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A Functional *RANKL* Polymorphism Associated with Younger Age at Onset of Rheumatoid Arthritis

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Abstract

Objective—We previously reported association of co-occurrence of *HLA-DRB1* shared epitope (SE) and *RANKL* SNPs with younger age of RA onset in 182 rheumatoid factor positive (RF) European American (EA) early RA patients. Here, we fine-mapped the 48 kb *RANKL* region in the extended 210 EA RF-positive early RA cohort, sought replication of RA-associated SNPs in additional 501 EA and 298 African-Americans (AA) RA cohorts, and explored functional consequences of RA-associated SNPs.

Methods—SNP genotyping was conducted using pyrosequencing or TaqMan PCR assays. Associations of rs7984870 with RANKL expression in plasma, PBMC and isolated T cells were quantified using ELISA and RT-PCR. Site-directed mutagenesis of rs7984870 within the 2kb *RANKL* promoter was performed to drive the luciferase reporter gene in osteoblast and stromal cell lines. Interaction of DNA and protein was determined by electrophoretic mobility shift assay.

Results—A single promoter SNP rs7984870 was consistently significantly associated with earlier age of RA onset in 3 independent seropositive (RF or anti-cyclic citrullinated peptide antibody positive) RA cohorts but not in seronegative RA patients. The risk C allele of rs7984870

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conferred 2-fold higher plasma RANKL levels in RF-positive RA patients, significantly elevated *RANKL* mRNA expression in activated normal T cells, and increased promoter activity after stimulation *in vitro* via differential binding to transcription factor SOX5.

Conclusion—The *RANKL* promoter allele that increased transcriptional levels upon stimulation might promote interaction between activated T cells and dendritic cells, predisposing to younger RA onset in seropositive EA and/or AA individuals.

Rheumatoid arthritis (RA) is characterized by chronic inflammation and destruction of cartilage and bone. The etiology of RA is not clearly defined, but it is presumed that environmental factors trigger the disease in genetically predisposed individuals. The strongest genetic contribution to RA susceptibility is the HLA - class II alleles that contain a common amino acid motif called the “shared epitope” (SE), which is also a risk factor for disease progression and development of extraarticular manifestations (1). In addition to the SE alleles, multiple gene variants, including *PTPN22*, *PADI4*, *FCRL3*, *CTLA4*, *STAT4*, *TRAF1*/C5, CD40, IL2RB, IL21, PRKCQ, KIF5A, AFF3, REL, TNFAIP3, CD28, PRDM1 and *CD2*/*CD58* have been associated with susceptibility to RA; however, causal variants of many of these loci remain elusive (2–5). Anti-cyclic citrullinated peptide antibody (ACPA) is highly specific for RA and can be detected years before the first clinical manifestations as a putative predictor for developing RA. ACPA production is associated with *HLA-DRB1*, *PTPN22*, *TRAF1*/*C5*, *CD40*, *FCGR3A*, *STAT4*, *REL* and *PADI4* alleles (6–11), as well as with environmental factors, primarily smoking (12), suggesting different pathophysiologic mechanisms underlying seropositive and seronegative RA.

Receptor activator of nuclear factor κB ligand (RANKL [OMIM 602642]), a type II membrane protein of the tumor necrosis factor family (*TNFSF11*), is expressed on osteoblasts, stromal cells, activated T cells, B cells (13) and megakaryocytes (14). The *RANKL*, located on chromosome 13q14, encodes three isoforms: RANKL1 and RANKL2 are both transmembrane proteins with the latter lacking an intracellular domain; RANKL3 is a soluble protein containing neither an intracellular nor a transmembrane domain (15). A deficiency of RANKL, the key molecule mediating osteoclast development, activity, and survival (16), leads to osteoporosis and severe skeletal abnormalities in the murine model (17). Apart from its importance in osteoclastogenesis, RANKL participates in immune regulation by mediating differentiation of T and B lymphocytes, prolonging survival of mature dendritic cells (DCs), promoting activation of monocytes, and participating in the development of lymph nodes and medullary thymic epithelial cells (18). In RA, local and systemic increased RANKL levels are associated with bone resorption, suggesting a pivotal role in mediating bone erosion (19). Importantly, co-localization of RANKL and RANK expression at the site of DC and T cell interaction in rheumatoid synovia and lymph nodes may represent a direct link between cell-mediated immunity and bone destruction in RA (20).

We have previously observed the association of younger age of RA onset with the presence of *HLA-DRB1*04* and *RANKL* polymorphisms in 182 RF+ EA RA patients (21). An interaction between SE and *RANKL* polymorphisms was associated with a mean 18 years younger age at RA onset in this cohort. Here, we used the enlarged EA RF+ RA cohort as a discovery panel to fine map *RANKL* variants predisposing to early RA onset, replicated in independent EA and AA early RA cohorts and tested this genetic association in EA RF+ polyarticular juvenile idiopathic arthritis (JIA) patients. Finally, we characterized functional properties of the identified and confirmed RA-associated SNP in three seropositive RA cohorts.

