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Research Article

Lack of Association of Bone Morphogenetic Protein 2 Gene Haplotypes with Bone Mineral Density, Bone Loss, or Risk of Fractures in Men

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Introduction. The association of bone morphogenetic protein 2 (BMP2) with BMD and risk of fracture was suggested by a recent linkage study, but subsequent studies have been contradictory. We report the results of a study of the relationship between BMP2 genotypes and BMD, annual change in BMD, and risk of fracture in male subjects. **Materials and Methods.** We tested three single-nucleotide polymorphisms (SNPs) across the BMP2 gene, including Ser37Ala SNP, in 342 Caucasian Englishmen, comprising 224 control and 118 osteoporotic subjects. **Results.** BMP2 SNP1 (Ser37Ala) genotypes were found to have similar low frequency in control subjects and men with osteoporosis. The major informative polymorphism, BMP2 SNP3 (Arg190Ser), showed no statistically significant association with weight, height, BMD, change in BMD at hip or lumbar spine, and risk of fracture. **Conclusion.** There were no genotypic or haplotypic effects of the BMP2 candidate gene on BMD, change in BMD, or fracture risk identified in this cohort.

1. Introduction

Osteoporosis is primarily a disease of older people and advancing age is one of the main determinants of osteoporosis and fragility fractures. The estimated lifetime risk of hip, spine, or distal forearm fracture for Caucasian man at the age of 50 years is 20% and approximately 30% of all hip fractures occur in men [1]. Heredity is now generally accepted as playing an important part in the pathogenesis of osteoporosis in both men and women. Indeed, after excluding secondary risk factors and age then the other main causal factor for osteoporosis in men is genetic [2]. This view is supported by genetic epidemiological studies on BMD, an important surrogate of fracture risk, which show that peak bone density has a substantial heritable component [3]. Variation in BMD

is determined by multiple genes; objective evidence for this is provided by candidate gene SNP studies, as well as quantitative trait locus (QTL) studies in inbred animals and humans [3]. QTL studies have identified multiple chromosomal regions which influence bone mass and are linked to osteoporosis-related phenotypes.

Studies of classical and novel candidate genes have only been able to account for a small proportion of the variance in bone mass. Candidate gene contributions to bone mass also show gender, geographic, and ethnic variability. Therefore, studies aimed at identifying the contribution of specific genes towards BMD and the heritable component to osteoporosis should ideally be gender and ethnic specific. One such candidate gene is bone morphogenetic protein 2 (BMP2), located on chromosome 20p12.3. It is a growth factor that

possesses osteoinductive properties and promotes osteoblast differentiation and bone formation [4–9]. Linkage analysis in extended families with osteoporosis in Iceland, using a phenotype that combines osteoporotic fractures and BMD measurements, showed linkage to chromosome 20p12.3 [4]. Three variants in the BMP2 gene, a missense polymorphism, and two anonymous single nucleotide polymorphisms and the resultant haplotypes have been associated with an osteoporotic phenotype, including osteoporotic fractures as well as low BMD, both before and after the menopause. A number of follow-up studies however have yielded inconsistent results [5–9]. To date no investigation has been performed in an exclusively male cohort and no follow-up study has investigated the association with risk of osteoporosis in men and rate of bone loss. In view of the inconclusive nature of the earlier study and to determine possible gender and geography-related variability, we therefore investigated the association of Ser37Ala and Arg190Ser genotypes/haplotypes with BMD, rate of bone loss, and fracture risk in men from the UK.

2. Materials and Methods

2.1. Participants. Participants comprised 342 male subjects, 224 controls, and 118 subjects with osteoporosis, who were all Caucasian men from England, UK [10–12]. The men with osteoporosis comprised men who had a BMD T score below -2.5 at either the femoral neck or lumbar spine or had a low trauma fracture and a T score below -1.0 at either the femoral neck or lumbar spine. Of the patients 89 also had a history of fractures (46 had distal forearm fracture, 33 symptomatic vertebral, 6 hip, and 4 other fractures). Their fractures had all occurred at least six months prior to their inclusion in the study, and all blood samples were taken before the initiation of therapy. Underlying secondary causes of osteoporosis were excluded by medical history, physical examination, and laboratory investigations. Controls were all volunteers without any history of low trauma fractures [10]. The laboratory investigations included full blood count, ESR, biochemical profile, thyroid function tests, serum testosterone, sex-hormone-binding globulin, gonadotrophins, and serum and urine electrophoresis [12].

