ETS1 polymorphism rs73013527 in relation to serum RANKL levels among patients with RA

Bin Yang, PhDa, Limei Luo, MMa, Lin Chen, MMb, Qian Niu, MDa, Junlong Zhang, PhDa, Huan Xu, BMa, Yifeng Wu, BMa, Zhuochun Huang, MDa,b

Abstract
We previously identified E26 transformation specific sequence 1 (ETS1) rs73013527 single nucleotide polymorphism associated with RA susceptibility and disease activity. In the present study, we aim to further investigate the association between ETS1 rs73013527 and receptor activator of nuclear factor kappa B ligand (RANKL), an index related to bone destruction and was reported to elevate in RA.

We determined genotypes of ETS1 rs73013527, serum RANKL concentration, clinical characteristics (disease duration, disease activity score for 28 painful/swollen joints), and laboratory markers (rheumatoid factor, anti-citrullinated protein antibody, anti-keratin antibody, c-reactive protein, erythrocyte sedimentation rate) of 254 RA cases. Univariate and multivariate analysis were employed to explore the association between ETS1 rs73013527 and serum RANKL levels in RA patients.

Univariate and multivariate analysis indicated no association of serum RANKL levels with patient age, gender, clinical characteristics, and laboratory markers. Univariate analysis, not multivariate analysis indicated genotype CT/TT of ETS1 rs73013527 was significantly associated with elevated RANKL levels in RA patients. ETS1 rs73013527 is in relation to serum RANKL levels among patients with RA. ETS1 probably might be an indirect factors involved in RANKL regulation in RA.

Abbreviations: ACPA = anti-citrullinated protein antibody, AKA = anti-keratin antibody, CRP = c-reactive protein, DAS28 = disease activity score for 28 painful/swollen joints, ESR = erythrocyte sedimentation rate, ETS1 = E26 transformation specific sequence 1, RANKL = receptor activator of nuclear factor kappa B ligand, RF = rheumatoid factor.

Keywords: E26 transformation specific sequence 1, receptor activator of nuclear factor kappa B ligand, rheumatoid arthritis

1. Introduction
Rheumatoid arthritis (RA) is a progressive systemic autoimmune disease, characterized by the destruction and remodeling of joints and surrounding tissues. Systemic inflammation and abnormality of bone metabolism are both relevant aspects in RA disease. Receptor activator of nuclear factor kappa B ligand (RANKL), a member of tumor necrosis factor (TNF) superfamily, is the most essential factor for osteoclastogenesis by stimulation of osteoclast precursor cells differentiation, prompting osteoclasts migration, fusion, activation, and survival. Meantime, RANKL, which also resides on the surface of activated T-cell lymphocytes, is considered to function in regulating adaptive immunity. In addition, RANKL-expressing T cells can influence osteoclastogenesis. RANKL is clarified to play roles in immunity and bone metabolism simultaneously. Dual function of RANKL in bone homeostasis and immunity seeded a rapidly growing field dubbed osteoimmunology. RANKL is a crucial member in osteoimmunology. Considering function of RANKL in regulating immunity and osteoclastogenesis, accumulation researches demonstrated RANKL involved in RA inflammation and bone erosion. In fact, increased serum RANKL concentration has been observed in RA patients.

E26 transformation specific sequence 1 (ETS1) is the founding member of ETS family of transcription factors and plays roles in a wide variety of cellular processes including angiogenesis, fibroblast activation, and regulating expression of cytokine and chemokine genes. Besides, ETS1 is also reported to be involved in bone metabolism. Numerous studies have demonstrated that ETS1 participates in osteoblast differentiation and bone development. ETS1 is elucidated to have pronounced relation to RA. Single nucleotide polymorphisms (SNP) in ETS1 were reported to associate with RA in Caucasians and
European.[19,20] We also revealed ETS1 polymorphism rs73013527 significantly related to RA susceptibility and disease activity in Chinese. Our published study demonstrated that percentages of allele T (26.9%) and genotypes CT/TT (44.9%) were significantly lower in Chinese Han population with RA than in healthy controls (T: 41.9% and CT/TT: 65.6% in healthy controls). Further correlation analysis indicated allele T of ETS1 rs73013527 might be a risk factor associated with disease activity in RA.[21,22] However, like many other risk alleles for complex diseases, little is known about the mechanism through which the genetic variants affect RA patients. Some studies suggested ETS1 may relate to joint destruction in RA.[11,22]

Therefore, in the present study, we assessed whether ETS1 rs73013527 correlated to RANKL levels in RA, aimed to explore the potential link between ETS1 and RANKL. For more precise analysis, putative confounders for RANKL levels, such as patient age, sex and anti-citrullinated protein antibody (ACPA) were also taken into consideration in the study.

