RELATIONSHIP OF SCLEROSTIN AND SECRETED FRIZZLED PROTEIN POLYMORPHISMS WITH BONE MINERAL DENSITY: AN ASSOCIATION STUDY WITH REPLICATION IN POSTMENOPAUSAL WOMEN

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ABSTRACT

Objectives.- Secreted frizzled-related protein and sclerostin, encoded by *FRZB* and *SOST* genes, respectively, are extracellular Wnt inhibitors that tend to decrease bone formation. The purpose of this study was to explore the association of sets of polymorphisms capturing common variations of these genes with bone mineral density (BMD).

Methods.- Twelve polymorphic loci of the *FRZB* gene and 7 of the *SOST* gene were genotyped in postmenopausal women from two Spanish regions (Cantabria, n=1043, and Valencia, n=342). The polymorphisms included tagging SNPs and SNPs with possible functional consequences assessed in silico.

Results.-The rs4666865 polymorphism of the *FRZB* gene was associated with spine BMD in the Cantabria cohort in the single-locus (p=0.008) and the haplotypic analysis. However, the results were not replicated in the Valencia cohort. Several polymorphisms at the 5´region of the *SOST* gene, and particularly rs851056, were associated with BMD in women from both cohorts (p=0.002 in Cantabria and 0.005 in Valencia). When the results of both cohorts were combined, the mean BMD difference across rs851056 genotypes was 47 mg/cm^2 or 0.31 standard deviations (p<0.001). No differences in *FRZB* and *SOST* expression was detected across genotypes.

Conclusions.- Polymorphisms in the 5' region of *SOST* gene are associated with BMD in postmenopausal women, and consequently contribute to explain in part the hereditary influence on bone mass.

KEYWORDS: Osteoporosis, sclerostin, *SOST*, *FRZB*, association study, polymorphisms.

INTRODUCTION

The Wnt pathway has emerged as an important player in skeletal homeostasis. Wnt ligands promote the differentiation of mesenchymal precursors towards the osteoblastic lineage and have a positive effect on bone formation. On the other hand, they decrease the expression of RANKL by osteoblastic cells, which in turn inhibits osteoclast differentiation and bone resorption [1-4]. Wnt receptors are membrane molecular complexes including LRP5/6 and a frizzled protein. A number of extracellular Wnt inhibitors have been identified. Dickkopf 1 is a Wnt inhibitor, encoded by the DKK1 gene, which appears to play an important role in multiple myeloma and other skeletal tumours [5]. Secreted frizzled-related protein 3 (SFRP3), the product of the *FRZB* gene, is related to membrane frizzled and binds Wnt ligands, thus preventing their binding to membrane receptors [6]. Sclerostin, the product of the *SOST* gene, appears to interact with LRP5/6, making it unable to bind Wnt's. It has been suggested that polymorphisms of the genes encoding SFRP3 and sclerostin influence bone mass and the risk of skeletal diseases such as osteoarthritis and osteoporosis [7;8]. Contradictory results were initially reported regarding their influence on bone mass [9;10], and no evidence for association between polymorphisms of these genes and bone mineral density (BMD) was reported in two genome-wide association studies (GWAS) [11;12]. However, some investigators later reported an association of *SOST* alleles and BMD [13-15]. Three markers located 23-57 kb downstream to the *SOST* gene were also recently found as genome-wide significant quantitative trait loci in an extended GWAS in Iceland population [16]. Differences in study subjects and the markers selected may explain these contradictory results. In fact, mixed populations, including men and women, pre and postmenopausal, or individuals with extreme BMD values were included in previous studies. Therefore, the aim of this study was to try to clarify the possible association of allelic variants of the *FRZB* and *SOST* genes with BMD in postmenopausal women by genotyping sets of SNPs located throughout these genes.

MATERIALS AND METHODS

Subjects.- We studied 1043 postmenopausal women over 50 years of age (mean 66±8) living in Cantabria, a region in Northern Spain. They included volunteers recruited by advertisements and women sent to our clinic because of osteoporosis concerns. Women with present or past diseases or treatments known to affect bone metabolism, or with non-Spanish ancestors (parents and grandparents) were excluded. BMD at the lumbar spine and femoral neck was measured by DXA using a Hologic QDR 4500 densitometer. Participants gave informed consent and the study was approved by the Clinical Research Ethical Committee of the Hospital U.M. Valdecilla.

