***JAG1* and *COL1A1* polymorphisms and haplotypes in relation to bone mineral density variations in postmenopausal Mexican-Mestizo Women**

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Received: 30 September 2011 / Accepted: 5 December 2011 / Published online: 16 December 2011
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Abstract Osteoporosis is characterized by low bone mineral density (BMD). One of the most important factors that influence BMD is the genetic contribution. The *collagen type 1 alpha 1 (COL1A1)* and the *JAGGED (JAG1)* have been investigated in relation to BMD. The aim of this study was to investigate the possible association between two single-nucleotide polymorphisms (SNPs) of *COL1A1*, their haplotypes, and one SNP of *JAG1* with BMD in postmenopausal Mexican-Mestizo women. Seven hundred and fifty

unrelated postmenopausal women were included. Risk factors were recorded and BMD was measured in lumbar spine, total hip, and femoral neck by dual-energy X-ray absorptiometry. DNA was obtained from blood leukocytes. Two SNPs in *COL1A1* (rs1800012 and rs1107946) and one in *JAG1* (rs2273061) were studied. Real-time PCR allelic discrimination was used for genotyping. The differences between the means of the BMDs according to genotype were analyzed with covariance. Deviations from Hardy–Weinberg equilibrium were

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tested. Pairwise linkage disequilibrium between single nucleotide polymorphisms was calculated by direct correlation r^2 , and haplotype analysis of *COL1A1* was conducted. Under a dominant model, the rs1800012 polymorphism of the *COL1A1* showed an association with BMD of the lumbar spine ($P=0.021$). In addition, analysis of the haplotype of *COL1A1* showed that the G–G haplotype presented a higher BMD in lumbar spine. We did not find an association between the rs1107946 and rs2273061 polymorphisms of the *COL1A1* and *JAG1*, respectively. Our results suggest that the rs1800012 polymorphism of the *COL1A1*, in addition to one haplotype, were significantly associated with BMD variation in Mexican-Mestizo postmenopausal women.

Keywords Bone mineral density · Polymorphisms · *COL1A1* · *JAG1* · Haplotypes · Postmenopausal Mexican-Mestizo women

Introduction

Osteoporosis is a complex disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone, which increases the incidence of fragility fractures and is one of the principal causes of diminished quality of life, morbidity, and mortality among postmenopausal women (NIH Consensus Development Panel on Osteoporosis 2001). Several factors influence BMD; however, one of the most important is a genetic contribution, with heritability >60% (Ralston and de Crombrughe 2006).

Several candidate genes have been investigated in relation to BMD variations. One of these is the *collagen type 1 alpha 1* gene (*COL1A1*) (Grant et al. 1996). This gene encodes the alpha 1 chain of type I collagen, the most abundant protein of the bone matrix (Byers 1990). Numerous association studies have been performed on the *COL1A1* and BMD variations, osteoporosis, and osteoporotic fracture. One of the most studied single-nucleotide polymorphisms (SNPs) in this gene is the rs1800012 (Sp1 binding sites in the first intron at position +1245 G/T of the *COL1A1*) (Grant et al. 1996; Jin et al. 2011). Furthermore, another polymorphism in the 5' flank of *COL1A1*, rs1107946 (–1997G/T), was significantly associated with lumbar spine BMD and femoral neck BMD in a cohort of Spanish postmenopausal women (Garcia-Giralt et al. 2002; Yamada et al. 2005). However, the results of studies seeking associations of

these polymorphisms with osteoporosis have not always been consistent in different population analyses (Wynne et al. 2002; Hubacek et al. 2006; Bustamante et al. 2007; Yazdanpanah et al. 2007; Jin et al. 2009, 2011).

The involvement of the *JAGGED 1* (*JAG1*) gene in bone formation has recently been reported. This gene encodes a cell surface protein called JAGGED 1, which belongs to the Delta/Serrate domain (DSL) family. Moreover, *in vitro* and *in vivo* assays have shown that *JAG1* gene is expressed in osteoblastic cells during bone regeneration and its activation is also associated with increased bone mineral deposition (Nobta et al. 2005). Interestingly, a recently published genome-wide association analysis identified that the rs2273061 polymorphism localized in intron 3 of the *JAG1* gene was associated with the presence of variations of BMD and osteoporotic fractures in subjects of European descent and in Asian populations. In addition, *in vitro* studies suggested that this polymorphism affects the expression of the gene (Kung et al. 2010).