2. Materials and methods

2.1. Participants

Our study recruited 254 RA patients in West China Hospital. All the patients fulfilled rheumatoid arthritis criteria 2010 released by ACR/EULAR[23] and signed informed consents. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of West China Hospital.

2.2. Ets-1 polymorphism genotyping

SNP rs73013527 of ETS1 was genotyped by using polymerase chain reaction-high resolution melting and genotype analysis was performed on Light Cycler 480 (Roche Diagnostics, Penzberg, Bavaria, Germany).

Genomic DNA extraction: Genomic DNA of every patient was extracted from peripheral blood sample by using Genomic DNA kit (Biotake Corporation, Beijing, China) and the concentration was checked by Nanodrop 2000c spectrophotometer (Thermo Scientific, DE).

Polymerase chain reaction-high resolution melting genotyping: The study adopted 20 μL reaction system contained 10 μL Roche Master Mix (Roche Applied Science, Mannheim, Germany) which comprised FastStart Taq DNA polymerase and the High Resolution Melting Dye in a reaction buffer, 2.4 μL 25 mM MgCl2, 0.2 μL 10 μmol/L Forward Primer and 0.2 μL 10 μmol/L Reverse Primer, 6.2 μL deionized water and 1 μL DNA sample as recommended by the manufacturer. The whole genotyping process encompassed 4 steps: pre-denaturation, amplification, high resolution melting, and cooling. When finished, the results were analyzed by the corresponding Gene Scanning Software v1.2 (Roche Diagnostics, Germany).

2.3. Laboratory assays

Serology markers of RA were measured by following methods. Rheumatoid factor (RF) and c-reactive protein (CRP) were tested using Beckman Coulter IMMAGE 800 immunoassay (Beckman Coulter, Inc, CA). ACPA were tested by the Elecsys Anti-CCP assay (second-generation assay for anti-cyclic citrullinated peptides, Roche Diagnostics, GmbH, Mannheim, Germany). Anti-keratin antibody was tested by indirect immunofluorescence (Euroimmun, Germany). Peripheral whole blood erythrocyte sedimentation rate (ESR) was tested by analyzer “test-1” (Alifax, Italy). All the tests were conducted in accordance with manufacturers’ instruction. Serum RANKL was tested by ELISA (Cat#:EHC162, NeoBioscience, China).

2.4. Statistical analysis

Genotype analysis and disease model analysis were executed. Allele T was the minor allele at ETS1 rs73013527. It also applied to disease models-dominant model (CT + TT vs CC) and recessive model (CC + CT vs TT). The difference of serum RANKL between/among groups was analyzed by using Mann-Whitney U or Kruskal-Wallis tests. Correlations between RANKL and RA clinical characteristics were tested with Spearman test. In order to investigate the association between SNP rs73013527 on RANKL serum levels with respect to gender, ACPA, and CRP, liner regression (method: inclusion) was applied. All statistical analyses were performed by using the Statistical Package for the Social Sciences (version 17.0 software, SPSS, SPSS Inc., Chicago, IL). A P value <.05 was considered significant.

3. Results

3.1. Characteristics of study population

A total of 254 RA patients were included in the study. Demographic and clinical characteristics as well as distribution of genotypes and alleles of ETS1 rs73013527 investigated are presented in Table 1. More female than male were shown in patients. Disease activity score for 28 painful/swollen joints (DAS28) ranged from 1.38 to 8.56, covering patients with disease remission and patients with disease active.

Lower frequencies for ETS1 rs73013527 allele T and genotype TT were found than for allele C or CC genotype. More than half patients were carriers of the CC genotype (Table 1).

3.2. Demographic, clinical, and genetic associations with serum RANKL levels

3.2.1. Univariate analyses. Serum RANKL concentration was examined with regard to demographic, clinical, and genetic factors. Univariate analyses shown in Table 2 suggested no influence of demographic and clinical parameters on RANKL serum levels in RA patients, except CRP. Though RANKL seemed to associate with CRP (P = .03), however, correlation coefficient between them was only 0.164, which also meant no correlation. Further, genotype factor analysis showed RANKL serum levels differed among CC, CT, and TT genotypes. Especially, dominant model analysis suggested patients with genotype CT/TT had elevated serum RANKL levels.

