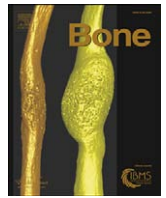


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Further genetic evidence suggesting a role for the RhoGTPase-RhoGEF pathway in osteoporosis

Ben H. Mullin^{a,b,c,*}, Richard L. Prince^{a,d}, Cyril Mamotte^{b,c}, Tim D. Spector^e, Deborah J. Hart^e, Frank Dudbridge^f, Scott G. Wilson^{a,d,e}

^a Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, WA, Australia

^b School of Biomedical Sciences, Curtin University of Technology, Bentley, WA, Australia

^c Curtin Health Innovation Research Institute, Curtin University of Technology, Bentley, WA, Australia

^d School of Medicine and Pharmacology, University of Western Australia, Nedlands, WA, Australia

^e Twin & Genetic Epidemiology Research Unit, St Thomas' Hospital Campus, King's College London, London, UK

^f MRC Biostatistics Unit, Institute of Public Health, Cambridge, UK

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ABSTRACT

Osteoporosis is a highly heritable trait that appears to be influenced by multiple genes. Genome-wide linkage studies have highlighted the chromosomal region 3p14-p21 as a quantitative trait locus for BMD. We have previously published evidence suggesting that the *ARHGEF3* gene from this region is associated with BMD in women. The product of this gene activates the RHOA GTPase, the gene for which is also located within this region. The aim of this study was to evaluate the influence of genetic polymorphism in *RHOA* on bone density in women. Sequence variation within the *RHOA* gene region was determined using 9 single nucleotide polymorphisms (SNPs) in a discovery cohort of 769 female sibs. Of the 9 SNPs, one was found to be monomorphic with the others representing 3 distinct linkage disequilibrium (LD) blocks. Using FBAT software, significant associations were found between two of these LD blocks and BMD Z-score of the spine and hip ($P = 0.001-0.036$). The LD block tagged by the SNP rs17595772 showed maximal association, with the more common G allele at rs17595772 associated with decreased BMD Z-score. Genotyping for rs17595772 in a replication cohort of 780 postmenopausal women confirmed an association with BMD Z-score ($P = 0.002-0.036$). Again, the G allele was found to be associated with a reduced hip and spine BMD Z-score. These results support the implication of the RhoGTPase-RhoGEF pathway in osteoporosis, and suggest that one or more genes in this pathway may be responsible for the linkage observed between 3p14-p21 and BMD.

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Introduction

Postmenopausal osteoporosis is a systemic bone disease that is characterised by low bone mass and disturbed micro architecture of bone tissue which results in increased fragility and a corresponding increase in risk of fracture [1]. Peak bone mass is attained in early adult life, but declines in postmenopausal women due to a reduction in oestrogen production which has direct effects on bone as well as intestinal and renal calcium handling [2]. However, there is a strong genetic effect on peak bone mass, bone loss and fracture rates in postmenopausal women in addition to the effects of oestrogen, calcium and environmental factors [3–6]. Twin and family studies suggest that 50%–90% of the variance in peak bone mass is heritable [3,4,6–10].

The whole-genome linkage scanning approach has identified many quantitative trait loci (QTL) for bone mineral density (BMD) [11–20], strongly suggesting that the genetic effect for common variation of the phenotype is under polygenic control. The 3p14-p21 region of the human genome has been identified as one of the most replicated QTLs for BMD in multiple studies, including our own [19] and a meta-analysis [21]. We have previously demonstrated significant associations between the gene *ARHGEF3*, which is located in this chromosomal region and encodes a RhoGEF that specifically activates the RHOA and RHOB GTPases [22], and BMD in Caucasian women [23]. The *RHOA* gene, which encodes the ras homolog gene family member A, is also situated within 3p14-p21.

This paper investigates the association between polymorphisms within the *RHOA* gene and BMD in a well-described large family-based cohort of women from Australia and the UK. We then sought to validate any effects observed by study of a second and independent cohort of postmenopausal women from the UK. These are the same populations in which we were able to demonstrate significant associations between the *ARHGEF3* gene and BMD.

* Corresponding author. Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, Western Australia 6009, Australia. Fax: +61 8 9346 3221.

E-mail address: ben.mullin@health.wa.gov.au (B.H. Mullin).