In a subset of 145 control subjects, there was a follow-up BMD estimation and annual change in BMD was determined, in order to test the association between BMP2 genotypes and change in BMD. A similar analysis of the annual change in the BMD measurement was not performed in the men with osteoporosis, because of the confounding effect of antiresorptive treatment.

2.2. Biochemical and BMD Measurements. Serum testosterone, sex-hormone-binding globulins, follicular stimulating hormone, and luteinizing hormone were measured by commercially available radioimmunoassay (SAS laboratory, Royal Victoria Infirmary, Newcastle upon Tyne).

All bone density measurements, for both the osteoporotic and control subjects, were performed by DXA using a Hologic QDR 2000 Bone Densitometer (Hologic, Waltham,

Mass). *In vivo* precision for measurement with this system is 1.0% at the lumbar spine (L1–L4) and 1.5% for the femoral neck. BMD results were obtained as an areal density in g/cm^2 , but were also given as T and Z scores. The T score is the number of standard deviation units above or below the mean for normal young men, whilst the Z score is the number of standard deviation units above or below the age-related normal men (calculated using the manufacturer's standard normal reference database).

2.3. BMP2 Genotyping. Three BMP2 SNPs previously identified were genotyped [4]. Individual PCR standardisation reactions were set up prior to setting up multiplex PCR for the three BMP2 SNPs. The three BMP2 SNP polymorphisms (SNP1, Ser37Ala; SNP2, Ala94Ser and SNP3, Arg190Ser) studied are shown in the schematic diagram (Figure 1). The multiplex reactions were transferred into 384 well plates and treated with shrimp alkaline phosphatase to remove the excess dNTPs in the reaction mixture. Extension primers designed to detect the three BMP2 SNPs were added to the samples and incubated as a PCR reaction for 2 h. Salt ions were removed from the reaction mixture using a desalting resin and finally resuspended with $16\ \mu\text{L}$ ddH₂O. 15 nL of each sample was spotted onto the chip and analysed on the Sequenom (Maldi-TOF mass spectrometer). The results were collected by Spectroanalyser software and the SNP information, represented by chromatogram peaks at G, T, A, GT, or AT, was sorted by the Typeranalyser software. The SNP information was then exported along with the sample details into MS-Excel file(s) for further statistical analysis.

2.4. Data Analysis. Results are presented as mean \pm standard deviation. Data were analyzed using appropriate statistical packages (Microsoft Excel XL, SPSS); the significance of differences in genotype and allele frequency between control subjects and men with vertebral fractures was determined using odds ratio and χ^2 tests. ANOVA and Student's unpaired *t*-test were used to determine significant differences in BMD and anthropometric measurements between different BMP2 genotypes and between control subjects and osteoporotic men, respectively. Genetic association analyses were carried out using an online HWE calculator (<http://ihg.gsf.de/cgi-bin/hw/hwa1.pl>) and SNPSTATS programme (<http://bioinfo.iconcologia.net/SNPstats>) which also worked out the Hardy-Weinberg equilibrium statistics. The haplotypes and associated statistics were derived from SNPSTATS programme. The binary logistic regression analyses were performed (SPSS, version 13.0) with disease or fracture outcome as the dependent variables. The genotypes as categorical variables were used as specific genotypes; the other variables included in the regression analysis were age, height, weight, BMI, and key relevant biochemical parameters.

