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ORIGINAL ARTICLE

Meta-analysis of genome-wide studies identifies *MEF2C* SNPs associated with bone mineral density at forearm

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ABSTRACT

Background Forearm fractures affect 1.7 million individuals worldwide each year and most occur earlier in life than hip fractures. While the heritability of forearm bone mineral density (BMD) and fracture is high, their genetic determinants are largely unknown.

Aim To identify genetic variants associated with forearm BMD and forearm fractures.

Methods BMD at distal radius, measured by dual-energy x-ray absorptiometry, was tested for association with common genetic variants. We conducted a meta-analysis of genome-wide association studies for BMD in 5866 subjects of European descent and then selected the variants for replication in 715 Mexican American samples. Gene-based association was carried out to supplement the single-nucleotide polymorphism (SNP) association test. We then tested the BMD-associated SNPs for association with forearm fracture in 2023 cases and 3740 controls.

Results We found that five SNPs in the introns of *MEF2C* were associated with forearm BMD at a genome-wide significance level ($p<5\times10^{-8}$) in meta-analysis (lead SNP, rs11951031[T] −0.20 SDs per allele, $p=9.01\times10^{-9}$). The gene-based association test suggested an association between *MEF2C* and forearm BMD (p=0.003). The association between *MEF2C* variants and risk of fracture did not achieve statistical significance (SNP rs12521522[A]: OR=1.14 (95% CI 0.92 to 1.35), p=0.14). Meta-analysis also revealed two genome-wide suggestive loci at CTNNA2 and 6q23.2.

Conclusions These findings demonstrate that variants at *MEF2C* were associated with forearm BMD, implicating this gene in the determination of BMD at forearm.

INTRODUCTION

Osteoporosis is a common disease characterised by low bone mineral density (BMD), resulting in an increased risk of fragility fracture.[1] BMD, the best clinical indicator of fracture risk, is a highly heritable trait, with heritability estimates of 60%–85%.[2] Forearm fractures are among the most common fractures, affecting 1.7 million individuals per year[3] and have heritability of 54%.[4]

Genome-wide association studies (GWAS) have identified more than 10 genes associated with BMD from the Wnt-signalling pathway, which is crucial to bone biology.[5 6] We recently conducted two separate GWAS meta-analyses for cortical bone thickness and forearm BMD, and reported *WNT16*, which encodes an important Wnt factor, to be associated with BMD, cortical bone thickness, bone strength and risk of osteoporotic fracture.[7] In the current study, we extended our study on forearm BMD by adding an additional GWAS cohort with BMD data, increasing our meta-analysis sample size for six GWAS cohorts to 5866 European-descended samples. In this new analysis, we detected an additional locus associated with forearm BMD and then replicated the association in an independent cohort comprising 715 Mexican American samples. We additionally conducted a gene-based association test to more fully characterise the association signals from the meta-analysis. Finally, we selected the most compelling SNPs from these analyses and genotyped them in three cohorts comprising 2023 forearm fracture cases and 3740 controls to test their effects on the risk of forearm fracture.

MATERIALS AND METHODS

The GWAS and fracture samples have been described previously.[7] Briefly, the six GWAS cohorts include the Amish Family Osteoporosis Study (AFOS), the Gothenburg Osteoporosis and Obesity Determinants (GOOD) study, the Anglo-Australasian Osteoporosis Genetics Consortium (AOGC) study, TwinsUK1, TwinsUK23 and TwinsUK4, comprising a total of 5866 European-descended samples. The TwinsUK4 cohort, which included 194 subjects phenotyped for forearm BMD, was not included in our previous GWAS,[7] nor was the Mexican American replication sample (see the next para). Genotyping of the TwinUK4 was done on the Illumina HumanHap650K platform. The quality control criteria are similar to TwinsUK23 described in Zheng *et al*.[7] Imputation was performed using the IMPUTE2[8] based on HapMap2, release 22. BMD at distal radius was measured in all cohorts by dual-energy x-ray absorptiometry following standard manufacturer protocols. The fracture cohorts include AOGC, the Umea Fracture and Osteoporosis (UFO) study, the Canadian Multi-centre Osteoporosis study

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(CaMos) and the Manitoba-McGill (ManMc) fracture study, comprising 2023 forearm fracture cases and 3740 controls. Forearm fracture was defined as fractures resulting from low trauma (such as a fall from standing height) occurring at the wrist, ulna, radius and forearm, as well as Colles' fractures. 594 individuals from AOGC fracture study are overlapped with the AOGC BMD study samples. There are no overlapping samples between other discovery cohorts and fracture cohorts. De novo genotyping of SNP rs12521522 in fracture cases and controls was undertaken at Kbiosciences (England). All study participants provided informed written consent. Approval by local institutional review boards was obtained in all studies.