PATIENTS AND METHODS

Study populations

The present study included 4 independent cohorts (3 RA and 1 JIA) (Table 1). The three RA cohorts were early RA long-term observational studies including 1) Discovery panel, 210 EA RF+ early RA patients were from the Western Consortium of Practicing Rheumatologists (WCPR cohort) (22), which had been enlarged from the 182 EA patients reported (21); 2) Replication panel 1, 501 independent EA early RA patients were from the Brigham Rheumatoid Arthritis Sequential Study registry (BRASS cohort); 3) Replication panel 2, 298 patients who were participating in the Consortium for the Evaluation of AAs with Early Rheumatoid Arthritis Registry (CLEAR cohort) (23). We also tested the genetic association in 80 EA RF+ polyarticular JIA patients recruited from Cincinnati's Children Hospital Medical Center, OH. All RA patients fulfilled the American College of Rheumatology 1987 revised classification criteria for RA (24). Early RA was defined as those patients who enrolled within 24 months after symptom onset and before disease-modifying antirheumatic drugs (DMARDs) therapy. This study was approved by appropriate institutional review boards.

Genotyping methods

SNPs spanning a 48 kb region from 7.3 kb upstream to 6.7 kb downstream of *RANKL* transcribed sequence were surveyed in the NCBI-dbSNP build 36 database. A total of 16 SNPs were genotyped in this study, including 5 promoter, 7 intronic, one 3' untranslated region (UTR), and three 3'downstream *RANKL* polymorphisms (Figure 1B). Among these 16 SNPs, 9 tag EA haplotypes throughout dbSNP database 124, 125 and 126, 1 promoter and 3 intronic SNPs create putative transcription factor binding sites, 1 SNP has been associated with low bone mineral density in men (25) and 2 SNPs were tested in our previous study (21). Genomic DNA was isolated from blood mononuclear cells by the standard protocol. Genotyping of *RANKL* SNPs was determined using PCR pyrosequencing as described (20), except rs5803141, which was genotyped using fluorescence-based PCR. In the BRASS cohort, SNPs rs7984870, rs9525641 and rs1054016 were genotyped by TaqMan real-time PCR assays (Applied Biosystems). HLA–DRB1 genotyping were determined by DNA-based sequence-specific oligonucleotide probe assay, AlleleSEQR HLA–DRB1 reagent kit or allele-specific polymerase chain reaction as described elsewhere (26–28).

ELISA

Concentrations of soluble RANKL levels in plasma of 23 RA patients at baseline and 24 normal controls were quantified using a commercial ELISA kit from ALPCO Diagnostics (Salem, NH).

Real-Time PCR

Two isoforms in *RANKL* transcript levels (isoform1: NM003701.2 and total *RANKL* transcript: NM033012.2) in PBMC from 38 EA normal controls were measured using ABI TaqMan assay. Relative *RANKL* expression in PBMCs and T cells was normalized using the expression level of CD4 and ribosomal protein (RPLPO), respectively and calculated by the $2^{-\Delta\Delta Ct}$ method.

Cells cultures

Primary T cells were purified from peripheral blood of 8 healthy volunteers (4 CC and 4 GG genotype for rs7984870) using a T-rosette purification kit (StemCell Technologies), which were 93% CD3+ by flow cytometry. 4×10^5 T cells were plated onto 12 well plates in the

presence or absence of 20ng/ml IL-2 (R&D Systems) for 24h or 48h, and then harvested to perform Real-time PCR assays.

Site-directed mutagenesis

The *RANKL* promoter region (−1 bp to −2 kb relative to the transcription start site) was previously cloned into the luciferase reporter pGL2 Basic vector (a kind gift from Dr. Sakamuri V. Reddy) (29). The plasmids for −1816 G/G in the *RANKL* promoter region were mutated into −1816 C/C using the QuikChange Lightning Site-Directed Mutagenesis kit (Stratagene). Accuracy of mutagenesis was confirmed by sequence analysis.

Cell transfection

Five microgrammes of *RANKL* promoter/luciferase fusion plasmid DNA or empty vector (pGL4) were transfected into human stromal cells SAKA-T and osteoblast cells hFOB 1.19 using Lipofect-AMINE kit (Invitrogen) and 100ng of pRL-SV40 control vector (Renilla luciferase) was cotransfected as an internal control for transfection efficiency. After cells treated with or without b-FGF (4ng/ml) and TNFα (10ng/ml) (R&D Systems) for 48h, cell lysates were prepared and assayed for firefly and control Renilla luciferase using the Dual-Luciferase reporter assay (Promega).

