Genetic Variations in Genes Encoding RANK, RANKL, and OPG in Rheumatoid Arthritis: A Case-Control Study

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ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joints, which may lead to structural damage of the cartilage and bone. The receptor activator of nuclear factor-κB (RANK) and the osteoprotegerin (OPG) cascade system have been reported to be essential in osteoclastogenesis. Genetic variations in the genes coding for RANK, RANK ligand (RANKL), and OPG are thought to play roles in the susceptibility to RA.

Methods. In our case-control study, genomic DNA was obtained from 534 patients with RA who fulfilled the American College of Rheumatology 1987 criteria and 516 healthy control blood donors (HC). We studied 7 single-nucleotide polymorphisms (SNP) in the genes of RANK (2 SNP: rs1805034, rs35211496), OPG (2 SNP: rs3102735, rs2073618), and RANKL (3 SNP: rs9533156, rs2277438, rs1054016) using TaqMan assay-guided polymerase chain reaction. Genotype and allelic frequencies comparing RA patients with HC were analyzed by chi-square test for 2×3 and 2×2 tables, respectively.

Results. Genotype distributions of the SNP rs35211496 in the RANK gene as well as the SNP rs2277438 in the RANKL gene differed significantly between patients with RA and HC. The frequency of the minor allele of rs9533156 of RANKL was significantly higher in patients with RA than in HC (OR 0.84, 95% CI 0.71–0.99, $p = 0.047$). Multivariate analysis adjusted to sex and investigating SNP demonstrated a significantly elevated risk for RA associated with the major allele in the RANK SNP rs35211496 ($p = 0.0231$) and with the minor allele in the RANKL SNP rs2277438 ($p = 0.0092$). No significantly increased risk was detected in the other SNP.

Conclusion. The minor allele of the RANK SNP rs35211496 may be protective against RA, while the minor alleles of the RANKL SNP rs2277438 may increase susceptibility to RA. (First Release March 15 2010; J Rheumatol 2010;37:900–4; doi:10.3899/jrheum.091110)

Key Indexing Terms:
POLYMORPHISM RANK RANKL OSTEOPROTEGERIN
RHEUMATOID ARTHRITIS SINGLE-NUCLEOTIDE POLYMORPHISM

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic inflammation of the joints, which may lead to structural damage of the cartilage and bone. Several lines of evidence have clearly established that osteoclasts and monocytic cells are key mediators of the bone loss during the course of disease[1-3]. The 3 tumor necrosis factor (TNF) family molecules, the receptor activator of nuclear factor-κB (RANK), its ligand RANKL, and the decoy receptor of RANK, osteoprotegerin (OPG), have pivotal roles as central regulators of osteoclast development and activation[4,5]. First, activated T lymphocytes express a primary soluble form and a cellular form of RANKL inducing osteoclast formation, survival, fusion, and activation and inhibiting osteoclast apoptosis, thus expanding the pool of osteoclasts that are capable of bone and cartilage decay[6,7]. Further, RA synovial fibroblasts have been found to produce RANKL, especially after interleukin 6 (IL-6) stimulation, and it has been proposed that this promotes osteoclast development and plays a major pathogenic role in RA-associated bone erosions[8,9]. Underlining these results, coculture of osteoclast precursors with rheumatoid synovial fibroblasts

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induces osteoclastogenesis by downregulation of OPG[10]. In addition, RA-derived synovial fibroblasts stimulated by the proinflammatory cytokine IL-1ß and TNF-α expressed higher levels of RANKL and OPG than fibroblasts from normal joints[11]. Moreover, specific treatment with disease-modifying antirheumatic drugs modulates the RANKL and OPG expression by synovial tissue of RA, possibly reflecting a successful treatment by avoiding bone and cartilage damage[12,13]. Altogether, the functional properties of the RANKL OPG network suggest an important effect of these genes on the pathogenesis of RA.

We investigated single-nucleotide polymorphisms (SNP) in the genes of RANK (2 SNP), OPG (2 SNP), and RANKL (3 SNP), located in the promoter region, the exon, or the 3' UTR and 5' UTR, all possibly associated with functional alterations. We conducted a case-control study to evaluate whether genetic variations — single SNP or SNP in combination — might be associated with RA.

