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Association between *RANK, RANKL* and *OPG* polymorphisms with ACPA and erosions in rheumatoid arthritis: results from a meta-analysis involving three French cohorts

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ABSTRACT

Objectives: The RANK/RANKL/osteoprotegerin (OPG) system plays a central role in the pathogenesis of bone erosions in rheumatoid arthritis (RA). The aim of this study was to test the association between 11 single-nucleotide polymorphisms (SNPs) located on *RANK, RANKL* and *OPG* genes and anticitrullinated peptide antibody (ACPA) presence or erosions in RA.

Methods: Patients: This work was performed on three independent samples of French patients with RA: the Etude de Suivi des PolyArthrites Indifférenciées Récentes (ESPOIR) (n=632), Rangueil Midi-Pyrénées (RMP) (n=249) and French Rheumatoid Arthritis Genetic Consortium (FRAGC) (n=590) cohorts. Genotyping: the genotyping of 11 SNPs located on *RANK*, *RANKL* and *OPG* were performed by PCR. Statistical analyses: The association between the genotypes with ACPA or erosions was first tested in the ESPOIR cohort using a χ^2 test and, in the case of significant association, replicated in the RMP and FRACG cohorts. A meta-analysis on the three cohorts was performed using the Mantel-Haenszel method.

Results: One SNP on *RANK* (rs8086340) and three SNPs on *RANKL* (rs7984870, rs7325635, rs1054016) were significantly associated with ACPA presence, while one SNP on *OPG* (rs2073618) and one SNP on *RANKL* (rs7325635) were significantly associated with erosions in the ESPOIR cohort. Following meta-analysis performed on the three samples, the SNP on *RANK* and the GGG haplotype of the three SNPs located on *RANKL* were both significantly associated with ACPA presence, while only the SNP on *OPG* remained significantly associated with erosions.

Conclusions: This study identified one SNP located on *RANK*, one haplotype on *RANKL* associated with ACPA presence, and one SNP located on *OPG* associated with erosions in three different samples of French patients with RA.

Key messages

What is already known about this subject?

- Some single nucleotide polymorphisms located on *RANK*, *RANKL* or *OPG* genes are associated with rheumatoid arthritis (RA) susceptibility.

What does this study add?

- In this study, we showed for the first time an association between one locus on *OPG* and bone erosions in three RA cohorts.
- We also identified a haplotype on *RANKL* and a locus on *RANK* associated with anticitrullinated peptide antibody presence.

How might this impact on clinical practice?

- These loci may be implicated in gene expression or protein function, explaining differences in RA phenotypes.

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune disorders, characterised by peripheral synovial joint inflammation, which ultimately leads to joint destruction and increases mortality.[1] RA is characterised by the presence of anticitrullinated peptide antibodies (ACPA) and erosions. However, ACPA presence and titre vary significantly among patients, as does structural damage, consequences of the interaction between individual and environmental factors. Among individual factors, genetic factors might explain about 50–60% of the risk of developing RA,[2] and also the risk of ACPA production and erosion development. Over the past few years, more than 100 RA genetic risk factors have been identified.[3] However, most of the studies identified

associations between genetic markers and ACPA-positive RA, suggesting a different genetic background that could explain the difference between outcomes involving ACPA-positive or ACPA-negative RA.[4 5]

The balance between osteoblast and osteoclast activity is disturbed in systemic or local conditions that affect the skeleton, such as osteoporosis or RA.[6] The activity of these cells is mediated by the receptor activator of nuclear factor κ B (RANK)/receptor activator of nuclear factor κ B ligand (RANKL)/osteoprotegerin (OPG) system. Since the genes encoding these proteins are highly implicated in erosion pathogenesis, numerous studies have examined the potential implications of certain single nucleotide polymorphisms (SNPs) located on these genes and RA risk or presence of erosions.[7–11] However, most of the associations were studied in Asian populations. Furthermore, some works suggested a RANK/RANKL pathway role in immunity since RANK and RANKL play a role in T-cell activation and dendritic cell survival.[12] Recent studies suggested that RANKL regulates the microenvironment of the thymus by activating the expression of autoimmune regulators (Aire).[13] Their role in autoimmune disease is still debated.

