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Association of a single nucleotide polymorphism of *RANK* gene with blood pressure in Spanish women

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Abstract

In addition to governing key functions in bone metabolism and the immune system, the RANK/RANKL/OPG system plays a role in the vascular system, particularly in vascular calcification and atherosclerosis.

Given that these 2 phenotypes are considered a major cause of high blood pressure (BP), in this study we analyzed the association of SNPs in *RANK* and *OPG* genes with blood pressure. An observational study was conducted of 2 SNPs in the *RANK* gene (rs884205 and rs78326403) and 1 in the *OPG* gene (rs4876869) with systolic (SBP) and diastolic blood pressure (DBP) in a cohort of 695 women.

Data analysis revealed a statistically significant association between the SNP rs884205 and BP pressure (SBP and DBP). Analyzing this relationship by the dominant inheritance model for this SNP (allele risk: A), women of the AA/AC genotype showed higher BP than women of the CC genotype, both for SBP ($P = .001$) and for DBP ($P = .003$), and these associations both surpassed the Bonferroni threshold for multiple comparisons. Multivariate regression analysis including known predictors of BP as independent variables was performed to evaluate the strength of this association, which in the case of the SNP rs884205 of the *RANK* gene remained statistically significant after adjustment for both SBP ($P = .0006$) and DBP ($P = .005$), demonstrating the key role of this SNP in BP.

We report a robust association between the SNP rs884205 in *RANK* gene and BP in women, and this SNP is validated as a candidate in cardiovascular risk studies.

Abbreviations: ALP = alkaline phosphatase, BMI = body mass index, BP = blood pressure, CI = confidence interval, DBP = diastolic blood pressure, HDL = high-density lipoprotein cholesterol, HOMA-IR = insulin resistance index (homeostasis model assessment), HWE = Hardy-Weinberg equilibrium, LDL = low-density lipoprotein cholesterol, MAF = minor allele frequency, mm Hg = millimeters of mercury, OPG = osteoprotegerin, RANK = receptor activator of nuclear factor-kappa B, RANKL = receptor activator of nuclear factor-kappa B ligand, SBP = systolic blood pressure, SD = standard deviation, SNP = single nucleotide polymorphism, UTR = untranslated region.

Keywords: association study, blood pressure, polymorphism, *RANK* and *osteoprotegerin* genes

1. Introduction

Hypertension represents a major global public health problem, owing both to its high prevalence, estimated at more than 1.6 billion people by 2025,[1] and its position as a leading risk factor for cardiovascular diseases such as heart failure, cardiac hypertrophy, ischemic stroke, and intracerebral hemorrhage.[2] In 2016, non-communicable diseases were responsible for 71% (40.5 million people) of all deaths worldwide, of which 44% (17.9 million) were due to cardiovascular disease, with hypertension among the main risk factors.[3]

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The data that support the findings of this study are available from a third party, but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are available from the authors upon reasonable request and with permission of the third party.

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With a multifactorial pathology, hypertension is governed by both genetic and environmental and lifestyle factors. Among the latter, modifiable and non-modifiable risk factors such as aging, sex, obesity, lack of exercise, smoking and alcohol, and salt consumption are crucial.[4] Regarding genetic factors, several studies have shown between 30% to 60% heritability for systolic blood pressure (SBP) and diastolic blood pressure (DBP),[5,6] and many have attempted to uncover loci associated with this phenotype.[7,8] However, far from identifying all the genes involved, as yet only a small proportion of total phenotypic variance of blood pressure or hypertension has been explained.[6]

The end of the 20th century saw the discovery of a series of cytokines belonging to the tumor necrosis factor superfamily, which are critical for the immune system and for differentiation and activation of osteoclast, the cell that resorbs bone. These are the receptor activator of nuclear factor-kappa B ligand (RANKL, codified by *TNFSF11* gene), its receptor RANK (*TNFRSF11A* gene) and the decoy receptor osteoprotegerin (OPG, *TNFRSF11B* gene).[9–12] In bone, RANKL expressed in stromal/osteoblastic cells, binding to RANK expressed in osteoclast precursors, initiates osteoclastogenesis and osteoclast activation. In bone, OPG is produced by stromal/osteoblastic cells and works as a "decoy receptor" for RANKL, competing with RANK for binding RANKL, and is therefore a potent inhibitor of osteoclastogenesis.[9,11,12]

