4–170Mb | rs13476905 | rs3143472 | 4.21 |
| 4 | Chromosome 4, 52–52.5Mb | rs6204339 | rs13477705 | 6.00 |
| | Chromosome 4, 82.2–82.3Mb | rs13477806 | rs3692661 | 4.21 |
| | Chromosome 4, 86.5–87.9Mb | rs13477823 | rs13477829 | 6.00 |
| 5 | Chromosome 5, 112.7–113.6 Mb | rs13478465 | rs13478469 | 6.00 |
| | Chromosome 5, 138.7–139.2Mb | rs4225536 | rs13478555 | 4.51 |
| 6 | Chromosome 6, 32.3–32.8Mb | rs13478695 | rs3699842 | 4.52 |
| 10 | Chromosome 10, 13.4–13.8Mb | rs4228122 | rs3658786 | 4.21 |
| | Chromosome 10, 26.8–27.1Mb | rs3679593 | rs13480559 | 4.21 |
| 11 | Chromosome 11, 71.2–71.7Mb | rs13481090 | rs13481091 | 4.21 |
| 12 | Chromosome 12, 7–8.7Mb | rs13481285 | rs13481292 | 6.00 |
| | Chromosome 12, 26.9–27.7Mb | rs4229294 | rs13481358 | 4.22 |
| | Chromosome 12, 27.9–28.6Mb | rs13481359 | rs13481363 | 6.00 |
| | Chromosome 12, 75.3–75.6Mb | rs13481531 | rs3655558 | 4.22 |
| | Chromosome 12, 99.7–100Mb | rs13481609 | rs13481611 | 4.21 |
| 14 | Chromosome 14, 77.1–78.5Mb | rs13482263 | rs3023413 | 6.00 |
| 16 | Chromosome 16, 14.4–15.3Mb | rs4164241 | rs4164780 | 6.00 |
| | Chromosome 16, 44.9–46.5Mb | rs4180154 | rs4182747 | 6.00 |

which is the minimum in the analysis; Fig. 1]. This shows that the mutation of *Cer1* accounts for a large proportion of the QTL variance on chromosome 4.

### *Cer1 is expressed in mouse growth plate*

*Cer1* encodes Cerberus related, which was originally described in 1999 as a multivalent growth factor that acts in the extracellular space that binds to Nodal, BMP, and Wnt proteins through independent sites during development.[22] Initial evidence suggested that the function of murine *Cer1* is transient, solely functions during an early stage of development, and does not have a function after development.[23] However, a more recent finding by Simic et al.[24] showed that administration of BMP6 in osteoporotic rats restores bone inductive activity and decreased expression of several BMP antagonists including *Cer1* in osteoblasts. Given this background, we were unclear how polymorphisms in *Cer1* may influence BMD and studied whether this might be a direct influence of *Cer1* expressed in the growth plate. We studied whether *Cer1* may be expressed in the growth plate and bone by three approaches: RT-PCR, in situ hybridization, and immunostaining (Fig. 2A). RT-PCR showed that in the 10-day-old wildtype mouse, *Cer1* is expressed in all tissues tested including bone marrow, heart, lungs, kidney, spleen, muscle, and throughout the growth plate (Fig. 2A). In situ hybridization (Fig. 2B) showed expression generally in the growth plate, but proliferating chondrocytes appeared to have particularly high levels of expression, whereas hypertrophic chondrocytes had lower levels of expression. The positive control of *Col10a1* showed expression restricted to the hypertrophic zone as expected (Fig. 2B),[20] whereas the negative control showed no expression. Immunostaining also showed general staining throughout the growth plate (Fig. 2C), consistent with the in situ data. Specificity of the *Cer1* antibody was tested by expressing *Cer1* with a V5 tag in 293T cells and observing a band at an identical mass when blotted with either the *Cer1* or V5 antibody. The expression patterns are consistent with polymorphisms potentially having an effect on BMD.