3. Results

The age, anthropometric, and BMD measurements for the men with osteoporosis and the control subjects are given in

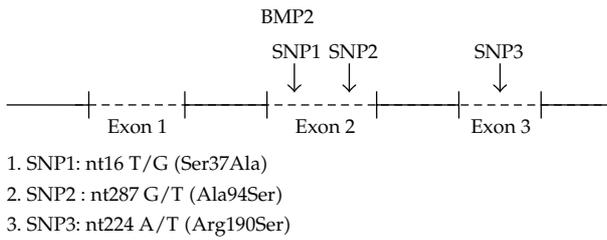


FIGURE 1: Schematic diagram of the three single nucleotide polymorphisms in BMP2 gene.

Table 1. The men with osteoporosis, who had age range of 30 to 86 yr, were on average 6 years younger than controls with the age range of 41 to 79 yr. For this reason when comparing bone density comparisons, to compensate for age-related decline in BMD, comparison with Z score was performed. The osteoporotic patients were also significantly shorter and lighter than the male control subjects, as well as having significantly lower BMD at the lumbar spine, total femoral and femoral neck ($P < 0.0001$).

3.1. Genotype and Allele Frequency. In both men with osteoporosis and control subjects the BMP2 gene SNP1 GG genotype was absent, and in the combined cohort only eight subjects were found to be heterozygotes, thus making this locus uninformative. SNP1 genotype, due to Ser37Ala variants that change a conserved amino acid, has been previously shown to have significant association with osteoporosis in both Danish and Icelandic cohorts [9]. However, in our cohorts the frequency of SNP1-related G allele was low in both the patients (0.004) and controls (0.015). SNP2, which gives rise to Ala to Ser change at 94th position, was rare, uninformative, and monomorphic, and of the three genotypes GG, GT, and TT, only GG was observed. SNP3 (Arg190Ser) was polymorphic, and distribution of genotypes and allele frequencies in both male controls and men with osteoporosis is shown in Table 2. In both cohorts the genotypes were in Hardy-Weinberg equilibrium. Genotype AA was slightly higher in controls (14.3% versus 10.2%, $P = 0.36$) while susceptibility genotype TT was higher in patients (38.1% versus 31.7%, $P = 0.28$). The odds ratio for BMP2 SNP3 genotypes and alleles *A and *T showed no statistically significant evidence of association with the risk of osteoporosis, although a trend towards carriers of the *T allele may be worth exploring.

Linkage disequilibrium (LD) was calculated for SNP1 and SNP3 using EM method. The D' prime is 0.989 (P value = 0.0264), suggesting that there is a significant linkage disequilibrium between SNP1 and SNP3, but caution is warranted in view of the relatively small numbers and low allele frequencies especially at SNP1 locus which led to a low r^2 value (0.02). Haplotype analysis based on two SNPs (SNP1 and SNP3) showed that two common haplotypes (TA and TT) accounted for >99% of the alleles at these BMP2 loci (Table 3). Haplotype-based association analysis indicated higher odds ratio for T-T haplotype; however, it was nonsignificant (OR = 1.43, CI 0.96–2.15, $P = 0.08$).

TABLE 1: Age, weight, height and bone mineral density of control male subjects and men with osteoporosis.

	Controls ($n = 224$)	Patients ($n = 118$)
Age (yr)	65.0 (7.7)	59.1 (12.6)***
Height (cm)	173 (6)	171 (8)*
Weight (kg)	81.5 (10.8)	75.9 (14.6)***
Spinal BMD (g/cm^2)	1.113 (0.160)	0.797 (0.100)***
Spinal Z score	0.96 (1.49)	-2.06 (0.96)***
Spinal T-score	0.14 (1.46)	-2.69 (0.90)***
Total femoral BMD (g/cm^2)	1.02 (0.13)	0.783 (0.099)***
Total femoral Z score	0.71 (0.96)	-1.25 (0.81)***
Total femoral T-score	-0.32 (0.98)	-2.18 (0.75)***
Femoral neck BMD (g/cm^2)	0.85 (0.12)	0.692 (0.100)***
Femoral neck Z score	0.71 (1.09)	-1.05 (0.90)***
Femoral neck T score	-1.11 (1.09)	-2.57 (0.86)***

* $P < 0.01$ and *** $P < 0.0001$ using unpaired Student's t -test.