EMSA and supershift assays

Electrophoretic mobility shift assay (EMSA) and supershift assays were performed as previously described (30). After Jurkat cells were stimulated with TNFα for 24h, nuclear extract from Jurkat cells was prepared by NE-PER nuclear extraction reagent (Thermo scientific). Biotin-labeled oligonucleotides were used to perform EMSA: 5'ACCCATTAAAAAC/GAATGGAAGACACTAT3' (forward) and 5'ATAGTGTCTTCCATTG/CTTTTTAATGGGT3' (reverse). EMSAs were performed with the LightShift Chemiluminescent EMSA Kit (Pierce). The antibody used in the supershift reactions was 2μl of polyclonal rabbit anti human SOX5 (Anti-SOX5) (Santa Cruz biotechnology).

Statistical analysis

Comparisons between patient groups were made by 1-way analysis of variance (ANOVA) for three groups (3 genotypes), and unpaired t-tests for two groups (dominant or recessive models) for age of RA symptom onset. For comparison of mean values, Student's t-test was performed. The strength of pair-wise linkage disequilibrium (LD) (r^2) between two genetic polymorphisms was assessed using Haploview 4.0. Statistical analyses were performed using software package Prism 4 (GraphPad Software). P values < 0.05 were considered to indicate statistical significance.

RESULTS

Fine-mapping *RANKL* SNPs for association with younger age of RA onset in EA patients (Extended RF+ early RA WCPR cohort)

We previously observed a strong association between the combined presence of *HLA-DRB1*04* and *RANKL* polymorphisms (rs9525641 in the promoter region and rs922996 in intron 4) with a younger age of RA onset in 182 RF+ EA early RA WCPR patients (21). Because these two RA-associated SNPs showed no potential biologic functions based on bioinformatic searches, we reasoned the observed association signals are likely to reflect potential functional variants within the *RANKL* locus. To test this hypothesis, we assessed 16 SNPs located in the 48 kb *RANKL* genomic region using the enlarged discovery panel of the 210 EA RF+ early RA WCPR patients.

The minor allele of 4 *RANKL* SNPs, mainly in promoter (rs5803141, rs7984870 and rs9525641 in promoter, and rs1054016 in 3'UTR) were associated with earlier age at RA onset in EA patients ($p < 0.05$) (Figure 1A and B). These 4 SNPs are in strong linkage disequilibrium (LD) ($r^2 = 0.75 \sim 0.94$) with each other but showed modest LD ($r^2 = 0.57 \sim 0.65$) with the formerly observed RA-associated *RANKL* rs922996, the association of which was not confirmed in this study.

The similarities in manifestations and genetic background between RF+ adult RA and RF+ polyarticular JIA (31) prompted us to test the potential association of these 4 SNPs with juvenile arthritis in 80 EA RF+ polyarticular JIA patients. No significant association between the minor allele of these SNPs and age of disease onset was observed in this cohort, which might be attributed to the modest sample size and narrow range in age of disease onset (median = 11.1 yrs; 25% to 75% percentile = 8.8 to 12.8 yrs).

Confirmation of the association of *RANKL* SNPs with younger age of RA onset in an additional EA cohort (BRASS cohort) and AA cohort (CLEAR cohort)

To seek replication of the significant association of *RANKL* genotypes with age of RA onset, we genotyped these four SNPs in two independent RA cohorts. One replication cohort was 501 independent EA early RA patients from BRASS. The strong LD of these 4 SNPs ($r^2 = 0.76 - 0.93$) observed in BRASS cohort was similar to the LD values in WCPR EA cohort, indicating BRASS is a well-matched independent cohort for replication study. Only the rs7984870 CC allele was associated with earlier RA onset in 344 ACPA+ EA patients (37.6 ± 12.8 yrs) compared to non-CC carriers (41.4 ± 14.6 yrs; $p = 0.04$) and a similar trend of association in RF+ patients (CC *vs.* non-CC carriers, 37.9 ± 13.0 yrs *vs.* 41.4 ± 14.5 yrs; $p = 0.08$) (Figure 1A, Table 2).

The second independent replication panel included 298 AA RA patients from CLEAR cohort. Genotypic association with younger age of RA onset was observed in two promoter SNPs (rs5803141 and rs7984870) in an ACPA+ subset (n = 172) and a trend of this association in RF+ AA RA patients (n = 211) (Figure 1A, Table 2), implicating a role of *RANKL* promoter SNPs in development of RA in multiple ethnic groups.