In the present study, we aimed to assess the association between 11 SNPs located on *RANK, RANKL* and *OPG;* and ACPA presence or erosions in 3 cohorts of French patients with RA.

METHODS

Study population

Three data sets of French patients with RA were included in this study: the Etude de Suivi des PolyArthrites Indifférenciées Récentes (ESPOIR) cohort (n=632), the Rangueil Midi-Pyrénées (RMP) cohort (n=249) and the French Rheumatoid Arthritis Genetic Consortium (FRAGC) sample (n=590). RA was defined according to the 2010 American College of Rheumatology European League Against Rheumatism (ACR/EULAR) criteria for RA[14] in the ESPOIR cohort and according to the 1987 ACR criteria[15] for the RMP and FRAGC cohorts. These cohorts have already been described elsewhere.[4 16 17] All participants provided written consent by signing an informed consent form as approved by the recruiting site review board at each of the affiliate institutions.

All patients underwent an ELISA test for anticyclic citrullinated peptide antibodies-2. Radiographs were centrally scored for the ESPOIR and RMP cohorts using the van der Heijde modified total Sharp score[18] and the details of the scoring have been described elsewhere.[17] Erosion was defined by a van der Heijde modified erosion Sharp score of 1 or more. In the FRAGC cohort, radiographs were locally read and patients were classified as having erosion or not according to the local investigator.

SNP selection and genotyping

After a literature search of studies involving an association between SNPs on RANK, RANKL or OPG and RA susceptibility or erosions, 11 SNPs located on *RANK* (rs8086340, rs1805034 and rs35211496), *RANKL* (rs2277438, rs1054016, rs7325635 and rs7984870) and *OPG* (rs2073618, rs2073617, rs10955911 and rs1485305) were genotyped in the ESPOIR cohort. The ESPOIR cohort was the discovery cohort; if it was significantly associated with ACPA or erosions with a $p<0.05$, then it was replicated in the RMP and FRAGC cohorts. All the SNPs were genotyped using allele-specific kinetic PCR analysis by LGC genomics (Herts, UK) using the KASPar method in the three cohorts.

Statistical analysis

Tests for deviation from the Hardy-Weinberg equilibrium were performed using a standard χ^2 test (1 d.f.).

Associations between the genotypes and ACPA presence or erosions were assessed by a χ^2 test and the Cochrane-Armitage test for trends in the ESPOIR cohort, in a first exploratory phase without correction for multiple tests. Significant SNPs with a p value of <0.05 from this first analysis were then replicated in the RMP and FRAGC cohorts. The meta-analysis of all three data sets was performed using a fixed-effects model with the Mantel-Haenszel method. Testing multiple loci in one data set leads to inflation of the p value, for which we applied the most conservative method, the Bonferroni method, to reduce the chance of false-positive findings as much as possible. Since the first phase was used for identification, this correction was applied to the number of variants tested in the meta-analysis.

When several SNPs located on the same gene were significantly associated with ACPA or erosion, haplotypes were built and analysed. Haplotypes were assigned to each individual using PLINK 1.08 requiring a probability of >0.8. Analyses of the haplotypes were performed with methods similar to those used to analyse the individual SNPs, by then testing for the presence or absence of a haplotype.

The epistatic interactions between the SNPs of the different genes were tested using three methods: the RERI method, a logistic regression with an interaction parameter and PLINK epistasis software.[19] Interactions with the *HLA-DRB1*Shared Epitope* allele carriage were also tested using the same methods.

RESULTS

In the ESPOIR cohort, all the SNPs had a minor allele frequency >10% and the genotype frequencies fit the Hardy-Weinberg Equilibrium expectations ($p>0.05$). The success rate for the genotyping was 94–98%.

The patient characteristics are summarised in table 1 below.

