Osteoprotegerin CGA Haplotype Protection against Cerebrovascular Complications in Anti-CCP Negative Patients with Rheumatoid Arthritis

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Abstract

Introduction: Rheumatoid arthritis is an inflammatory disease with high incidence of cardiovascular disease due to accelerated atherosclerosis. Osteoprotegerin (OPG) has been associated with increased risk of atherosclerotic disease in the general population. Several polymorphisms in the *OPG* gene with functional effects on cardiovascular disease in non-rheumatic individuals have been described. Therefore, we aimed to analyze the effect of three of these functional *OPG* polymorphisms on the risk of cardiovascular disease in a large and well-characterized cohort of Spanish patients with rheumatoid arthritis.

Methods: Three *OPG* gene variants (rs3134063, rs2073618 and rs3134069) were genotyped by TaqMan assays in 2027 Spanish patients with rheumatoid arthritis. Anti-cyclic citrullinated peptide (anti-CCP) antibody testing was positive in 997 of 1714 tested. Also, 18.3% of the whole series had experienced cardiovascular events, including 5.4% with cerebrovascular accidents. The relationship between *OPG* variants and cardiovascular events was assessed using Cox regression.

Results: No association between *OPG* gene variants and cardiovascular disease was observed in the whole group of rheumatoid arthritis patients or in anti-CCP positive patients. Nevertheless, a protective effect of CGA haplotype on the risk of cardiovascular disease in general, and specifically in the risk of cerebrovascular complications after adjusting for sex, age at disease diagnosis and traditional cardiovascular risk factors was disclosed in anti-CCP negative patients (HR = 0.54; 95%CI: 0.31–0.95; p = 0.032 and HR = 0.17; 95%CI: 0.04–0.78; p = 0.022, respectively).

Conclusion: Our results indicate a protective effect of the *OPG* CGA haplotype on cardiovascular risk, mainly due to a protective effect against cerebrovascular events in anti-CCP negative rheumatoid arthritis patients.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory rheumatic disease associated with high incidence of cardiovascular (CV) morbidity and mortality compared to the general population [1–4], similarly to what occurs in type 2 diabetes [5,6]. Specifically, it has been shown a high incidence of coronary heart disease and a high rate of CV events in RA [7,8] due to accelerated atherosclerosis [9]. Besides traditional CV risk factors [4,10] and the magnitude and severity of the chronic inflammatory response [8], genetic factors located inside [8] and outside the Human Leukocyte Antigen (HLA) region [11,12] play a pivotal role in the development of atherogenesis in RA [13–15].

Osteoprotegerin (OPG) belongs to the TNF receptor super-family and is implicated in bone remodeling and in the atherosclerotic process. This molecule acts as a decoy receptor for the receptor activator of nuclear factor-κB ligand (RANKL), inhibiting binding of RANKL to its receptor, RANK [16,17]. Binding of RANKL to OPG inhibits osteoclastogenesis, although it is also well known that both molecules are involved in vascular wall mineralization [16]. Additionally, OPG acts as a soluble neutralizing receptor of TNF-related apoptosis-inducing ligand (TRAIL), an anti-inflammatory molecule with anti-atherosclerotic properties [18–20]. Despite having a paradoxically protective effect on vascular calcification [21,22], OPG has been associated with increased risk of atherosclerotic disease in the general population [23].

The human OPG gene (also called TNFRSF11B) is located on chromosome 8q24. This gene is affected by genetic polymorphisms with functional consequences on CV disease and bone metabolism [24,25]. Recently, several groups have reported that a single-nucleotide OPG polymorphism (SNP) located in the 5′ UTR region (rs2073617), as well as one in exon 1 (rs2073618) and another in the promoter region (rs3134069) were associated with atherosclerosis and risk of cerebrovascular disease in non-rheumatic individuals [26,27].

Considering the functional involvement of the above mentioned OPG polymorphisms in the CV disease, in the present study we aimed to analyze the potential association of these gene variations on the risk of developing CV disease in a large and well-characterized cohort of patients with RA, also evaluating their combined effect on this risk.