3.2.2. Multivariate analysis. Univariate analyses only suggested ETS1 rs73013527 genotype CT+TT were associated with RANKL serum levels. CRP seemed to correlate to RANKL levels for P value was .03 despite correlation coefficient was 0.164. Though univariate analyses showed no correlation of gender and ACPA with RANKL serum levels, it was suggested in publications that female sex hormones could influence RANKL axis and RANKL elevated in ACPA-positive patients. Therefore, gender (female), ACPA (≥ 51 U/ml), CRP, and ETS1 rs73013527 (CT+TT) were included in multivariate analysis in our study. Nevertheless, none of above parameters showed correlation to serum RANKL levels in multivariate analysis (Table 3).
Table 1
Important characteristics of the patients with RA.

| Characteristics       | RA patients (n = 254) |
|----------------------|----------------------|
| Demographic parameters |                      |
| Age, mean ± SD (yrs)  | 53.5 ± 12.7          |
| Female (%)/Male (%)  | 76.4/23.6            |
| Clinical characteristics |                      |
| Age at onset, mean ± SD (yrs) | 43.6 ± 14.7  |
| Disease duration, mean ± SD (yrs) | 10.1 ± 9.8  |
| DAS28, mean ± SD | 6.12 ± 1.43          |
| RF > 20 IU/ml (%)    | 62.28                |
| ACPA > 51 U/ml (%)  | 79.92                |
| AKA-positive (%)     | 42.91                |
| CRP (mg/L), median (interquartile range) | 17.20 (6.47–50.30) |
| ESR (mm/h), median (interquartile range) | 62.50 (42.00–99.75) |
| ETS1 rs73013527      |                      |
| Genotype frequencies (%) |                |
| CC                   | 54.3                 |
| CT                   | 37.0                 |
| TT                   | 8.7                  |
| Allele frequencies   |                      |
| C                    | 72.8                 |
| T                    | 27.2                 |
| Disease model        |                      |
| Dominant model       |                      |
| CT + TT              | 45.7                 |
| CC                   | 54.3                 |
| Recessive model      |                      |
| TT                   | 8.7                  |
| CC + CT              | 91.3                 |

ACPA = anti-citrullinated protein antibody, AKA = anti-keratin antibody, CRP = C-reactive protein, DAS28 = disease activity score for 28 painful/swollen joints, ESR = erythrocyte sedimentation rate, RF = rheumatoid factor.

4. Discussion

In previous studies, ETS1 rs73013527 was identified to associate to RA susceptibility.[20,21] The allele T of ETS1 rs73013527 plays a risk factor in the high disease activity in RA patients.[21] As increased RANKL level was observed and recognized to stand a pivotal role in dysregulating the bone remodeling cycle, and causing joint destruction in the pathological processes of RA,[12,11,24,23] our study aimed to investigate whether ETS1 rs73013527 is associated with RANKL concentrations among patients with RA. Other factors possibly influencing RANKL levels needed to be taken into consideration, too.

Univariate analyses (Table 2) investigated some possible predictors for RANKL and suggested no association of serum RANKL concentration with patient age, gender, clinical characteristics (disease duration, DAS28), and laboratory markers (RF, ACPA, AKA, ESR). Though P value of correlation between RANKL and CRP was 0.03, the correlation coefficient was 0.164 only, which also meant no correlation. These findings were similar with Aase Haj Hensvold group’s report, which suggested age, sex, DAS28, ESR, RF, and CRP were not significant predictors for serum RANKL level in RA patients.[11] However, they reported RANKL elevated in ACPA-positive patients.[11] This finding was opposite to our univariate analysis results. Additionally, it is accepted the expression and function of RANKL axis are influenced by the female sex hormones.[26]

Consider publication report and significant P value of correlation analysis between CRP and RANKL, gender (female), ACPA, and CRP were included in multivariate analysis in our study. Data in Table 3 confirmed no association of gender (female), ACPA, and CRP with serum RANKL levels. Patient disease duration less than 1 year (in contrast to medium disease duration 6.5 years in our study) and actually detection of anti-cyclic citrullinated peptide version 2 (anti-CCP2, CCPlus Immunoscan, Malmö, Sweden) test (in contrast to detection of the Elecsys Anti-CCP assay/second-generation assay for anti-cyclic citrullinated peptides, Roche Diagnostics, GmbH, Mannheim, Germany in our study) might be partially explanations for this discrepancy between our findings and Aase Haj Hensvold group’s report. Furthermore, our study showed no association between serum RANKL concentration and patient gender. This may be partially explained by Bord S et al’s study reported estrogen influenced osteoclastogenesis by directly increasing the level of OPG and to a lesser extent that of RANKL mRNA and protein, in human osteoblasts.[27]