The replication cohort is from the San Antonio Family Osteoporosis Study (SAFOS), which was designed as a study of cardiovascular and bone health in a representative sample of multigenerational Mexican American families.[9] Probands aged 40–60 years were recruited from low-income neighbourhoods in San Antonio, Texas regardless of the health status. The SAFOS samples were genotyped using the Illumina 550 HumanHap BeadChip by the Texas Biomedical Research Institute as part of the San Antonio Family Heart Study. Association analysis was conducted using the SOLAR software program[10] to account for family structure. To minimise the risk of false associations due to stratification in this admixed sample, we performed a principal component analysis using approximately one million genotypes to capture the total genetic variation in the sample as previously described.[11] We then included the first four principal components as covariates into the association analysis. A total of 715 samples with forearm BMD data were analysed in the current study; the mean age, height and weight of these study subjects was 42±14.7 (year), 161.9±9.2 (centimetre) and 81.6±21.5 (kilogram), respectively.

Statistical methods for the meta-analysis were similar to those used in the previous analysis.[7] Briefly, all cohorts independently conducted the association analysis of SNP allele dosage with standardised BMD residuals, while adjusting for age, age^2, gender, height, weight and population substructure where applicable, for centre of recruitment (AOGC), and for family structure in cohorts with family members. A meta-analysis of the GWAS results was conducted using the GWAMA software (genome-wide association meta analysis) (http://www.well.ox.ac.uk/gwama/)[12] with fixed-effects inverse-variance meta-analysis.[13]

We next performed a gene-based association test following the procedure proposed by Liu *et al*[14] as implemented in the software VEGAS, a computationally feasible method for analysing meta-analytic results. We included all SNPs within genes (including ±50 kb from the 5′- and 3′-UTR) with a maximum of 1×10^6 simulations to account for the linkage disequilibrium (LD) structure among SNPs within a gene. Conditional analysis was conducted using GCTA V. 0.93.9,[15] an approximate conditional analysis method using summary-level statistics from the meta-analysis and LD corrections between SNPs estimated from a reference sample.[16] We used TwinsUK23 as the reference sample to calculate the LD information of SNPs due to its size.

SNPs that were associated with BMD were assessed for association with fracture risk using logistic regression models adjusted for age, gender, height and weight. We used CatS[17] for power calculation.

RESULTS

GWAS analyses were performed in the six cohorts for forearm BMD applying cohort-specific genomic controls. The cohort-specific results were meta-analysed using fixed-effects meta-analysis, applying the overall meta-analytic genomic control (overall, λ=1.012 and 1.051 for AFOS; 0.990 for AOGC; 1.014 for GOOD; 1.0089 for TwinsUK1; 1.0037 for TwinsUK23; 1.170 for TwinsUK4). A quantile–quantile plot of the observed p values showed a clear deviation at the tail of the distribution from the null distribution (the distribution expected if there were no association) even after 648 SNPs were removed from the *WNT16* region, which was reported previously.[7] This suggests that the observed p values, particularly the ones within the tail of the distribution, are smaller than those expected by chance and probably reflect true genetic association (figure 1).