Positive results were replicated in a cohort of women attending a menopause clinic in Valencia, a region in Eastern Spain. After applying similar exclusion criteria, the cohort included 342 Caucasian postmenopausal women (more than 6 months since the last menstruation) aged 41-69 years (mean 52). BMD was measured by DXA using either a DPX (GE Lunar Corporation, Madison, WI, USA) or a Norland XR-36 (Norland Medical Systems Inc; Fort Atkinson, WI, USA). The results from different densitometers were standardized as proposed by Lu and Hui [17;18].

Genotyping.- The Hapmap database was explored to identify SNPs of the *FRZB* and *SOST* regions in the Caucasian population. Then, tag-SNPs were selected using the algorithms available in Haploview with the "aggressive tagging" option [19]. Minor allele frequency (MAF) of 0.05 and r^2 0.8 were used as criteria. In addition, we included two SNPs (rs7775 and rs1230395 in *FRZB* and *SOST* genes, respectively) with potential functional consequences, as assessed by PUPASUITE, a web tool that explores a variety of databases (coding SNPs, SNPs disrupting potential transcription factors binding sites, intron/exon boundaries) [20;21]. DNA was isolated from peripheral blood by using column-based commercial methods and quantified with the Qubit procedure (Invitrogen, Carlsbad, CA, USA). Then alleles at each locus were analyzed on a mass-array Sequenom platform at the Centro Nacional de Genotipado (Santiago de Compostela, Spain). Polymorphisms associated with BMD in the

Cantabria cohort were analyzed in Valencia cohort by using similar procedures for genotyping at Unidad Central de Investigación (Facultad de Medicina, Valencia, Spain).

Gene expression.- Trabecular bone samples were obtained from the central part of the femoral heads of 27 patients undergoing hip replacement because of osteoarthritis, as previously reported [22]. RNA was isolated with Trizol (Invitrogen), and further purified by using a column adsorption procedure (Qiagen, Hilden, Germany). Gene expression was analyzed by reverse transcription (RT) real-time polymerase chain reaction (PCR). Aliquots of RNA (250 ng) were reverse-transcribed with the Superscript III kit (Invitrogen). After RT, a real-time PCR was done in an ABI7300 apparatus (Applied Biosystems, Foster City, CA), using specific primers and FAM-labelled probes for *FRZB* and *SOST* (Taqman gene expression assays, Applied Biosystems). The results were then normalized to the expression of the housekeeping gene TATA box protein (TBP) as $2^{-\Delta Ct}$, where ΔCt is the difference between either the *FRZB* or *SOST* threshold cycle and TBP threshold cycle [23].

Statistical analysis.- Haplotypic blocks were estimated by the method of Gabriel, implemented in Haploview [19]. The departure from Hardy-Weinberg equilibrium (HWE) was tested with Plink software [24]. The association of alleles with BMD was studied at the single-locus level, assuming codominant and recessive models, with Plink. The significance threshold after multiple test correction for each gene was estimated by considering the effective number of independent marker loci, as proposed by Li and Ji, using the single nucleotide spectral decomposition software (SNPSpD), developed by Nyholt [25]. Haplotypic analyses were performed using the sliding window procedure including 3 consecutive SNPs. The presence of population stratification was explored with STRUCTURE software, running several datasets of 5-68 markers [26]. Results from different cohorts were combined with MIX software [27].Gene expression data, normalized by housekeeping gene expression, were log-transformed and compared by t-test. Association between SNP genotypes and gene expression was also explored in silico comparing expression data in immortalized B-lymphocytes, using a database from the Sanger Institute GENEVAR project [28] and HapMap project, explored with WGAViever software [29]. The study power to detect an a polymorphism explaining at least

1.5% variance of BMD under an additive model was more than 96% in the discovery cohort and more than 60% in the replication cohort (estimates obtained with QUANTO v1.2.3 (available at available at http://hydra.usc.edu/gxe/). Uncorrected nominal p-values are shown, unless otherwise indicated. p-values <0.05 were considered as statistically significant.