The principal aim of this study was to analyze the possible association between rs1800012 and rs1107946 polymorphisms and their haplotypes of the *COL1A1* gene as well as rs2273061 polymorphism of the *JAG1* gene with the presence of BMD variations in Mexican-Mestizo postmenopausal women.

Subjects and methods

Subjects

This study was performed as part of a population-based osteoporosis study. One thousand consecutive unselected postmenopausal women of Mexican-Mestizo ethnic origin who visited the outpatient clinic for general medical evaluation were invited to participate, and 750 women accepted to participate in the study. All women were unrelated and considered postmenopausal if they had not had a menstrual period within the 12 months prior to the examination.

A research physician administered a structured questionnaire to all women on a face-to-face basis to define the potential risk factors for osteoporosis. Exclusion criteria, as well as the risk factors for osteoporosis, were described previously (Rojano-Mejía et al. 2011). Body height and weight were measured at baseline examination with the subject in a standing position with heavy outer clothes and without shoes.

The study was approved by the Institute's Human Research Committee. Informed consent was obtained from all women.

Methods

BMD (in grams per square centimeter) was measured in total hip (trochanter, Ward's area, and femoral neck) and lumbar spine (L2–L4) by dual-energy X-ray absorptiometry (DXA) with a Hologic QDR 4500 (Hologic Inc., Waltham, MA, USA). *T* score was used to analyze BMD data, which is a deviation from BMD weight-adjusted average peak of a race- and gender-matched healthy Latin American population. The stability of the equipment was verified each morning using an anthropometric spine phantom provided by the manufacturer (Hologic). The coefficient of variation (CV%) of the DXA equipment was 0.6–1.0% for L2–L4 and <1.5% for the femoral neck.

Genotyping

Peripheral blood samples were obtained from all individuals, and genomic DNA was purified using the salting out procedure described by Miller et al. (1998). The rs1800012 and rs1107946 polymorphisms in *COL1A1* gene and rs2273061 polymorphism in *JAG1* gene were studied.

Real-time PCR allelic discrimination TaqMan assay (AB) was used for genotyping analysis. All PCR reactions contained 10 ng of DNA, 5.0 μ l TaqMan Universal Master Mix (AB) (2 $\times$), 0.25 μ l primers and probes (10 $\times$) and water for a final volume of 10 μ l, including the appropriate negative controls in all assays. For the two polymorphisms, the assay used probes and primers designed by assay-on-demand services from Applied Biosystems: for rs1800012, assay ID: C__7477170_30; for rs1107946, assay ID: C__7477171_10; and for rs2273061, assay ID: C__16177763_10.

Real-time PCR was performed on an ABI Prism 7500 Fast (Applied Biosystems, Foster City, CA, USA). Conditions for the three polymorphisms were 95°C for 10 min and 40 cycles of amplification (95°C for 15 s and 62°C for 1 min). For each cycle, the software determined the fluorescent signal from the VIC- or FAM-labeled probe (Applied Biosystems). Allelic discrimination was performed using specific primers and probes for each allele. A 5% random

sample from each genotype was independently verified twice for quality control.

Statistical analysis

Data from the overall patient population in the study were summarized as mean and standard deviation in the case of quantitative variables and absolute and relative frequencies for qualitative variables.

We compared subjects' characteristics by genotype and used one-way analysis of covariance (ANCOVA) for continuous variables [BMD, body mass index (BMI), years since menopause, number of pregnancies, number of children, and duration of breastfeeding]. χ^2 test was used for categorical variables (alcohol consumption, tobacco use, caffeine and cola beverage consumption, calcium supplement, estrogen replacement therapy, and physical activity). Statistical analyses were carried out using SPSS v.16.0 (SPSS Inc., Chicago, IL, USA); *P* value <0.05 was accepted as statistically significant.