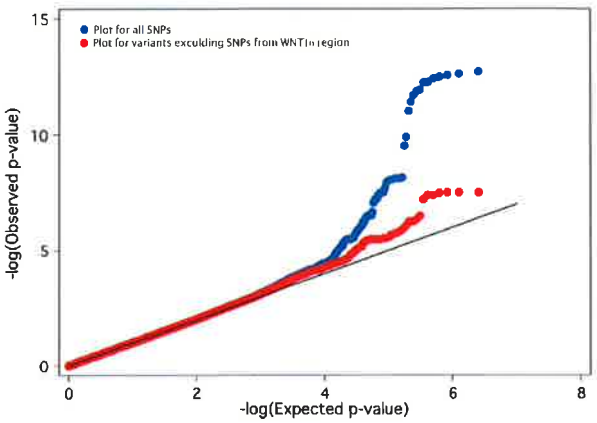

Figure 1 Quantile–quantile plots of the observed p values versus the expected p values for association. The dots in blue were plotted on the entire set of SNPs, whereas the dots in red were obtained after removing 648 SNPs in *WNT16* region (±400 kb either side of rs2908004). The black line was the distribution expected if there were no association.

Genome-wide associations with forearm BMD were observed at two loci, *WNT16* (7q31) and *MEF2C* (5q14.3). At *WNT16*, significant associations were observed, with 30 SNPs ($3.26\times10^{-8}\geq p\geq1.87\times10^{-13}$), replicating an association we have previously observed[7] (figure 2). At *MEF2C*, five of the eight SNPs were significantly associated with forearm BMD, with the other three SNPs showing suggestive levels of association ($4.55\times10^{-7}>p>3.15\times10^{-8}$) on meta-analysis (figures 2 and 3). The most significantly associated SNP was rs12521522 (−0.20 SDs per A allele, $p=3.15\times10^{-8}$) (table 1). These eight SNPs were highly correlated with each other (HapMap CEU LD calculation: $1>R^2>0.85$). Using association results from the GWAS meta-analysis, we next sought to determine whether there were any gene-based signals arising when GWAS summary statistics were collapsed across the genes[14]; The gene-based test results support the single-SNP findings of the meta-analysis, with collapsing p values of 0.003 for gene *MEF2C*.

Meta-analysis also revealed two genome-wide suggestive loci at *CTNNA2* and 6q23.2: 34 genome-wide suggestive SNPs in the region of *CTNNA2* ($1.73\times10^{-6}<p<5\times10^{-6}$) and 10 genome-wide suggestive SNPs at 6q23.2 ($5.52\times10^{-7}<p<3.76\times10^{-6}$) (figure 2).

We attempted an in silico replication on the eight SNPs associated with forearm BMD at *MEF2C* in the Mexican American population. Four of the eight SNPs were monomorphic in the replication population. Of the remaining four polymorphic SNPs, three had effect sizes in the same direction as, and even slightly larger than, those observed in the meta-analysis, including two SNPs for which the associations in Mexican Americans achieved statistical significance at the 0.05 threshold (rs12522630 and rs17494872) (table 1). In the joint analysis of

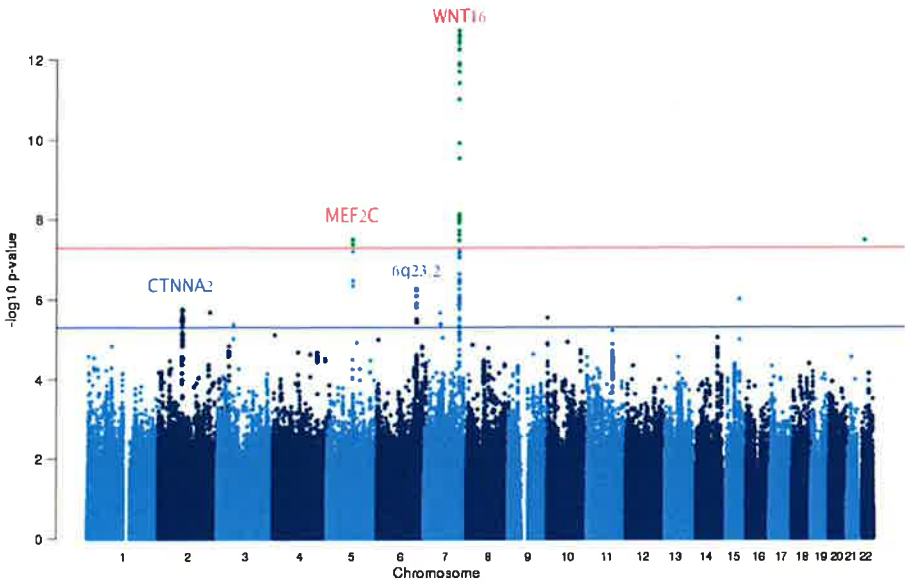

Figure 2 Manhattan plot for genome-wide association studies meta-analysis of forearm bone mineral density. Genome-wide p values (−log10 p) of the linear regression analysis plotted against position on each chromosome.

discovery and replication populations, evidence of association improved for the three SNPs, with the most significant association at rs11951031 (−0.20 SD per T allele, $p=9.01\times10^{-9}$; table 1 and figure 4).