TABLE 2: The distribution of BMP2 SNP3 genotype and allele frequencies and odds ratio.

(a)		
Genotypes	Patients ($n, \%$)	Controls ($n, \%$)
AA	12 (10.2)	32 (14.3)
AT	61 (51.7)	121 (54.0)
TT	45 (38.1)	71 (31.7)
Total	118	224
HWE P value	0.19	0.09
Alleles		
*A	0.36 (± 0.029)	0.41 (± 0.022)
*T	0.64	0.59
(b)		
Allele/genotype	Odd ratios (95% confidence interval), P value	
*A versus *T	1.25 (0.90–1.73) $P = 0.18$	
AA versus AT	1.34 (0.65–2.79), $P = 0.43$	
AA versus TT	1.69 (0.79–3.62), $P = 0.17$	
AA versus AT + TT	1.47 (0.73–2.98), $P = 0.28$	
Armitage's trend test	1.29, $P = 0.15$	

3.2. BMP2 Genotype Association with Phenotype. The effect of BMP2 gene SNP3 genotypes on weight, height, and BMD at lumbar spine, femoral neck, and total femoral was analysed by ANOVA in both the male controls and men with osteoporosis, and genotype was found to lack any significant association (Table 4). The analysis of the pooled data, comprising controls and patients, also failed to demonstrate any significant association of SNP3 genotype with the BMD or anthropometric indices (data not shown).

3.3. Logistic Regression Analysis. The association of the SNP3 genotype was also determined by employing a binary logistic

TABLE 3: Basic haplotypes of SNP1 and SNP3 at BMP2 gene in men with osteoporosis and controls.

Haplotype	Cases no., freq.	Control no., freq.	Odds ratio	Chi	<i>P</i> value
T-A	0.344	0.431	0.69 (0.46–1.04)	3.16	0.07
T-T	0.651	0.565	1.43 (0.96–2.15)	3.09	0.08
G-A	0.00	0.004		0.809	0.36
G-T	0.005	0.000	19.01 (19.01–65938)*	0.984	0.321

D' prime is 0.989 (*P* value = 0.0264) suggesting that there is a significant linkage disequilibrium between SNP1 and SNP3 but caution is warranted as numbers are small.

*This range is due to the small numbers involved.

TABLE 4: BMP2 SNP3 genotypes and age, weight, height, BMD and annualised bone loss in male controls and men with osteoporosis.

	Control	Control	Control	Patients	Patients	Patients
	AA (<i>n</i> = 32)	AT (<i>n</i> = 121)	TT (<i>n</i> = 71)	AA (<i>n</i> = 12)	AT (<i>n</i> = 61)	TT (<i>n</i> = 45)
Age (yr)	66.1 (7.2)	64.0 (9.4)	65.4 (7.4)	65.7 (9.2)	59.0 (12.6)	57.1 (12.9)
Height (cm)	173 (7)	173 (7)	172 (5)	174 (9)	172 (8)	171 (7)
Weight (kg)	81.9 (9.6)	80.8 (12.4)	81.2 (12.5)	83.9 (25.9)	75.7 (11.8)	74.0 (14)
Spinal BMD (g/cm ²)	1.132 (0.195)	1.112 (0.156)	1.105 (0.152)	0.826 (0.058)	0.785 (0.095)	0.807 (0.113)
Spinal Z score	1.15 (1.85)	0.92 (1.46)	0.93 (1.38)	−1.71 (0.55)	−2.16 (0.88)	−2.03 (1.13)
Spinal T score	0.14 (1.46)	0.14 (1.46)	0.14 (1.46)	−2.43 (0.50)	−2.81 (0.86)	−2.60 (1.03)
Total femoral BMD (g/cm ²)	1.012 (0.133)	1.014 (0.119)	1.039 (0.135)	0.821 (0.121)	0.782 (0.091)	0.773 (0.114)
Total femoral Z score	0.65 (1.04)	0.64 (0.90)	0.86 (1.01)	−0.91 (0.77)	−1.24 (0.79)	−1.39 (0.87)
Total femoral T score	−0.39 (1.03)	−0.37 (0.93)	−0.21 (1.05)	−1.91 (0.95)	−2.19 (0.68)	−2.28 (0.83)
Femoral neck BMD (g/cm ²)	0.860 (0.132)	0.840 (0.132)	0.861 (0.125)	0.708 (0.095)	0.687 (0.087)	0.695 (0.114)
Femoral neck Z score	0.82 (1.24)	0.59 (1.03)	0.85 (1.10)	−0.74 (0.67)	−1.07 (0.87)	−1.10 (1.00)
Femoral neck T score	−0.39 (1.03)	−0.37 (0.93)	−0.20 (1.05)	−2.42 (0.87)	−2.62 (0.76)	−2.54 (1.00)
Spinal bone loss (% per annum)	−0.37 (1.14)	−0.51 (1.47)	−0.45 (1.05)			
	(<i>n</i> = 20)	(<i>n</i> = 69)	(<i>n</i> = 56)			
Femoral neck bone loss (% per annum)	−0.03 (1.77)	0.20 (1.59)	0.33 (1.42)			
	(<i>n</i> = 20)	(<i>n</i> = 69)	(<i>n</i> = 56)			
Total femoral bone loss (% per annum)	0.00 (0.80)	0.16 (1.70)	0.15 (1.07)			
	(<i>n</i> = 20)	(<i>n</i> = 69)	(<i>n</i> = 56)			