Association between the SNPs located on *RANK, RANKL* and *OPG* and ACPA or erosions in the ESPOIR cohort: first exploratory phase

Of the 11 SNPs tested, 1 SNP located on *RANK* (rs8086340) and 3 SNPs located on *RANKL* (rs7984870,

Table 1 Patient characteristics from all three data sets

	ESPOIR n=632	RMP n=249	FRAGC n=590
Age at disease onset, years, med (IQR)	50 (39–57)	50 (39–61)	45 (34–55)
Female (%)	490 (78)	188 (75)	448 (76)
Disease duration, year, med (IQR)	0.4 (0.2–0.6)	0.6 (0.3–0.9)	9.0 (4.7–18.0)
ACPA, number (%)*	308 (49)	177 (73)	311 (61)
Patients with a ESS≥1, number (%)†	219 (37)	92 (39)	366 (67)

*Number of missing data: ESPOIR: n=0, RMP: n=8, FRAGC: n=76.
†Number of missing radiographs: ESPOIR: n=33, RMP: n=12, FRAGC: n=47.
ACPA, anticitrullinated peptide antibodies; ESPOIR, Etude de Suivi des PolyArthrites Indifférenciées Récentes; ESS, Erosion Sharp Score; FRAGC, French Rheumatoid Arthritis Genetic Consortium; Med, median; RMP, Rangueil Midi-Pyrénées.

rs7325635, rs1054016) were significantly associated with a p<0.05 with ACPA in the ESPOIR cohort and were selected for the replication and meta-analysis phase (table 2).

Only one SNP located on *OPG* (rs2073618) was significantly associated with erosions with a p<0.05 and thus selected for the replication and meta-analysis phase. Furthermore, an SNP located on *RANKL* (rs7325635) had a trend for significant association with erosions with a p=0.06 on the χ^2 test and a p value of 0.05 on the Cochran-Armitage test. Thus, it was also added to the meta-analysis.

Association between the SNPs located on *RANK, RANKL* and *OPG* and ACPA or erosions in the three cohorts: results of the meta-analysis

The results of the first explanatory genotype analysis conducted us to analyse the effects of the SNPs located on *RANK, RANKL* and *OPG* on ACPA presence and erosions in a meta-analysis with an allele dominant effect model.

The SNPs located on *RANKL* were in linkage disequilibrium (r^2 comprising 0.83–0.94) and thus haplotypes were built using PLINK software. The association of the protective haplotype GGG was assessed in comparison to non-*RANKL*-GGG haplotype carriers. rs8086340 on *RANK* and the GGG haplotype of *RANKL* were tested for association with ACPA and rs2073618 on OPG and rs7325635 on *RANKL* were tested for association with erosions. A Bonferroni correction was applied to the results of the meta-analysis and the threshold of the p value was set at 0.013. The results of the meta-analysis are summarised in table 3.

The SNP on *RANK* and the GGG haplotype on *RANKL* were both significantly associated with ACPA presence after meta-analysis. Furthermore, the SNP on *OPG* was associated with erosions, while the SNP on *RANKL* was not associated, after meta-analysis.

The association between the SNP on OPG was tested for erosions after stratification on ACPA status. The association was not significant after meta-analysis in ACPA-positive RA (p=0.09), while it remained significant in ACPA-negative RA (p=0.05).

Interactions between the SNPs and between SNPs and *HLADRB1*SE* on ACPA presence and erosions were tested. No interaction was significant after using three different methods (data not shown).

DISCUSSION

In this meta-analysis performed on 1471 French patients with RA, one SNP located on *RANK* and one haplotype of three SNPs located on *RANKL* were associated with ACPA presence while one SNP on *OPG* was associated with erosions. No epistatic interaction between genes was identified for ACPA presence or erosions. The association between the SNP on OPG was statistically significant in ACPA-negative patients and almost significant in ACPA-positive patients.

Furuya *et al*[8] previously showed that rs2277438 located on *RANKL* was associated with the 2-year radiographic progression in a cohort of 72 Japanese patients, while Xu *et al*[11] reported that AG genotype of rs2277438 could influence joint erosions. However, in the ESPOIR cohort, we failed to observe any association between this SNP and erosions. Tan *et al*[10] further showed that rs7984870 located on the promoter of *RANKL* modulated the expression of RANKL via activated T cells only in rheumatic factor positive patients with RA. In a recent study, Knevel *et al*[9] identified an SNP (rs1485305) on *OPG* associated with radiographic progression in a meta-analysis of 4 independent data sets including 1418 patients with RA. In our study, this SNP was not associated with erosions in the ESPOIR cohort and thus not included in the meta-analysis.