Materials and methods

Subjects

Discovery cohort

A total of 769 women from 335 families were recruited in Australia and the UK as described previously [23]. All subjects from the study provided written informed consent, and the institutional ethics committees of participating institutions approved the experimental protocols.

At a clinic visit data including age, height, weight, medical, gynaecological, and lifestyle data were recorded and a blood sample was collected. Dual energy X-ray absorptiometry (DXA) BMD was assessed (Hologic Inc., Bedford, MA, USA) at the lumbar spine L1–L4 and the total hip. Due to the wide range of ages in this cohort, the BMD data was adjusted for age prior to analysis by conversion to BMD Z-scores.

Replication cohort

This group of subjects was recruited in 1988 to participate in a longitudinal epidemiological study of rheumatic diseases (The Chingford Study). Women aged between 45 and 64 were recruited from a single large general practice in Chingford, North-East London using a population-based method as described previously [23]. DNA samples were obtained from 780 individuals. Bone density measurements were undertaken using a Hologic QDR-2000 densitometer (Hologic Inc., Bedford, MA, USA) in 1998 approximately 10 years after the subjects were initially recruited. Hip and spine DXA BMD data was obtained from 775 and 779 individuals respectively. To maintain consistency with the discovery cohort, BMD data was adjusted for age prior to analysis by conversion to BMD Z-scores. Subjects were categorised as fracture-free or having had a previous fracture as described previously [24], with fractures sustained at any skeletal site up to 2003 included in the analysis, excluding those caused by high-impact trauma. Informed consent was obtained from each individual and the study was approved by the local ethics committee.

Genotyping

Genomic DNA was extracted and purified from EDTA whole blood obtained from each subject [25]. Genotyping in the discovery cohort was performed with genomic whole-genome amplified (Repli-g) DNA using the Illumina GoldenGate assay on an Illumina BeadStation 500 GX, utilising bead array hybridisation [26]. The genotype call rate using this technique was 99.6% with an error rate of <0.1%.

Genotyping in the replication cohort was performed using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry as described previously [27]. For this technique the genotype call rate was 97.1% and the estimated error rate was <0.1%.

SNP selection

9 SNPs were selected in the region of the *RHOA* gene for genotyping in the discovery cohort. Tagging SNPs (tSNPs) were initially selected

Table 1
Demographics and bone density of the discovery and replication populations.

Variable	Discovery	Replication
Age (years)	54.2 ± 12.7 (769)	62.5 ± 5.9 (780)
Weight (kg)	62.7 ± 11.27 (699)	69.1 ± 12.6 (778)
Prevalent fractures (%)	–	34 (780)
Total hip DXA BMD (mg/cm ²)	801 ± 136 (760)	869 ± 128 (775)
Total hip BMD Z-score	–0.4 ± 1.0 (760)	0.5 ± 1.0 (775)
Femoral neck DXA BMD (mg/cm ²)	700 ± 133 (749)	747 ± 119 (775)
Femoral neck BMD Z-score	–0.4 ± 1.1 (749)	0.3 ± 1.0 (775)
Spine L1–L4 DXA BMD (mg/cm ²)	855 ± 158 (767)	955 ± 155 (779)
Spine BMD Z-score	–0.7 ± 1.3 (767)	0.7 ± 1.4 (779)

Results are given as mean ± SD (number of measurements).

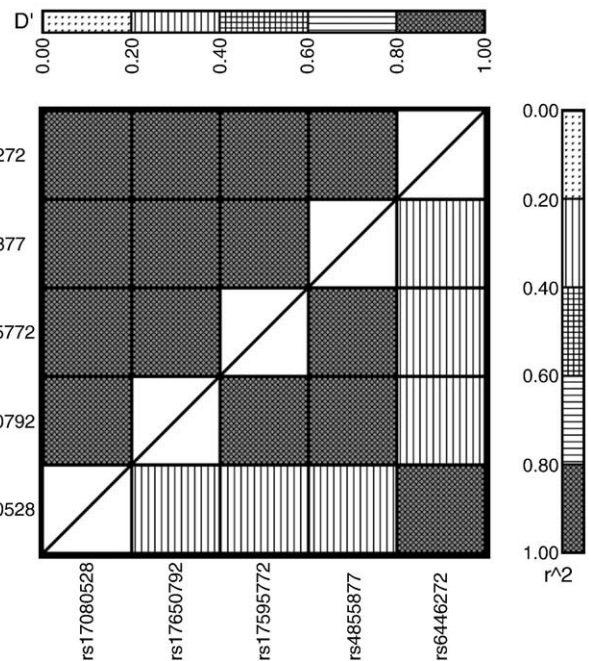


Fig. 1. LD analysis of the 5 SNPs significantly associated with BMD parameters in the discovery cohort.

