CLINICAL STUDY

Nuclear receptor NR5A2 and bone: gene expression and association with bone mineral density

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Abstract

Objective: There is growing evidence for a link between energy and bone metabolism. The nuclear receptor subfamily 5 member A2 (NR5A2) is involved in lipid metabolism and modulates the expression of estrogen-related genes in some tissues. The objective of this study was to explore the influence of NR5A2 on bone cells and to determine whether its allelic variations are associated with bone mineral density (BMD).

Design: Analyses of gene expression by quantitative PCR and inhibition of NR5A2 expression by siRNAs were used to explore the effects of NR5A2 in osteoblasts. Femoral neck BMD and 30 single nucleotide polymorphisms (SNPs) were first analyzed in 935 postmenopausal women and the association of NR5A2 genetic variants with BMD was explored in other 1284 women in replication cohorts.

Results: NR5A2 was highly expressed in bone. The inhibition of NR5A2 confirmed that it modulates the expression of osteocalcin, osteoprotegerin, and podoplanin in osteoblasts. Two SNPs were associated with BMD in the Spanish discovery cohort (rs6663479, $P=0.0014$, and rs2816948, $P=0.0012$). A similar trend was observed in another Spanish cohort, with statistically significant differences across genotypes in the combined analysis ($P=0.03$). However, the association in a cohort from the United States was rather weak. Electrophoretic mobility assays and studies with luciferase reporter vectors confirmed the existence of differences in the binding of nuclear proteins and the transcriptional activity of rs2816948 alleles.

Conclusions: NR5A2 modulates gene expression in osteoblasts and some allelic variants are associated with bone mass in Spanish postmenopausal women.

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Introduction

Epidemiological and experimental studies suggest a link between energy metabolism and bone metabolism (1–3), which may also share genetic influences. For instance, Zhao *et al.* (4) recently reported that polymorphisms at the osterix locus, a critical gene for osteoblast development, are associated with childhood adiposity. The nuclear receptor subfamily 5 member A2 (NR5A2) is highly expressed in the liver and gastrointestinal tract and is involved in the regulation of lipid metabolism (5). Moreover, it modulates the expression of genes coding for aromatase and other enzymes participating in estrogen metabolism (6–8). Therefore, we hypothesized that NR5A2 might play a role in skeletal homeostasis. The aims of this study were to

explore the influence of NR5A2 on bone cells and to analyze whether the allelic variations in the NR5A2 gene are associated with bone mineral density (BMD).

Materials and methods

Analysis of gene expression in bone

Trabecular bone samples were obtained from the femoral heads of patients undergoing hip arthroplasty for osteoarthritis (age 76 ± 6 years, $n=10$) or fractures (age 77 ± 4 years, $n=9$), after obtaining informed written informed consent. They had no other disorders affecting bone metabolism revealed by clinical examination and routine blood tests. The osteoblastic cell line

HOS-TE85 was obtained from EACC and maintained in Minimum Essential Medium supplemented with antibiotics and 10% fetal bovine serum. RNA was isolated from the bone pieces or confluent cell cultures and reverse transcribed with Superscript III reverse transcriptase (Invitrogen- Life Technologies). Gene expression was analyzed by quantitative real-time PCR using specific primers and Taqman probes (Applied Biosystems, Foster City, CA, USA). The results were normalized by the expression of the constitutive gene TATA box-binding protein (*TBP*). For comparison purposes, *NR5A2* expression was also measured in a human reference RNA mixture derived from ten different cell lines (Stratagene, La Jolla, CA, USA).

NR5A2 inhibition

To explore the influence of *NR5A2* on the transcription of other genes, we generated HOS-TE85 clones with stable inhibition of *NR5A2*. Two different commercial plasmids coding for shRNAs already validated for the specific inhibition of human *NR5A2* gene expression (TRCN0000019654 and TRCN0000019656) were purchased from Sigma-Aldrich. The viral particles were prepared according to the manufacturer's protocol. The amount of viruses necessary for obtaining a 50% infection of HOS-TE85 cultures was determined by using GFP-encoding viruses. A similar amount of *NR5A2* shRNA viruses was used for infecting HOS-TE85 cultures grown to 50% confluence. Puromycin (1.5 µg/ml) was added to the cultures 48 h after infection and ten resistant clones were selected after several medium changes and were used in subsequent experiments. The studies of the effect of *NR5A2* inhibition on the expression of other osteoblastic genes were done with two of them, resulting from the infection with virus prepared from TRCN0000019656. They were cultured in the presence or absence of 1,25-dihydroxyvitamin D₃ (10⁻⁸ M), a well-known stimulus for the osteocalcin gene. HOS-TE85 clones infected with virus preparations derived from plasmid pLKO.1-TRC control (Sigma) were used as a negative control. Gene expression was analyzed using specific primers and Taqman probes (Applied Biosystems). The results were normalized by the expression of the constitutive gene *TBP*. Experiments were repeated three times.

