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BRIEF REPORT

Associations between *HLA-DRB1*, *RANK*, *RANKL*, *OPG*, and *IL-17* genotypes and disease severity phenotypes in Japanese patients with early rheumatoid arthritis

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Abstract We examined associations between human leukocyte antigen DRB1 (*HLA-DRB1*) shared epitope (SE), receptor activator of nuclear factor-kappaB (*RANK*), RANK ligand (*RANKL*), osteoprotegerin (*OPG*), and interleukin 17 (*IL-17*) genotypes with age of disease onset and radiographic progression in Japanese patients with early rheumatoid arthritis (RA). *HLA-DRB1* genotypes were evaluated in 123 patients with early RA (98 female, 25 male) within 1 year of symptom onset. In 72 patients, radiographic progression over a 2-year period was evaluated using Larsen's methods, and genotypes of three polymorphic sites in *RANK*, five sites in *RANKL*, two sites in *OPG*, and three sites in *IL-17* were determined by direct polymerase chain reaction sequencing. Possession of an SE allele was significantly associated with earlier disease onset in females (median 46.9 vs 51.9 years in SE− patients; P=0.04). Single nucleotide polymorphisms (SNPs) in *RANKL* (rs2277438, P=0.028) and *IL-17* (rs3804513, P=0.049) were significantly associated with radiographic progression at 2 years. *RANKL*-G−, SE− patients (n=12) had significantly less joint damage than did *RANKL*-G+, SE− patients (n=11; P=0.0038), *RANKL*-G−, SE+ patients (n=21; P=0.0018) and *RANKL*-G+, SE+ patients (n=28; P=0.0024). In Japanese RA patients, *HLA-DRB1* SE alleles are associated with disease onset at an earlier age, as has been observed in Caucasian RA patients. In addition, SNPs in *RANKL* and *IL-17* may be associated with radiographic progression in Japanese patients with early RA.

Keywords IL-17 · Japanese · RANKL · Rheumatoid arthritis · Shared epitope · Single nucleotide polymorphism

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Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease that primarily affects the joints of the hands and feet. Many studies have suggested that at least some of the genetic risk for RA can be explained by the presence or absence of a 'shared epitope' (SE) in various class II human leukocyte antigen (HLA)-DR molecules [1]. We previously reported associations between SE and susceptibility to polyarthritis [2] and between SE and production of anti-cyclic citrullinated peptide (CCP) antibodies in Japanese patients with early RA [3]. Although previous studies have demonstrated a correlation between the presence of SE and RA onset at an earlier age [4–7], no studies have examined this relation in Japanese RA patients.

Despite the strong association with HLA [1–3], it is clear that multiple other genes are also involved in determining RA risk, as genome scan analyses have provided evidence

for multiple non-HLA susceptibility loci on many chromosomes [8–10]. We reported in previous studies that the receptor activator of nuclear factor-kappaB (RANK), RANK ligand (RANKL), osteoprotegerin (OPG), and interleukin 17 (IL-17) contribute to osteoclastic bone resorption in RA patients [11, 12]. *RANK*, which is located at 18q22, appears to be one of the candidate genes for RA susceptibility/severity, based on the results of genome-wide linkage studies of RA sibpairs [8, 9] and an animal RA model [13]. Wu et al. [7] reported that in RA patients, an intronic single nucleotide polymorphism (SNP; rs922996) in the *RANKL* gene was associated with an earlier age of disease onset in SE+ patients but not with radiographic progression.

In the present study, we examined the correlations between SE and age at RA onset, SNPs of the above-mentioned genes, and radiographic progression at 2 years in Japanese patients with early RA. Herein, we demonstrated significant associations between SE alleles and disease onset at an earlier age, as well as between three SNPs in the *RANKL* and *IL-17* genes and radiographic progression.