for the region using the Perlegen Genome Browser Version 1 [28], which identifies tSNPs as being in linkage disequilibrium (LD) of $r^2 \geq 0.8$ with all other SNPs in the LD bin. We were able to tag all 3 LD bins identified by the Perlegen Genome Browser Version 1 within the immediate vicinity of the *RHOA* gene (Perlegen Version 1 LD bins 1050962, 1046850 and 1046281). The remaining SNPs genotyped were selected from dbSNP.

Statistical analysis

All SNPs were in Hardy–Weinberg equilibrium (χ^2 test, $\alpha = 0.05$). Analysis of the data from the discovery cohort was performed using the FBAT (Family Based Association Tests) software [29]. Throughout, two-tailed *P*-values are reported, with adjusted $P \leq 0.05$ considered significant. LD between the different SNPs was evaluated using the JLIN software [30].

Correction for multiple testing was performed by randomly permuting phenotypes within sibships and repeating all FBAT tests on the permuted datasets. Empirical significance was estimated as the proportion of permuted datasets in which the minimum *P*-value was at least as small as that seen in the observed data. This approach was used to correct for tests of multiple SNPs within each phenotype, and also for tests of multiple SNPs across multiple phenotypes. For the latter, correlation between phenotypes was preserved by permuting the whole phenotype vector for each subject.

To estimate the genetic effect size, we followed a recent suggestion to find the genotype-specific adjustment to the trait values that leads

Table 2
Position and allele distribution of rs17080528 and rs17595772.

SNP	rs17080528	rs17595772
Chromosome position ^a	49364846	49384708
Function/location ^a	3' region	Intron 2
Allele distribution (%)	CC (45.8) ^b (50.9) ^c TC (45.2) ^b (41.3) ^c TT (9.0) ^b (7.8) ^c	GG (32.1) ^b (29.9) ^c AG (50.9) ^b (50.0) ^c AA (17.0) ^b (20.1) ^c

^a From GenBank reference sequence NM_001664, Genome Build 36.3.

^b Allele distribution in the discovery cohort.

^c Allele distribution in the replication cohort.

Table 3

A. Genetic data for rs17080528 relevant to BMD Z-score in the discovery cohort	
BMD Z-score phenotype	Additive effect of <i>T</i> allele
Total hip	−0.1
Femoral neck	−0.2
Spine	−0.1
B. Genetic data for rs17595772 relevant to BMD Z-score in the discovery cohort	
BMD Z-score phenotype	Additive effect of <i>G</i> allele
Total hip	−0.2
Femoral neck	−0.2
Spine	−0.2

Results are given as the effect size that gives a residual FBAT statistic of zero [31].

to an FBAT statistic of zero for the residuals [31]. This gives an estimate of the additive effect on the mean that is consistent with the testing method we used.

Statistical analysis of the data from the replication cohort was performed in STATISTICA Ver 8.0 (StatSoft, Tulsa, OK, USA) using one-way analysis of variance (ANOVA) for differences between genotype groups. BMD Z-score data was adjusted for weight by analysis of covariance (ANCOVA). Genotype effects on fracture rate were examined using a Chi-square test.

Results

Discovery cohort

The demographic and morphometric characteristics of the populations are detailed in Table 1. Due to the high proportion of osteoporotic individuals in the discovery cohort, it recorded a lower mean BMD than the replication cohort at every site studied.

