RESEARCH ARTICLE

Quantitative trait loci for tibial bone strength in C57BL/6J and C3H/HeJ inbred strains of mice

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Abstract

Three-point bending technology has been widely used in the measurement of bone strength. Quantitative trait loci (QTLs) for bone strength have been identified using mouse femurs. In this study, we investigate the use of mouse tibiae in identification of QTLs that regulate bone strength. Mouse tibiae were from a F_2 population derived from C57BL/6J (B6) and C3H/HeJ (C3H). Three-point bending was measured using ISO 4049, with the support width adjustable to accommodate specimen sizes outside the scope of ISO 4049. The strain rate is selectable from 0.05 to 500 mm per min. All stress strain diagrams are recorded and retrieved in digital electronic form. Genome scan was performed in The Jackson Laboratory (TJL). QTL mapping was conducted using Map Manager QTX software. Data show that (i) both elastic modulus (stiffness) and maximum loading (strength) value appear as normal distributions, suggesting that multiple genetic factors control the bone strength; (ii) 11 QTLs, accounting for 90% of variation for strength, have been detected. More than half QTLs of three-point bending are located on the same locations of bone density earlier identified from mouse femurs; (iii) a major QTL of femoral and vertebral bone mineral density (BMD) was not detected for bone strength of tibiae; (iv) the QTL on chromosome 4 has extremely high LOD score of 31.8 and represents 60% of the variation of bone strength; and (v) four QTLs of stiffness (chromosomes 2, 11, 15 and 19) have been identified.

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Introduction

In the last decade, mouse model has increasingly been used in the identification of genetic factors that regulate osteoporosis related traits such as bone mineral density (BMD). A F_2 population derived from the cross between C57BL/6J (B6) and C3H/HeJ (C3H) (B×C F_2) has been widely used in the identification of QTLs of BMD (Beamer *et al*. 2001, 2005; Sheng *et al*. 2002; Koller *et al*. 2003; Sheng *et al*. 2004). In an early study, Beamer *et al*. (1996) compared the BMD of 11 inbred mouse strains using peripheral quantitative computed tomography (pQCT) and found that the C3H has the highest bone density while the B6 has the lowest. Since then, these two strains have been used in identification of more than 10 QTLs of femoral and lumbar vertebral BMD (Beamer *et al*. 2001, 2005; Koller *et al*. 2003; Bouxsein *et al*. 2004). The molecular mechanism of regulation of difference of BMD between these two strains has also been extensively investigated (Bouxsein *et al*. 2002, 2004; Li *et al*. 2002a; Sheng *et al*. 2002; Koller *et al*. 2003).

Risk of osteoporosis or fractures is largely dependent on the bone quality. Although BMD is an important factor in determining the bulk bone strength, the bulk bone strength is

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ultimately determined by both material and geometric properties (van der Meulen *et al*. 2001), which are regulated by complex interaction between multiple genetic factors and environment. Accordingly, fully characterization of osteoporosis related traits in addition to BMD between B6 and C3H will provide a wealth of information for the understanding of genetics of osteoporosis. While most of QTL identified are for BMD, other osteoporosis related traits have been characterized from B6 and C3H crosses. Those traits include relationships among serum insulin-like growth factor I, bone mineral density, and skeletal morphology (Bouxsein *et al*. 2002), genetic locus that modulates serum insulin-like growth factor I and bone acquisition (Adamo *et al*. 2006; Delahunty *et al*. 2006), QTL for vertebral trabecular bone volume fraction and microarchitecture (Bouxsein *et al*. 2004), site-specific regulation of trabecular and cortical bone morphology (Judex *et al*. 2004), osteocalcin levels in bone versus serum (Li *et al*. 2002a) and bone adaptive response to mechanical loading (Kesavan *et al*. 2006).