Confirmation that the interaction between HLA-DRB1 SE and *RANKL* promoter contributes to earlier age of RA onset in WCPR, BRASS and CLEAR RA cohorts

We previously reported a significant association of the co-occurrence of *RANKL* polymorphisms and HLA-DRB1*04 with younger age at onset of RA in the original WCPR cohort (21). In the present study, we defined the SE as containing any one of the following HLA-DRB1 alleles: *0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001, *1402, *1406. The mean age at disease onset in RA patients harboring SE was an average of 4.7 and 4.5 years younger than that in patients without SE in seropositive (RF+ and/or ACPA+) EA WCPR and BRASS RA cohort ($p = 0.03$ and 0.01, respectively), but this difference was not observed in AA CLEAR RA cohort (Figure 2A). Among RF+ RA patients, co-occurrence of SE and the CC allele of rs7984870 was associated with younger age at disease onset with an average of 8.2 yrs (WCPR cohort), 4.7 yrs (BRASS cohort) and 6.7 yrs (CLEAR cohort) younger than non-CC SE positive carriers ($p = 0.004$, 0.01 and 0.01, respectively) (Figure 2 B–D). A similar trend of younger age at onset also was seen in ACPA+ patients in BRASS and CLEAR cohorts (mean 5.1 and 5.7 yrs earlier; $p = 0.01$ and 0.02) (Figure 2 C–D), suggesting co-occurrence of SE and *RANKL* risk alleles had a stronger impact on the earlier age at disease onset than each gene variant individually. However, these genetic associations were not observed in seronegative RA patients. Stratifying RA patients into 0, 1 and 2 copies of SE subgroups, only ACPA+ BRASS EA RA showed a trend toward a younger RA onset (supplementary Table 1). Of note, *RANKL*

CC allele was not associated with early age at RA onset in 0 copy SE RA patients, which might be attributed to the small sample size (n= 8~26 patients in each CC plus SE 0 copy subgroup) and/ or in part because the effect of rs7984870 CC on age of RA onset might depend on HLA-DRB1 (supplementary Table 1).

The HLA– DRB1*04 alleles represent a considerably stronger susceptibility factor than other SE alleles in RA (32). When SE was defined as only HLA-DRB1*04 alleles (included *0401, *0404, *0405, *0408, and *0410), the combined presence of SE-containing DRB1*04 and rs7984870 CC genotype displayed an even earlier age of RA onset in RF+ or ACPA+ patients (mean 13.3 yrs earlier in WCPR cohort, P =0.0003, mean 5.8 and 6.3 yrs earlier in BRASS, P = 0.01 and 0.007, and mean 9 and 9.4 yrs earlier in CLEAR, P =0.01, respectively) (Figure 2 B–D). Multiple regression analysis testing the interaction on age at RA onset with the presence of SE and risk allele in rs7984870, confirmed a significant interaction only in WCPR and BRASS EA RA patients ($p < 0.05$, respectively), indicating that the interaction only existed in EA patients.

Our data indicated among the 4 SNPs identified in the initial discovery WCPR cohort, only rs7984870 showed genetic association with younger age onset in 2 independent early RA replication cohorts of EA and AA ancestries. The consistent association of rs7984870 with RA led us to investigate its potential functional effect.

RANKL promoter SNP rs7984870 might affect RANKL expression level

Plasma levels of soluble RANKL (derived from membrane forms and measured by ELISA) were compared to genotypes. Frozen plasma samples had been collected from the baseline evaluation of WCPR RF+ EA RA patients who were still within 1 year of symptom onset and had not yet been treated with DMARDs. Similar to a previous report (33), our study found that soluble RANKL levels in plasma were significantly higher in RA patients (0.64 ± 0.61 pmol/L) compared to healthy controls (HC) (0.26 ± 0.18 pmol/L; p = 0.004). No significant allelic-specific difference in RANKL levels was observed in HC. However, soluble RANKL levels were approximately 2 - fold higher in RA patients carrying rs7984870 CC genotype (0.87 ± 0.53 pmol/L) than in those carrying GG genotype (0.46 ± 0.67 pmol/L; p = 0.002; Figure 3A).

We subsequently tested whether rs7984870 variant would impact on *RANKL* transcription. The relative expression of *RANKL* isoform 1 transcript (encodes RANKL1 and RANKL2 by alternative splicing) and total *RANKL* transcript (isoform 1 plus isoform 2 that encodes RANKL3) in PBMC were specifically measured using real-time PCR, and normalized using the expression level of CD4 because RANKL is mainly expressed in activated T helper cells in peripheral blood. Compared to isoform 1, isoform 2 has an upstream transcription start site, suggesting it may be regulated differently. Transcript levels of *RANKL* isoform 2 were generated from transcripts levels of total expression minus isoform 1 in this study. We did not observe any allele-specific difference in mRNA expression of the two isoforms in PBMC from HC (Figure 3B). *RANKL* mRNA expression in 22 RA patients (11 CC and 11 GG genotype for rs7984870) was also examined, but the allelic difference was not conclusive, perhaps owing to a small sample size and/or disease activity (data not shown).