RESULTS

Cantabria cohort

The polymorphisms studied are shown in table 1. Allelic frequencies were similar to those reported in the Hapmap database for the Caucasian population. There was no evidence for departure from HWE; some SNPs were associated with p-values in the 0.03-0.05 range, well above the multiple test significance threshold (see below). The characteristics of women included in the study are shown in table 2.

FRZB

Lumbar spine.-The rs4666865 polymorphism of the *FRZB* gene was associated with lumbar spine BMD (p=0.008; figure 1), but it did not reach the significance threshold after multiple test adjustment, estimated as 0.0051. The average spine BMD in women with different genotypes was: GG, 0.850±0.156; GC, 0.861±0.140; CC, 0.884±0.149 g/cm^2. Similar results were obtained including age and weight as covariates (not shown). In the multimarker analysis, haplotypes including this SNP and two neighbour downstream polymorphisms (rs7775 and rs6710705) showed the most significant association with BMD. Among the individual haplotypes, the frequent ACC haplotype showed the strongest association and explained 0.9% of the overall variance in BMD (table 3). Spine BMD was 0.853±0.009, 0.867±0.145, and 0.900±0.141 g/cm^2, in women with 0, 1 and 2 copies of the ACC haplotype, respectively (p=0.002). In the haplotype conditional analysis, rs4666865 was independently associated with BMD (p=0.004), but rs7775 and rs6710705 were not (p=0.13 and 0.28, respectively).

Femoral neck.- In the single locus analysis, rs4666865 showed a marginally significant association with femoral neck BMD. Average values were 0.678±0.120, 0.681±0.109 and

0.697±0.121 g/cm^2 in women with GG, GC and CC genotypes, respectively (nominal p-value=0.048). Several haplotypes including the rs4666865 locus were also associated with femoral neck BMD. As with spine BMD, the most significant one included rs4666865, rs7775 and rs6710705 polymorphisms. Femoral neck BMD was 0.677±0.115, 0.683±0.110, and 0.714±0.0.125 g/cm^2, in women with 0, 1 and 2 copies of the ACC haplotype, respectively (p=0.0017) (table 3).

When the association of haplotypic blocks (defined according to the Gabriel method, see figure 1) with BMD was studied, similar but slightly less significant results were found (lowest p-values were 0.004 and 0.005, for the association of block 2 haplotypes with spine and femoral BMD, respectively).

SOST

Lumbar spine.- There was an association between several linked polymorphisms in the 5' region of the *SOST* gene and spine BMD, best fitting a recessive genetic model (figure 2). Thus, women homozygotes for the G minor allele at rs851056 had significantly lower BMD than women with other genotypes (p=0.0022), in the crude analysis and after adjustment by age and weight (not shown). The p-value was below the significance threshold after multiple test correction, which was estimated as 0.0085. Furthermore, the haplotype analysis confirmed an association with BMD, but it did not increase the statistical significance in comparison with the single locus analysis. Thus, the CTT haplotype at loci rs851056, rs12346012 and rs1230395 was significantly associated with spine BMD. The average BMD values were 0.851±0.147, 0.880±0.150 and 0.873±0.152 g/cm^2, in women with 0, 1 and 2 CTT copies, respectively (p=0.031, table 4).

Femoral neck.- There was no evidence for association of either SNPs or haplotypes with femoral neck BMD, although rs851056 alleles almost reached nominal statistical significance (p=0.06).

Haplotypic blocks (defined according to the Gabriel method, see figure 2) were not significantly associated with either spine or femoral BMD (p>0.07 and >0.10, respectively).

Valencia cohort

The allele frequencies were similar in both cohorts (table 1). In the Valencia cohort we could not replicate the association of *FRZB* polymorphisms with either spine or femoral neck BMD. However, the rs851056 polymorphism of the *SOST* gene was associated with lumbar spine BMD (p=0.005).

The combination of the results of both cohorts confirmed a significant association of rs851056 genotypes with BMD, with a weighted mean difference of 47 mg/cm^2 (95% confidence interval 24-70) between women with GG genotypes and those with other genotypes (GC or CC). As shown in table 5, the standardized mean weighted difference was 0.31 standard deviations (95% CI 0.16-0.46; p<0.0001).