It is not known whether the same set of QTL controls BMD in both femurs and tibiae. Like many other mammals, in mice, the femur is used in a pendulum-like motion to propel the leg forward and backwards, while tibia and foot segments engage in bending and accordion-like movements which serve to lift the foot during the recovery phase of the step. It is obvious that there are difference in structural and material properties, and morphological parameters of the mouse femur and tibia. Previously, we have reported that QTLs controlling bone density and bone strength are different (Li *et al*. 2002b). In a study of genetic loci influencing femur-breaking strength (FBS), which was measured by three-point bending, we identified six significant QTLs affecting bone breaking strength on chromosomes 1, 2, 8, 9, 10 and 17, which together explained 23% of F_2 variance. Of these, the QTLs on chromosomes 2, 8 and 10 seem to be unique to bone breaking strength, whereas the remaining three QTLs are concordant with the femur BMD QTLs. Genetic analysis suggests that, of these six FBS QTLs, three influence BMD, two influence bone material properties, and one influences bone size. In this study, with the same B×C F_2 population, the femurs of which were used to identify QTLs of BMD, we measured the bone strength of tibiae and identified QTL that regulate bone strength. Here, we report the QTL and compared them with that of three-point bending.

Materials and methods

Animals and tibia

All tibiae used in this study were from female mice. Mouse strains of B6 and C3H mice were obtained from The Jackson Laboratory (TJL). Before sample collections, mice were housed at the animal research facility, UM, Memphis, TN, Egypt. Experimental animal procedures for this study were approved by the IACUC of the UM. Mouse tibiae from B×C F_2 population used in this study were provided by WGB from TJL. Mouse tibiae were from the same mice that have been well described for QTLs of femoral and vertebral volumetric (v)BMD (Beamer *et al*. 2001). All tibiae were harvested from female mice at four months of age and stored at room temperature in 95% ethanol to eliminate bacterial and fungal growth, and to dehydrate and defat bone prior to use in original vBMD studies and for the three-point bending measurements at TJL.

Three-point bending measurement of bone strength

Three-point bending was measured using ISO 4049, with the support width adjustable to accommodate specimen sizes outside the scope of ISO 4049. The strain rate is selectable from 0.05 to 500 mm per min. All stress strain diagrams are recorded and retrieved in digital electronic form. All bones were taken out of the formalin solution and kept in saline at least one day before testing. A testing head with a span of 9 mm was used to conduct the measurement. The bone was oriented with the distal end towards the left of the machine and the proximal side towards the right of the machine. The nostral side of the bone was pointed towards the ground. The Instron 1550 was set to stop after load decreased by 20% from the maximum load. From this test, we recorded maximum load, stiffness and modulus.

Genotyping of the parental strains and F_2 population

Genotype of each individual of F_2 population was done using microsatellite markers in TJL (Beamer *et al*. 2001). Briefly, genomic DNA was extracted from kidney using chloroform/phenol and high salt methods. Genotyping of individual mouse DNAs was accomplished by polymerase chain reaction (PCR) using oligonucleotide primer pairs and electrophoresis. Primer pairs identifying simple sequence length polymorphisms between B6 and C3H were selected from more than 60,001 available from TJL genomic information (http://www.informatics.jax.org/menus/marker_menu.shtml). Name of the markers are the same as previously reported (Beamer *et al*. 2001). Those molecular markers have been used for the genetic mapping of bone mineral density (Beamer *et al*. 2001) and for the nanoindentation property (Jiao *et al*. 2007) of the same F_2 population as in this study.

QTL analysis

QTX (http://www.mapmanager.org/mmQTX.html) was used to analyse the QTLs that regulate bone strength. Data from three-point bending and genotypes were organized in patterns requested by the software. Briefly, data on phenotypes and genotypes (in F_2 or RI strains) were organized in a text file, and then the file was opened with the import command. We formatted the file according to the specific instructions. For example, the text file was formatted with each locus represented by a line of information, and items of information in that line were separated by a specific character, usually a tab.

To determine if any genes were acting together to influence bone strength, we conducted mapping using both the maximum load strait and the elastic modulus. The text file of phenotype was uploaded first into the QTX. Then, data of genotype corresponding to each phenotype were uploaded in a second file. With marker regression analysis, a likelihood-ratio statistic (LRS) correlating genotype with each phenotype was listed for each of the 107 marker genotypes with a $P < 0.05$. Next, each set of significant markers in a chromosomal region (starting with the set with the highest significance) in each trait was studied by interval mapping of all the markers on that chromosome.