Aside from its function in bone and the immune system, the RANKL/RANK /OPG system is also involved in the vascular system. Indeed, almost immediately after its discovery, OPG-deficient mice were shown to suffer from arterial calcification in addition to severe osteoporosis.[13] Since then, substantial evidence has accumulated regarding the role of these molecules in the vascular system.[14,15] The RANK/RANKL/OPG system in general, and particularly OPG, has been associated with several phenotypes related to the vascular system, such as metabolic syndrome and cardiovascular risk in osteoporotic patients,[16] serum cholesterol levels,[17] atherosclerosis,[18] vascular calcification,[19] angiogenesis,[20] hypertension,[21] and heart failure.[22]

Against this background, the present study aims to investigate if polymorphisms in genes such as *RANK* and *OPG* are associated with blood pressure in a female population, given that these molecules are linked to vascular calcification and atherosclerosis and the phenotypes are considered a major cause of high blood pressure and hypertension.[23]

2. Material and methods

2.1. Study subjects

The present study is an observational study that included Spanish female residents of the city of Valencia located in eastern Spain who regularly participate in studies related to womens health at the Hospital Clínico Universitario de Valencia.[24–26] The women were recruited consecutively from among patients receiving regular health services at the Menopause Unit for our hospital, which was the only inclusion criterion for the study. A total of 748 women gave informed consent to be included in this study.

The exclusion criteria were coronary heart disease, cardiomyopathies, secondary hypertension due to kidney or endocrine diseases,[27] active oncological process, severe disability, or severe psychiatric disorders. After applying exclusion criteria, 695 women were finally included in the study.

During their hospital visit, the women completed a cardiovascular risk questionnaire which collected data such as age, weight, height, waist and hip circumference, parity, smoking, and use of antihypertensive or antidiabetic drugs. In the present study diabetes was defined as fasting blood glucose >126 mg/dl or receiving anti-diabetic treatment.[28]

The Medicaments Research Ethics Committee (CEIm) of the Hospital Clínico Universitario of Valencia approved the study in accordance with the principles of the Declaration of Helsinki, and written informed consent was obtained from participants in accordance with the standards of INCLIVA Health Research Institute guidelines.

2.2. Anthropometric and biochemical measurement

The women attended hospital between 8:00 and 10:00 AM after overnight fast. A blood sample was obtained for biochemical determinations and DNA extraction. On the same visit anthropometric characteristics were recorded and blood pressure was taken after sitting for at least 5 minutes.

Blood pressure was measured with an Omron M6 (HEM-7001-E) oscillometric blood pressure monitor (Omron Healthcare, Kyoto, Japan). Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2).

The serum was separated and freshly used for most biochemical determinations, and kept frozen at −80°C for tests not performed on the same day. Insulin levels were measured with electrochemiluminescence immunoassay (ECLIA, Elecsys Insulin kit, Roche Cobas, Mannheim, Germany) in a Modular Analytics E170 analyzer. Levels of glucose, total calcium and phosphate, total alkaline phosphatase (ALP), triglycerides (TG), total cholesterol (TC), and high-density lipoprotein cholesterol (HDL) were measured using a spectrophotometer (Olympus 5400, Olympus, Melville, NY, USA).

Low-density lipoprotein cholesterol (LDL; mg/dl)) was calculated as TC − (HDL+TG/5),[29] and the $HOMA_{IR}$ (homeostasis model assessment) insulin resistance index was estimated as fasting serum insulin in μIU/ml × (fasting serum glucose in mg/dl × 0.05551)/22.5.[30]

2.3. Single nucleotide polymorphism (SNP) selection and genotyping

We selected SNPs following the criteria of minor allele frequency (MAF) over 0.05 in the HapMap-CEU population and according to their possible function, using the PubMed and dbSNP (National Center for Biotechnology Information) and GTEx-Portal (https://gtexportal.org/home/) databases.