Materials and methods

Patients

One hundred and twenty-three RA patients (98 female, 25 male) who visited the outpatient clinic of the Institute of the Rheumatology, Tokyo Women's Medical University, within 1 year of symptom onset were consecutively enrolled in this study, as described previously [2]. Associations between their *HLA-DRB1* genotypes and radiographic progression over a 1-year period [2] and between *HLA-DRB1* genotypes and expression of anti-CCP antibodies [3] have been reported previously. All patients satisfied the 1987 classification criteria for RA [14] at presentation (n=51) or during the follow-up period (n=72). Patients were enrolled in a prospective follow-up study from 1991 to 1995, and each provided informed consent before participation. Patients with active disease were offered treatment with disease-modifying antirheumatic drugs (DMARDs) throughout the 2-year study duration. During the 2 years, D-penicillamine (36%) was most commonly used among DMARDs. Only a small portion (12.5%) and none of RA patients received methotrexate and biologicals because these agents were not officially approved for RA in Japan until 1999 and 2003, respectively.

Radiographic progression

Follow-up visits generally occurred at 4-week intervals. Radiographs of the hands/wrists and feet were taken at the first visit, at 1 year, and at 2 years. Radiographs of the hands and feet at baseline and at 2 years were available for 72 (58 female, 14 male) of the 123 RA patients included in this cohort. Radiographs were assessed using Larsen's methods [15], as described in our previous study [2]. A Larsen score greater than or equal to grade 2 was selected as the criterion for the presence of erosions. Progression of radiographic damage, ΔLarsen score, was calculated by subtracting the baseline score from the 2-year follow-up score for each of the 72 patients.

Biochemical analysis

Laboratory parameters were measured for each patient every 4 weeks; these included the erythrocyte sedimentation rate (mm/h; Westergren method), C-reactive protein level (in mg/dL), and rheumatoid factor (RF). RF status was assessed using a particle agglutination test (RAPA test; Fujizoki Pharmaceutical, Tokyo, Japan). Plasma samples obtained between 1992 and 1995, which had been stored at −20°C, were used for analysis of anti-CCP antibodies and the bone resorption marker tartrate-resistant acid phosphatase (TRACP) 5b [16]. A second-generation anti-CCP enzyme-linked immunosorbent assay kit (DIASTAT Anti-CCP) was purchased from Axis-Shield, Dundee, UK. A cutoff value of greater than 5 U/mL was used to indicate a positive result. Plasma concentrations of TRACP 5b were determined using a commercial immunoassay kit (Bone-TRAP; SBA-Sciences, Turku, Finland). Assays were conducted according to the manufacturer's instructions. Anti-CCP antibodies and TRACP 5b were not evaluated in 13 patients because of a lack of plasma samples.

Genotyping

After obtaining informed consent from all subjects, peripheral blood was drawn. Genomic deoxyribonucleic acid was extracted from leukocytes using a standard phenol–chloroform extraction procedure. The *HLA-DRB1* genotype was determined in all patients using the polymerase chain reaction (PCR)–restriction fragment length polymorphism method [17]. Using the Japanese public database (Japanese Single Nucleotide Polymorphism [JSNP], http://snp.ims.u-tokyo.ac.jp) [18], we selected three SNPs in the *RANK* gene (rs3810024, rs3826618, and rs3826619), five in the *RANKL* gene (rs2296533, rs2277438, rs2277439, rs3742257, and rs3742258), two in the *OPG* gene (rs2073617 and rs2073618), and three in the *IL-17* gene (rs3804513, rs3748067, and rs1974226) for genotyping. All of these SNPs were detected by direct PCR sequencing according to the methods of the JSNP in the 72 patients whose radiographs of the hands and feet at baseline and 2 years were available.

Statistical analysis

Statistical significance of differences between groups was determined using a Mann–Whitney *U* test and Kruskal–Wallis test (continuous variables). Pearson's product moment correlation coefficient was used to access correlations between pairs of continuous variables. *P* value less than 0.05 was considered significant.

Results

Baseline characteristics

Baseline patient characteristics are shown in Table 1. Median patient age was 51.0 years, 79.7% were women, median disease duration was 5.4 months, and 87.0% were RF positive. Anti-CCP antibodies were present in 82 (74.5%) patients at baseline. The median level of plasma TRACP 5b was 1.5 U/L.

SE and age at RA onset

The presence of SE was significantly associated with disease onset at an earlier age in female patients (P=0.04; Table 2). In male patients, the age at RA onset tended to be earlier in patients with SE compared to those without SE, although the difference was not statistically significant. The age at RA onset was not significantly different between males and females among patients with SE, although the difference was significant (P=0.01) among patients without SE.

