Single nucleotide polymorphism of RANKL and OPG genes may play a role in bone and joint injury in rheumatoid arthritis

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Abstract

Objective
This paper aims to investigate the influence of single-nucleotide polymorphisms (SNPs) in the receptor of activator of nuclear factor kappaB ligand (RANKL) gene (TNFSF11) and osteoprotegerin (OPG) gene (TNFRSF11B) on bone and joint injury in patients with rheumatoid arthritis (RA).

Methods
Two hundred RA patients and 201 matched controls were analysed by case-control design, and their samples were genotyped. Bone mineral density (BMD) and serum OPG and RANKL levels were measured. Clinical and laboratory parameters were recorded, and the radiographic changes in both hands of RA were evaluated by Sharp's method.

Results
Our results showed no significant differences in the distribution frequency of the alleles and genotypes of TNFRSF11B (rs2073618 and rs3102735) and TNFSF11 (rs2277438) between the RA group and controls (p>0.05). Compared to patients with TNFSF11 (rs2277438) AA or GG genotype, RA with TNFSF11 (rs2277438) AG genotype had significantly decreased BMD values at lumbar spine 3, lumbar spine 4, lumbar spine 2–4 (p<0.05–0.01), and apparently elevated Sharp scores (p<0.05), respectively. The RA group showed significantly higher serum levels of RANKL, RANKL/OPG ratio and a lower serum level of OPG than that of the controls (p<0.05–0.0001). RA patients with RANKL-rs2277438 heterozygotic genotype (AG) had significantly increased serum levels of RANKL (p<0.05), compared to homozygotic genotype (AA or GG).

Conclusion
These results indicate that SNP of TNFRSF11B (rs2073618 and rs3102735) and TNFSF11 (rs2277438) may not be susceptibility factors for RA in Chinese Han population. SNP of TNFSF11 (rs2277438) may have an important influence on bone and joint injury in RA.

Key words
OPG gene (*TNFRSF11B*), RANKL gene (*TNFSF11*), single nucleotide polymorphism, rheumatoid arthritis, bone and joint injury

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterised by chronic inflammation of the joints, which may lead to invasion of synovium into the adjacent cartilage matrix with bone erosion as a consequence. In addition, RA may have negative effects on bone metabolism, resulting in a low bone mineral density (BMD) both at the adjoining site of involved joints and at sites far from the inflammatory articulations, such as the vertebra. As a result, RA patients may have a higher risk of developing osteoporosis and osteoporotic fracture, particularly at the femur or lumbar spine (1).

Local bone erosion and general bone loss, which are always found simultaneously in individuals with RA, are the major and characteristic manifestations of bone and joint injury in RA. The pathophysiological mechanisms of this kind of bone and joint damage in RA are not completely understood. It is now clear that abundant generation and activation of osteoclasts at the cartilage-pannus junction is the key mediator in the degeneration of joint cartilage and destruction of bone in patients with RA (2-4). Cumulative studies have indicated that RANKL, the ligand of receptor of activator of nuclear factor kappa B (RANK), is an essential factor for osteoclast formation by cells in the rheumatic joints and that osteoprotegerin (OPG), the decoy receptor of RANK, could prevent the bone erosion in the joints of RA by binding to RANK and hence inhibiting RANKL (5-7).

Genetic factors have been demonstrated to play an important role in susceptibility and bone metabolism in RA (8-9). Several single nucleotide polymorphisms (SNPs) in the RANKL gene (*TNFSF11*) and OPG gene (*TNFRSF11B*), which might affect the binding of transcription factors, have recently been shown to be significantly associated with bone metabolism and susceptibility in multiple autoimmune diseases including RA (9-10). Appraising the relationship between these SNPs and RA might be helpful in targeting preventive measures to individuals with higher risk of developing RA and inhibiting the progression of serious clinical outcomes such as bone destruction and the development of osteoporosis. To our knowledge, no studies published thus far have described an association between genetic polymorphisms of RANKL and OPG genes and RA in the Han Chinese, particularly its possible potential linkage with bone and joint injury in RA. The aim of our study was to investigate the possible association between RANKL and OPG gene polymorphisms and susceptibility to RA, and more importantly to discover whether SNPs of RANKL and OPG genes play a role in bone and joint injury in RA or not.