MATERIALS AND METHODS

Study participants. We recruited 534 patients with RA from the clinics of the Department of Internal Medicine I at the hospital of Saarland University Medical School, Homburg/Saar, Germany, and from the Hospital of Orthopaedics and Rheumatology, Vogelsang-Gommern, Germany. Blood donors from the Institute for Transfusion Medicine, University of Saarland Medical School, served as controls (n = 519, women 35.8% vs men 64.2%; mean age 34.8 yrs, SD ± 11.3, median 37, range 18–65). Patients and controls were of central European white ethnicity. The ethics committees of the medical association of the Saarland, Germany, approved the study, and all study participants gave written informed consent. All patients fulfilled ≥ 4 of the 1987 American College of Rheumatology criteria for RA[14]. Their mean age was 49.1 years, SD ± 14.4, median 50, range 21–82. Their mean disease duration was 8.2 ± 8.3 years, range 0–44 years. Most patients (73.4%) were women. A total of 75.6% of the patients tested positive for rheumatoid factor (RF); anticyclic citrullinated peptide (anti-CCP) antibodies were determined in 175 patients and detected in 67.4%. Antibodies were tested by RF IgM ELISA (positive > 20 U/ml) and anti-CCP IgG ELISA (positive > 5 U/ml; Euroimmun, Luebeck, Germany).

Studied SNP. The 3 genes of interest together span more than 120 kb pairs and show only weak to moderate linkage disequilibrium patterns, according to the HapMap data. This makes it quite laborious to cover complete genomic regions, e.g., by a tagging SNP approach. We therefore preferentially selected those SNP for each gene from the US National Center for Biotechnology Information database (Genome Build 36.3; www.ncbi.nlm.nih.gov/gene) that we expected to be of functional relevance, either by altering the amino acid sequence of the respective gene product or by its location within a potentially regulatory region (promoter or untranslated region).

Altogether, 7 known SNP were genotyped. All 3 known SNP of the RANKL gene (*TNFSF11*, OMIM*602642) on chromosome 13q14.11 were noncoding and comprised SNP in the promoter region (rs9533156), in the 3' untranslated region (UTR; rs1054016), and in intron 1 (close to the intron 1/exon 2 boundary; rs2277438). The 2 known SNP of RANK (*TNFRSF11A*, OMIM*603499) on chromosome 18q22.1 were both missense SNP leading to amino acid exchanges in the RANK peptide: rs1805034 (Ala192Val) and rs35211496 (Thr141His). For OPG (*TNFRSF11B*, OMIM*602643) on chromosome 8q24, an SNP in the promoter region (rs3102735) as well as a missense SNP (rs2073618, Leu3Asn) were selected.

Genotyping. Three SNP (rs2277438, rs1054016, rs1805034) were genotyped using commercial TaqMan assays (all genotyping tools, Applied Biosystems, Darmstadt, Germany). For 4 SNP (rs9533156, rs35211496, rs2073618, rs3102735), TaqMan genotyping assays were designed with Primer Express 3.0. All TaqMan assays were performed with TaqMan Genotyping Master Mix on a StepOne Plus real-time PCR cycler according to the manufacturer's instructions.

Statistical analysis. For statistical analysis, SAS statistical software was used. The differences in genotype and allele frequencies between patients and controls were analyzed using chi-square tests for 2 × 3 tables and 2 × 2 tables, respectively. We tested for Hardy-Weinberg equilibrium in controls by the appropriate chi-square test with 1° of freedom. Differences in allele frequencies were quantified by OR and 95% CI. Haplotype frequencies for sets of SNP belonging to a gene were estimated by the maximum likelihood method using the EM-algorithm as implemented in the SAS procedure Haplotype. The test comparing cases to controls with respect to haplotype frequencies was performed by the likelihood ratio chi-square test. Interactions between SNP genotypes of different genes were assessed by fitting a logistic model to the case-control status variable. One interaction effect in turn was entered in addition to sex and all 7 SNP genotypes, and tested by Wald chi-square test. Starting from this model, SNP were removed if they were not significant by likelihood ratio test. The remaining RANK SNP rs35211496 and RANKL SNP rs2277438 were adjusted to the other SNP and sex was included in a parsimonious model.

All p values are 2-sided and p values < 0.05 were referred to as statistically significant.

No adjustment for multiple testing was made, because analyses were considered exploratory and needing confirmation by an independent set of data.

RESULTS

Among the controls, the genotype distributions of all 7 SNP were in Hardy-Weinberg equilibrium. No significant sex-specific differences were detected between the SNP genotype distributions derived from the controls. Table 1 summarizes the results obtained from all SNP analyses of the genes coding for RANK (2 SNP: rs1805034 and rs35211496), OPG (2 SNP: rs3102735 and rs2073618), and RANKL (3 SNP: rs9533156, rs2277438, and rs1054016). Allele frequencies and genotype distributions in patients with RA were compared to healthy controls.