### *Examination of polymorphisms of CER1 in the southern Chinese female population*

In the second stage, we attempted to examine whether the common variation of *CER1* was associated with BMD in humans. We studied a cohort of pre- and postmenopausal southern Chinese women using 12 SNPs of *CER1* that covered the whole 3-kb *CER1* gene (these comprised all SNPs of *CER1* that were available in the Hapmap Database [phase I, Release 18] and three nonsynonymous SNPs [rs3747532, rs17289263, and rs7036635] in the SNP Database [NCBI]). The heritability estimates for BMD were 63–71% in our female population.[25] The demographic data of the studied population are shown in Supplementary Table S2. After exclusion of those SNPs with a low call rate (call rate < 90%, two SNPs) and low heterozygosity (minor allele frequency [MAF] < 0.05, eight SNPs), two SNPs (rs3747532 and rs1494360) were further analyzed. Both were in Hardy-Weinberg equilibrium within our study group: our estimated genotyping error rate was <1%. The data for frequency distribution of SNPs showed a slight variation from the HapMap data (Supplementary Table S4).

LD analysis showed that these two SNPs were in high LD with $r^2 = 0.97$ and $D' = 1$, and therefore, only three haplotypes were present in the population: GG (88.5%), CT (11.2%), and CG (0.3%). We also studied the LD block structure of *CER1* with the adjacent genes using the Chinese population data from the HapMap database as described by

**TABLE 2.** Genotypes and Inferred Haplotype of the Five Nonsynonymous SNPs in *Cer1* and Mean BMD of 38 Mouse Female Strains

| *Strain* | *Observations* | *Mean BMD* | *Haplotype* | *rs32341805 (M232I)* | *rs31776352 (M220R)* | *rs28072586 (R136Q)* | *rs28072584 (V60M)* | *rs28072583 (V6F)* |
|---|---|---|---|---|---|---|---|---|
| KK/HlJ | 10 | 0.0662 | GTCCC | G | T | C | C | C |
| LP/J | 10 | 0.0633 | GGCCC | G | G | C | C | C |
| NON/LtJ | 10 | 0.0631 | GTCCC | G | T | C | C | C |
| NZB/BlNJ | 10 | 0.0623 | GGCCC | G | G | C | C | C |
| C57BR/cdJ | 15 | 0.0618 | GGCCC | G | G | C | C | C |
| C57L/J | 10 | 0.0618 | GGCCC | G | G | C | C | C |
| AKR/J | 9 | 0.0608 | GTCCC | G | T | C | C | C |
| BTBR_T+_tf/J | 10 | 0.0593 | GGCCC | G | G | C | C | C |
| NZW/LacJ | 10 | 0.0590 | GGCCC | G | G | C | C | C |
| SJL/J | 10 | 0.0586 | GTCCC | G | T | C | C | C |
| C3H/HeJ | 10 | 0.0584 | GGCCC | G | G | C | C | C |
| 129S1/SvImJ | 10 | 0.0579 | GGCCC | G | G | C | C | C |
| BALB/cByJ | 9 | 0.0574 | TGTTC | T | G | T | T | C |
| NOD/LtJ | 10 | 0.0573 | GTCCC | G | T | C | C | C |
| CBA/J | 10 | 0.0568 | GTCCC | G | T | C | C | C |
| FVB/NJ | 10 | 0.0564 | GTCCC | G | T | C | C | C |
| BUB/BnJ | 10 | 0.0559 | GGCCC | G | G | C | C | C |
| RIIIS/J | 10 | 0.0556 | GTCCC | G | T | C | C | C |
| SPRET/EiJ | 9 | 0.0545 | GGCCA | G | G | C | C | A |
| C57BL/6J | 9 | 0.0544 | GTCCC | G | T | C | C | C |
| SEA/GnJ | 10 | 0.0543 | TGTTC | T | G | T | T | C |
| C58/J | 9 | 0.0537 | GTCCC | G | T | C | C | C |
| SWR/J | 10 | 0.0536 | TGTTA | T | G | T | T | A |
| CE/J | 9 | 0.0525 | GGCCC | G | G | C | C | C |
| PL/J | 10 | 0.0512 | GTCCC | G | T | C | C | C |
| DBA/2J | 10 | 0.0509 | TGTTC | T | G | T | T | C |
| C57BL/10J | 10 | 0.0498 | GTCCC | G | T | C | C | C |
| MA/MyJ | 8 | 0.0494 | GTCCC | G | T | C | C | C |
| A/J | 10 | 0.0493 | TGTTC | T | G | T | T | C |
| I/LnJ | 10 | 0.0492 | TGTTC | T | G | T | T | C |
| C57BLKS/J | 10 | 0.0478 | GTCCC | G | T | C | C | C |
| CAST/EiJ | 11 | 0.0477 | GGCCC | G | G | C | C | C |
| SM/J | 10 | 0.0471 | GTCCC | G | T | C | C | C |
| MOLF/EiJ | 9 | 0.0468 | TGCCC | T | G | C | C | C |
| WSB/EiJ | 10 | 0.0467 | TGCTA | T | G | C | T | A |
| PWK/PhJ | 10 | 0.0465 | TGCCC | T | G | C | C | C |
| PERA/EiJ | 10 | 0.0465 | TGCTA | T | G | C | T | A |
| CZECHII/EiJ | 9 | 0.0460 | TGCCC | T | G | C | C | C |
| AVONA | Adjusted $R^2$ | | 0.302669 | 0.265697 | −0.01609 | −0.00311 | 0.076576 | 0.37447 |
| | *F* ratio | | 3.6766 | 14.3879 | 0.4141 | 0.8853 | 4.0679 | 2.4397 |
| | *p* | | 0.0071 | 0.0005 | 0.524 | 0.353 | 0.0512 | 0.1271 |