rs8086340 located in the intronic region of *RANK* should have no functional effect on RANK, but could play a role in regulating gene transcription or could be in linkage disequilibrium with another SNP with potential functional effects. rs7325635 and rs7984870 are both located in the intronic region of *RANKL* while rs1054016 is located after the last exon in a non-coding region of *RANKL*. The haplotype might be in linkage disequilibrium with another polymorphism that could potentially affect *RANKL* expression or function. rs2073618 is located on the first exon of *OPG* and the polymorphism substitutes an Asparagine for a Lysine. This SNP could have a functional effect and thus could be implied in the pathophysiology of bone erosions. All

Table 2 Association of the SNPs located on *RANK, RANKL* and *OPG;* and ACPA presence or erosions in 632 patients in the ESPOIR cohort

		Association with ACPA			Association with erosions		
Rs	Genotypes	Number of ACPA+ (%)	χ^2 test p value	Trend-test p value	Number of patients with erosions (%)	χ^2 test p value	Trend-test p value
RANK							
rs8086340	GG	106 (56.1)	0.02	0.02	68 (37.8)	0.8	0.8
	CG	148 (46.7)			108 (35.5)		
	CC	49 (40.8)			42 (38.2)		
rs1805034	TT	103 (51.8)	0.4	0.4	63 (32.8)	0.2	0.2
	CT	154 (48.3)			121 (40.6)		
	CC	46 (43.8)			34 (33.7)		
rs35211496	TT	18 (56.3)	0.3	0.3	11 (35.5)	1.0	1.0
	CT	98 (51.8)			68 (37.6)		
	CC	187 (46.3)			139 (36.6)		
RANKL							
rs2277438	GG	7 (63.6)	0.3	0.3	2 (18.2)	0.2	0.2
	AG	80 (44.9)			66 (40.2)		
	AA	217 (49.7)			147 (35.1)		
rs1054016	GG	101 (45.7)	0.04	0.05	81 (38.9)	0.3	0.2
	GT	142 (46.4)			109 (37.3)		
	TT	57 (60.0)			27 (29.7)		
rs7325635	GG	101 (46.3)	0.04	0.05	81 (39.5)	0.06	0.05
	AG	140 (46.5)			109 (38.0)		
	AA	64 (59.8)			27 (26.5)		
rs7984870	GG	88 (45.4)	0.03	0.03	73 (39.7)	0.1	0.06
	CG	142 (46.0)			111 (38.0)		
	CC	74 (59.2)			34 (38.3)		
OPG							
rs2073618	CC	82 (50.0)	0.8	0.8	67 (42.4)	0.03	0.03
	CG	145 (49.0)			106 (38.1)		
	GG	80 (46.8)			46 (28.4)		
rs2073617	TT	79 (43.9)	0.3	0.3	52 (30.6)	0.2	0.2
	CT	146 (50.2)			106 (38.7)		
	CC	74 (51.4)			54 (38.9)		
rs10955911	GG	219 (51.3)	0.2	0.2	159 (39.0)	0.1	0.1
	AG	76 (43.2)			54 (33.1)		
	AA	12 (46.4)			6 (22.2)		
rs1485305	TT	86 (50.0)	0.8	0.8	65 (39.4)	0.3	0.3
	AT	144 (48.00)			107 (38.1)		
	AA	65 (46.4)			42 (31.1)		

ACPA, anticitrullinated peptide antibodies; *OPG,* osteoprotegerin; *RANK,* receptor activator of nuclear factor κ B; *RANKL,* receptor activator of nuclear factor κ B ligand; SNP, single-nucleotide polymorphisms.

SIFT, POLYPHEN2 and Mutation taster indicated that this locus has a benign influence on OPG and is thus probably harmless with a high security of prediction (p=0.999). This SNP had no influence on the OPG serum level in the ESPOIR cohort (Kruskal-Wallis test for comparison of medians across the genotypes: p=0.7). In this study, this was the only SNP associated with erosions in the ESPOIR cohort, and this finding was confirmed by the meta-analysis.