Methods
Study participants
We studied a total of 200 RA patients from the Han Chinese population, who fulfilled the 1987 revised criteria of the American College of Rheumatology (11), and had never accepted therapy with disease-modifying anti-rheumatic drugs before (28 men and 172 women, age range 23–82 years, mean age 53.5±11.9 years, body mass index [BMI], 22.2±3.4 kg/m^2). They were all from the Department of Rheumatology and Immunology of the First Affiliated Hospital of Anhui Medical University in China. Patients with thyroid disease or parathyroid glands disease, other endocrine disorders, serious liver or kidney disease, radiological abnormalities (scoliosis, platyspondyly, and others) were excluded from the study. Patients were also excluded if they had concomitant use of steroid, oestrogen, androgen, anticonvulsant or anticoagulant drugs. Moreover, we recruited 201 healthy volunteer subjects matched for age, sex, height and weight to the patients (30 men and 171 women, age range 25–78 years, mean age 51.9±10.2 years, BMI 21.6±3.6 kg/m^2).

Our study had been approved by the ethics committee of Anhui Medical University and had therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. According to principles of the Declaration of Helsinki, written permission was obtained from all individuals who were enrolled in our study

before measurements. After providing informed consent, patients underwent physical examination and laboratory testing at screening. They were questioned about age, gender, height, weight and duration of disease. Joint counts for tenderness and swelling, Health Assessment Questionnaire (HAQ) score, Visual Analogue Score (VAS) of overall pain, disease activity scores (DAS28), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF) and anti-cyclic citrulline polypeptide (anti-CCP) values were recorded simultaneously by rheumatologists. Radiographs were assessed using Sharp's method by two independent readers blinded to each other; this method evaluated each hand, including 17 areas for bone erosions and 18 areas for joint space narrowing (JSN) with a maximum total Sharp score of 314 (12).

Genotyping

Two SNPs of the OPG gene (*TNFRSF11B*) and one SNP of the RANKL gene (*TNFSF11*) were selected from NCBI dbSNP (the dbSNP homepage) in our study because they are often selected for investigation with positive results in many other fields such as osteoporosis. SNP rs2073618 (G/C transition) is located in exon 1 (amino acid position 1181) of the OPG gene (*TNFRSF11B*), while SNP rs3102735 (A/G transition) is located in promoter (amino acid position 163) of the OPG gene (*TNFRSF11B*). SNP rs2277438 (A/G transition) is located in intron (amino acid position 401) of the RANKL gene (*TNFSF11*). The genotyping of SNPs (rs2073618, rs3102735, rs2277438) was conducted by the Shanghai Biowing Applied Biotechnology Company (http://www.biowing.com.cn) using ligase detection reactions (LDR) (13).

The polymorphic regions of the OPG and RANKL genes were amplified by a multiplex polymerase chain reaction (PCR) method with specific forward primers (CCAAGCCCCTGAG-GTTTCC for rs2073618, TTCCTTC-CCTTGAATCTGGTG for rs3102735, and ACATGTGATTTCCTTGGTGCC for rs2277438) and with specific reverse primers (CCCAGGGACTTAC-CACGAG for rs2073618, CTAAAGC-

CCGTGCTATTCTGC for rs3102735 and TGGAGTCTCAAAATTCTATA-AATGCAG for rs2277438). After the amplification, we performed a second-round PCR, of which the reaction mixture consisted of 1 μl genome DNA (50 ng/μl), 2 μl buffer (1×), 1.2 μl Mg^{2+} (3 mmol/l), 2 μl dNTP (2 mmol/l), 0.3 μl 1 U Taq DNA polymerase (Qiagen hotstar), 7.5 μl H$_2$O, 4 μl Q-solution (1×) and 2 μl primer mix. The amplification procedure consisted of initial denaturation at 95°C for 15 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 90 s and extension at 72°C for 60 s, followed by a final extension at 72°C for 7 min. A Perkin Elmer Gene Amp PCR system 9600 was used. The ligation reaction was carried out in a final volume of 10 μl containing 1 μl buffer (1×), 1 μl Prob mix (12.5 pmol/μl), 1 μl of multi-PCR product (100 ng/μl), 12.5 pmol/μl of each common probe and 0.05 μl of 2 U/μl Taq DNA ligase (New England Biolabs, Ipswich, MA, USA). The LDR was performed using 35 cycles of denaturation at 95°C for 2 min, annealing at 94°C for 30 s and extension at 60°C for 2 min. The LDR fluorescent product was analysed by ABI sequencer 377.