No significant effect of BMP2 genotypes on height, weight or BMD was seen using ANOVA (*P* < 0.05). Annualized loss was studied in a subgroups of subjects, the numbers indicated in appropriate cells. Results are given as mean, and SDs are given in parentheses.

regression model, which accounted for between 44.5% and 62.6% of the variance in disease status and the overall model successfully predicted 72% of osteoporotic cases correctly (Table 5). An analysis of fracture incidence with reference to genotypes and age, age at fracture, and biochemical parameters was performed. Age at fracture, weight, biochemical parameters, and genotypes did not show any interaction or independent effect in the fracture prediction. The haplotype analysis did not reveal any significant influence on BMD via logistic regression. The addition of covariates to the model resulted in very sparse data categories and led to wide confidence intervals.

4. Discussion

This investigation is the only one from the UK that has sought an association between BMP2 gene polymorphisms and BMD and susceptibility to osteoporosis. As far as we know this is the first such investigation carried out exclusively in men with idiopathic osteoporosis. However, the power of

this study is low; therefore any conclusions drawn should be treated with caution. Post hoc power analysis, calculated using a Quanto programme and based on observed allele frequencies, sample size, and odds ratios, showed that the study had 67% power to detect an odds ratio of 1.50 (moderate level) and above. In order to achieve an odds ratio of 1.25 one would require a case-control sample size of 507 individuals in each group. In the original Icelandic study a significant effect of BMP2 gene SNP1 polymorphism, Ser37Ala, was demonstrated in a cohort of 201 subjects, comprising 153 controls and 58 subjects with fracture [4]. Just like the earlier observation we too found it to be a rare variant in our cohort with an allele frequency of 1.25% for Ala37. In previous reports, the allele frequency for Ala37 in the Icelandic cohort was 0.8% in controls and 3% to 4.9% in osteoporotic patients and 2.5% in the Rotterdam study. However, unlike the Icelandic study, we did not see a significant association or increase in the relative risk (RR) with the SNP1 genotype. In our cohort we did not see any GT or TT genotypes of the rare variant of the SNP2 genotype that arises from Ala94Ser. The gene frequency for

TABLE 5: Logistic analysis of BMP2 SNP2 genotypes.