As shown in Figure 3B, higher expression of isoform 1 than isoform 2 was observed in PBMC, similar to a previous report (15), consistent with isoform 1 being the major *RANKL* transcript in blood circulation. We next tested the allelic expression levels of the major *RANKL* isoform in T cells *in vitro*. The primary T cells from 8 normal controls (4 CC and 4 GG genotype for rs7984870) were treated with or without IL-2 20ng/ml for 24 or 48 h and harvested to perform real-time PCR assays. *RANKL* expression was normalized using the expression level of *RPLPO*. *RANKL* transcript levels were significantly increased after *in*

vitro activation of T cells by co-culturing with IL-2 for 48h. Interestingly, T cells carrying the rs798470 CC genotype conferred significantly higher *RANKL* transcript levels than those carrying GG genotype after activation by IL-2 ($p = 0.04$, Figure 3C).

RANKL promoter SNP rs7984870 may affect the promoter activity

2-kb *RANKL* promoter luciferase constructs expressing either the major G allele or the minor C allele at position −1816 were transiently transfected into the human stromal cell line SAKA-T and osteoblast cell line hFOB1.19. Basic fibroblast growth factor (b-FGF) and TNFα have been reported to upregulate *RANKL* expression (29;34). Transfection of the rs7984870 CC *RANKL* promoter displayed a 40% increaseing transcription activity in stromal cell lines and a 30% increase in osteoblast cell lines compared with GG genotype after stimulation with b-FGF; similarly, the promoter activity was increased significantly more after stimulation with TNFα when these two cell lines were transfected with rs7984870 CC construct compared with GG construct ($p < 0.05$, Figure 4 A–B).

rs7984870 CC genotype may create a binding site to transcription factor *SOX5*

To investigate the effect of the polymorphism on transcription factor binding, EMSA assay was performed. The 28-bp oligonucleotide containing the homozygous minor C allele or the major G allele of rs7984870 was incubated with nuclear extracts prepared from Jurkat cells after stimulation with TNFα for 24h. Binding of nuclear proteins was observed with the oligonucleotide probe containing either the GG or the CC allele (Figure 4C, lane 2 and 5). *In silico* analysis indicated that transcription factor *SOX5* might bind to the rs7984870 CC allele but not to the GG allele. The addition of commercial polyclonal rabbit IgG antibodies to SOX5 produced a supershift band only in CC allele (Figure 4C lane 8) but not in GG allele (Figure 4C, lane 10), suggesting only rs7984870 CC allele could bind to the *SOX5* transcription factor. The addition of rabbit IgG failed to produce a supershift band (Figure 4C, lanes 7 and 9). Binding to each oligonucleotide probe was competed by a 200-fold excess of unlabeled specific oligonucleotide (Figure 4C, lanes 3 and 6).

DISCUSSION

Although the role of *RANKL* in RA has been well established, little is known about the contribution of its genetic variations to RA onset. We previously observed a strong association between the combined presence of *HLA-DRB1*04* and *RANKL* polymorphisms with a younger age of RA onset in 182 RF+ EA early RA WCPR patients (21). In this extended study of 210 RF+ RA WCPR patients, we fine mapped the *RANKL* locus and observed association of the minor allele of 4 SNPs (rs5803141, rs7984870, rs9525641 and rs1054016) with younger age at RA onset. Subsequent replication studies of these 4 SNPs showed association of a single SNP in the promoter, the minor C allele of rs7984870, with younger age of RA onset in two independent cohorts (ACPA+ EA BRASS and AA CLEAR). These two independent RA cohorts were early RA (<24 months after symptom onset) long-term observational cohorts, and have an accurately documented date of RA onset similar to WCPR cohort.

In both the previous (21) and extended WCPR cohort, the association between rs9525641 and younger age of RA onset was present; however, the formerly observed RA-associated rs922996 was not confirmed. Recently, a *RANKL* intron 1 rs2277438 has been associated with RA in European population (534 cases and 516 controls) (35). This SNP was also tested in our WCPR EA cohort, but had weak LD with promoter SNP rs7984870 ($r^2 = 0.17$) and did not show the association with age of disease onset. Possible explanations for the inability to replicate *RANKL* SNPs (rs5803141, rs9525641, rs922996, rs1054016 and rs2277438) in some of the studied cohorts include genetic, ethnic, and clinical heterogeneity