Of the 9 SNPs genotyped in the discovery cohort, one was found to be monomorphic (rs6446270). The remaining 8 fell into 3 distinct LD blocks as defined by a tagging strength of $r^2 \geq 0.8$ (Fig. 1). Block 1 contained the SNPs rs6446272 and rs17080528 (Perlegen Version 1 LD bin 1050962); block 2 contained rs4855877, rs17595772 and rs17650792 (Perlegen Version 1 LD bin 1046850); block 3 contained rs940045, rs10155014 and rs4955426 (Perlegen Version 1 LD bin 1046281). Using FBAT, significant associations were observed between various measures of BMD Z-score and the SNPs in block 1 (rs6446272, rs17080528) and block 2 (rs4855877, rs17595772, rs17650792) ($P=0.001$ – 0.036). No significant associations were seen between the SNPs in block 3 (rs940045, rs10155014, rs4955426) and BMD phenotypes ($P=0.46$ – 0.8). rs17080528 was selected to represent block 1 and rs17595772 was selected to represent block 2 (Table 2). rs17080528 was significantly associated with BMD Z-scores for the total hip ($P=0.026$) and femoral neck ($P=0.003$) whereas rs17595772 was significantly associated with BMD Z-scores for the total hip ($P=0.014$), femoral neck ($P=0.001$) and spine sites ($P=0.036$). Significant associations for rs17595772 with total hip and femoral neck sites were maintained after correction for testing multiple SNPs ($P=0.038$ and 0.002 respectively), and the association with femoral neck was maintained after further correction for testing multiple anatomical sites ($P=0.007$). Estimates of the additive genetic effect suggest that the less common *T* allele at

Table 4

BMD Z-scores in relation to the allele distribution of rs17595772 in the replication cohort.

Phenotype	AA genotype	AG genotype	GG genotype	P-value
Total hip BMD Z-score	0.7 ± 1.1^a (149)	0.5 ± 1.0^b (371)	0.4 ± 0.9^b (225)	0.034
Femoral neck BMD Z-score	0.4 ± 1.1^a (149)	0.3 ± 1.0 (371)	0.2 ± 0.9^b (225)	0.036
Spine BMD Z-score	1.1 ± 1.5^a (150)	0.8 ± 1.5^b (374)	0.6 ± 1.2^b (225)	0.002

Results are given as mean \pm SD (number of measurements).

The BMD Z-score data is adjusted for weight.

^a Significantly different from ^b in post hoc analysis ($P < 0.05$).

rs17080528 and the more common *G* allele at rs17595772 are associated with a reduced BMD Z-score (Table 3).

A 2-SNP haplotype analysis was undertaken on rs17080528 and rs17595772 to determine whether haplotypes of the LD blocks tagged by each SNP would prove to be more significantly associated with BMD Z-scores than either block individually. The *TA* haplotype had an estimated frequency of zero, suggesting a lack of historical recombination. The association of the *CA* haplotype was therefore equivalent to that of the *A* allele of rs17595772, and the association of *TC* equivalent to that of the *T* allele of rs17080528. The *CG* haplotype had no significant association, so we conclude that no additional information is obtained from the haplotype analysis.

Replication cohort

rs17080528 and rs17595772 were genotyped in the replication cohort. No significant associations between rs17080528 and BMD Z-scores were observed ($P=0.21$ – 0.42). However, rs17595772 demonstrated significant associations with BMD Z-scores for the total hip, femoral neck and spine sites after adjustment of the BMD Z-score data for the covariate weight (Table 4). Consistent with the results for the discovery cohort, subjects homozygous for the *G* allele at rs17595772 compared to individuals homozygous for the *A* allele had lower BMD at the total hip, femoral neck and spine sites (-3.4% , -3.8% , and -5.9% respectively). Compared to heterozygous individuals with the *AG* genotype, *GG* individuals again had lower BMD at the three sites (-1.1% , -1.8% and -2.7% respectively). No significant associations between rs17595772 genotype and the covariate weight were found.

In the replication cohort 265 subjects had suffered a fracture prior to 2004, giving a prevalent fracture rate of 34%. Neither rs17080528 nor rs17595772 were significantly associated with fracture rate.