To determine significance levels for these LRS scores, 500 permutations of all marker genotypes together from the set of 485 F_2 progeny were tested. The resulting data at suggestive, significant, or highly significant levels were then marked to QTL as references for consideration by readers.

Results

Genotypes of B6C3H-F_2 progeny

A total of 116 microsatellite markers with an average of 13 cM coverage of the whole genome have been previously genotyped (Beamer *et al*. 2001; Jiao *et al*. 2007) and have been used to genotype this B6C3H-F_2 population.

Bone strength properties from B6C3H-F_2 population

Bone density has been proved to be a quantitatively inherited trait. We assumed that bone property measured by three-point bending is also controlled by QTLs. We therefore used the F_2 mice derived from B6 and C3H to map QTL that regulate the bone property measured by three-point bending. According to the genetic principle, if the bone three-point bending properties are controlled by QTLs, then the bone three-point bending properties of the F_2 population will show a normal distribution pattern. We examined 683 F_2 mice, and compared data to the two progenitors. Figure 1 shows the distribution of properties of three-point bending of the F_2 population. Statistical analysis indicates that the distribution of maximum load appears fitting into the normal distribution well with skewness of 0.63 and kurtosis of −1.14. However, the elastic modulus appears skewed, with skewness of 1.97 and kurtosis of 3.28.

QTL loci obtained from measurement of three-point bending from the same set of mice

Because the data from three-point bending are largely dependent on the bone mineral density, we expect that many QTLs to be same between the three-point bending and the bone density. Tables 1 and 2 show QTLs from the elastic modulus and the maximum loading. More than half QTLs of three-point bending are located on the same locations of bone density, suggesting that the hardness measures the

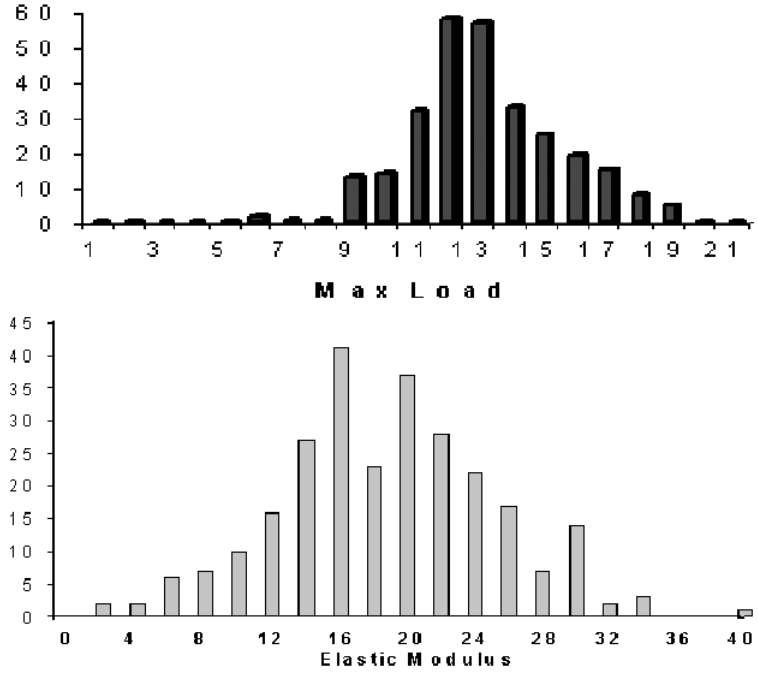

Figure 1. The distribution of two parameters from 500 measurements from a B6C3H-F_2 population. The distribution curve shows that both elastic modulus (GPa) (figure 2a) and maximum loading (LPa) (figure 2b) are controlled by multiple genes/loci.

Table 1. QTLs determining elastic modulus, determined by three-point bending.

Chromosome	Name of QTL	Locus	LOD score	% Changes	Size (cM)	Coverage (cM)
2*	EM1	D2mit15- D2mit103	12.8	10	6	50 cM–56 cM
11*	EM2	D11mit71-D11mit70	12.5	11	70	1.1 cM–71 cM
15*	EM3	D15mit175-D15mit42	7.3	3	36	9.9 cM–55.5 cM
19	EM4	D19mit19-D19Mit137	6.3	2	29	26 cM–55.7 cM

*QTL position is located in the QTL region of bone mineral density.