There was no evidence for association between *SOST* polymorphisms and femoral neck BMD in the single locus analysis (p=0.10 for the rs801056 polymorphism).

FRZB and *SOST* expression

FRZB and *SOST* transcripts were readily detected in bone samples by RT-qPCR. However, no significant association between rs4666865 alleles and *FRZB* expression was found in either bone samples or in the lymphoblastoid cell line database. Likewise. there were no significant differences in *SOST* gene expression across the genotypes of the *SOST* rs851056 locus.

DISCUSSION

The binding of Wnt ligands to their membrane receptors activate a number of intracellular signals, the best known of which constitute the so-called canonical pathway and involves an increase in ß-catenin, which induces the transcription of target genes [30;31]. SFRP3, encoded by *FRZB* gene, is usually considered a Wnt antagonist. Nevertheless, it is still unclear whether it may promote Wnt actions on target cells under certain conditions [6]. Targeting deletion of *FRZB* in mice appears to accelerate osteoarthritis [32], and *FRZB* polymorphisms, including rs7775, have been associated with large joint osteoarthritis in humans [8;33]. In the present

study we found an association of rs4666865 and neighbour SNPs, including rs7775, with BMD in postmenopausal women from the Cantabria cohort. However, the results could not be replicated in the Valencia cohort. Gao et al recently studied four SNPs of the *FRZB* gene in Chinese families and explored their association with peak BMD. They found some suggestion for association of rs4666865 alleles and lumbar spine BMD in the whole male offspring population (p=0.02), but no within-family evidence for association [34]. Therefore, the possible association of *FRZB* allelic variants with either peak bone mass or bone loss in later life is still unclear.

Sclerostin, the product of the *SOST* gene, inhibits Wnt signaling. Its role is emphasized by studies showing that the inhibition of sclerostin by means of targeted gene deletion or neutralizing antibodies increases bone mass [35;36]. Osteocytes are the main source of sclerostin in the bone microenvironment, but little is known about the mechanisms regulating sclerostin synthesis [37;38]. The human *SOST* gene has two exons and spans about 5 kb on chromosome 17. A 52 kb deletion located in a noncoding region, approximately 35 kb downstream of *SOST* gene (the Van Buchen region) may act as a gene transcription enhancer [9].

In the present study we found a consistent association of the rs851056 polymorphism, located in the 5' region of the gene, and lumbar spine BMD in two Spanish cohorts. These results are consistent with previous reports showing an association of several SNPs in this region with BMD [9;13;15;39]. Although some negative results have also been reported [10], our data, showing an association in two different populations, add further support to the contention that allelic variants of the proximal region of the *SOST* gene are associated with BMD. Some investigators also found evidence for association of bone mass with the so-called Van Buchen region, located 35-87 downstream of the *SOST* gene. It was usually reported in studies with male individuals [9;39], suggesting that there might be some sex-specific influence. However, in a large GWAS study including both men and women, three SNPs located between 23 kb and 57 kb 3' to the *SOST* gene were also associated with BMD [16]. Therefore, further studies are

needed to clarify whether *SOST* variants show sex-dependent associations with bone mass. We did not include Van Buchen region in our study.

This rs851056 polymorphism is located around 1100 bp upstream of the *SOST* translation start site. Therefore, it can be speculated that its allelic variants influence gene transcription. In fact, the Fast SNP bioinformatics tool (http://fastsnp.ibms.sinica.edu.tw/pages /input_SNPListAnalysis.jsp) suggested that a putative binding site for c-Myc was lost in G alleles. We found no genotype-related differences in the abundance of RNA transcripts in a lymphoblastoid cell expression database. However, the relevance of those non-skeletal data is questionable. In studies of *SOST* expression in bone samples, we did not find differences across genotypes either. These data may be more relevant, but, given the relatively small number of samples studied, they should be interpreted with caution. It is also possible that rs801056 is linked to other polymorphisms which are the actual regulatory loci.