Electrophoretic shift and reporter vector assays

Nuclear extracts were prepared from subconfluent HepG2 cell cultures. Binding reactions and electrophoresis mobility shift assays (EMSA) were performed as described previously (9), using two 20 nt-long, 5'-fluorescein-labeled, double-stranded oligonucleotides including either C or G alleles of the rs2816948 polymorphism and the surrounding region. Gel analysis was carried out in an FLA-5100 fluorescent image

analyzing system (FujiFilm). Competition studies were carried out with an unlabeled C allele-specific oligonucleotide. The specificity of the retarded band was tested by adding a 50-fold excess of an unrelated oligonucleotide to the reaction mix.

For the construction of luciferase reporter plasmids, a 248 bp fragment of the *NR5A2* gene promoter extending from position -212 to position +36 was amplified by PCR using primers modified to contain KpnI and XhoI sites and cloned in pGEMT-easy vector (Promega), subcloned into the promoterless pGL3- basic vector (Promega) and then used in subsequent transient transfection assays of HepG2 cultures. The procedures for transfection and measurement of luciferase activity have been described previously (10).

Genetic association study

Discovery cohort We selected 935 Caucasian postmenopausal women over 50 years of age (range 50–88, mean 66) living in Cantabria, a region in Northern Spain. They included volunteers recruited by advertisements, taking part in a population study on the epidemiology of osteoporosis, and women sent to our clinic because of osteoporosis concerns. Participants gave informed consent and the study was approved by the Clinical Research Ethics Committee of the Hospital U.M. Valdecilla. Women with diseases that might potentially affect bone metabolism were excluded. These diseases/conditions included chronic disorders involving vital organs (e.g. heart, lung, liver, and kidney), serious metabolic diseases (e.g. hypo- and hyper-parathyroidism, hyperthyroidism, etc.), skeletal diseases other than primary osteoporosis (e.g. Paget disease, osteogenesis imperfecta, rheumatoid arthritis, etc.), and use of drugs affecting bone metabolism (e.g. hormone replacement therapy, corticosteroids, anticonvulsant drugs, and anti-resorptive drugs). In addition, women with non-Spanish ancestors were excluded (11). BMD was measured with a Hologic densitometer. Results at the femoral neck are reported, which had a coefficient of variation of 1.4% in normal subjects after repositioning.

Tagging single nucleotide polymorphisms (SNPs) covering the common variations of the *NR5A2* gene, as well as the 16 kb upstream and downstream regions, and with a minor allele frequency >5% were selected with Tagger Software <http://www.broadinstitute.org/mpg/tagger>. SNPs with a potential regulatory role suggested by bioinformatic analyses (Math Inspector <http://www.genomatix.de> and, Mapper <http://www.mapper.chip.org>) were also included. DNA was isolated from peripheral blood and a 30 SNP set was analyzed by iPLEX technology on a Mass-Array platform (Sequenom, Hamburg, Germany) or by using Taqman assays. Reproducibility was confirmed by obtaining consistent genotypes in 30 replicate samples.

Figure 1 Gene expression in unstimulated and 1,25-dihydroxyvitamin D₃-stimulated HOS-TE85 osteoblastic cells. The results represent the mean and s.d. of gene expression in two clones with stable inhibition of *NR5A2*, shown as the percentage of gene expression in unstimulated control cells. Similar results were obtained in three independent experiments.

Replication cohorts The Valencia cohort included 324 Caucasian postmenopausal women aged 41–69 years (mean 52) attending a menopause clinic and living in Valencia, a region in eastern Spain. BMD was measured using either a Lunar or a Norland densitometer. The results obtained from different densitometers were standardized as proposed by Lu *et al.* (12).