across study populations as well as the probability that these SNPs may be linked to the actual *RANKL* causal allele. Several genome-wide association studies (GWAS) in RA have identified and replicated more than 20 risk loci (2;5), but have not included the RA-associated *RANKL* SNP described here. Given that the association with younger age of RA onset was only observed in CC homozygotes, we used a recessive genetic model to estimate the power of GWAS to capture this risk allele under the assumption of relative risk of 1.3 (36). A sample size of 10,000 cases and 10,000 controls could provide 65% power to reach a p value of 10^{-7}, which might explain why this SNP was not identified in previous GWAS in RA (2;5;36;37). However, the consistent presence of the observed genetic association in 3 independent panels and the functional properties of the rs7984870 allele in transcription regulation of *RANKL* in our study make this promoter polymorphism an excellent candidate for a causal variant contributing to younger age of RA onset.

SE is a recognized genetic risk predisposing to RA onset and serves as a binding site for arthritogenic peptides, allowing their presentation to CD4+ T cells. Similar to our previous report, the co-occurrence of rs7984870 risk allele and SE displayed the earliest age of RA onset in RF+ or ACPA+ RA patients. One possible hypothesis is that co-occurrence of *RANKL* polymorphisms and SE contributed to younger RA onset in ACPA+/RF+ patients by causing abnormally stimulated *RANKL* expression, which dysregulates dendritic/T cell communication and breaks the immune tolerance to self-components in the joint, thereby enhancing T-cell reactivity towards arthritogenic antigens.

A growing number of genetic variants have been reported to contribute to ACPA+ RA (6–11). In our replication studies performed in 298 AA and 501 additional EA patients, the significant genetic association of rs7984870 with earlier RA onset was only shown in the ACPA+ patients but not in ACPA− RA, suggesting that the *RANKL* allele might be a novel disease predisposing variant in ACPA+ RA subset. Alternatively, the lack of a significant association might be attributed to the modest sample size of seronegative RA patients used in these studies.

Our study provides evidence that risk C allele of rs7984870 conferred a 2-fold higher plasma soluble RANKL level in RF+ RA patients and significantly elevated *RANKL* isoform 1 mRNAs expression by activated control T cells, as well as increased promoter activity after cytokine stimulation *in vitro*. Over expression of *RANKL* results in functional alteration of epidermal DCs (38) and enhances survival of DCs (13). Transfer of RANKL-stimulated MRL/lpr DCs pulsed with collagen into MRL/lpr mice have been shown acceleration autoreactivity of T and B cells and enhance the productions of pathogenic cytokines in the recipients (13). The minor C allele of rs7984870 contributed to higher *RANKL* expression and protein level upon activation and thus, might affect initiation of immune responses between DCs and T cells. In addition, immunoreceptor tyrosine-based activation motif (ITAM) associated receptor networks have been implicated in providing co-stimulation for RANKL signaling (39); thus, another possible explanation is that genetic variants causing differences in *RANK*L expression could therefore result in abnormal cross-talk in signaling networks and alteration of cellular responses to various extracellular stimuli.

An important issue is how this *RANKL* variant affects the expression of *RANKL*. Our data suggests that the minor C allele of rs7984870 creates a binding site for transcription factor SOX5, a member of the SOX family, which might explain observed alterations in *RANKL* transcription. SOX family have been primarily characterized as important transcriptional regulators involved in determination of cell fate and tissue specification during a number of developmental processes (40). Elevated *SOX5* transcripts have been related to invasive growth and reduced apoptosis in tumors and have been associated with progression of certain cancers (41–43). Recently, *SOX6*, another member of the SOX family, which shares

91% amino acid homology in the leucine-zipper motif with SOX5, was identified as a low bone mineral density locus in a large-scale meta-analysis of GWAS studies (44). Taken together, the differential binding of the minor C allele of rs7984870 to *SOX5* transcriptional regulator might influence bone metabolism, and facilitate invasive growth and resistance to apoptosis in the synovium, leading to RA manifestations.