Gabriel et al.,[26] and the results showed that the *CER1* gene region fell into a single LD block (Supplementary Fig. S1) because the gene only contained two exons and was <3 kb.

Binary logistic regression with adjustment for age, height, and weight was performed on the high and low BMD subjects to assess the risk of low BMD. The *G* allele of rs3747532 showed marginal significant association with lower risk of low spine BMD in premenopausal women, but not postmenopausal women, in an additive fashion (OR = 0.5, $p = 0.05$). Using a recessive model, *G* alleles of rs3747532 and rs1494360 showed lower risk in developing low spine BMD with an OR of 0.45 ($p = 0.045$) and 0.48 ($p = 0.047$), respectively (Table 3). These results suggest a significant association of *CER1* with peak bone mass variation, which further supports the association result using inbred mice. In the power calculation, our group of premenopausal women with BMD variation in the lumbar spine had >80% power in detecting a QTL that contributed to at least 1.5% of the final variance in BMD in an additive model, assuming that the marker itself was a true causal allele.[27] The association of *CER1* with BMD in pre- but not postmenopausal women suggests that *CER1* was associated with peak bone mass variation among the young rather than bone loss in later life.

### *CER1 polymorphism and prevalent vertebral fracture in southern Chinese*

Because overexpression of another related BMP antagonist, gremlin, led to spontaneous fracture,[28] we hypothesized that common polymorphisms in *CER1* may alter fracture risk. In the postmenopausal cohort ($n = 620$) with 130 prevalent fractures (Supplementary Table S3), we observed an association between minor allele *G* of rs1494360