In order to investigate whether the variants showing association with forearm BMD also have an effect on the risk of forearm fracture, we tested SNP rs12521522 for de novo genotyping in samples with forearm fracture and their controls. The meta-analysis for fracture comprised 2023 forearm fracture cases and 3740 controls from three cohorts. The association between rs12521522 and the risk of fracture did not achieve statistical significance (OR=1.14 (95% CI 0.92 to 1.35), p=0.14) (table 1). The fracture associations for the other seven SNPs at the *MEF2C* locus were tested in silico in the much smaller AOGC fracture GWAS cohort in 155 cases and 1672 controls and the results showed no evidence of association (table 1).

Because SNP rs1366594, which is located upstream of *MEF2C* gene (figure 3), has been previously reported to be associated with femoral neck (FN) BMD,[18] we evaluated whether this SNP or signals from this region could explain the observed association with forearm BMD. First, the minor allele frequency (MAF) of forearm BMD-associated SNP in our study (rs11951031, MAF=0.06) was considerably lower than that of FN BMD-associated SNP (rs1366594, MAF=0.45), and the effect size of rs11951031 (−0.20 SD per T allele) was much larger than rs1366594 (−0.085 SD per C allele). Second, these two SNPs were only very weakly correlated with each other (HapMap CEU LD calculation: $R^2=0.087$). Third, after conditioning on the effect of rs1366594, the effect size for

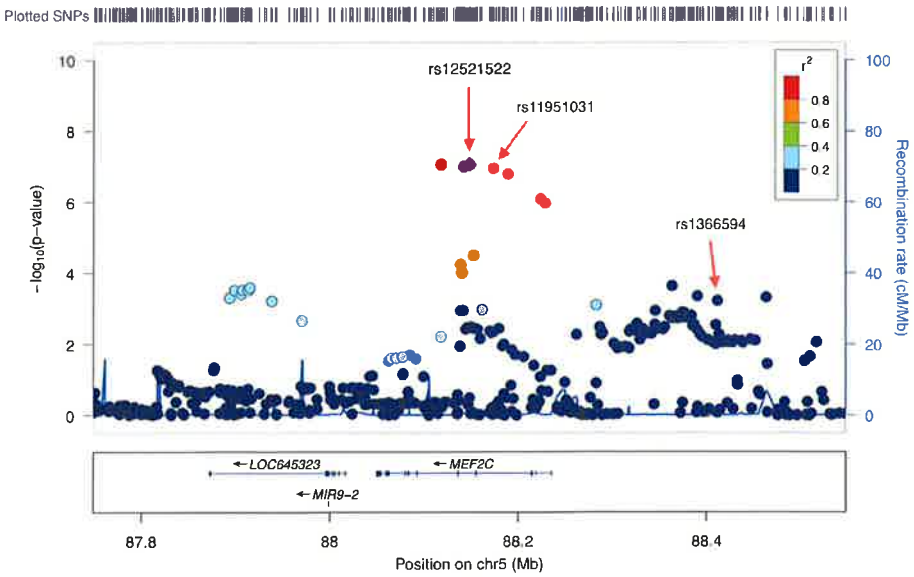

Figure 3 Scatter plots of the observed association with forearm bone mineral density in the 800 kb wide region around rs12521522 in *MEF2C* locus. The p values of SNPs (shown as −log 10 values in y-axis, from the genome-wide single-marker association analysis using the linear regression model) are plotted against their map position (b36) (x-axis). The colour of each SNP spot reflects its r2 with rs12521522. SNPs rs11951031 and rs12521522 are in perfect linkage disequilibrium, and rs1366594 is ~237 kb away from rs11951031.