Deviations from Hardy–Weinberg equilibrium were tested using the χ^2 test. Linkage disequilibrium (LD) among polymorphisms was tested using Haploview 4.2 (Barrett et al. 2005). Sample size and power were calculated with 80% power and an alpha risk of 0.05 in a two-sided test based on a dominant genetic model as well as by frequency of the minor allele of rs1800012 polymorphism equal to 0.08. Our sample size allowed us to recognize, as statistically significant, differences in BMD of lumbar spine between groups ≥ 0.30 g/cm², assuming the common standard deviation 0.13.

Differences between means of the BMDs of the lumbar spine, total hip, and femoral neck according to genotype were analyzed with ANCOVA adjusted for body mass index, years since menopause, and total duration of breastfeeding.

We estimated the haplotype frequencies using Haploview 4.2 (Barrett et al. 2005), PHASE 2.0.2 (Stephens and Donnelly 2003), and PLINK (Purcell et al. 2007). These programs are for association analyses of the multiloci haplotypes from unphased genotype data. The programs used likelihood ratio tests in a generalized linear model and the expectation maximization algorithm to obtain maximum likelihood frequency estimates (Dudbridge 2003). In addition, we used only haplotype data in which the probability of correct haplotype assignment by PHASE in an individual participant was estimated to be $\geq 95\%$. In addition, we

separately analyzed BMD measurements of lumbar spine, total hip, femoral neck, and haplotypes using ANCOVA to adjust significant variables in our study (BMI, years since menopause, and total duration of breastfeeding).

Results

General characteristics of the 750 postmenopausal women are shown in Table 1. Only 14.8% of the postmenopausal women were current users of hormone replacement therapy, 33.5% of women exercised regularly, 33.2% reported intake of carbonated beverages, 15.5% smoked cigarettes, and only 0.7% of women consumed alcohol.

Hardy–Weinberg equilibrium test was performed for the polymorphisms under study and showed that the distribution of the observed genotypes did not differ from that expected in postmenopausal women (Table 2). The two SNPs of *COL1A1* and the SNP of the *JAG1* genotype and allele frequencies are presented in Table 2.

The Sp1 polymorphism was significantly associated only with lumbar spine BMD under the dominant model (*GG* vs. *GT+TT*); $P=0.029$. Analysis of the data using ANCOVA with adjustment for covariates yielded similar results as the unadjusted analyses ($P=0.021$) (Table 3).

On the other hand, genotype and allele distributions of the rs1107946 polymorphism of *COL1A1* and rs2273061 polymorphism of the *JAG1* showed no significant differences under any model tested (recessive

and dominant models), as well as after adjustment for covariates (Table 3).

Furthermore, the two SNPs of *COL1A1* were at high linkage disequilibrium ($D'=0.84$). Data from the genotyping analysis show the presence of four haplotypes in the population analyzed in this study. One of these (*T–T*) was rare with a frequency of 0.002 and, thus, was excluded from further analyses. The three haplotypes, *G–G*, *G–T*, and *T–G*, had frequencies of 0.624, 0.294, and 0.082, respectively (Table 4). Interestingly, a significant association was observed between *G–G* haplotype and BMD at the lumbar spine ($P=0.022$). Analysis of the data of the haplotypes using ANCOVA with adjustment for covariates showed that this haplotype remained significantly associated ($P=0.017$) (Table 4). However, the femoral neck BMD as well as the total hip BMD was not significant, even after adjusting for covariates ($P=0.053$ and $P=0.588$, respectively) (Table 4).

Discussion

In the present study, we analyzed the association of two polymorphisms of the *COL1A1* gene (rs1800012 and rs1107946) and their haplotypes as well as the rs2273061 polymorphism of the *JAG1* gene with the presence of BMD variations in postmenopausal Mexican-Mestizo women. We found that the rs1800012 polymorphism and one haplotype were associated with BMD variations in lumbar spine.