Table 1 Baseline characteristics of 123 Japanese patients with early rheumatoid arthritis

Patient characteristics	Value
Age, years (range)	51.0 (41.4 to 58.7)
Gender (% female)	98 (79.7%)
Age at rheumatoid arthritis (RA) onset, years (range)	50.8 (41.2 to 58.2)
Disease duration, months (range)	5.4 (3.0 to 8.5)
Rheumatoid factor positive (%)	107 (87.0%)
Erythrocyte sedimentation rate, mm/h (range)	45.5 (28.9 to 63.9)
C-reactive protein, mg/dL (range)	0.7 (0.4 to 3.1)
Anti-CCP[a] present (%)	82 (74.5%)
TRACP 5b level[a], U/L (range)	1.5 (1.2 to 2.0)
Larsen score 0–180 (range)	4 (1.0 to 5.25)
Number of positive RA criteria at baseline (%)	51 (41.5%)

Values are median (25th to 75th percentile) or *n* (%).
Anti-CCP Antibodies to cyclic citrullinated peptides, *TRACP* tartrate-resistant acid phosphatase
[a] Anti-CCP antibodies and TRACP 5b levels were examined in 110 patients.

SNPs in the *RANK*, *RANKL*, *OPG*, and *IL-17* genes as disease progression markers

Among the 13 SNPs in the *RANK*, *RANKL*, *OPG*, and *IL-17* genes, three—two in a *RANKL* intron (rs2277438 and rs2277439) and one in an *IL-17* intron (rs3804513)—were significantly associated with joint destruction (Table 3). The two polymorphic sequences of the *RANKL* gene were found to be in strong linkage disequilibrium. Significantly increased joint destruction was observed in RA patients with *RANKL*-rs2277438 AG plus GG genotypes (n=39) compared to patients with the AA genotype (n=33; P=0.028). Patients with the *IL-17*-rs3804513 AA genotype (n=2) had significantly more destruction compared to those with AT plus TT genotypes (n=70; P=0.049). Plasma TRACP 5b levels of the two patients with the AA genotype were 0.5 and 1.3 U/L, respectively. The amount of joint destruction did not differ significantly between different genotypes, alleles, or haplotypes of the *RANK* or *OPG* genes (data not shown). Plasma TRACP 5b levels did not differ significantly between different genotypes, alleles, or haplotypes of the *RANK*, *RANKL*, *OPG*, or *IL-17* genes (data not shown). TRACP 5b levels negatively correlated with ΔLarsen score (r=−0.39, P=0.0017) in our study (data not shown). Treatment strategy, mean age, and gender were approximately equally distributed among each group. The number of the patients who had been treated with methotrexate was not significantly different between in the patients with *RANKL*-rs2277438 AG plus GG genotypes (n=6, 15.4%) compared to those with the AA genotype (n=3, 9.1%).

We analyzed the interaction between SE and *RANKL* haplotype in predicting joint damage progression, as a previous report demonstrated an interaction between SE and another SNP in the *RANKL* gene [7] (Table 3). *RANKL*-G−, SE− patients (n=12) had significantly lower joint damage than did *RANKL*-G+, SE− patients (n=11; P=0.0038), *RANKL*-G−, SE+ patients (n=21; P=0.0018), and *RANKL*-G+, SE+ patients (n=28; P=0.0024). Among these four patient groups, the number of the patients who had been treated with methotrexate was not significantly different, and mean age and gender were approximately equally distributed (data not shown).