Table 1 Association results of forearm bone mineral density meta-analysis and fracture of the top SNPs

					GWAS meta-analysis				SAFOS				Joint analysis*			Fracture analysis†	
CHR	SNP	Position	EA	NEA	EAF	BETA	SE	p Value	EAF	BETA	SE	p Value	BETA	SE	p Value	OR	p Value
5	rs17558256	88119209	C	T	0.06	−0.20	0.04	$\mathbf{3.15\times10^{-8}}$	0	NA	NA	NA	NA	NA	NA	1.20 (0.82 to 1.43)	0.33
5	rs11958689	88142609	G	C	0.06	−0.20	0.04	$\mathbf{4.16\times10^{-8}}$	0.08	0.04	0.11	0.72	−0.18	0.03	3.35×10^{-7}	1.21 (0.83 to 1.45)	0.31
5	rs12521522	88148517	A	T	0.06	−0.20	0.04	$\mathbf{3.15\times10^{-8}}$	0	NA	NA	NA	NA	NA	NA	1.14 (0.92 to 1.35)	0.14
5	rs11955542	88148984	T	C	0.06	−0.20	0.04	$\mathbf{3.15\times10^{-8}}$	0	NA	NA	NA	NA	NA	NA	1.20 (0.82 to 1.43)	0.33
5	rs11951031	88174487	T	C	0.06	−0.20	0.04	$\mathbf{4.16\times10^{-8}}$	0.04	−0.23	0.14	0.10	−0.20	0.04	$\mathbf{9.01\times10^{-9}}$	1.20 (0.81to 1.45)	0.33
5	rs12515983	88189831	A	T	0.06	−0.20	0.04	6.12×10^{-8}	0	NA	NA	NA	NA	NA	NA	1.19 (0.81to 1.44)	0.34
5	rs12522630	88224123	A	G	0.07	−0.18	0.04	3.35×10^{-7}	0.04	−0.29	0.14	0.04	−0.19	0.03	5.19×10^{-8}	1.25 (0.83to 1.49)	0.22
5	rs17494872	88228915	A	G	0.07	−0.18	0.04	4.55×10^{-7}	0.04	−0.29	0.14	0.04	−0.19	0.03	6.82×10^{-8}	1.25 (0.83to 1.49)	0.23

These SNPs were not polymorphic in Mexican Americans (rs17558256, rs12521522, rs11955542 and rs12515983).
Boldface indicates the genome-wide significant SNPs.
*Combined results of GWAS meta-analysis and SAFOS replication study.
†SNP rs12521522 was tested in 2023 cases and 3740 controls; the other seven SNPs were tested in 155 cases and 1672 controls.
EA, effect allele; EAF, effect allele frequency; GWAS, genome-wide association studies; NA, not applicable; NEA, non-effect allele; SAFOS, San Antonio Family Osteoporosis Study.

rs11951031 on forearm BMD decreased from −0.20 SD per T allele ($P=4.16\times10^{-8}$) to −0.18 SD per T allele ($P=1.35\times10^{-6}$). Therefore, the SNPs we have found to be associated with forearm BMD are distinct from those found previously.[18]

DISCUSSION

We identified gene *MEF2C*, a member of the Wnt-signalling pathway, to be associated with forearm BMD in the meta-analysis in a collection of 6584 individuals. In addition, we observed a non-significant trend towards risk of fracture at this locus.

The Wnt/β-catenin signalling pathway is known to play an important role in the regulation of bone mass and bone turnover.[19] *MEF2C* is an important member of this pathway.[5 6] In fact, in the large GEFOS consortium, a SNP (rs1366594) located upstream from this gene, was associated with FN BMD, although not with lumbar spine BMD.[18] We report in this study that intronic variants in *MEF2C* are associated with forearm BMD, a clinically distinct phenotype from that at FN.