One of the most extensively studied polymorphisms in *COL1A1* is the Sp1 binding site. There have been several association studies and meta-analyses of this SNP and its relation to BMD variations and vertebral fractures; furthermore, functional studies have shown that the *T* allele of this polymorphism is associated with effects on DNA binding, gene transcription, protein production, and defects in bone mineralization (Grant et al. 1996; Efstathiadou et al. 2001; Mann et al. 2001; Wynne et al. 2002; Mann and Ralston 2003; Stewart et al. 2005; Ralston et al. 2006; Bustamante et al. 2007; Yazdanpanah et al. 2007; Jin et al. 2009, 2011).

The majority of the studies found an association of the Sp1 polymorphism with BMD of the lumbar spine and femoral neck; however, we found only an association of this SNP with BMD at the lumbar spine in a dominant model. It is unclear why we did not find differences in the BMD of the femoral neck as compared with the lumbar spine. This may be due to the low

Table 1 General characteristics of postmenopausal Mexican-Mestizo women

Variable	Mean (SD)
Age (years)	60.0 (7.55)
Height (cm)	1.51 (0.59)
Weight (kg)	67.43 (11.7)
BMI (kg/m ²)	29.25 (4.8)
BMD lumbar spine (g/cm ²)	0.849 (0.139)
BMD total hip (g/cm ²)	0.914 (0.136)
BMD femoral neck (g/cm ²)	0.742 (0.123)
Years since menopause	11.7 (8.3)

BMI body mass index, BMD body mineral density, SD standard deviation

Table 2 Hardy–Weinberg equilibrium, genotype, and allele frequencies for the two SNPs of *COL1A1* and *JAG1* in postmenopausal Mexican-Mestizo women

SNP dbSNP	<i>COL1A1</i>			<i>COL1A1</i>			<i>JAG1</i>		
Reference number	rs1800012			rs1107946			rs2273061		
	Genotype	<i>n</i>	%	Genotype	<i>n</i>	%	Genotype	<i>n</i>	%
Genotype frequencies	<i>GG</i>	635	84.7	<i>GG</i>	380	50.7	<i>AA</i>	261	34.8
	<i>GT</i>	106	14.1	<i>GT</i>	298	39.7	<i>AG</i>	360	48
	<i>TT</i>	9	1.2	<i>TT</i>	72	9.6	<i>GG</i>	129	17.2
Allele frequency	<i>G</i>	0.92		<i>G</i>	0.71		<i>A</i>	0.59	
	<i>T</i>	0.08		<i>T</i>	0.29		<i>G</i>	0.41	
HWE	<i>P</i> >0.05			<i>P</i> >0.05			<i>P</i> >0.05		

HWE Hardy–Weinberg equilibrium

percentage of the *TT* genotype, which was not sufficient to show an impact in this bone region. Moreover, the low number of *TT* homozygotes (Table 2) could limit the power to rigorously assess the association between this relatively rare genotype and other modes of inheritance (i.e., recessive).

Ralston et al. (2006) investigated the association of *COL1A1* Sp1 alleles and BMD in a multicenter collaborative study from various European countries, finding that the *TT* genotype was associated with BMD both at the lumbar spine and femoral neck with

a recessive pattern of inheritance. The average of the percentage reported by these authors for genotype *TT* in the Caucasian population was ~4% vs. 1.2% in our study. Despite the low percentage of the *TT* genotype observed in our population, BMD at the lumbar spine was significantly lower than the carriers of the *GG* genotype. Noteworthy, the effect size for *TT* was 59 mg/cm², whereas for *GT* it was 31 mg/cm². This provides evidence of gene-dose effects as previously described (Uitterlinden et al. 1998). Furthermore, these differences remained even after adjustment for covariates