For the *OPG* gene (*TNFRSF11B*) SNP rs4876869 was selected, as it has been reported as a functional variant.[31] For the *RANK* gene (*TNFRSF11A*), we selected SNPs rs884205 and rs78326403 for their previous association with bone phenotypes and specifically the SNP rs884205 for its link to increased risk of cerebral infarction.[32]

We performed DNA extraction from peripheral blood, rs4876869 and rs884205 SNP genotyping by allelic discrimination using TaqMan SNP Genotyping Assays (Applied Biosystems) and quality control as previously described.[33] The SNP rs78326403 of *RANK* gene was analyzed using iPlex technology on a Mass-Array platform (Sequenom, Hamburg, Germany) at the Central Medical Research Unit (Faculty of Medicine, Valencia, Spain).

Table 1

Analysis of the association between genotypes and blood pressure uncorrected for covariates. Values are the mean ± SD.

Gene SNP (rs)	Genotype	N	SBP (mm Hg)	DBP (mm Hg)
OPG rs4876869	AA	242	138.3 ± 21.9	83.6 ± 12.2
	AG	340	134.1 ± 20.2	82.1 ± 11.1
	GG	100	134.9 ± 20.4	82.2 ± 10.7
	P-value		.057	.287
RANK rs884205	AA	36	144.4 ± 24.2	84.1 ± 12.0
	AC	224	138.4 ± 21.4	84.4 ± 11.7
	CC	427	133.8 ± 20.1	81.7 ± 11.1
	P-value		.0013	.012
RANK rs78326403	AA	543	135.9 ± 20.1	83.0 ± 11.2
	AT	98	134.0 ± 21.9	82.0 ± 11.6
	TT	2	117.5 ± 37.5	77.5 ± 14.8
	P-value		.326	.594

DBP = diastolic blood pressure, mm Hg = millimeters of mercury, SBP = systolic blood pressure, SD = standard deviation, SNP = single nucleotide polymorphism.

2.4. Statistical analysis

The SNPStats web tool (www.snpstats.net/start.htm) was used to estimate allelic and genotypic frequencies, to ascertain if these frequencies met the Hardy-Weinberg equilibrium (HWE), to determine which inheritance mode (co-dominant, dominant, overdominant, and recessive) best fit our frequencies, and to study association of haplotypes of the *RANK* gene with blood pressure.

Fixed effect analysis of variance (ANOVA) was used to undercover correlation between genotypes and blood pressure (Table 1) and to compare anthropometric and biochemical data of the study cohort by inheritance pattern of SNPs.

Multiple linear regression models with stepwise variable selection were performed to detect linear relationships between dependent variables (SBP or DBP), adjusted for the independent blood pressure variables listed in Table 2. The variables were introduced as quantitative independent variables (age, BMI, etc.), or as dummy variables, which were codified as 0 or 1: smoking (0 nonsmoker, and 1 smoker), diabetes (0 non-diabetic, and 1 diabetic) and SNP rs884205 of *RANK* gene (0 genotype CC, and 1 genotype AA/AC, using the inheritance model). To avoid multicollinearity we removed highly correlated predictors from the model, excluding all independent variables whose variance inflation factor (VIF) was ≥ 5 (LDL, $HOMA\text{-}_{IR}$, waist and hip circumference and weight). After this initial selection, all independent variables that were analyzed in the regression study showed VIF values <2, indicating low/moderate collinearity.[34]

In the present study, a sample size of 578 was considered sufficient to attain a statistical power of 90%, estimated to detect a gene effect of $R^2 = 0.018$ (by regression analysis between SBP and SNP rs884205 of *RANK* gene), according to mean ± SD for SBP 136.0 ± 21.0 mm Hg for the cohort of women and a frequency for the A allele of 0.22 (Table 3) under a dominant model of inheritance. We used the QUANTO software package (https://preventivemedicine.usc.edu/) for the analysis.