The genotype RANKL SNP rs2277438 was significantly associated with RA (p = 0.039), resulting in a 3-fold higher susceptibility to RA for the homozygous genotype of the minor allele (homozygous minor vs major allele: OR 0.35, 95% CI 0.15–0.80; homozygous minor allele vs heterozygous: OR 1.10, 95% CI 0.78–1.55; Table 1).

The RANK SNP rs35211496 showed a significant difference in the genotype distribution between RA and controls (p = 0.034) and a trend toward higher frequency of the major allele in patients with RA: 864 (83.2%) vs 825 (79.9%) (OR 1.25, 95% CI 1.00–1.25, p = 0.053). The RANKL SNP rs9533156 showed a borderline significantly higher frequency of the minor allele in the RA study population compared to controls (OR 0.84, 95% CI 0.71–0.99, p = 0.047), while the genotype distribution failed to show significant differences (p = 0.144). Similarly, all OPG SNP failed to show significant effects.

Further, a multivariate analysis of different genotypes adjusted for sex and the nonsignificant SNP revealed that a

Table 1. Allelic and genotype frequencies of the genetic variations in the genes for RANK, OPG, and RANKL in patients with RA and healthy controls (chi-square tests for 2 × 2 tables and 2 × 3 tables).

Allelic and Genotype Frequencies	Alleles	RA, n = 534 (%)	Controls, n = 519 (%)	OR (95% CI)	p
RANK rs1805034					
Alleles	C (Ala)	499 (48)	472 (46)	C vs T: 1.09 (0.92–1.30)	0.326
	T (Val)	543 (52)	560 (54)		
Genotypes	CC	132 (25)	104 (20)	CC vs CT: 1.39 (0.95–2.03)	
	CT	235 (45)	264 (51)	CC vs TT: 1.31 (0.85–2.01)	0.079
	TT	154 (30)	148 (29)		
RANK rs35211496					
Alleles	C (His)	864 (83)	825 (80)	C vs T: 1.25 (1.00–1.56)	0.053
	T (Tyr)	174 (17)	207 (20)		
Genotypes	CC	368 (71)	330 (64)	CC vs CT: 1.37 (0.98–1.92)	
	CT	128 (25)	165 (32)	CC vs TT: 0.72 (0.34–1.52)	0.034
	TT	23 (4)	21 (4)		
OPG rs3102735					
Alleles	C	130 (12)	109 (11)	C vs T: 1.17 (0.89–1.53)	0.254
	T	936 (88)	919 (89)		
Genotypes	CC	7 (1)	5 (1)	CC vs CT: 1.22 (0.28–5.39)	
	CT	116 (22)	99 (19)	CC vs TT: 1.62 (0.38–6.97)	0.514
	TT	410 (77)	410 (80)		
OPG rs2073618					
Alleles	C (Asn)	584 (55)	552 (54)	C vs G: 1.06 (0.89–1.26)	0.522
	G (Lys)	472 (45)	472 (46)		
Genotypes	CC	154 (29)	143 (28)	CC vs CG: 1.04 (0.74–1.45)	
	CG	276 (52)	266 (52)	CC vs GG: 0.96 (0.63–1.46)	0.791
	GG	98 (19)	103 (20)		
RANKL rs9533156					
Alleles	C	450 (44)	495 (48)	C vs T: 0.84 (0.71–0.99)	0.047
	T	578 (56)	533 (52)		
Genotypes	CC	101 (20)	123 (24)	CC vs CT: 0.75 (0.34–1.66)	
	CT	248 (48)	249 (48)	CC vs TT: 0.42 (0.14–1.31)	0.144
	TT	165 (32)	142 (28)		
RANKL rs2277438					
Alleles	A	820 (81)	860 (84)	A vs G: 0.82 (0.65–1.03)	0.079
	G	192 (19)	164 (16)		
Genotypes	AA	342 (68)	360 (70)	AA vs AG: 1.10 (0.78–1.55)	0.039
	AG	136 (27)	140 (27)	AA vs GG: 0.35 (0.15–0.81)	
	GG	28 (6)	12 (2)		
RANKL rs1054016					
Alleles	G	598 (58)	564 (55)	G vs T: 1.10 (0.93–1.31)	0.267
	T	438 (42)	456 (45)		
Genotypes	GG	178 (34)	161 (32)	GG vs GT: 0.69 (0.32–1.51)	
	GT	242 (47)	242 (47)	GG vs TT: 0.48 (0.16–1.47)	0.554
	TT	98 (19)	107 (21)		

RANK: receptor activator of nuclear factor-κB; RANKL: RANK ligand; OPG: osteoprotegerin; RA: rheumatoid arthritis.

significantly elevated risk for RA is associated with the major allele in the RANK SNP rs35211496 (p = 0.0231) and the minor allele in the RANKL SNP rs2277438 (p = 0.0092; Table 2).