Materials and Methods

Patients and Study Protocol

A set of 2027 Spanish patients with RA were included in the present study. Blood samples were obtained from patients recruited from Hospital Lucus Augusti (Lugo), Hospital Marqués de Valdecilla (Santander), Hospital de Bellvitge (Barcelona), and Hospital Clínico San Carlos, Hospital La Paz, Hospital La Princesa, Hospital Gregorio Marañón and Hospital 12 de Octubre (Madrid). A subject's written consent was obtained according to the declaration of Helsinki, and the study was approved by the Ethics Committee of Galicia for Hospital Lucus Augusti, of Cantabria for Hospital Universitario Marqués de Valdecilla, of Cataluña for Hospital de Bellvitge and of Madrid for Hospital Clínico San Carlos, Hospital La Paz, Hospital La Princesa, Hospital Gregorio Marañón and Hospital 12 de Octubre. All the patients fulfilled the 1987 American College of Rheumatology (ACR) and also the 2010 classification criteria for RA [28,29]. In all the cases, the samples were assessed for OPG rs2073618 and rs3134069 polymorphisms. Additionally, rs2073617 polymorphism was assessed with a pre-designed Taqman probe for the
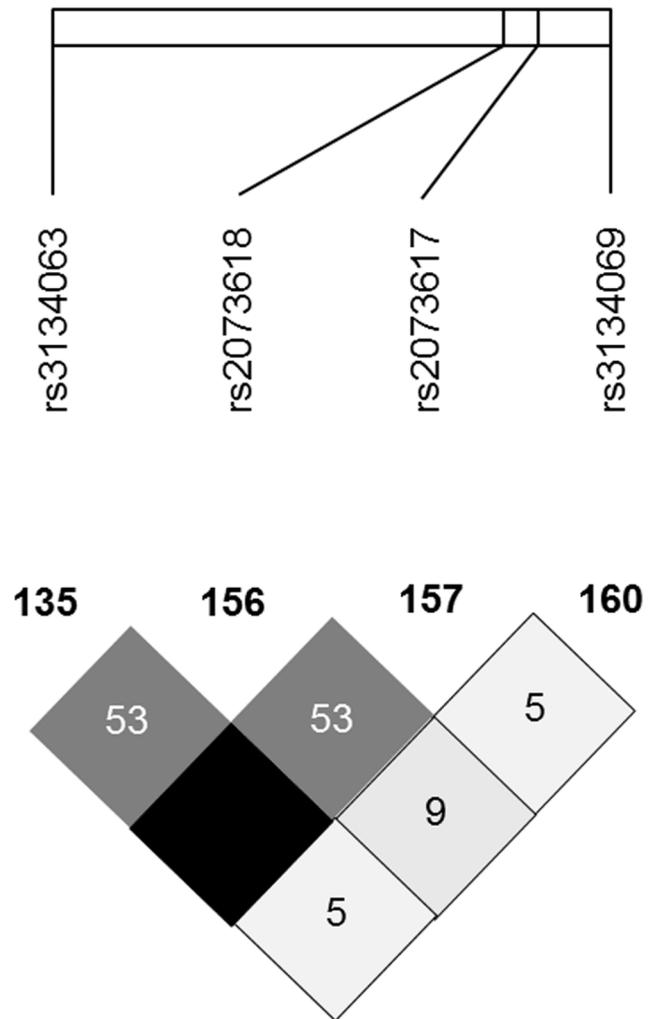
Figure 1. Linkage disequilibrium (LD) pattern of the OPG polymorphisms analyzed in our study (in European population). Data obtained by HapMap Project phase I, II and III and HAPLOVIEW (v.4.2) software. The LD between the OPG polymorphisms studied is shown in a scale from minimum (white) to maximum (black).
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rs3134063 polymorphism, which is in complete linkage disequilibrium with rs2073617 ($r^2 = 1$, http://hapmap.ncbi.nlm.nih.gov/). The linkage disequilibrium (LD) pattern of the OPG polymorphisms analyzed in our study obtained by HapMap Project phase I, II and III (in the European population) and HAPLOVIEW (v.4.2) software is displayed in **Figure 1**.