This study has several limitations. First, it included only Caucasian women and therefore it is unclear if the results can be extrapolated to women from a different ethnicity or to men. Second, the size of the replication cohort was small. It is also worth to mention that women in the replication cohort were younger than in the discovery cohort. Therefore, the lack of replication of the association of *FRZB* polymorphisms may reflect a chance association in the discovery cohort, but it might also be the consequence of the limited statistical power for replication. Alternatively, if *FRZB* variants influence postmenopausal bone loss, rather than peak bone mass, genotypic differences in BMD might be more evident in women with longer duration of menopause. The study design was cross-sectional, clinic and volunteer-based, not population based. Therefore, although we tried to avoid potential biases, some may remain. For instance, individuals with musculoskeletal complaints are likely to be over-represented. We excluded women with diseases or treatments known to have a powerful influence on bone mass, which may confound the genetic influence. We also excluded women with severe osteoarthritis, but we did not control for subtle skeletal degenerative changes. Therefore, since we found a stronger association of *FRZB* and *SOST* polymorphisms with spine BMD than with femoral BMD, it could be questioned whether the associations were actually the

consequence of a genetic influence on spine osteoarthritis, particularly for *FRZB* polymorphisms, which have been previously associated with large-joint osteoarthritis [8]. Further studies with a prospective design are needed to clarify this issue, because osteoarthritis may spuriously influence BMD measurements, but, on the other hand, a true association between bone mass and osteoarthritis has also been suggested [40].

In summary, this study confirms that polymorphisms in the 5' region of *SOST* gene are associated with BMD in postmenopausal women. The molecular mechanisms involved remain to be elucidated. We also found an association of some *FRZB* polymorphisms with BMD, but it could not be replicated in an independent cohort. Therefore, its actual biological relevance is uncertain.

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FIGURE LEGENDS

Figure 1. Association of *FRZB* polymorphisms with lumbar spine and femoral neck BMD (Cantabria cohort). In the lower part, the haplotypic structure is shown.

Figure 2. Association of *SOST* polymorphisms with lumbar spine and femoral neck BMD (Cantabria cohort). In the lower part, the haplotypic structure is shown.

Tables 1-5

Table 1. Polymorphisms studied

Chr	SNP	Gene	Position	Location	Minor alelle	Major allele	p-HWE (Cantabria)	p-HWE (Valencia)	MAF (Cantabria)	MAF (Valencia)
2	rs288316	*FRZB*	183403694	Intergenic	A	G	0.49		0.22	-
2	rs17265803	*FRZB*	183404764	Intergenic	C	T	0.34		0.08	-
2	rs4666865	*FRZB*	183406336	3'	G	A	0.50	0.59	0.41	0.39
2	rs7775	*FRZB*	183407829	Exon 6 (Gly/Arg)	G	C	0.64	0.71	0.11	0.11
2	rs6710705	*FRZB*	183408338	Intron 5	T	C	0.14	0.13	0.08	0.08
2	rs288324	*FRZB*	183409833	Intron 5	A	G	0.54		0.48	-
2	rs288326	*FRZB*	183411581	Exon 4 (Trp/Arg)	A	G	0.04		0.14	-
2	rs7602601	*FRZB*	183431211	Intron 2	C	T	0.52		0.15	-
2	rs6433993	*FRZB*	183432193	Intron 1	G	A	0.18		0.30	-
2	rs7592998	*FRZB*	183432783	Intron 1	C	T	0.17		0.06	-
2	rs13026760	*FRZB*	183434958	Intron 1	C	T	0.04		0.47	-
2	rs12469777	*FRZB*	183440609	Intergenic	C	T	0.78		0.45	-
17	rs17610444	*SOST*	39181383	Intergenic	C	T	0.04		0.04	-
17	rs865429	*SOST*	39190741	Intron 1	C	T	0.99		0.12	-
17	rs17882143	*SOST*	39191608	Exon 1 (Ile/Val)	A	G	0.11		0.03	-
17	rs851054	*SOST*	39192149	5'	G	A	0.73		0.38	-
17	rs851056	*SOST*	39192708	5'	G	C	0.96	0.24	0.38	0.38
17	rs1234612	*SOST*	39196328	Intergenic	C	T	0.06	0.55	0.32	0.32
17	rs1230395	*SOST*	39198463	Intergenic	C	T	0.03	0.99	0.08	0.07

MAF: minor allele frequency

Table 2. Characteristics of women included in the study (mean±SD)