Table 2. QTLs determining maximum loading, determined by three-point bending.

Chromosome	Name of QTL	Locus	LOD score	% Changes	Size (cM)	Coverage (cM)
1	ML1	Dmit231-D1MIT410	6.7	3	5	12 cM–17cM
4*	ML2	D4mit214-D4mit33	31.8	60	61.9	17.9 cM–79.0 cM
5	ML3	D5mit24-D5mit101	6.8	3	21	60 cM–81.0 cM
6*	ML4	D6mit44-D6Mit15	4.7	2	23	51.5 cM–74.0 cM
7*	ML5	D7mit36-D7mit332	4.2	2	16	50 cM–65.6 cM
9	ML6	D9mit34-D9Mit18	8.4	3	21	50 cM–71.0 cM
13*	ML7	D13Mit218-D13mit54	4.5	4	45	9 cM–54 cM
15*	ML8	D15Mit070	6.1	3	36	9.9 cM–55.5 cM
16	ML9	D16mit173-D16mit120	4.1	4	13	43 cM–56 cM
17	ML10	D17mit164-D17mit274	6.4	4	29	4.1 cM–33.5 cM
18*	ML11	D18mit20-D18mit120	3.9	2	9	5 cM–16 cM

*QTL position is located in the QTL region of bone mineral density.

similar properties of bone density. QTLs that are not located in the same region of QTLs from femurs represent either the difference between bone properties measured by three-point bending and bone mineral density or the difference between femurs and tibia.

Four QTLs on chromosomes 2, 11, 15 and 19 were detected for elastic modulus (table 1), which together explained 26% of F_2 variance. The QTL on chromosome 11 has similar location with the QTL from femurs of the same cross (Beamer *et al*. 2001) and other crosses (Shimizu *et al*. 1999; Klein *et al*. 1998). QTL on chromosome 15 has similar location as that of a QTL using femur from F_2 population derived from C57BL/6J and CAST/EiJ (Beamer *et al*. 1999). QTL on chromosome 2 has been reported using peak whole body BMD from a F_2 cross created by C57BL/6 × DBA/2 (Klein *et al*. 1998, 2001). QTL 19 has not been reported earlier; however, it represents only a small percentage of variation.

Permutation test for elastic modulus indicated that $P < 0.001$ was 21.2 (highly significant or HS); $P < 0.05$ was 16.0 (significant or S), and $P < 0.67$ was 9.6 (suggestive or sugg) (Suzuki *et al*. 2002; Liu *et al*. 2008). By this standard, QTLs on chromosomes 2 and 11 fall between the significant and suggestive levels.

Genetic control of the maximum loading was found extremely high. Eleven QTLs on different chromosomes 1, 4, 5, 6, 7, 9, 13, 15, 16, 17 and 18 were detected for maximum loading (table 2), and together they explained 90% of the F_2 variance. QTLs on chromosomes 4, 6, 13 and 18 were located on the similar locations to those detected using femurs BMD from the same cross (Beamer *et al*. 2001). Location of QTL on chromosome 7 was near the QTL using peak whole body BMD from a F_2 cross created by C57BL/6 × DBA/2 (Klein *et al*. 1998). The location of QTL of maximum loading on chromosome 15 was the same region of elastic modulus, which is also the same region of QTL of BMD detected using femurs from a F_2 population derived from C57BL/6J and CAST/EiJ (Beamer *et al*. 1999). QTLs on chromosomes 1 5, 7, 16 and 17 appeared only here. These four QTLs contributed about 13% of the variation.

Permutation test for maximum loading indicated that $P < 0.001$ was 19.3 (HS); $P < 0.05$ was 15.2 (S), and $P < 0.67$ was 9.3 (sugg). By this standard, the QTL on chromosome 4 reached high significant level, and the QTL on chromosome 18 reached the significant level.