Missing data were entirely attributable to random and unpredictable factors in sample collection and analysis, and were thus disregarded in the statistical analysis. Values shown are means ± SD, percentages or frequencies. Statistical significance was defined as $P < .05$, except for multiple comparisons, where the cut-off value for the Bonferroni correction was estimated as $P < .0083$ (0.05/6; 2 outcomes, SBP and DBP, and 3 comparisons or SNPs). Data was analyzed using IBM SPSS statistics for Windows (version 26.0; Armonk, NY: IBM Corp.).

Table 2

Participants anthropometric and biochemical characteristics (mean ± SD or percentage).

	Number	Values
Age (years)	695	56.2 ± 7.8
Weight (kg)	694	66.3 ± 11.1
Height (cm	693	157.6 ± 6.2
BMI (kg/m^2)	693	26.7 ± 4.3
Waist circumference (cm)	640	85.7 ± 10.6
Hip circumference (cm)	630	102.3 ± 8.8
Waist-to-hip ratio	630	0.84 ± 0.07
Parity	691	1.9 ± 1.2
Systolic blood pressure (mm Hg)	695	136.0 ± 21.0
Diastolic blood pressure (mm Hg)	695	82.8 ± 11.4
Diabetes (%)	670	13.9
Smoking (%)	679	25.2
Total cholesterol (mg/dl)	677	221.4 ± 33.5
HDL-Cholesterol (mg/dl)	677	66.4 ± 13.7
LDL- Cholesterol (mg/dl)	677	134.5 ± 30.0
Triglycerides (mg/dl)	677	104.0 ± 49.5
Total-ALP (U/L)	622	173.8 ± 54.0
Calcium (mg/dl)	655	9.7 ± 0.4
Phosphate (mg/dl)	671	3.6 ± 0.5
Glucose (mg/dl)	671	101.7 ± 22.0
Insulin (μU/ml)	620	8.6 ± 6.7
$HOMA_{IR}$ index	614	2.3 ± 2.4

ALP = alkaline phosphatase, BMI = body mass index, HDL = high density lipoprotein, $HOMA_{IR}$ = insulin resistance index (homeostasis model assessment), LDL = low density lipoprotein, SD = standard deviation.

3. Results

Table 3 shows the data and genomic location of the studied SNPs. Our population does not show stratification, as previously published.[26] The allele frequencies are similar to those described in the HapMap database for Europeans and the genotypic frequencies met the Hardy-Weinberg equilibrium (HWE).

Table 2 shows the anthropometric and biochemical characteristics of the studied population, all potential predictors of blood pressure.

Table 1 shows the relationship between the genotypes of each SNP and blood pressure (SBP and DBP). The SNP rs4876869 of the *OPG* gene was tentatively associated with SBP ($P = .057$),

Table 3

Single nucleotide polymorphisms (SNPs) studied.

SNP	Chromosome	Gene	Position*	Location	Major allele	Minor allele	MAF	*p*-HWE
rs4876869	8	*TNFRSF11B*	118929038	Intron Variant	A	G	0.396	0.27
rs78326403	18	*TNFRSF11A*	62387207	3'-UTR Variant	A	T	0.079	0.42
rs884205	18	*TNFRSF11A*	62387624	3'-UTR Variant	C	A	0.215	0.37

* Genomic coordinates according to Genome Reference Consortium Human Build 38 patch release 12 (GRCh38.p12).
HWE = Hardy-Weinberg equilibrium, MAF = minor allele frequency, UTR = Untranslated region.

which was confirmed analyzing this SNP using the inheritance model (AA vs AG/GG). However, although the difference between genotypes for SBP was statistically significant (nominal $P = .018$), it did not meet the cut-off imposed by the Bonferroni test for multiple comparisons ($P = .008$).

Regarding the SNPs of the *RANK* gene, the SNP rs78326403 was not associated with blood pressure. In contrast, SNP rs884205 was correlated with both SBP ($P = .0013$) and DBP (0.012), although only association with SBP met the Bonferroni threshold ($P = .008$). When the association of the SNP rs884205 was studied using the best inheritance model (dominant for both SBP and DBP; risk allele A), AA/AC genotype women showed higher blood pressure than CC genotype women, both for SBP (AA/AC:139.2 ± 21.9 mm Hg vs CC: 133.8 ± 20.1 mm Hg; $P = .0011$) and for DBP (AA/AC: 84.4 ± 11.7 mm Hg vs CC: 81.7 ± 11.1 mm Hg; $P = .0029$). In this case, both associations surpassed the Bonferroni threshold.