In summary, the association of CC genotype of rs7984870 with younger RA onset was identified and replicated in several independent cohorts in this study. This novel genetic risk factor, *RANKL* rs7984870 CC genotype, conferred elevated promoter activity after stimulation by cytokines, potentially via binding to transcription factor SOX5. Elevated inducible *RANKL* mRNA and protein levels might result in enhanced interaction between activated T cells presenting arthritogenic peptides to DCs, predisposing to earlier development of seropositive (RF+ or ACPA+) RA in EA and AA patients. Our evidence suggests that the combination of CC genotype of rs7984870 *RANKL* polymorphism, SE, and abnormal humoral immune responses interacts to permit an earlier onset of RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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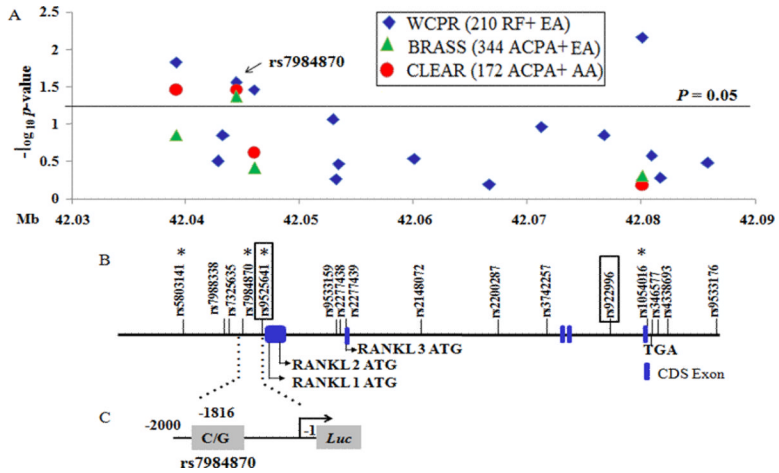

Figure 1.

RANKL structure with tested SNPs and haplotype blocks in EA and AA patients. A. The −log10 base of genotypic p value for each SNP (comparing those homozygous for the minor allele vs. those homozygous for the major allele plus heterozygote) is shown as diamond (210 RF + WCPR EA), triangle (344 ACPA + BRASS EA) and circle (172 ACPA + CLEAR AA). The 2 SNPs (rs9525641 and rs922996) showed with box is reported in our previous study. Four *RANKL* SNPs (rs5803141, rs7984870, rs9525641 and rs1054016) in WCPR EA RA patients showed significant association with early age RA onset, and these 4 SNPS were selected for replication studies. B. The relative position of 5 exons of *RANKL* is depicted. The start (ATG) and the end (TGA) of the ORF (Open Reading Frame) are indicated on *RANKL*1, 2 and *RANKL*3. C. Schematic structure of *RANKL* promoter luciferase (Luc) reporter gene construct.

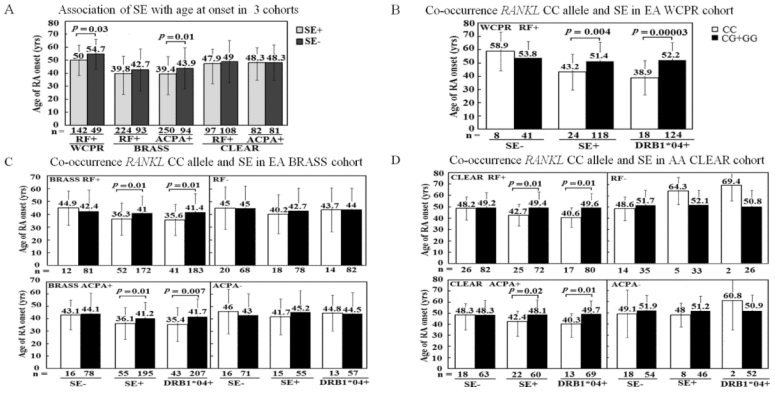

Figure 2.

Association of shared epitope (SE) and *RANKL* rs7984870 CC allele with age of RA onset. A. Association of SE with age of RA onset in seropositive WCPR, BRASS and CLEAR early RA cohort. B–D. Co-occurrence rs7984870 CC allele and SE negative, SE positive, as well as DRB1*04 positive with age at RA onset in seropositive and seronegative WCPR (B), BRASS (C) and CLEAR (D) early RA cohort. SE was defined as presence of any one the following HLA-DRB1 alleles: *0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001, *1402 and *1406. HLA–DRB1*04 alleles included *0401,*0404, *0405, *0408, and *0410. Mean age of RA onset in each group is shown on the top of column.

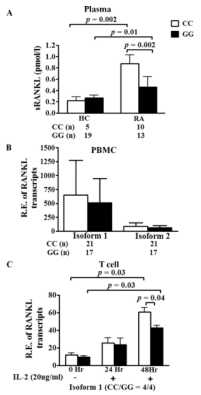

Figure 3.

Association of *RANKL* genotypes with expression levels of mRNA and secreted protein. A. Soluble RANKL levels from serum samples of healthy controls (HC) who were homozygote for either the minor C allele or the major G allele of *RANKL* promoter SNP rs7984870 (CC = 5 vs. GG = 19), and those from WCPR RA patients at study entry before DMARD treatments (CC = 10 vs. GG = 13). Data are means of triplicate experiments ± SD. B. Relative mRNA expression levels of two *RANKL* isoforms in PBMCs from HC who were homozygous for the minor C allele or the major G allele of rs7984870 (CC = 17 vs. GG = 21). C. Relative mRNA expression levels of *RANKL* isoform 1 in isolated T cells from HC, who were homozygous for the minor C allele or the major G allele of rs7984870 (CC = 4 vs. GG = 4), were determined at various time points during co-culture with or without IL-2. The mRNA expression results represent the mean ± SD of two independent experiments performed in triplicate.