Discussion

The results presented here suggest that genetic variation in the *RHOA* gene as well as the *ARHGGEF3* gene may influence BMD in women. These genes are both situated in the 3p14–p21 region of the genome, an area that has been linked with BMD in multiple studies [19,21]. Other genes from this chromosomal region have also been reported as associated with BMD in women, including *PTH1R* [32] and *FLNB* [33]. This certainly raises the possibility that more than one gene from this region is responsible for the linkage observed, a phenomenon that has been reported previously with genetic susceptibility to tuberculosis [34]. This could explain why a single gene from this

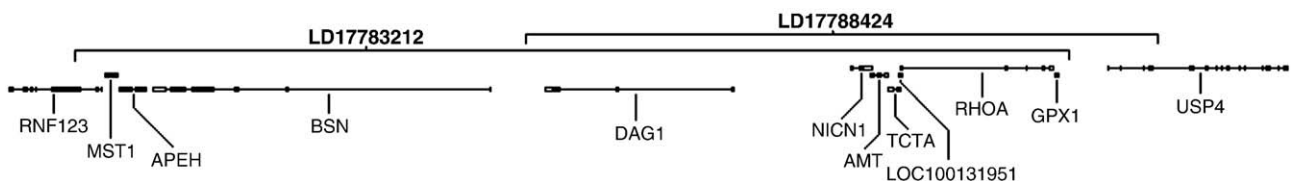


Fig. 2. LD bins tagged by rs17080528 (LD17783212) and rs17595772 (LD17788424) according to the Perlegen Genome Browser Version 2.

region with a large influence on BMD has not been found, an outcome that perhaps would have been originally anticipated judging from the strength of the linkage observed. Indeed, this phenomenon could hold true for other regions of the genome linked with BMD in which no single gene with a large influence on BMD has been identified, a plausible theory considering the large number of genes thought to have a minor influence on the BMD phenotype.

Updated LD data presented by the Perlegen Genome Browser Version 2 [28] reveals that the polymorphisms within *RHOA* that we have identified as associated with BMD are part of 2 large LD blocks that span approximately 370 kb on chromosome 3 (Fig. 2). Although 12 genes have been identified within this region, *RHOA* is considered to be the most likely candidate supporting the hypothesis that the RhoGTPase-RhoGEF pathway plays an important role in bone cell biology and therefore osteoporosis.

Firstly variation within *ARHGEF3*, a gene encoding a RhoGEF that specifically activates the RHOA protein, is associated with BMD in these same populations [23]. Next the human *RHOA* gene regulates intracellular actin dynamics [35] and polymerisation [36], and has been shown to have a role in bone cell biology. McBeath et al. [37] found that cell shape regulates the commitment of human mesenchymal stem cells (hMSCs) to adipocyte or osteoblast fate by modulating endogenous RHOA activity. hMSCs expressing dominant-negative RHOA became adipocytes whereas those expressing constitutively active RHOA became osteoblasts, overriding the presence of differentiation factors in the media. A study by Meyers et al. [38] produced supporting evidence for this when they demonstrated that overexpression of RHOA restored actin cytoskeletal arrangement, enhanced the expression of osteoblastic genes and suppressed the expression of adipocytic genes in hMSCs cultured in modelled microgravity.

Osteoclasts are highly motile cells that rely on rapid changes to their cytoskeleton to achieve the movement and attachment that is required for bone resorption [39–41]. Studies by Chellaiah et al. [42] suggest that RHOA may play a major role in these activities. By transducing active and inactive RHOA into avian osteoclasts they demonstrated that the protein is essential for podosome assembly, stress fibre formation, osteoclast motility and bone resorption. In addition, Zhang et al. [43] found that the actin ring structure that is typical of a resorbing osteoclast is disrupted by C3 exoenzyme, which is produced by *Clostridium botulinum* and specifically inactivates the Rho proteins.

The RHOA protein also appears to be very highly conserved across multiple species. Indeed, the dbSNP database does not presently contain any record of confirmed transcribed polymorphism within the gene. This suggests that if the *RHOA* gene is responsible for the associations seen between the SNPs genotyped in this study and BMD, the effect of polymorphism on the gene would be regulatory as opposed to a structural change to the protein.

In conclusion, we have shown that common genetic variation within the *RHOA* gene region is associated with BMD in Caucasian women. There is a significant amount of evidence to suggest that RHOA has a role in the biology of osteoblasts and osteoclasts. These data further support a role for the RhoGTPase-RhoGEF pathway in osteoporosis, particularly considering the fact that an association between another gene in this pathway, *ARHGEF3*, and BMD has also been demonstrated.

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