Association of polymorphisms of the *TNFRSF11B* and *TNFSF11* genes with bone mineral density in postmenopausal women from western Mexico

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Osteoporosis (OP) is a disease with reduced bone mass and deterioration of the spatial distribution of the trabecular bone structure, leading in consequence to increased bone fragility and risk of fractures [1]. In Mexico its frequency in postmenopausal women is 8.3% to 31% [2]. OP is a multifactorial disease because its development involves physiological, environmental and genetic factors [3]. Genes of the tumor necrosis factor (TNF) family like *TNFRSF11B* (TNF receptor superfamily member 11b) and *TNFSF11* (TNF superfamily member 11) encode for osteoprotegerin (OPG) and receptor activator for nuclear factor κB ligand (RANKL) proteins, respectively, that participate in the signaling pathway OPG/RANK/RANKL, balancing the activity between osteoblasts and osteoclasts to prevent bone loss and to ensure normal bone turnover [4, 5]. Different single nucleotide polymorphisms (SNPs) in genes of this pathway are related to bone mineral density (BMD) or OP [6, 7]. In Mexican postmenopausal women, SNPs of the *TNFRSF11B* gene were studied but no association with BMD [8, 9] or OP [10] was observed; SNPs of the *TNFSF11* gene have not been studied for OP.

The aim of this study was to investigate the association of the polymorphisms 1181 G>C (rs2073618) and 1217 C>T (rs3102734) of *TNFRSF11B*; as well as -708 T>A, -693 C>G (rs9533155), -657 C>A, -643 C>T (rs9533156) and -290 C>T (rs9525641) of *TNFSF11* with BMD.

Five hundred and thirteen postmenopausal Mexican-Mestizo women from western Mexico agreed to participate in the study. They were recruited from Hospital General Regional No. 110 of the Instituto Mexicano del Seguro Social in Guadalajara city. BMD of the lumbar spinal L1-L4 region and of the femoral neck (left and right) was determined by means of DEXA (dual-energy X-ray absorptiometry, Prodigy Advance, GE). A hundred and seventy-four women were matched by age (range was between 42 to 69 years) and body mass index (BMI) (range from 19.7 to 42.1 kg/m^2) in two groups by the WHO criteria: women with values of the T-score less than –2.5 SD (OP group, $n = 87$) and those with values of the T-score above -1 SD (non-OP group, $n = 87$). A questionnaire that included differ-

ent biological (age, BMI, age at onset of menarche and menopause, family history of OP, personal and family history of fractures) and lifestyle (smoking, alcoholism, coffee consumption and sedentarism) variables was applied to all participants. This study was approved by the local ethical committee; informed consent was obtained from all women.

Identification of polymorphisms was performed by PCR followed by Sanger DNA sequencing with the BigDye terminator kit v3.1 (Applied Biosystems Foster City). To identify the rs2073618 and rs3102734 variants, a fragment of 274 bp was amplified using the primers forward 5´-GATCAAAGG-CAGGCGATACTTCC-3´ and reverse 5´-CTGGGAG-GGAGCGAGTGGAGC-3´. The PCR mixture (25 μl) contained: genomic DNA (200 ng), 10 pmol each primer, *Taq* polymerase 1 U, $MgCl_2$ 2.0 mM, dNTPs 250 μmol and buffer 1X. The thermocycling conditions were: initial denaturation 95°C/5 min, denaturation 95°C/1 min, annealing 57°C/1 min, extension 72°C/1:30 min, 35 cycles and a final extension 72°C/5 min. The -708 T>A, rs9533155, -657 C>A, rs9533156 and rs9525641 polymorphisms were identified according to Mencej *et al.* [11].

For categorical variables, the frequencies or percentages were obtained; for continuous variables, the average and standard deviation were calculated. Chi square and Fisher exact tests were used for comparison of genotypic and allelic frequencies of each polymorphism between both groups. The risk for OP was estimated through odds ratio, considering classic models of inheritance. Haplotypes were established inferring phase of family segregation. Linkage disequilibrium (LD) was significant when $r^2 \geq 0.33$, using Arlequin v3.01 software. ANOVA and Student *t*-tests were used to relate quantitative variables with genotypes and alleles, respectively. Other tests used were Spearman and Pearson correlations and linear regression models. Statistical analyses were carried out using SPSS version 20.0 and $p < 0.05$ was significant.