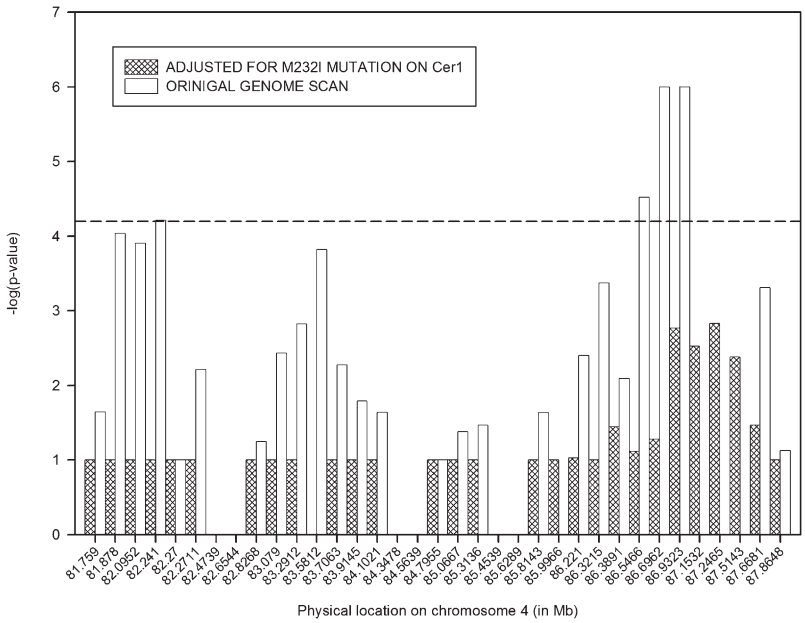


**FIG. 1.** A zoom-in view of HAP on chromosome 4: 82.2–87.9MB QTL (NCBI 36.1).

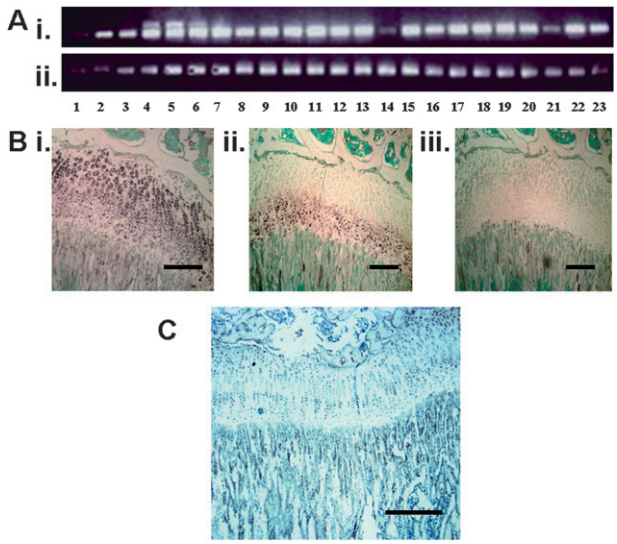


**FIG. 2.** Expression of *Cer1* in a 10-day-old wildtype mouse.

and prevalent vertebral fracture risk in recessive modeling, with increased odds of vertebral fractures (OR = 1.92, $p$ = 0.016; Table 4). Not surprisingly, a similar association was also observed between the *G* allele of nonsynonymous SNP rs3747532 and vertebral fracture, with an increased odds of vertebral fracture (OR = 1.82, $p$ = 0.025). rs3747532 is associated with vertebral fracture before and after adjustment of BMD, suggesting the effects of *CER1* polymorphisms may affect fracture risk through a mechanism that is independent of BMD.

## DISCUSSION

Identification of candidate genes underlying peak bone mass variation and fracture risk has been challenging in the field. Using HAM in mice and subsequent replication in humans, we found evidence of an association between peak BMD variation and the *Cer1* polymorphism in mice. *Cer1* is close to the linkage peak of the mouse chromosome 4 region, which is syntenic with chromosome 9p22–23 in humans. In addition, using vertebral fracture per se as the phenotype, a significant association between *CER1* polymorphism and fracture suggested significant clinical potential of the *CER1* gene or its polymorphism in future fracture management.

According to the community's view of identification of QTLs and candidate genes by the Complex Trait Consortium, we showed different lines of evidence in this study that support *Cer1* as a gene for peak bone mass variation.[29] First, we sequenced exons of *Cer1* in mice that eventually identified an SNP that codes for an amino acid change (M232I) that was significantly associated with BMD. M232I is located in the cystine knot, which potentially affects the interaction of *Cer1* with other interacting proteins such as TGFβ and the BMPs. Subsequently, a nonsynonymous SNP, rs3747532, which changes the amino acid alanine to glycine at position 65, was found to be significantly associated with spine BMD and vertebral fracture in humans, further suggesting that these nonsynonymous SNPs might have a functional impact on the gene product. Second, we showed in our own experiments that *Cer1* is expressed throughout the growth plate at both the RNA and protein levels, consistent with the gene playing a role in bone. Our data were supported by public database expression data for this gene in quantitative trait–related bone cells, which showed expression in osteoblasts and their mesenchymal precursor cells. Third and most convincingly, after showing an association of *Cer1* with the phenotype in the mouse, there was a successful replication in humans, showing strong evidence that *CER1* is one of the genes regulating human peak bone mass and fracture risk.