Discussion

In the current study, we have demonstrated that RA patients with the *RANKL*-G allele (rs2277438) have significantly more severe joint damage than those who do not possess this allele (Table 3). This result is particularly interesting in light of our previous finding that RANKL contributes to osteoclastic bone resorption in RA patients [11]. Recently, Hsu et al. [19] found a significant correlation between the

Table 2 Age of disease onset according to the presence or absence of SE in 123 Japanese patients with early rheumatoid arthritis

	All	SE		P^b
		+	–	
Female patients (range)	49.9 (40.6 to 55.6) (n=98)	46.9 (38.4 to 54.5) (n=63)	51.9 (43.7 to 58.4) (n=35)	0.04
Male patients (range)	56.0 (47.7 to 65.7) (n=25)	54.1 (41.3 to 63.7) (n=15)	61.9 (54.0 to 66.5) (n=10)	NS
P^a	0.005	NS	0.01	

Values are median (25th to 75th percentile).
SE Shared epitope, *NS* not significant
[a] For comparisons between women and men
[b] For comparisons between patients with and without SE

rs2277438 SNP in the *RANKL* gene and bone mineral density. Thus, these results together suggest that this SNP may have some effect on bone, although future studies are required to confirm this.

In this study, we have also shown that RA patients lacking the T allele of rs3804513 SNP in the *IL-17* gene had more destruction compared to patients with this allele (P=0.049; Table 3). These results suggest that the T allele may have some protective effect against radiographic progression in RA patients. Previously, we reported that IL-17 is a crucial cytokine for osteoclastic bone resorption in RA patients [12]. It may therefore be interesting to analyze the functional significance of this *IL-17* polymorphism, although herein we did not find any significant correlation between TRACP 5b values and this polymorphism. A larger-sample size study is required for such an analysis, as it appears most Japanese patients (e.g., 70 of 72 in this study) have the T allele.

We have confirmed other reports of an association between SE and disease onset at an earlier age, which has previously only been reported in Caucasian [4, 6, 7] and Spanish [5] patients. We found that in female RA patients, the age of disease onset is 5 years earlier in patients with SE compared to patients without SE (Table 2). This is the first study to show an association between age of RA onset and SE in Japanese patients, although Yukioka et al. [20] reported positive associations between *DRB1*0101*, **0405*, and elderly-onset RA in Japanese.

Wu et al. [7] reported that anther intronic SNP (rs922996) in the *RANKL* gene is associated with an earlier age of RA onset in SE+ patients but not with radiographic progression. We did not analyze this SNP in this study because we could not find any information about it in the JSNP [18]. However, we did not find any associations between other SNPs in the *RANKL* gene and age of disease onset (data not shown).

Despite the advantages of this prospective cohort study conducted at a single institute, this study also has some limitations. First, we did not analyze the association of disease activity score 28 (DAS28) [21] or health assessment questionnaire (HAQ) [22] with SNPs, as we did not collect DAS28 or HAQ data during the baseline period (1991–1995). Second, the number of patients in our study was smaller than the previous study that evaluated the association between *RANKL* genotypes and RA severity [7]. Future studies with large sample sizes are required to demonstrated firm conclusions. Third, we used Larsen methods [15] to evaluate radiographic progression of early RA, as has been done in previous studies [2, 3, 23]. However, this method is reported to be less sensitive than Sharp/van der Heijde methods [24], which have become more commonly used in recent years [25].

Table 3 Associations between *RANKL* and *IL-17* genotypes and presence/absence of SE with ΔLarsen score in Japanese patients with early rheumatoid arthritis (n=72)

	Number	Median ΔLarsen score (IQR)	P
RANKL (rs2277438)			
AG plus GG	39	12.0 (7.0, 22.0)	
AA	33	8.0 (3.0, 14.0)	0.028
IL-17 (rs3804513)			
AA	2	34 (26.5, 41.5)	
AT plus TT	70	9 (4.25, 15)	0.049
RANKL-G (rs2277438) and SE			
RANKL-G–, SE–	12	3.0 (1.0, 7.3)	
RANKL-G+, SE–	11	12.0 (7.0, 24.0)	0.0038[a]
RANKL-G–, SE+	21	11.0 (5.0, 15.0)	0.0018[a]
RANKL-G+, SE+	28	12.0 (6.8, 21.5)	0.0024[a]

SE Shared epitopes, *IQR* interquartile range (25th, 75th percentiles)
[a] Versus *RANKL-G–*, SE–

In conclusion, in Japanese patients with RA, SE alleles are associated with an earlier age at disease onset, as has been previously observed in Caucasian RA patients. In addition, SNPs in genes related to osteoclastic bone resorption may be predictors of radiographic progression in Japanese patients with early RA.

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