In this study, the definition of erosive disease was an Erosion Sharp Score ≥1 for the ESPOIR and RMP cohorts. In a recent paper, Knevel *et al*[20] showed that the definition of ≥5 joints with erosions was the definition with the higher specificity. Applying this cut-off, only 84 patients in the ESPOIR cohort and 17 in the RMP cohort were classified as erosive, which did not allow us to test the association between our candidate SNPs and erosive disease because of a lack of power. However, when assessing the number of erosive joints as an outcome, CC genotype of rs2073618 was still associated with a severe disease in ACPA+ patients with a number of erosive joints median about 2 (IQR: 0–4) in CC genotype comparison of medians across genotypes with Kruskall-Wallis test: p=0.02; test for a trend across genotypes: Cuzick trend test: p=0.013. In the FRACG cohort, erosive disease was defined according to the physician's opinion, with no quantification of the number of erosions on hand and foot radiographs, which did not allow us to test the association between our candidate SNPs and erosive disease defined on different cut-offs for the

Table 3 Results of the meta-analysis on the three cohorts assessing the association of a SNP on *RANK*, a haplotype on *RANKL* with ACPA; and a SNP on *RANKL,* a SNP on *OPG* with erosions

	ESPOIR OR (95% CI)	FRAGC OR (95% CI)	RMP OR (95% CI)	Meta-analysis OR (95% CI)	p Value
Association with ACPA					
RANK	0.64 (0.45 to 0.91)	0.71 (0.47 to 1.06)	0.46 (0.23 to 0.92)	0.64 (0.50 to 0.81)	0.0003
C allele of rs8086340					
RANKL	0.58 (0.38 to 0.89)	0.60 (0.34 to 1.03)	0.93 (0.43 to 1.98)	0.63 (0.46 to 0.86)	0.003
GGG Haplotype					
Association with erosions					
RANKL	1.75 (1.18 to 2.82)	0.67 (0.39 to 1.16)	0.50 (0.26 to 0.98)	0.97 (0.71 to 1.32)	0.85
G allele of rs7325635					
OPG	0.72 (0.49 to 1.04)	0.49 (0.31 to 0.78)	1.10 (0.59 to 2.05)	0.68 (0.52 to 0.88)	0.004
G allele of rs2073618					

ACPA, anticitrullinated peptide antibodies; *RANK,* receptor activator of nuclear factor κ B; *RANKL,* receptor activator of nuclear factor κ B ligand; *OPG,* osteoprotegerin; SNP, single-nucleotide polymorphisms.

number of erosive joints. Long-term structural progression could be another way to test the association of genetic polymorphisms and structural damage. Long-term structural progression could be another way to test the association of genetic polymorphisms and structural damage. In the ESPOIR cohort, the median of progression within the first year was about 0 (IQR: 0–1) with no difference across the genotypes of rs2073618. Furthermore, the potential association between SNPs and long-term structural damage progression is impaired because of disease-modifying antirheumatic drug protective effect on structural damage progression and a lack of association should be interpreted with caution.[21]

Interestingly, while an association between genetic factors in the *OPG* gene and the risk of erosions could be suspected, we also found some associations between a polymorphism on *RANK* and a haplotype on *RANKL* and ACPA presence. RANKL is expressed by various immunity cells including T cells or dendritic cells and can be induced by inflammatory factors such as interleukin 1, tumour necrosis factor α, transforming growth factor β, Wnt ligand and lipopolysaccharides (LPS).[22 23] RANK message is detected in the thymus, liver, colon, mammary glands, prostate, pancreas, bone marrow, heart, lung, brain, skeletal muscle, kidney, liver and skin and is strongly induced by M-CSF. RANK interacts with TRAF6 for transducing the signal via MAP Kinases p38, JNK or the NF-κB pathway.[24] RANKL-deficient and RANK-deficient mice have abnormal development of secondary lymphoid tissues, including lymph nodes, Peyer's patches, cryptopatches and the spleen.[25–27] Furthermore, a critical role for RANKL–RANK signalling has been established in thymic organ development, and specifically for the epithelial lineage cells required for negative selection of T cells.[28–30] We might assume that some polymorphisms on *RANK* and *RANKL* could play a role in the rupture of tolerance and explain the association with RA susceptibility and ACPA production.

In this study, we identified one SNP located on *RANK* and one haplotype on *RANKL* associated with ACPA presence as well as one SNP located on *OPG* associated with erosions in three cohorts of French patients with RA. The challenge will now be to identify and characterise the causal variants of the *RANK, RANKL* and *OPG* genes and their functional consequences to better understand the role played by the RANK, RANKL and OPG system in ACPA production and in structural damages, which characterise severe RA.

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