Information on the main demographic data, clinical characteristics, CV risk factors and CV events of patients enrolled in the study is shown in **Table 1**. Anti-cyclic citrullinated peptide (anti-CCP) antibody testing were positive in 997 (58.2%) of 1714 RA patients in whom this result was available. Three hundred and seventy (18.3%) of these 2027 patients had experienced CV events. One-hundred and nine (5.4%) of the 2027 patients had suffered cerebrovascular accidents. Definitions of CV events and traditional CV risk factors were established as previously described [8,30].

Genotyping

DNA from patients was obtained from peripheral blood using standard methods. The OPG rs3134063, rs2073618 and

Table 1. Demographic and clinical characteristics of the Spanish patients with RA included in the study.

Clinical Feature		% (n/N)
Patients		2027
Main characteristics	Age at the time of disease onset (years, mean ± SD)	51.2±14.9
	Follow-up (years, mean ± SD)	11.7±8.4
	Female gender	75.3 (1527/2027)
	Rheumatoid factor positive*	68.6 (1344/1958)
	Anti-CCP antibodies positive	58.2 (997/1714)
	Shared epitope positive	63.7 (726/1139)
Cardiovascular risk factors	Hypertension	38.2 (763/1996)
	Diabetes mellitus	12.3 (246/1995)
	Dyslipidemia	35.9 (713/1984)
	Obesity	18.8 (345/1840)
	Smoking habit	24.9 (491/1974)
Patients with cardiovascular events		18.3 (370/2027)
	Ischemic heart disease	8.9 (180/2020)
	Heart failure	5.6 (113/2027)
	Cerebrovascular accident	5.4 (109/2027)
	Peripheral arteriopathy	2.6 (52/2025)

CCP: cyclic citrullinated protein/peptide antibodies; RA: rheumatoid arthritis; SD: standard deviation.
*At least two determinations at different times were required.
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rs3134069 polymorphisms were genotyped with TaqMan SNP pre-designed genotyping assays (C__32324439_10, C___1971047_1_, and C__27464534_10, respectively) in a 7900 HT real-time polymerase chain reaction (PCR) system, according to the conditions recommended by the manufacturer (Applied Biosystem, Foster City, CA, USA). Negative controls and duplicate samples were included to check the accuracy of genotyping.

Statistical analysis

The genotype data were checked for deviation from Hardy-Weinberg equilibrium (HWE) using http://ihg.gsf.de/cgi-bin/hw/hwa1.pl.

The relationship between alleles, genotypes and haplotypes, and CV events that occurred in the follow-up was tested using Cox regression adjusting for sex, age at RA diagnosis and traditional CV risk factors. For that purpose, we used the most frequent allele, genotype and haplotype as reference; the end of follow-up was the first date among the end of the study, date of death or date of CV event. Follow-up time was estimated as the difference between the

Table 2. Results of haplotype analysis in anti-CCP negative RA patients in association with CV events.

Variable		HR (95% CI)	p
	Age at RA diagnosis (by each year)	**1.08 (1.06–1.10)**	**<0.001**
	Hypertension	0.98 (0.63–1.51)	0.929
	Diabetes mellitus	**1.58 (1.01–2.45)**	**0.043**
	Obesity	1.22 (0.78–1.89)	0.390
	Dyslipidemia	1.27 (0.84–1.93)	0.254
	Smoking	1.12 (0.85–1.48)	0.417
Haplotypes (rs3134063, rs2073618, rs3134069)		*HR (95% CI)**	*p**
	TCA	1 (reference)	-
	TGA	0.94 (0.55–1.60)	0.825
	CCA	1.09 (0.68–1.76)	0.724
	CGA	**0.54 (0.31–0.95)**	**0.032**

CCP: cyclic citrullinated protein/peptide antibodies; CI: confidence interval; CV: cardiovascular; HR: hazard ratios; RA: rheumatoid arthritis.
*Adjusted for sex, age at RA diagnosis and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit).
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Table 3. Association between *OPG* polymorphisms and the risk to develop cerebrovascular events in anti-CCP negative RA patients.