Genetic polymorphism analysis in univariate analyses suggested probable correlation of ETS1 polymorphism rs73013527 to serum RANKL concentration in RA patients (Table 2).
RANKL expression differed among CC, CT, and TT genotypes. RA patients carrying allele T of ETS1 rs73013527 were observed to have higher serum level of RANKL. Disease model analysis further validated this finding. In dominant model, RA patients carrying rs7301527 CT and TT genotypes had higher serum level of RANKL compared with carrying CC genotype. In recessive model, though statistical difference did not suggest, patients carrying TT genotype also had increased serum level of RANKL than those carrying CC and CT genotypes. In conclusion, univariate analyses did suggest ETS1 rs73013527, especially genotype CT+TT, associated with RANKL serum levels in RA patients. However, surprisingly, when taken into multivariate analysis, the significant correlation between ETS1 rs73013527 and RANKL level disappeared. The contradiction between univariate and multivariate analysis made us realize that the influence of ETS1 rs73013527 on RANKL serum levels was indirect. There might be other factors that could directly influence RANKL serum levels and associated with ETS1 rs73013527. The probable factors might be interleukin 17 (IL-17) and IL-2. Accumulated evidences pointed out that ETS1 played pivotal role in regulating expression of cytokine and chemokine genes in a wide variety of different cell lineages. Meantime, studies suggested an essential role for the cytokine network in terms of RANKL induction.\(^{25,28,29}\) ETS1 can negatively regulate the expression of RANKL and osteoprotegerin (OPG) genes, which are involved in RANKL regulation in RA, which may supply new ideas to explore the regulatory role of ETS1 in RA disease and are also beneficial for bone erosion study in RA.

**Table 3**

Multivariate analysis for serum RANKL levels.

| Model 1 | Non-standardised coefficients | Standard error | Standardized coefficients | t | Sig. |
|---------|--------------------------------|----------------|--------------------------|---|------|
| (constant) | 26.222 | 8.679 | 3.021 | .003 |
| Gender (male) | 8.050 | 2.079 | 3.869 | .002 |
| ACPA >51 (U/ml) | –3.057 | 1.711 | –0.041 | –0.502 | .62 |
| CRP (mg/L) | 7.171 | 0.954 | 0.091 | 1.116 | .27 |
| ETS1 rs73013527 CT+TT | 6.049 | 4.989 | 0.098 | 1.212 | .22 |

Dependent variable: RANKL (pg/ml).

In fact, till now, studies on ETS1 in RA are few, little is known about how ETS1 plays role in RA disease. Our study explored the association between ETS1 SNP and RANKL serum levels. Combined univariate and multivariate analysis, our results suggested an indirect influence of ETS1 rs73013527 on RANKL serum levels in RA. Probably, ETS1 might influence RANKL serum levels via cytokine network, especially IL-17 which notably elevated in RA patients. Certainly, the definitive association between ETS1 and RANKL in RA demands larger sample size and multicenter study. And the exact mechanism underlying influence of ETS1 on RANKL still requires careful and rigorous investigation. However, our study makes a meaningful attempt to explore the association between ETS1 and RANKL serum levels. Our results indicated ETS1 probably might be an indirect factor involved in RANKL regulation in RA, which may supply new idea to explore the regulatory role of ETS1 in RA disease and are also beneficial for bone erosion study in RA.

**Author contributions**

Conceptualization: Bin Yang, Lin Chen, Qian Niu, Zhuochun Huang.

Data curation: Limei Luo, Lin Chen, Junlong Zhang, Huan Xu, Yifeng Wu.

Formal analysis: Limei Luo, Qian Niu, Junlong Zhang, Zhuochun Huang.

Funding acquisition: Bin Yang, Zhuochun Huang.

Investigation: Limei Luo, Zhuochun Huang.

Methodology: Bin Yang, Lin Chen, Zhuochun Huang.

Project administration: Bin Yang.

Software: Zhuochun Huang.

Supervision: Zhuochun Huang.

Writing – original draft: Bin Yang, Limei Luo.

Writing – review & editing: Lin Chen, Qian Niu, Junlong Zhang, Huan Xu, Yifeng Wu, Zhuochun Huang.

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