Since the 2 SNPs of the *RANK* gene are 417 nt apart, we next performed a haplotype analysis (Table 4). According to the results obtained using the SNPStats software, the values of the linkage disequilibrium parameters between both SNPs were D' = 1 and $R^2 = 0.024$, consistent with the LDLINK web tool results (ldlink.nci.nih.gov) for the Iberian population in Spain, indicating that both SNPs are in linkage equilibrium. Despite this, the A-A haplotype representing 22% of the chromosomes in our population (Table 4) showed a strong association with both SBP and DBP.

When the Table 2 variables were compared between women of AA/AC and CC genotypes for the SNP rs884205 of the *RANK* gene, in addition to the differences already shown regarding blood pressure, we detected differences in HDL level (AA/AC = 64.9 ± 12.5 mg/dl vs CC = 67.3 ± 14.4 mg/dl; $P = .033$), and waist-to-hip ratio (AA/AC = 0.84 ± 0.07 vs CC = 0.83 ± 0.07; $P = .028$), whereby women with lower HDL levels and a higher waist-to-hip ratio show higher blood pressure.

To test the strength of association between the SNP rs884205 of the *RANK* gene and blood pressure, we next performed a multivariate stepwise linear regression analysis, using variables in Table 2 with VIF <5 as independent variables (Table 5). Age, BMI, diabetes, ALP, and the SNP rs884205 (genotype AA/AC) remained positively associated with SBP. The negative value of the smoking variable is artifactual, since the group of smokers (codified as 1) have lower age and blood pressure ($P < .001$) than the non-smoker group (codified as 0). Regarding DBP, we found that BMI, ALP, SNP rs884205, diabetes, parity, and insulin remained positively associated, whereas height was negatively associated. Interestingly, the SNP rs884205 of the *RANK* gene was significantly associated with blood pressure in both phenotypes, even after Bonferroni correction.

4. Discussion

The present study demonstrates that the SNP rs884205 of the *RANK* gene (*TNFRSF11A*) was robustly associated with both SBP and DBP in a population of Spanish women. Furthermore, this association surpassed the threshold imposed by the Bonferroni correction for multiple comparisons. On the other hand, although the SNP rs4876869 of the *OPG* gene (*TNFRSF11B*) was tentatively associated with SBP, it failed to pass the Bonferroni correction.

To our knowledge, this is the first time that a statistically significant association has been found between SNPs in the *RANK* gene and blood pressure. Several mainly intronic SNPs of the RANK/RANKL/OPG system were associated with blood pressure in Chinese women in a previous study,[21] in which correlations with SBP and DBP were detected in SNPs of the *RANK*, but not the *RANKL* and *OPG* genes. However, these had low nominal statistical significance ($P < .05$), so did not pass the Bonferroni correction. The results were nonetheless along the lines of our findings, in that only SNPs in the *RANK* gene were associated with blood pressure, despite being different SNPs to the ones in our study (Table 3).[21]

In the present study, no statistically significant association between the SNP of the *OPG* gene and blood pressure was detected, despite positive findings in previous studies, at least in men.[35] Furthermore, SNPs in both the *OPG* and *RANKL* genes have previously been linked to arterial calcification and atherosclerosis,[36,37] highlighting the importance of this cytokine

Table 4

Haplotypes of the *RANK* gene and association with SBP and DBP.