Our finding adds three novel pieces to the genetics of BMD puzzle. First, bone at the forearm is structurally different from that at the FN insofar as the forearm bone contains a much higher proportion of cortical bone. BMD at both sites predicts fracture at their respective anatomical sites better than at other sites. Second, the associated variants for forearm BMD appear to be quite distinct from that associated with FN BMD. Not only are they located over ~237 kb from each other (figure 3), but they have very different allele frequencies (0.06 vs 0.45) and very different effect sizes (−0.20 SD vs −0.085 SD), and they are not correlated. Moreover, conditional analyses reveal that the effect of rs11951031 on forearm BMD are largely

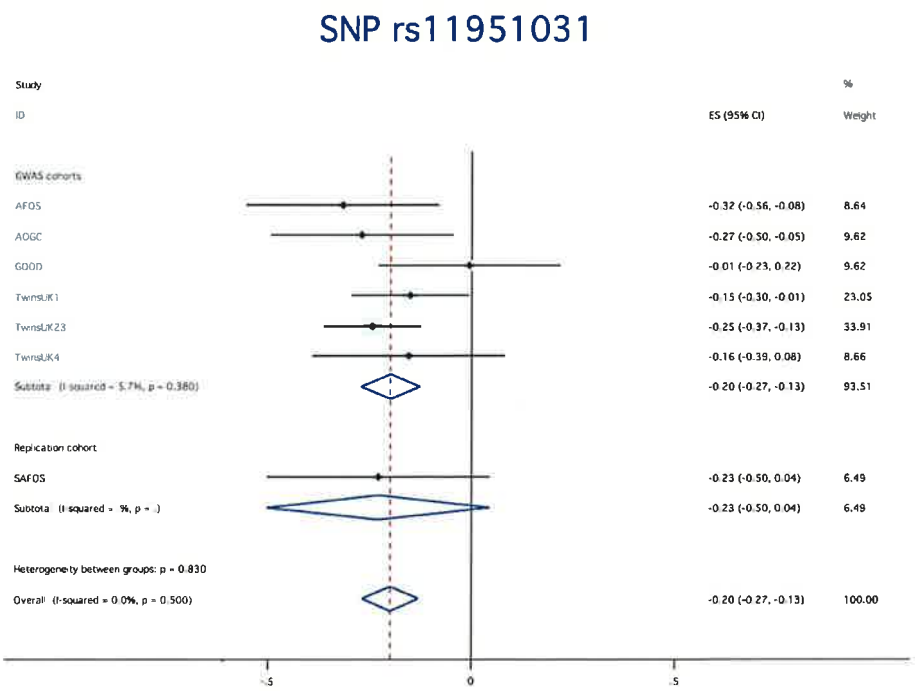

Figure 4 Forest plot of association of rs11951031 (effect allele T) with forearm bone mineral density.

independent of any effect of rs1366594. We postulate that these common variants are likely independent signals that have different independent effects on the two BMD phenotypes. It is also possible that both associations arise from several rare causal variants on the same haplotype background;[20] however, this hypothesis will likely be tested as more sequencing studies emerge for BMD.

In the current study, we did not observe a statistically significant association of *MEF2C* SNP (rs12521522) with osteoporotic fracture. Our sample size (2023 cases and 3740 controls) provided 44% power to detect an OR of 1.14 for a risk allele having a frequency of 0.06. However, the direction of effect of the alleles that decreased BMD was associated with an increase in fracture risk across the study cohorts. Given the sample size for fracture in this study, these results should be interpreted cautiously and require further replication. Additionally, rs1366594 which was reported in Rivadeneira *et al*[18] showed no evidence of association with forearm fracture in the AOGC in silico analysis too (155 cases and 1672 controls, p=0.27).

In summary, our data provide first evidence that intronic variants at the *MEF2C* locus, a member of the Wnt-signaling pathway, are associated with forearm BMD. These findings expand our understanding of the genetic determinants of forearm BMD, a clinically relevant skeletal site.

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Contributors H-FZ and JBR conceived and designed the study; H-FZ, ED, LMY-A, JE, PJL, UB, EAS, BDM and JBR analysed the data; WDL, DG, TDS, JB, ML, CO, UP-K, MAC, DAH, MAB and JBR made contributions of reagents/materials/phenotype; H-FZ, BDM and JBR wrote the paper; and all authors revised the manuscript.

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Competing interests None.

Patient consent obtained.

Ethics approval Approval by local institutional review boards was obtained in all studies.

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