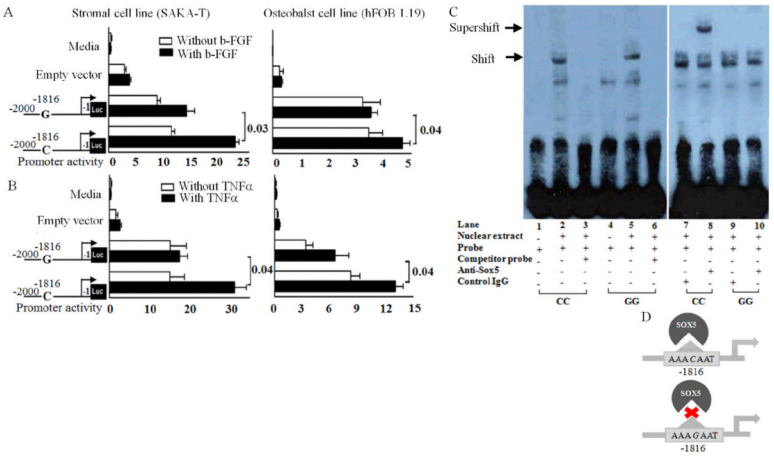

Figure 4.

Allelic differences in transcriptional and DNA-binding properties of rs7984870 variants. A–B. SAKA-T (left) and hFOB 1.19 (right) cell lines were transfected with a luciferase (*Luc*) reporter gene driven by *RANKL* promoter sequences (−2000/−1) containing either the CC or GG allele of rs7984870 and cultured in the presence and absence of b-FGF (4 ng/ml) (A) or TNFα (10 ng/ml) (B). Luciferase activity is displayed as the mean ± SD of three independent experiments performed in triplicate. C. Shift, and supershift and competition experiments with Jurkat nuclear cell extract and allelic variants of rs7984870. The 28-bp oligonucleotide containing the rs7984870 CC or GG allele were incubated with nuclear extracts prepared after stimulation with TNFα for 24h. The band that was either a shift (below) or supershift (above) was indicated by an arrow. D. Proposed model of interaction between the allelic variants of rs7984870 with the transcription factor *SOX5*.

Table 1

Clinical characteristics of the RA and JIA patients

	RA			JIA
Clinical characteristics	**EA WCPR (n=210)**	**EA BRASS (n=501)**	**AA CLEAR (n=298)**	**EA RF+ Polyarticular JIA (n=80)**
Female (n, %)	157 (74.8%)	403 (80.4%)	246 (82.6%)	69 (86.3%)
Age at disease onset (yrs)*	51.4 ± 13.1	41.8 ± 15.2	49.8 ± 13.3	10.4 ± 4.0
Baseline disease duration* (months)	6.1 ± 3.3	9.9 ± 6.2	13.2 ± 7.4	-
RF+ (n, %)	210 (100%)	317 (63.3%)	211 (70.8%)	80 (100%)
ACPA+ (n, %)	-§	344 (68.7%)	172 (57.8%)	-

*Mean ± SD

§Patients recruited from 1993, no ACPA record.

Table 2

Association of *RANKL* rs7984870 risk allele with younger age at RA onset in WCPR, CLEAR and BRASS cohorts

RA Cohort (n)	Age of onset Mean ± SD (yrs)					
	Seropositive			Seronegative		
	CC (n)	CG+GG (n)	p	CC (n)	CG+GG (n)	p
	RF +			RF −		
WCPR (210)	47.1 ± 14.6 (37)	52.3 ± 12.6 (173)	0.03		-	
BRASS (317)	37.9 ± 13.0 (64)	41.4 ± 14.5 (253)	0.08	42.3 ± 15.9 (38)	44.0 ± 16.9 (146)	0.58
CLEAR (211)	46.3 ± 10.9 (53)	49.8 ± 13.6 (158)	0.09	53.9 ± 12.3 (21)	51.1 ± 14.6 (50)	0.42
Joint analysis			0.01			-
	ACPA +			ACPA −		
BRASS (344)	37.6 ± 12.8 (71)	41.4 ± 14.6 (273)	0.04	43.4 ± 17.1 (31)	43.9 ± 16.2 (126)	0.90
CLEAR (172)	44.8 ± 10.6 (40)	49.6 ± 12.9 (132)	0.04	49.6 ± 13.5 (31)	52.5 ± 14.8 (82)	0.43
Joint analysis			0.01			-