Table 3 Bone mineral density in postmenopausal Mexican-Mestizo women in different genotype subgroups of the *COL1A1* and *JAG1* genes

dbSNP	Genotype	LS (g/cm ²)		TH (g/cm ²)		FN (g/cm ²)	
		Mean	SD	Mean	SD	Mean	SD
rs1800012 of <i>COL1A1</i>	G/G	0.854	0.141	0.915	0.137	0.745	0.126
	G/T T/T	0.823	0.126	0.908	0.13	0.725	0.101
		<i>P</i> =0.021		<i>P</i> =0.697		<i>P</i> =0.106	
rs1107946 of <i>COL1A1</i>	G/G	0.856	0.136	0.917	0.135	0.745	0.126
	G/T	0.841	0.143	0.91	0.134	0.727	0.053
	T/T	0.848	0.139	0.908	0.147	0.724	0.104
		<i>P</i> =0.489		<i>P</i> =0.683		<i>P</i> =0.305	
rs2273061 of <i>JAG1</i>	A/A	0.840	0.14	0.910	0.128	0.748	0.119
	A/G	0.853	0.144	0.919	0.144	0.742	0.129
	G/G	0.861	0.125	0.908	0.127	0.729	0.112
		<i>P</i> =0.174		<i>P</i> =0.419		<i>P</i> =0.332	

Values are mean and standard deviation (SD) for bone mineral densities (g/cm²). *P* values <0.05 were considered statistically significant

LS lumbar spine, TH total hip, FN femoral neck

Table 4 Bone mineral density in postmenopausal Mexican-Mestizo women in different haplotypes of the *COL1A1* gene

Haplotype	LS (g/cm ²)			TH (g/cm ²)		FN (g/cm ²)	
	Frequency (%)	Mean	SD	Mean	SD	Mean	SD
G–G	936 (62.4)	0.856	0.140	0.918	0.136	0.748	0.127
G–T	441 (29.4)	0.844	0.142	0.911	0.139	0.736	0.123
T–G	123 (8.2)	0.822	0.125	0.907	0.128	0.724	0.099
		<i>P</i> =0.017		<i>P</i> =0.588		<i>P</i> =0.053	

Frequency of haplotypes with their percentages is shown. Values are mean and standard deviation (SD) for bone mineral densities (g/cm²). *P* values shown are after adjustment for covariates

LS lumbar spine, TH total hip, FN femoral neck

shown in our population: BMI, years since menopause, and total duration of breastfeeding (Rojano-Mejía et al. 2011).

Garcia-Giralt et al. (2002) analyzed, for the first time, polymorphism rs1107946 (−1997 G/T) located in the promoter region of *COL1A1* in postmenopausal Spanish women, finding that this polymorphism was associated with BMD of the lumbar spine and femoral neck as well as modifying the transcriptional activity of the gene. These results were confirmed in other ethnic groups (i.e., Chinese, Japanese, and Caucasian women from the USA and from the UK) (Liu et al. 2004; Yamada et al. 2005; Zhang et al. 2005; Stewart et al. 2005). In regard to our results with this SNP, frequency of the *T* allele was higher in comparison to that reported by Garcia-Giralt et al. (2002) (29% vs. 13%, respectively). Despite this, we found no significant differences in BMD. Nevertheless, our results are in accordance with those described in a population-based study of unrelated Chinese women that showed no association between −1997 G/T alleles and BMD (Lau et al. 2005).

We analyzed the haplotypes of the rs1800012 and rs1107946 polymorphisms of *COL1A1*, and found that the G–G haplotype was associated with a higher BMD in the lumbar spine. The effect size for this haplotype was 34 mg/cm². This is important because this is the most common haplotype in our study population (62.4%). In addition, we observed that this haplotype shows a trend toward a higher BMD of the femoral neck (24 mg/cm²), although this was not significant (*P*=0.053).

When we analyzed the linkage disequilibrium (LD) among the two SNPs of *COL1A1* in our population, we observed that these were in high LD (*D'*=0.84),

different from the values described by Liu et al. (2004) for the Caucasian population (*D'*=0.918). This difference may be due to the fact that the Mestizo-Mexican population is a relatively young group. In this regard, it has been suggested that the population-specific haplotypes may be particularly relevant when a common disease is explained by a common variant where the age of the causative mutation allows for different recombination histories in different populations compounded by different migration histories (Weiss and Clark 2002; Neale and Sham 2004).