SNP	Genotype/Allele	HR (95%CI)	p*
rs3134063	TT	1 (reference)	-
	CT	0.61 (0.21–1.76)	0.362
	CC	0.14 (0.02–1.23)	0.077
	T	1 (reference)	-
	C	0.52 (0.26–1.06)	0.072
rs2073618	CC	1 (reference)	-
	CG	0.60 (0.20–1.82)	0.368
	GG	**0.17 (0.03–0.89)**	**0.035**
	C	1 (reference)	-
	G	**0.45 (0.22–0.91)**	**0.025**
rs3134069	AA	1 (reference)	-
	AC	1.20 (0.26–5.64)	0.814
	CC	-	-
	A	1 (reference)	-
	C	1.18 (0.27–5.09)	0.823

CCP: cyclic citrullinated protein/peptide antibodies; CI: confidence interval; HR: hazard ratios; RA: rheumatoid arthritis.
*Adjusted for sex, age at RA diagnosis and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit).
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RA diagnosis date and the end of follow-up. Patients without CV events in the follow-up time or dying by any non-CV cause were considered as censored. Results were expressed as hazard ratios (HR) with 95% confidence interval (CI). Statistical significance was defined as p≤0.05, and all analyses were performed using STATA statistical software 12/SE (Stata Corp., College Station, TX, USA).

Results

OPG rs3134063, rs2073618 and rs3134069 genotype distribution was in Hardy-Weinberg equilibrium (p>0.05). Genotyping success was greater than 96% in all the cases. Genotype and allele frequencies of the *OPG* rs3134063, rs2073618 and rs3134069 polymorphisms were in agreement with the data of the HapMap project (http://hapmap.ncbi.nlm.nih.gov/).

There was no association between *OPG* gene variants and CV disease when all the RA patients were assessed as a whole. It was also the case for the group of anti-CCP positive patients (data not shown). Nevertheless, an association between *OPG* gene polymorphisms and CV disease was observed in those who were anti-CCP negative (**Table 2**). In this regard, even if no association was observed between allelic and genotypic *OPG* variants and CV disease (not shown), in the haplotype analysis, a protective effect of

Table 4. Results of haplotype analysis in anti-CCP negative RA patients in association with cerebrovascular events.

Variable		HR (95% CI)	p
	Age at RA diagnosis (by each year)	**1.09 (1.05–1.13)**	**<0.001**
	Hypertension	1.13 (0.51–2.48)	0.769
	Diabetes mellitus	**3.71 (1.78–7.70)**	**<0.001**
	Obesity	1.28 (0.58–2.83)	0.547
	Dyslipidemia	0.80 (0.39–1.66)	0.556
	Smoking	1.13 (0.68–1.88)	0.632
Haplotypes (rs3134063, rs2073618, rs3134069)		HR (95% CI)*	p*
	TCA	1 (reference)	-
	TGA	1.38 (0.56–3.42)	0.487
	CCA	1.16 (0.49–2.76)	0.740
	CGA	**0.17 (0.04–0.78)**	**0.022**

CCP: cyclic citrullinated protein/peptide antibodies; CI: confidence interval; CV: cardiovascular; HR: hazard ratios; RA: rheumatoid arthritis.
*Adjusted for sex, age at RA diagnosis and traditional CV risk factors (hypertension, diabetes mellitus, dyslipidemia, obesity, and smoking habit).
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the CGA haplotype on the risk of CV disease after adjusting for sex, age at RA diagnosis and traditional CV risk factors was disclosed in the group of anti-CCP negative RA patients (HR = 0.54; 95% CI: 0.31–0.95; p = 0.032) (**Table 2**).

In a further step, we aimed to determine the type of CV event associated with *OPG* gene variants in the group of anti-CCP negative patients. Whereas no association was observed with ischemic heart disease (data not shown), we found an association of cerebrovascular complications with rs2073618 *OPG* polymorphism in this group of patients. In this respect, the risk of cerebrovascular complications was statistically decreased in the group of anti-CCP negative patients who carried the *OPG* rs2073618 GG genotype after adjusting the results for potential confounder factors (HR = 0.17; 95% CI: 0.03–0.89; p = 0.035) (**Table 3**). In accordance with these results, a protective effect of the CGA haplotype on the risk of cerebrovascular events after adjusting for sex, age at RA diagnosis and traditional CV risk factors was observed in the anti-CCP negative RA patients (HR = 0.17; 95% CI: 0.04–0.78; p = 0.022) (**Table 4**).