As expected, BMD values in the group of postmenopausal women with OP (0.834 ±0.052 g/cm² in lumbar spine L1–L4; 0.788 ±0.086 g/cm² in left femoral neck; 0.787 ±0.085 g/cm² in right femoral neck) were lower than in the non-OP group (1.173 ±0.109 g/cm² in lumbar spine L1-L4; 1.005 ±0.087 g/cm² in left femoral neck; 1.003 ±0.083 g/cm² in right femoral neck) ($p < 0.001$). The other biological and lifestyle variables did not show significant differences between the groups, even when they have been reported as high risk for the development of OP in some populations [12, 13].

The distribution of genotypic and allelic frequencies of the seven SNPs was similar between the groups ($p > 0.05$). The SNPs −708 T>A and −657 C>A were observed with low frequencies

Table I. Genotypic and allelic frequencies of the seven polymorphisms studied of the *TNFRSF11B* and *TNFSF11* genes

Variable	TNFRSF11B gene								TNFSF11 gene					
	1181 G>C		1217 C>T		−708 T>A		−693 C>G		−657 C>A		−643 C>T		−290 C>T	
	OP %	Non-OP %	OP %	Non-OP %	OP %	Non-OP %	OP %	Non-OP %	OP %	Non-OP %	OP %	Non-OP %	OP %	Non-OP %
Genotypic frequencies:														
WW	24.2	24.1	86.2	80.5	69.0	78.2	42.5	36.8	77.0	85.1	16.1	13.8	17.3	14.9
WM	42.5	43.7	13.8	19.5	31.0	21.8	43.7	50.6	23.0	14.9	39.1	48.3	40.2	48.3
MM	33.3	32.2	–	–	–	–	13.8	12.6	–	–	44.8	37.9	42.5	36.8
P-value	0.98		0.31		0.39		0.66		0.40		0.472		0.566	
Allelic frequencies:														
W	45.4	46.0	93.1	90.2	84.5	89.1	64.4	62.1	88.5	92.5	35.6	37.9	37.4	39.1
M	54.6	54.0	6.9	9.8	15.5	10.9	35.6	37.9	11.5	7.5	64.4	62.1	62.6	60.9
P-value	0.91		0.33		0.21		0.66		0.20		0.66		0.74	

OP – osteoporosis, WW – wild homozygote genotype, WM – heterozygote genotype, MM – mutated homozygote genotype, W – wild allele, M – mutated allele. N = 87 OP group, n = 87 non-OP group.

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in both groups, and the −708A and −657A alleles were not detected in homozygote state in the study (Table I). The most widely studied polymorphism of *TNFRSF11B* is 1181 G>C; the distribution of allelic frequencies is variable in women with OP from several populations [14–16]. Under classical models of inheritance (dominant, recessive, codominant or additive) neither genotypes nor alleles were associated with risk for OP ($p > 0.05$). Regarding *TNFSF11*, in the Egyptian population, the −290T allele was associated with increased risk of developing OP (OR = 1.7, IC: 1.1–2.7, $p = 0.019$) [17].

Haplotypes for *TNFRSF11B* were constructed with two polymorphisms (1181 G>C and 1217 C>T) and three haplotypes were observed; the most frequent was CC (OP 54.6% and non-OP 54.0%). In *TNFSF11*, the haplotypes were constructed with five SNPs: −708 T>A, −693 C>G, −657 C>A, −643 C>T and −290 C>T, and 13 haplotypes were observed, the most frequent in both groups being TCCTT (OP = 49.4% and non-OP = 54.1%). The distribution of haplotype frequencies was compared between the two groups and no statistically significant differences were found ($p > 0.05$), with the exception of ACATT haplotype (OP group = 9.8%, non-OP group = 4.0%; $p = 0.03$). The same combinations of SNPs for the two genes are not reported in the literature.

The SNPs of *TNFRSF11B* were in linkage equilibrium in the two groups (OP: $r^2 = 0.089$; non-OP: $r^2 = 0.127$). For the five sites of *TNFSF11*, we found that four pairs of loci are in LD in both groups, since r^2 values ranged from 0.6587 to 0.9058 with $p < 0.001$: 1) −708 T>A/-657 C>A, 2) −693 C>G/ −643 C>T, 3) −693 C>G/-290 C>T and 4) −643 C>T/ −290 C>T. In women with OP and non-OP from Slovenia, LD was also observed between pairs 2, 3 and 4 [18, 19].