	B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I. for Exp(B)	
							Lower	Upper
AGE	-.069	.021	10.994	1	.001	.933	.896	.972
HT	-.054	.171	.101	1	.751	.947	.677	1.325
WT	-.944	16.025	.003	1	.953	.389	.000	2.0E + 13
BMI	.166	.539	.095	1	.758	1.181	.410	3.398
FNBM	-3.359	2.205	2.319	1	.128	.035	.000	2.622
Spinal BMD	-20.656	2.929	49.731	1	.000	.000	.000	.000
SNP3 (AA-Ref)			1.213	2	.545			
SNP3 (AT)	-.518	.654	.626	1	.429	.596	.165	2.148
SNP3 (TT)	-.039	.697	.003	1	.955	.961	.245	3.770
Constant	26.453	27.867	.901	1	.342	2.0E + 11		

SNP3 polymorphism, due to Arg190Ser variants, constituting three genotypes, TT (Arg190Arg), AT (Arg190Ser), and AA (Ser190Ser), was akin to earlier reports in European populations. In both male controls and men with osteoporosis the genotypes were in Hardy-Weinberg equilibrium. In addition to studying the association of the BMP2 to risk of fracture and BMD, in a subset of subjects we also investigated the association of the genotype with annual bone loss at the lumbar spine, total femoral, and femoral neck. Both initial BMD as well as annual change in the BMD showed no association with the BMP2 genotype. This could be partly due to allele frequency differences in different geographical and gender cohorts. In our study we observed a higher frequency of susceptible T (Ser) allele (64% in patients and 59% in controls) which is comparable to HAPMAP frequencies (58%–73% in European populations), but other populations like Turkish, Korean, and African populations show a lower frequency [13–15].

The desirability of having a large cohort for SNP studies investigating polygenic disease association with putative candidate genes with phenotype or disease state is well established. This is due to the fact that in a polygenic disorder like osteoporosis many genes make a small, but significant contribution towards the attainment and maintenance of BMD. Therefore, many genes may be involved in the pathogenesis of osteoporosis and risk of osteoporotic fractures. In view of this small contribution of a large number of genes, the contribution of the genotypic influence is likely to be more clearly demonstrated in a sufficiently large cohort with the required level of statistical power. It is, however, becoming increasingly evident that the association of a large number of candidate gene SNPs with BMD and osteoporosis risk shows race-, geography- and gender-related differences [3, 16, 17]. We would also contend that, if a given genotype does make a substantial contribution to the attainment and maintenance of BMD and pathophysiology of disease then it should be possible to demonstrate such effects even in smaller homogenous cohorts. Indeed, in the original study on BMP2 the genotype association with osteoporosis was established in a small cohort [4]. We have been able to demonstrate associations of some novel genotypes in smaller cohorts and which were subsequently verified in much larger studies [5, 18, 19].

There have been only a handful of studies following the first report of bone morphogenetic protein 2 (BMP2) as a susceptibility gene for osteoporotic fractures and low BMD in Icelandic and Danish populations [9]. Even these relatively few studies of polymorphisms, within the BMP2 gene in relation to bone mineral density (BMD) and fracture, have produced inconsistent findings [4–9]. Neither a Rotterdam study of a large population-based cohort of Dutch whites nor a study in healthy American whites could find any contribution to BMD by variations in BMP2 genotypes [7, 8]. On the other hand, a SNP- and haplotype-based US family study showed highly suggestive associations with BMP2 [5]. In another analysis on a European cohort, variation in BMP2 genotype showed no association with BMD; it did, however, find a role in aspects of bone quality, which may be age and site dependent [9]. The 3' region of the gene was significantly associated with the ultrasound parameters speed of sound and stiffness. Similarly in Turkish women study BMP2 polymorphisms did not substantially contribute to lumbar spine bone mineral density [14]. Overall, association studies have shown that Arg190Ser SNP is not associated with BMD in different populations and the initial study may have been a false positive association. Alternatively, it is entirely possible that the association may show gender-related variation, as the initial study had shown association in females and our study was carried out in an exclusively male population.

In conclusion, in a BMP2 candidate gene polymorphism study in the UK, comprising osteoporotic men and male control subjects, there were no genotypic or haplotypic effects on phenotype and fracture risk identified.

Abbreviations

BMP2: Bone morphogenetic protein 2
 SNP: Single nucleotide polymorphism.

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