QTL on mouse chromosome 4

QTLs on chromosome 4 for the maximum loading, ML2, surprisingly dominate over QTLs on all other chromosomes with the LOD score as high as 31 and represent about 60% of changes. Although the QTL are spread almost all over the chromosome, major contributions seem to be in the region between marker D4mit27 and D4mit13. Figure 2 shows the QTLs along chromosome 4. It appears that multiple genes contribute to the QTL on the chromosome. However, considering the high percentage it explains, it is also possible a major gene that contributes to the bone property measured by three-point bending. The biggest QTL of BMD of femurs was also located on chromosome 4 from the same cross

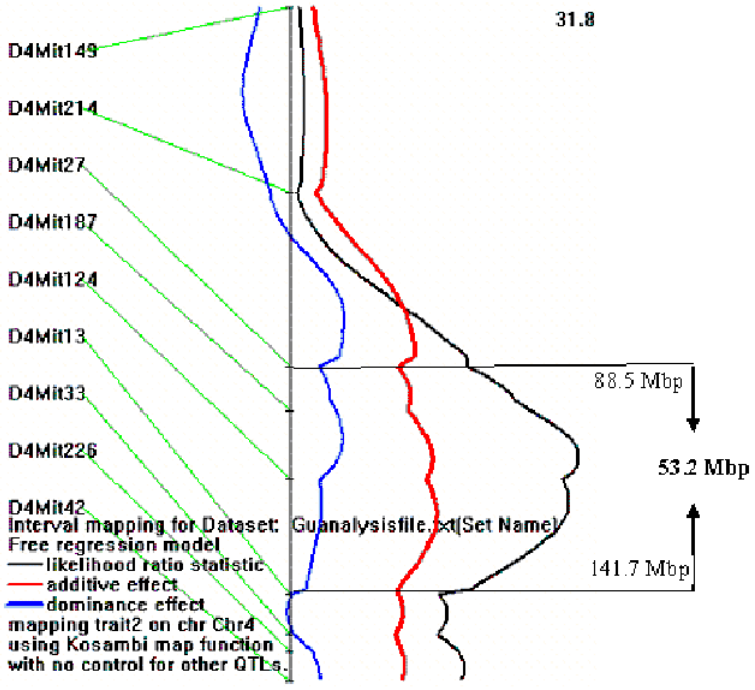

Figure 2. QTL detected for maximum loading on chromosome 4. The black vertical dashed line indicates the cut off LOD score of 31.8. The two horizontal black lines indicate the critical region of the QTL flanked by microsatellite markers D4mit27 and D4mit13.

(Beamer *et al*. 2001), although the percentage of BMD that the QTL explained was not as high as that of max loading that the QTL does in this study.

Discussion and conclusion

Most of the studies on the QTLs of bone strength using animals have been done on femurs. This study mapped QTLs of bone strength, an indicator of bone quality, using the tibiae. The F_2 population in this study has been used in the study of bone mineral density using femurs (Beamer *et al*. 2001). Therefore, this study provides opportunity for the comparison of QTLs between femurs and tibiae. Our study revealed two sets of QTLs; one set is shared between femurs and tibiae, and the other set is tibia specific. Together the tibia-specific QTL contribute to 13% of the variation on the maximum load of bone strength.

An extremely high genetic component of bone strength measured by maximum loading in tibiae was detected. QTL on 11 chromosomes regulate 90% of the tibial maximum loading. A simple interpretation for this data is that the strength of tibiae is genetically fixed without much influence by environment. Thus, the development of tibiae is not as flexible as that of femurs. However, our data obtained from mice which were produced at a fixed environmental condition at TJL. It appears that the influence of environment on the tibiae at TJL may be at a minimum level. In contrast, a relatively low genetic variation of elastic modulus (26%) was detected; indicating environment may play a larger role in determinant of elastic modulus of the tibiae.

A significant difference between QTL from tibial strength and that from BMD of femurs and vertebrae is the locus on chromosome 1. Previously, collectively, the BMD QTLs for femurs accounted for 35.1% and for vertebrae accounted for 23.7% of variances detected from the same F_2 population (Beamer *et al*. 2005). Table 3 summarizes all QTL detected in tibiae, femurs and vertebrae from the B×C F_2 population in different studies. Major QTLs from femurs, on chromosomes 4, 7 and 18 have been detected from tibial strength except the one on chromosome 1, which has the largest impact on femoral as well as vertebral BMD. Only a QTL with small impact on tibial strength was detected at the proximal end of the chromosome, which is different from previously reported QTL on the chromosome 1.