The relationship between genotypes and alleles of the studied polymorphisms with the biological variables in each group was analyzed; only the BMD and BMI presented statistically significant results with some SNPs (Table II). For BMD, no association was found with 1181 G>C and 1217 C>T SNPs of *TNFRSF11B*; similar results were observed in postmenopausal women from Australia [20], Ireland [14, 21], and Malta [22]; however, the 1181GG genotype was associated with lower BMD in Chinese [7, 16], Slovenian [15, 23], and Koreans [24]; in China, the 1217T allele was related to increased risk of osteoporotic fracture (OR = 1.35; 95% CI: 1.17–1.55; Bonferroni $p = 2.6 \times 10^{-4}$) [25]. Regarding BMI, in the present study, the 1181GG genotype was significantly associated with lower BMI in women with OP (Table II). Although this relationship has not been reported, we can suggest that this polymorphism could have an indirect effect on the variation of the BMD, as was demonstrated by Mendez *et al.* [26], in which a high BMI is positively correlated with higher BMD. It has been proposed that obesity protects against bone loss through mechanical load, and it has been suggested that the adipose tissue is involved in the homeostasis of the skeleton, possibly through the role of some adipokines in bone remodeling. Also, adipocytes secrete estrogens that help to regulate homeostasis and contribute to the increase of bone mass [26].

On the other hand, the −693CC, −643TT and −290CT genotypes of *TNFSF11* reveal lower BMD in the left and/or right femoral neck than the other genotypes in postmenopausal women with OP, which demonstrates the important role

Table II. Association between genotypes and alleles of the studied polymorphisms with biological variables

Gene	Polymorphism	Variable	Genotype/allele	Genotype/allele	*P*-value	Group
TNFRSF11B	1181 G>C	BMI [kg/cm²]	GG 26.6	CC 28.7	0.05	OP
				GC 29.3	0.02	
TNFSF11	−693 C>G	BMD LFN [g/cm²]	GG 0.840	CC 0.781	0.05	OP
				CG 0.781	0.03	
		BMD RFN [g/cm²]	GG 0.846	CC 0.774	0.02	OP
			G 0.806	C 0.776	0.03	OP
	−643 C>T	BMI [kg/cm²]	TT 28.0	CT 30.2	0.03	Non-OP
		BMD LFN [g/cm²]	CC 0.833	TT 0.779	0.05	OP
				CT 0.782	0.04	
		BMD RFN [g/cm²]	CC 0.830	TT 0.774	0.04	OP
			C 0.804	T 0.777	0.04	OP
	−290 C>T	BMI [kg/cm²]	TT 27.7	CT 30.4	0.01	Non-OP
		BMD RFN [g/cm²]	CC 0.828	CT 0.778	0.03	OP

BMI – body mass index, OP – osteoporosis, BMD – bone mineral density, LFN – left femoral neck, RFN – right femoral neck. n = 87 OP group, n = 87 non-OP group.

of these SNPs in the variation of BMD (Table II). The linear regression analysis with all studied polymorphisms and BMD of the lumbar spine, and the right and left femoral necks, showed only a significant model between the −693G allele and BMD of the right femoral neck (constant = 0.738, β = 0.028, *p* = 0.032), indicating that there is a positive relationship since the BMD is increased slightly. These three polymorphisms in Slovenian women with OP were associated with BMD of the lumbar spine and −693 C>G also with BMD of the femoral neck [18]. In the Chinese population −693 C>G or −643 C>T polymorphism was not related to BMD in peri- and postmenopausal women [7]. The −643TT and −290TT genotypes of *TNFSF11* in the non-OP group were related to lower BMI, which could suggest a possible influence on the BMD variation as explained above for 1181 G>C [26].

In conclusion, the 1181 G>C and 1217 C>T polymorphisms of *TNFRSF11B* and −708 T>A, −693 C>G, −657 C>A, −643 C>T and −290 C>T of *TNFSF11* were not associated with OP. However, −693CC, −643TT and −290CT genotypes have an effect of lowering BMD in the left and/or right femoral neck. The 1181GG genotype was significantly associated with lower BMI in women with OP as well as −643TT and −290TT genotypes of *TNFSF11* in the non-OP group. The −708 T>A and −657 C>A polymorphisms have not been described in the literature.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

R e f e r e n c e s

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