**TABLE 3.** Association of *CER1* Polymorphisms With BMD Variation in Premenopausal Women

| SNP | BMD group | AA | Aa | aa | p | OR* | 95% CI |
|---|---|---|---|---|---|---|---|
| rs3747532 | Low BMD | 74.8 | 23.7 | 1.5 | 0.04† | 0.45 | 0.2–1.0 |
| | High BMD | 82.2 | 15.8 | 2.0 | 0.05‡ | 0.5 | 0.3–1.0 |
| rs1494360 | Low BMD | 74.6 | 23.9 | 1.5 | 0.05§ | 0.45 | 0.2–1.0 |
| | High BMD | 81.7 | 16.3 | 2 | | | |

A, major allele; a, minor allele.
* Results are OR for risk of low BMD.
† Recessive model of *G* allele.
† Additive model of *G* allele.
§ Recessive model of *G*.

**TABLE 4.** Results of Association of *CER1* SNPs for Fracture at Any Site With and Without Adjustment of BMD

| | | Without adjustment of BMD* | | | | Adjustment of BMD† | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 95% CI | | | | 95% CI | |
| SNP | Allele | p | OR | Lower | Upper | p | OR | Lower | Upper |
| rs1494360 | G | 0.016 | 1.93 | 1.13 | 3.29 | 0.034 | 1.79 | 1.04 | 3.06 |
| rs3747532 | G | 0.025 | 1.82 | 1.08 | 3.08 | 0.045 | 1.71 | 1.01 | 2.90 |

* *p* value with adjustment of age, height, weight, sex, and history of fall.
† *p* value was further adjusted with lumbar spine $L_1$–$L_4$ BMD, femoral neck BMD, trochanter BMD, and total hip BMD.

In the initial HAM analysis, we used the information from 30 strains instead of all available strains (38 strains) based on the grounds of accuracy and robustness. Before the HAM analysis, we performed phylogenetic analysis of all 38 strains. The distance between every two strains was defined as the number of genotypes that they differed at a SNP. The distance was normalized by dividing the numbers of SNPs that had no missing genotypes information for the two strains. A phylogenetic tree was constructed using a neighbor-joining algorithm. The topology of the tree was compared with that of a recent study,[30] which divided the strains into six clusters. Any strains that fell into the wrong cluster were removed from the genome-wide HAM study to retain the robustness of the subsequent HAM analysis. Therefore, 30 strains that fulfilled both of the above two criteria were included in the final analysis. Although using 30 selected strains was a robust way to screen out false-positive findings, it was somewhat less powerful. Therefore, to improve the power of the association analysis between *Cer1* and BMD, 38 mice strain data were used for the association analysis.[15] The successful replication study in humans suggested that our gene-mapping strategy was feasible. In addition, we observed that several positional candidate genes within the 22 QTLs were previously known to be involved in bone development (NCBI mouse build 36.1); for example, Indian hedgehog (*Ihh*) on chromosome 1 (74.8–75.4 Mb) regulates the site of hypertrophic differentiation and determines the site of bone collar formation.[31] Furthermore *Hdac4,* a key component in chondrocyte hypertrophy and skeletogenesis,[32] and *Twist2,* a marker for osteoblast differentiation,[33] were found on chromosome 1 (93.5–94.8 Mb); *Notch1*, found on chromosome 2 (25.7–26.3 Mb), was also found to inhibit osteoblastogenesis through the Wnt/β-catenin pathway.[34] Future association studies focusing on these 22 QTLs may be fruitful in identification of other candidate genes that underlie BMD variation. A few regions were mapped to the previously reported QTLs at other bone sites (Supplementary Table S1). However, none of the HAM QTLs were the same as the previously reported QTLs for whole BMD variation in mice.[4,5] This may be explained by different strategies in gene mapping, whereas the HAM method applied here was expected to have a high resolution down to the single gene level.[13–16]

*Cer1* is a homolog of Cerberus in *Xenopus*, which belongs to a cystine knot superfamily containing a cystine knot motif in the C-terminal cysteine-rich region.[35] A previous study suggested that *Cer1* is an antagonist of the BMPs and Wnt.[35] Because both BMPs and Wnt signaling are very important in bone development, it is not surprising that variations in BMP antagonists may affect skeletogenesis and BMD variation in humans (e.g., the sclerosteosis/van Buchem disease gene, which is caused by mutations in *SOST*, the gene coding for the cysteine-knot protein sclerostin).[36] We performed experiments to study expression showing that *Cer1* was expressed in mouse growth plate by RT-PCR, in situ hybridization, and immunostaining. In addition, according to a public repository for high-throughput gene expression data,[37] *Cer1* is expressed in mouse osteoblasts (NCBI GEO; accession no. GDS928, GDS1631) and mesenchymal stem cells (GDS1288). Taken together with our association analysis in human, *Cer1* is an excellent positional candidate gene that may be important in bone development.