The Kansas City cohort included 930 unrelated Caucasian postmenopausal women aged 45–87 years (mean 65), recruited from Midwestern U.S. (mainly in Kansas City and vicinity areas) (13). BMD was measured at the femoral neck using a Hologic densitometer. Alleles of 50 polymorphisms of the *NR5A2* region were extracted from a Genomewide Association Study (GWAS) with the Affymetrix Human SNP Assay 6.0 to carry out an *in silico* replication.

Exclusion criteria in the Kansas City and Valencia cohorts were similar to those in the discovery cohort. Studies were approved by the Institutional Review Committees. Informed consent was obtained from the participants.

Statistical analyses

The Hardy–Weinberg equilibrium (HWE) and the association of alleles with BMD were studied at the single-locus level and adjusted by age and weight as covariates using Plink (14) and SPSS Software (SPSS, Chicago, IL, USA). Haplotypic blocks were built by the method of Gabriel, implemented in Haploview (15). The association between haplotypes and BMD was explored by testing 2-SNP haplotypes using the sliding window procedure, as well as haplotypes including SNPs that were part of a single haplotypic block. Multiple test correction was performed with the software developed by Nyholt (16). Power analysis was done using Quanto Software (available at <http://hydra.usc.edu/gxe/>). We estimated a 86% power to detect a polymorphism

explaining at least 1% of the BMD variance under an additive model (approximately equivalent to a 0.25 s.d. difference between opposite homozygotes) in the discovery cohort.

Results

NR5A2 is expressed in bone and modulates gene expression in osteoblasts

Abundant *NR5A2* transcripts (up to 1000-fold in comparison with the reference RNA) were detected in all bone samples. They tended to be slightly more abundant in fracture samples than in controls, but the difference was not statistically significant ($P=0.2$, not shown). In order to investigate the potential effects of *NR5A2* in osteoblasts, we generated HOS-TE85 clones with stable inhibition of *NR5A2* expression, down to $9 \pm 2\%$ of the controls. *NR5A2* inhibition resulted in a marked increase in osteoprotegerin and osteocalcin expression, up to tenfold, both in basal cultures and in 1,25-dihydroxyvitamin D₃ cultures (Fig. 1). On the other hand, the expression of podoplanin was markedly inhibited (Fig. 1) and there were inconsistent effects on other genes, such as alkaline phosphatase, estrogen receptors, *LRP5*, *LRP6*, and *FRZB*. The baseline

Figure 2 Relationship between *NR5A2* and aromatase expression in bone samples (A, total aromatase; B, 1,4-transcripts). Arbitrary units after normalization by *TBP* expression.

Table 1 Single nucleotide polymorphisms (SNPs) studied in the discovery cohort.

SNP	Chromosome position (HG18)	Gene region	Allele 1	Allele 2	Minor allele frequency	P (HWE)
rs6663479	198247395	5'	C	T	0.103	0.630
rs2816937	198249733	5'	C	G	0.157	0.116
rs2816948	198263308	5'	G	C	0.143	0.905
rs10919803	198295054	Intron	T	C	0.119	0.671
rs2821388	198300392	Intron	G	A	0.297	0.177
rs6658424	198315925	Intron	A	T	0.347	0.169
rs2821313	198317896	Intron	A	G	0.482	0.553
rs2816928	198321700	Intron	G	T	0.497	0.354
rs2821320	198331838	Intron	T	C	0.297	0.213
rs2737668	198332240	Intron	G	A	0.430	0.333
rs10800666	198340353	Intron	C	A	0.484	0.153
rs2816977	198346209	Intron	T	C	0.101	0.098
rs2248967	198351516	Intron	A	G	0.412	0.221
rs2821332	198352337	Intron	A	T	0.414	0.315
rs12041297	198353047	Intron	C	A	0.160	0.640
rs10919811	198356291	Intron	C	A	0.468	0.084
rs3762397	198356842	Intron	A	G	0.462	0.056
rs2737682	198358975	Intron	A	G	0.110	0.127
rs2816959	198368104	Intron	C	T	0.165	0.284
rs10919814	198371595	Intron	C	A	0.295	0.154
rs2816953	198374173	Intron	T	C	0.370	0.610
rs3828107	198387650	Intron	C	T	0.033	0.112
rs12078096	198396692	Intron	C	A	0.015	0.001
rs16846169	198401961	Intron	C	T	0.061	<0.0001
rs2246923	198405973	Intron	T	C	0.320	0.633
rs2737631	198408800	Intron	A	G	0.320	<0.0001
rs1060060	198409904	Exon	A	G	0.255	0.756
rs4551624	198418447	3'	C	T	0.362	0.563
rs2885821	198429406	3'	A	G	0.126	0.421
rs4915414	198429626	3'	A	G	0.194	0.635