Liu et al. (2004) reported the association among the same two polymorphisms and haplotypes of *COL1A1* and BMD in elderly Caucasian women. In accordance with our results, the authors found a significant association only of the haplotype G–G with BMD. Carriers of the G–G haplotype had, on average, a 2.7% higher BMD than non-carriers. Noteworthy, in our population, frequency of the same haplotype was higher compared with Caucasian women (3.94%).

It has been proposed that haplotypes consisting of polymorphisms that may or may not be functional could provide a better tool than single marker analysis in genome-wide association studies (GWAS). This is attributable to the ancestral established haplotype structure, which may define functional units of genes or the haplotype may modify the structure or interaction of the protein as well as its function (Akey et al. 2001; Clark 2004; Schaid 2004).

Moreover, Kung et al. (2010) performed GWAS of BMD variations and identified that the rs2273061 of *JAG1* was associated with BMD. This association remains significant in the second-step replication studies. The *in silico* replication analysis in three independent cohorts of European descent consistently showed

that the minor allele *G* of the rs2273061 polymorphism of this gene was associated with the presence of high BMD, and this association was also replicated in the Hong Kong Osteoporosis Study prospective cohort. Additionally, these authors documented a significant relationship between allele *G* of rs2273061 polymorphism with higher *JAG1* expression. On this basis, they propose this gene as a candidate in the regulation of BMD. Regarding the data obtained in our study population, we did not find any association of this SNP with BMD variations. Similar data were reported by Kung et al. (2010) in the Northern Chinese population. Both results may be due to the low power of the studies. On the other hand, differences in allelic frequencies were reported by Kung et al. (2010) in different ethnic groups. The findings observed in the study by Kung et al. (2010) may be due to genetic background because the Mexican-Mestizo population is comprised of a mixture of Europeans and Africans with native indigenous individuals (Lisker et al. 1990).

This study also has limitations. One limitation is the lack of ancestry confirmation studies; nevertheless, because Mexico City has been a site of massive immigration during the last century receiving inhabitants from throughout the country, this group can be considered representative of the overall Mexican population. In this study, we analyzed only women native to México whose ancestors throughout three generations were born in Mexico. This is according to the National Institute of Anthropology, which defines the Mexican Mestizo as a person who (1) was born in Mexico, (2) has a Spanish-derived last name, and (3) has a family history of Mexican ancestors dating back at least three generations (Gorodezky et al. 2001). Another limitation was that we did not have the power to detect the effect of an association of the rs1107946 polymorphism of the *COL1A1* and rs2273061 polymorphism of the *JAG1* in BMD variations.

Despite these limitations, the strengths of the study are the recruitment of consecutive patients and the statistical power of the study according to the frequency of the rs1800012 polymorphism as well as the haplotypes (>80%, $P<0.05$). Moreover, this association remained after adjusting for covariates.

In conclusion, low BMD is a major public health problem due to the high risk of fracture, decrease in the quality of life of affected individuals, and high healthcare costs. Because low BMD is an opportunity for intervention to reduce the risk of fracture, one of

the major challenges in the management of this condition is to identify women at high risk of bone loss and fragility fractures. Identification of risk factors associated with low BMD includes genetic factors; thus, our results confirm that the rs1800012 polymorphism and the haplotype with rs1107946 polymorphism of *COL1A1* were significantly associated with low BMD at the lumbar spine. For that reason, it is important to carry out association studies in different populations worldwide in order to determine whether genetic markers associated with BMD variations are the same or different, depending on the studied populations.

Acknowledgments This work was supported by a grant from the Instituto de Ciencia y Tecnología del Distrito Federal (#PICDS08-34, México). D.R. was supported by Consejo Nacional de Ciencia y Tecnología (CONACYT) and by a fellowship award from the Coordinación de Investigación en Salud, Instituto Mexicano del Seguro Social. G.L. was supported by a fellowship award from the Instituto de Ciencia y Tecnología del Distrito Federal. We thank M. Salas-Rojas (Endocrinology Service, Centro Médico Nacional 20 de Noviembre, Instituto de Seguridad y Servicios Sociales de los Trabajadores del Estado) for technical assistance. S. Morey, Executive Editor, Scientific Communications, assisted in the English review of the manuscript.

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