Discussion

RA is associated with increased morbidity and mortality attributable to accelerated atherosclerosis and CV events [7,9], in a similar fashion to what is observed in other autoimmune diseases such as diabetes [6]. Therefore, the search of new genetic markers which could help physicians to stratify RA patients according to their CV risk, leading thus to an improved and more personalized treatment, has become a main goal for several groups of research.

OPG has been proposed as a potential biomarker of CV risk since increased levels of this protein have been associated with CV disease [23]. In this regard, OPG levels were associated with biomarkers of endothelial activation (intercellular adhesion molecule-1), carotid intima-media wall thickness and carotid plaques in RA patients with severe disease [31]. In keeping with these results, an independent correlation of OPG levels with asymmetric dimethylarginine (ADMA), another biomarker of endothelial cell activation, has been disclosed in ankylosing spondylitis patients undergoing anti-TNF-α therapy [32]. Increasing concentrations of OPG have also been associated with the severity of CV complications in diabetic patients [33–37], reinforcing the idea that OPG could be used as a prognostic factor of CV disease in the clinic.

Several groups have described sequence variations in the gene that codifies OPG [24–26]. As elegantly proposed by Soufi et al., variations in the different regions of the *OPG* gene could combine to affect its transcription, intracellular trafficking or secretion [24]. To the best of our knowledge, our study constitutes the first attempt to assess the potential effect of rs3134063, rs2073618 and rs3134069 on the risk to develop CV disease in a large and well-characterized cohort of RA patients.

When we studied the influence of the different *OPG* polymorphisms on the risk of CV disease, we found a protective effect of the CGA haplotype on the risk of CV events in the subgroup of RA patients who were anti-CCP negative. Interestingly, further analyses disclosed that this protective effect was specifically focused

on the risk of developing cerebrovascular accidents. In this regard, anti-CCP negative RA patients who carried an *OPG* rs20730618 GG genotype had a lower risk of developing cerebrovascular complications. Additionally, when we combined the different genetic variants to create haplotypes, our results also revealed a protective effect of the CGA haplotype (that carries the G allele of the *OPG* rs2073618) against the risk of cerebrovascular events in the subgroup of anti-CCP negative RA patients. These results are in line with those obtained by Biscetti et al., who found a synergistic effect of rs3134069, rs2073617 and rs2073618 associated with cerebral ischemic events in a cohort of diabetic patients [27]. Likewise, our results are in accordance with those obtained by Mankoč Ramuš et al., who also found an association between a combination of two of the *OPG* gene variants studied by our group and diabetic retinopathy [38].

Interestingly, the protective effect of the *OPG* CGA haplotype was found in anti-CCP negative but not anti-CCP positive RA patients. This could be explained by the fact that anti-CCP positive and anti-CCP negative RA are considered different disease entities [39]. Several pieces of evidence disclose that anti-CCP antibodies are markers of severe disease, and that risk factors such as HLA class II alleles associate with anti-CCP status and a more severe disease course [40]. In this regard, anti-CCP positive RA patients display a more severe radiological destruction and elevated DAS28 and CRP values than anti-CCP negative patients [39,41].

Taken all these considerations together, our results and those previously mentioned support the idea that, in an attempt to establish the potential association between multiple genetic markers in a chromosomal region and traits of interest in complex diseases, haplotype analyses appear to provide more useful information than the separate assessment of individual gene variants [27,38,42–45]. Hence, combination analyses often help to disclose hidden signals and may tag other regional polymorphic sites.

Conclusion

Our results indicate a protective effect of the *OPG* CGA haplotype on CV risk, mainly due to a protective effect against cerebrovascular events in anti-CCP negative RA patients.

Author Contributions

Conceived and designed the experiments: FG RLM MGB JM MAGG. Performed the experiments: FG RLM MGB BU. Analyzed the data: FG RLM MGB JL. Contributed reagents/materials/analysis tools: FG RLM MGB SC CGJ JL AC BU JAMF TP CGV LRR BFG AB DPS FJLL PC RB IGA JM MAGG. Contributed to the writing of the manuscript: FG RLM MGB JM MAGG. Involved in the acquisition and interpretation of data and in revising it critically for important intellectual content: SC BFG.

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