expression of aromatase in HOS-TE85 cells was low and tended to decrease slightly with *NR5A2* inhibition. On the contrary, aromatase expression was increased by *NR5A2* inhibition in 1,25-dihydroxyvitamin D₃-stimulated cultures (not shown). The gene encoding aromatase has several alternative promoters and first exons. In bone tissue samples, *NR5A2* expression was correlated with total aromatase transcripts and with I.4-containing transcripts, which is the major form of first exon found in bone (Fig. 2).

Association of *NR5A2* polymorphisms with bone mass

Three out of the 30 SNPs did not follow HWE. The remaining 27 SNPs were included in the analysis (Table 1). The average genotyping rate was 97.9%. Polymorphisms were grouped into seven haplotypic blocks. Five SNPs were associated with femoral neck BMD at the nominal *P* value of 0.05 (Fig. 3). The strongest association was found with three SNPs within the first haplotypic block, at positions 198247395–198295054. Two of them were associated with BMD with *P* values below the multiple-test corrected threshold of 0.0026: rs6663479 (*P*=0.0014) and rs2816948 (*P*=0.0012). The haplotypic analysis confirmed the results of the single-locus analysis. SNP pairs showing a significant association with BMD

are depicted in Fig. 3. Likewise, haplotypes including the three SNPs in block 1 or the five SNPs in block 5 were also associated with BMD (*P*=0.0013 and 0.0160 respectively). Similar results were found after age and weight adjustment (not shown).

Figure 3 Association of *NR5A2* polymorphisms with femoral neck BMD in the Cantabria cohort. *P* values for the single-locus and haplotypic analyses (only significant haplotypes are shown). The haplotypic structure of the *NR5A2* gene is also shown. Full colour version of this figure available via <http://dx.doi.org/10.1530/EJE-11-0571>.

Table 2 Standardized femoral neck BMD (g/cm²), according to rs2816948 genotypes in Spanish cohorts. Mean $\pm$ s.d. (n).

	GG	GC	CC	P value
Cantabria	0.829 $\pm$ 0.101 (21)	0.804 $\pm$ 0.129 (228)	0.777 $\pm$ 0.123 (657)	0.0012
Valencia	0.833 $\pm$ 0.216 (6)	0.792 $\pm$ 0.110 (66)	0.806 $\pm$ 0.114 (252)	NS
Global	0.830 $\pm$ 0.130 (27)	0.801 $\pm$ 0.125 (294)	0.785 $\pm$ 0.121 (909)	0.03

Three SNPs with the most significant *P* values (rs6663479, rs2816937, and rs2816948) were genotyped in a different cohort of postmenopausal women from Valencia (Eastern Spain). There was a trend for similar BMD differences across rs6663479 and rs2816948 genotypes. The latter are shown in Table 2.

The NR5A2 rs2816948 polymorphism was not included in the Affymetrix Human SNP Assay 6.0 used to genotype Kansas City cohort. In this cohort, we found one SNP, rs2821320, located at position chromosome 1: 198331838 (build 36), marginally associated with femoral neck BMD (*P* = 0.04; Fig. 4).

Polymorphisms of NR5A2 promoter influence protein binding and transcriptional activity

EMSA suggested a difference in the ability of rs2816948 alleles to bind nuclear proteins. Competition experiments carried out with nonspecific oligonucleotides revealed the presence of a specific binding. The band intensity of the G allele was on average $53 \pm 14\%$ of the intensity of that of the C allele (*P* = 0.016) and disappeared very easily when competed with increasing amounts of an unlabeled C-specific oligonucleotide (Fig. 5A). Also, the luciferase assays showed that the C to G change resulted in a 24% decrease in the activity of the promoter (*P* < 0.005; Fig. 5B).

Discussion

We have shown that NR5A2 is highly expressed in bone and modulates the expression of some osteoblastic genes, thus suggesting that NR5A2 may play a role in skeletal homeostasis.