	Cantabria	Valencia
Age, yr	66±8	52±5
BMI, kg/m^2	27.9±4.3	26.5±14.3
Years since menopause	16±10	4±4
Spine BMD, T-score	-1.7±1.3	-1.1±1.4
Spine BMD, Z-score	-0.1±1.4	-0.2 ±1.2
Femur BMD, T-score	-1.5±1.2	-0.9±1.0
Femur BMD, Z-score	0.2±1.1	-0.1±1.0

Table 3. Association of haplotypes of the *FRZB* gene (rs466865-rs7775-rs6710705 loci) with spine and femoral BMD

Haplotype	Frequency %	p-value Spine	p-value Femoral neck
GCT	1.5	0.71	0.72
ACT	6.2	0.72	0.41
AGC	11.3	0.76	0.26
GCC	39.3	**0.0058**	**0.053**
ACC	41.7	**0.0021**	**0.0017**

Table 4. Association of haplotypes of the *SOST* gene (rs851056-rs1234612-rs1230395) with spine and femoral BMD

Haplotype	Frequency %	p-value Spine	p-value Femoral neck
GCC	7.6	0.72	0.28
CCT	24.3	0.26	0.18
GTT	30.3	0.34	0.14
CTT	37.8	**0.031**	**0.034**

Table 5. Lumbar spine BMD according to rs851056 (*SOST*) genotypes in both cohorts. Mean±SD [n] and standardized weighted difference (SWD, SD units and confidence interval).

Cohort	CC	CT/TT	SWD	p
Cantabria	0.899±0.153 (157)	0.940±0.152 (823)	0.27 (0.09-0.44)	0.002
Valencia	0.946±0.159 (48)	1.023±0.140 (273)	0.46 (0.15-0.77)	0.005
Combined			0.31 (0.16-0.46)	<0.001

Figure_1
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Fig 1

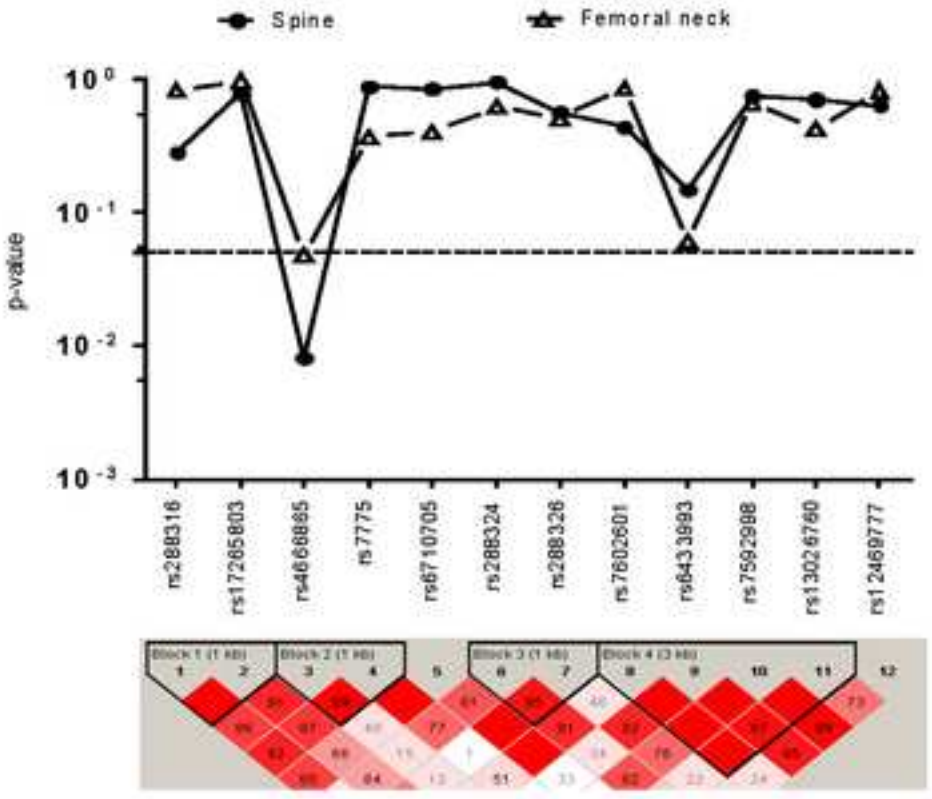

Figure_2
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