While the major QTL of femoral as well as vertebral BMD is on chromosome 1, bone strength of tibiae is mostly regulated by a QTL on chromosome 4. The QTL ML2 on chromosome 4 detected for tibial strength has several unique features (figure 3). It has the LOD score of 31, the highest LOD score among all QTLs detected from this cross. It represents 60% of variance of the bone strength, the largest ever among QTLs in BMD and strength detected from mouse models. The chromosomal region of this QTL is also very large. It stretches almost to the entire chromosome, although the peak seems near the distal end of the

Table 3. QTLs of maximum loading of tibiae and BMD of femurs from F_2 population derived from B6 × C3H.

	Tibial maximum load		Femoral BMD		Vertebral BMD	
Chromosome	LOD score	% Changes	LOD score	% Changes	LOD score	% Changes
1	6.7	3	24.44	9.98	14.02	6.18
2	–	–	3.14	1.06	Ns	–
4*	31.8	60	16.3	6.71	14.85	6.54
5	6.8	3	–	–	–	–
6*	4.7	2	4.56	1.84	Ns	–
7*	4.2	2	Ns	–	5.01	2.13
9	8.4	3	Ns	–	5.12	2.18
11	–	–	6.76	2.65	2.98	1.21
12	–	–	2.89	1.07	Ns	–
13*	4.5	4	7.73	2.96	Ns	–
14	–	–	4.30	1.72	4.48	1.94
15	6.1	3	–	–	–	–
16	4.1	4	4.07	1.43	Ns	–
17	6.4	4	–	–	–	–
18*	3.9	2	13.67	5.65	8.35	3.52
Total	87.6	90	87.86	35.07	54.84	23.7

*Loci that are located on the same chromosomal regions between tibiae and femurs or tibiae and vertebrae. Ns, nonsignificant.

chromosome. The QTL of BMD detected from both femurs and vertebrae were located in the peak region of the ML2. It is possible that one or several major genes regulate all those three traits. According to the mouse genome sequence database of Ensembl, this region contains 53.2 Mbp nucleotides (from D4mit27 at 88535063–88535210 to D4mit13 bp at 141770989–141771077, data obtained on 1 October 2006). It is a huge region with more than 665 known genes and 105 novel genes or pseudogenes. Therefore, further studies on fine mapping such as congenic breeding are needed to narrow down the QTL region and reduce the list of candidate genes.

Previously, BMD for femural and vertebral bones have been identified but regulates a relatively small portion of variation (Beamer *et al*. 2001, 2005). The peak region of QTL of femoral and vertebral BMD was located on the same location as of that from tibiae, at position D14mit124 (Beamer *et al*. 2001). However, they have lower LOD scores (16.3 and 14.8, for femoral and vertebral BMD, respectively) and explained a smaller percentage of variance (6.7 and 6.5 for femoral and vertebral BMD, respectively) compared to QTL from three-point bending detected from tibiae (table 3). As tibial strength appears much less affected by environment, phenotypes of QTL may be easy to measure using tibiae. When a QTL of femurs and tibiae is at the same location, phenotype the tibiae in the breeding process for dissection of QTL and cloning the QT genes provides an ideal alternative approach. Notably, there is another report on detection of QTL of long bone length using bone tibia and femurs (Norgard *et al*. 2008).

The strong QTL of bone strength on chromosome 4 may be responsible for multiple bone traits. In addition to the BMD, the same chromosomal region is also responsible for skeletal mechanical adaptation. There is also an indication that this QTL region regulates bone size. Major portion of ML2 on chromosome 4 is homologous to human chromosomes 1p31, 1p32, 1p33, and up to 1p36 region while a small portion to 9p21. QTL of bone size from human population has been mapped onto 1p35-p34 (Chen *et al*. 2006). These lines of evidence together strongly suggest that it is of great importance to study the genetic mechanism of ML2.

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