BMD is a trait with a strong genetic component (17). Genomewide association studies searching for genes associated with BMD have identified several loci at genes known to influence bone metabolism, such as estrogen receptors, osteoprotegerin, lipoprotein receptor-related protein, or sclerostin (18–21). Candidate gene studies have refined the gene regions involved in the association (22). However, the combined effect of those loci explains only a minor proportion of BMD variation in the population. Therefore, much more research is needed to elucidate the genetic factors underlying the hereditary influence on BMD. We found that several SNPs in the 5' region of the NR5A2 gene were associated with BMD. Among them, the rs2816948 polymorphism showed that allelic differences in the interaction with nuclear

proteins and the transcriptional activity of reporter gene constructs. C allele, which displayed higher transcriptional activity, was associated with lower BMD, suggesting that the net result of NR5A2 effect is to decrease bone mass. Consistent with this idea, there was a nonsignificant trend for higher NR5A2 expression in bone samples from patients with hip fractures than in control samples from osteoarthritic patients. The inhibition of NR5A2 resulted in increased expression of osteocalcin and osteoprotegerin, thus suggesting that NR5A2 tends to decrease both factors. Osteocalcin is a well-known marker of differentiated osteoblast function, whereas osteoprotegerin is an inhibitor of osteoclast differentiation. Therefore, NR5A2 might inhibit bone formation and stimulate bone resorption. NR5A2 modulates the expression of aromatase and estrogen receptors in some tissues (6, 8, 23). However, the inhibition of NR5A2 had inconsistent effects on the expression of these genes in the osteoblastic cell line HOS-TE85. Therefore, further studies are needed to clarify whether NR5A2 actually regulates estrogen-related genes in bone.

Genetic association studies have a number of drawbacks, including the difficulty to distinguish between true and casual associations, due to chance, population stratification, or genotyping errors. Decreasing the *P* value threshold for significance helps to reduce the number of false associations but increases type II error. The replication in different cohorts and functional studies are other approaches to identify true associations. Nevertheless, a number of factors may hamper the replication of true genetic association data (24). Our *in vitro* studies support the regulatory role of the

Figure 4 Association of NR5A2 polymorphisms with femoral neck BMD in postmenopausal women of the Kansas City cohort. The chromosome position of the SNPs included in the array is shown in the horizontal axis (from 198247394 through 198429625, which is the region explored in the discovery cohort with the SNPs included in Fig. 3).

Figure 5 Functional studies. (A) The six lanes on the left correspond to experiments done with a labeled oligonucleotide specific for allele C of the rs2816948 polymorphism, whereas in the six lanes on the right a labeled oligonucleotide specific for allele G was used. NE indicates that no extract was added to the labeled probe. In lanes labeled C and G, nuclear extracts were added to labeled C- and G-specific probes respectively. In lanes labeled 10×, 25×, and 50×, a 10-, 25-, and 50-fold excess of an unlabeled C-specific oligonucleotide was used for interfering the formation of the complexes by either C-specific or G-specific probes. In the lanes labeled as NS, a 50-fold excess of a nonspecific probe was used for competition. The specific complex is indicated with an arrow. (B) Differences in the transcriptional activities of C- and G-specific fragments when cloned in a luciferase reporter vector. The results are the average of four different experiments (*** $P < 0.005$).

upstream rs2816948 polymorphism. However, the association was only partially replicated in other cohorts. Alleles at the rs2816948 locus tended to be associated with BMD in the same direction in the two Spanish cohorts, and in fact, they were significant in the global analyses and in the Cantabria cohort, but not in the Valencia cohort, perhaps due to the smaller number of women included. This SNP was not studied in the Kansas City cohort, but other SNPs in the region were not associated with BMD. The role of population differences and environmental influences in those results is presently unclear, but it is interesting to notice that a weak association between BMD and a NR5A2 SNP was reported in a GWAS of other North American population (25).

In conclusion, NR5A2 gene is expressed in bone and modulates gene expression in osteoblasts. Some common polymorphisms in the 5'-region of the gene, which show allelic differences in functional assays *in vitro*, are associated with femoral neck BMD in Spanish postmenopausal women. These results suggest that the allelic variations in the NR5A2 gene may contribute to explain the genetic influence on bone mass, thus pointing toward NR5A2 as a novel osteoporosis candidate gene.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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