Haplotype	rs78326403	rs884205	Frequency	SBP Difference* (95% CI)	SBP *P* value	DBP Difference* (95% CI)	DBP *P* value
1	A	C	0.70	0.0	–	0.0	–
2	A	A	0.22	4.76 (2.11–7.42)	.0005	1.93 (0.47–3.39)	.0097
3	T	C	0.08	−0.98 (−5.46–3.5)	.67	−0.5 (−2.91–1.92)	.69
4	T	A	–	–	–	–	–

* Difference in blood pressure (in mm Hg) between the given haplotype and the reference haplotype (haplotype 1).
CI = confidence interval, DBP = diastolic blood pressure, mm Hg = millimeters of mercury, SBP = systolic blood pressure.

Table 5

SBP and DBP predictors determined by stepwise multiple linear regression analysis.

Dependent variable	Independent variables	Unstandardized coefficients		t	P value	Adjusted R^2
		B	SE			
SBP	Intercept	48.746	8.246	5.912	<.00001	0.318
	Age	0.847	0.114	7.404	<.00001	
	BMI	1.003	0.191	5.253	<.00001	
	Diabetes	10.518	2.404	4.375	.00002	
	RANK rs884205	5.717	1.658	3.448	.0006	
	ALP	0.054	0.015	3.648	.0003	
	Smoking	−5.100	1.965	−2.595	.010	
DBP	Intercept	86.645	13.748	6.302	<.00001	0.199
	BMI	0.618	0.130	4.748	<.00001	
	ALP	0.023	0.009	2.672	.008	
	RANK rs884205	2.719	0.972	2.798	.005	
	Diabetes	2.900	1.405	2.064	.040	
	Parity	0.890	0.409	2.176	.030	
	Insulin	0.261	0.107	2.430	.015	
	Height	−0.190	0.079	−2.397	.017	

ALP = total alkaline phosphatase, BMI = body mass index, DBP = diastolic blood pressure, SBP = systolic blood pressure.

system in phenotypes that cause hypertension and cardiovascular disease.

No data were found regarding the potential function of the SNP rs884205. This SNP does not seem to be an expression quantitative trait loci (eQTL) of the *RANK* gene, at least according to data obtained from GTExPortal (https://gtexportal.org/), but does appear to be an eQTL of the *PIGN* gene (Phosphatidylinositol Glycan Anchor Biosynthesis Class N), in which allele A is associated with higher expression of this gene (GTExPortal). The PIGN gene encodes a protein involved in the biosynthesis of glycosylphosphatidylinositol (GPI)-anchor glycolipid, which is found on many blood cells and serves to anchor proteins to the cell surface (GeneCards). Loss of function mutations in the *PIGN* gene cause multiple congenital anomalies-hypotonia-seizures syndrome 1 (OMIM: # 614080), a disease that affects neurodevelopment, but does not cause blood pressure-related problems. Therefore, if the SNP rs884205 is a functional variant, its effect on blood pressure does not appear mediated by the *PIGN* gene.

As noted above, the SNP rs884205 of the *RANK* gene has not previously been associated with blood pressure or hypertension, but has been linked to BMD,[38] and in a more recent study to cerebral infarction in a Japanese population.[32] In the present study, the A allele (risk allele) was associated with higher blood pressure, and was also associated with lower bone mineral density in the meta-analysis published by Rivadeneira et al.[38] This is to be expected, since low bone mass has been associated with calcification and atherosclerosis[39] and with high blood pressure.[40] However, in Yasukochi et al[32] the A allele is proposed as protective against cerebral infarction, although the subgroup with cerebral infarction showed higher blood pressure values than controls. The reasons for this possible discrepancy are unknown, and in any case, the limited data available precludes insight into whether the SNP rs884205 of the *RANK* gene is a functional polymorphism or is in linkage disequilibrium with any functional variant in its genomic region.

Our study has certain limitations that should be taken into account when interpreting the data. Firstly, as a study carried out on women, the results cannot be extrapolated to men; additionally, no data was retrieved about dietary habits or physical activity, and finally, the sample size is medium, although with sufficient statistical power to detect the described effects.

To conclude, we report an association between a SNP in *RANK* gene and blood pressure, underlining the important role of the RANK/RANKL/OPG system in this phenotype. Given the strength of this association and the fact that it has resisted adjustment for known predictors of blood pressure in linear regression analysis, this SNP is validated as a candidate in cardiovascular risk studies.

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