The haplotype analyses for the RANK SNP on chromosome 18, the RANKL SNP on chromosome 13, and OPG SNP on chromosome 8 showed no significant differences between the RA study population and controls (likelihood ratio chi-square test with 7˚ of freedom). All allele frequencies and genotype distributions were also stratified to sex and RF seropositivity. No significant differences were found (data not shown).

DISCUSSION

To our knowledge, this is the first study showing a significant association between the SNP rs2277438 of the RANKL gene and the susceptibility of RA in the white population. The SNP is associated with a 3-fold increased risk of the homozygous carrier state of the minor allele. Further, a significantly increased risk for RA was also associated with the

Table 2. Multivariable analysis of genotypes in SNP RANKL rs2277438 and RANK rs35211496 using a logistic model adjusted for investigated SNP and sex. The Wald chi-square test was used for genotype effect. Model was obtained by backward selection starting with a model with sex and all 7 SNP genotypes.

Model: Adjusting to Investigated SNP and gender [a]	OR (95% CI)		
Genotypes SNP locus	CT vs CC	CC vs TT	CT vs TT
RANK rs35211496	0.676 (0.484–0.917)	0.741 (0.365–1.505)	0.493 (0.235–1.034)
Genotypes SNP locus	AG vs AA	AA vs GG	AG vs GG
RANKL rs2277438	0.926 (0.673–1.274)	0.302 (0.137–0.667)	0.280 (0.123–0.637)
		p = 0.0092	

SNP: single-nucleotide polymorphisms; RANK: receptor activator of nuclear factor-κB; RANKL: RANK ligand.

major allele in the RANK SNP rs35211496 and the minor allele in the RANKL SNP rs2277438, using a multivariate Cox regression analysis adjusted for sex. In addition, the minor allele of the RANK SNP rs35211496 appears to be protective against RA; however, only 6% of the patients with RA and only 2% of the controls showed the homozygous minor genotype. All other SNP in the RANK, RANKL, and OPG genes we analyzed were not associated with an increased risk for RA.

Our results thus suggest that the minor allele of the RANKL SNP rs2277438 increases the risk for RA. This finding supports the observation of Furuya, *et al*[15] of a higher frequency of bone erosions during RA progression in a Japanese cohort that was remarkably independent of the shared epitope status. The same study also investigated the OPG SNP rs2073618 without finding a significant correlation with RA severity[15]. However, the shared epitope status together with other RANKL SNP appears to correlate with earlier ages of RA onset[16].

Inflammatory osteoporosis is a frequent finding in RA joints that is mediated by accelerated osteoclast recruitment and activation, induced through the interactions with RANK and its ligand, RANKL[10]. In the context of arthritis and osteoporosis, the study of Hsu, *et al* investigating men with decreased bone mineral density and without RA is notable. The authors observed an association with the minor allele of RANKL SNP, rs2277438[17]. Together with our results, this association points to RANKL as an important part of bone metabolism. However, the effect of SNP rs2277438 located in intron 1, a potentially regulatory region of the RANKL gene, on the regulation of RANKL expression is unclear. In the white population, RA has been linked with chromosome 18q, a region containing the RANK gene[18]. Further, a haplotype that includes RANK has been reported to be associated with RA in another study[19]. In accord with this, our data indicate a correlation between susceptibility to RA and the major allele of the RANK SNP rs35211496. This genetic variation causes an alteration in the amino acid sequence (Thr141His) in the RANK peptide; however, its functional significance remains to be shown.

Our results must be interpreted with caution: the effects we observed are moderate and significant only without adjustment for multiple testing. Another limitation is the insufficient power for the haplotype analyses to detect effects of the same order of magnitude as those observed in Table 1, outlining allelic and genotype frequencies of all 7 SNP. Thus, there may be haplotype effects in spite of nonsignificant results.

Our case-control study points to a moderate association of the RANKL SNP rs2277438 and the RANK SNP rs35211496 with RA that remains significant in a multivariate analysis adjusting for sex. Further studies are needed, including confirmation of our observation, in an independent validation set. Whether these SNP do indeed lead to a functional alteration of RANK and/or its ligand RANKL remains to be shown, e.g., by gene expression studies in cell culture models.

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