In this study, the current finding suggested that *G* alleles of rs3747532 and rs1494360 were associated with high spine BMD in premenopausal women but higher vertebral fracture risk in postmenopausal women. This could be explained by gene–environmental interactions with different estrogen levels as we reported previously[8] or the balance between BMPs and BMP antagonists. BMP is a potent osteogenic molecule, and overexpression of certain BMP antagonists (noggin, gremlin, etc.) have previously been suggested to be catabolic, resulting in low bone mass.[28,38,39] However, the BMP antagonist noggin was recently found to play a role in human mesenchymal stem cell differentiation whereby noggin controls BMP-2 and BMP-2 receptor expression, eventually leading to bone formation.[40] Thus far, no definite conclusion can be drawn regarding the roles of BMP and BMP antagonists in bone metabolism. BMP is a potent osteogenic molecule; however, it is also needed for osteoclast formation.[41,42] Because BMP signaling is controlled by several BMP antagonists, there is likely to be strict control of the interplay between BMP and BMP antagonists essential for formation of high-quality bone, whereby overexpression of either BMP or a BMP antagonist may also result in susceptibility to fracture.[43] Therefore, *G* alleles of rs3747532 and rs1494360 associated with high BMD in premenopausal women together with a higher risk of vertebral fracture in postmenopausal women might be explained in terms of

bone remodeling instead of BMD. High BMD is not necessarily equal to a high quality of bone in terms of microarchitecture, a major determinant of fracture; therefore, the higher risk of fracture associated with *G* alleles in both SNPs may be caused by deterioration of microarchitecture of bone, possibly independent of BMD. This postulation is consistent with the observation that the association between *CER1* SNP and vertebral fracture remained significant after adjustment of BMD in all sites.

There are several limitations of this study. Although we identified *CER1* as a gene for BMD variation, further functional confirmations such as transgenesis, knock-ins, and deficiency-complement tests are needed to examine the molecular role of *CER1* in bone metabolism.[29] In addition, because we only studied one positional candidate gene under the QTL, we cannot exclude the possibility of the existence of other candidate genes on chromosome 4 in mice. Nevertheless, removal of the peak signal in chromosome 4 QTL after adjustment of *Cer1* genotype suggested that *Cer1* variation explained a great proportion of chromosome 4 QTL (i.e., *Cer1* is an important positional candidate gene in that region). In addition, a recent 100K genome-wide association[44] showed a highly significant association between the SNP rs1552896 in *FREM1*, which is in close proximity with *CER1*, and femoral neck shaft angle, with a $p$ of $8.87 \times 10^{-6}$, implying that *FREM1* may also be an important candidate gene in bone metabolism. Therefore, a future QTL-wide fine mapping study will not only validate our findings but also show more candidate genes under the QTL. In addition, the association signal observed in the human study was modest: it became insignificant after Bonferroni correction for multiple traits tested. However, the modest $p$ value may be resulted from power issue, especially when the marker tested is only in modest LD with the causal variant; therefore, replication in larger number of subjects would be a better approach to validate this finding.

In conclusion, using HAM analysis, we identified several QTLs underlying peak BMD variation. Our results also showed that *Cer1* is a candidate gene underlying peak BMD variation in mice and probably in humans. The association of *CER1* with peak BMD and vertebral fracture in human further suggests there is a potential role for this gene in bone metabolism. Future functional and replication studies of *CER1* will be important in understanding the etiology of osteoporosis and in improving fracture management, such as the current use of BMP-2 as a therapeutic agent for fracture healing. In addition, our study also showed that use of publicly available mice resources with careful design and interpretation can facilitate the identification of genes underlying complex traits in humans. With the ongoing interest in genetic studies of inbred mice, a comprehensive phenome and dense genome will soon be available for accurate and quick surveys for complex traits.

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