Measurement of BMD

The BMD of the lumbar spine 2–4 (L2–4) and proximal femur, including femoral neck, Wards triangle, greater trochanter, and total hip was measured in 116 RA patients who were chosen at random from the whole enrolled RA group and 120 controls who were well matched for age, sex, height and weight to RA patients. BMD was measured using dual-energy x-ray absorptiometry (DXA) (Lunar Prodigy DF +310504, GE Healthcare, USA). BMD was automatically calculated from the bone area (cm^2) and bone mineral content (g) and expressed absolutely in g/cm^2. The default diagnostic threshold for osteoporosis was a T-score calculated from gender-matched BMD data in young adults derived in China (14). After acquisition of the BMD T-score, both RA patients and healthy individuals were divided into normal, osteopenic, and osteoporotic groups according to WHO criteria (15).

Detection of serum OPG and RANKL

Serum concentrations of OPG and RANKL from 116 RA patients and 120 controls were both measured by ELISA using reagents supplied by Yaji Biological Technology Limited Company (Shanghai, China). The ELISA tests were performed according to the manufacturer's instructions. The RANKL/OPG ratio was obtained for all individuals. The optical density was measured at 450 nm using an automatic ELISA reader (Sunrise, Austria). One hundred and thirteen eligible samples from patients and 100 qualified control specimens from all the individuals who had BMD measurements had valid levels of OPG and RANKL. The intra- and inter-assay CVs were less than 10% for both tests. All the specimens were measured in duplicate according to the manufacturer's instruction and then averaged.

Statistical analysis

Statistical analyses were performed using SPSS software (version 13.0). All the allele and genotype frequencies between patients and controls and Hardy–Weinberg equilibrium analysis were computed online using http://analysis.bio-x.cn (16). Normally distributed parameters were presented as mean (x) ± standard deviation (SD). Data of serum OPG, RANKL, ratio of RANKL/OPG and Sharp score were skewed parameters and presented as median (M) and quartile range (Q). The two-tailed independent samples t-test (for normally distributed data) or two-tailed independent samples non-parametric test (Mann-Whitney U-test for skewed distributed data) was performed to evaluate the diversity of parameters between different groups. Comparison of distribution frequencies of allele, genotype, bone metabolic state between RA and control were conducted using Pearson chi-square test or *ridit* analysis. Multiple linear regression and logistic regression analysis were used as appropriate. All p-values are two-sided, and values <0.05 were considered to be statistically significant.

Results

The research included 200 Chinese women or men with RA and 201 Chinese women or men normal subjects

Table I. Characteristics of RA patients and controls.

	Control	RA	p-value
n	201	200	
Age (years)	51.9 ± 10.2	53.5 ± 11.9	0.623
Weight (kg)	57.5 ± 9.1	56.8 ± 10.9	0.257
Height (cm)	163.2 ± 15.2	160.4 ± 18.1	0.167
BMI (kg/m²)	21.6 ± 3.6	22.2 ± 3.4	0.355
Male/female	30:171	28:172	0.792
Pre-menopausal/menopausal	50:121	58:114	0.372

Values are mean ± standard deviation or number.

who were all enrolled from March 2010 to February 2011. The characteristic of the subjects in the RA and control groups are shown in Table I. The subjects in both groups were well matched. Among the cases or controls, the genotype distributions of all three SNPs were in Hardy-Weinberg equilibrium. Hardy-Weinberg equilibrium test for case: $\chi^2=0.044$, Fisher's $p=0.835$ (*TNFRSF11B*, rs2073618), $\chi^2=0.143$, Fisher's $p=0.706$ (*TNFRSF11B*, rs3102735), $\chi^2=0.338$, Fisher's $p=0.561$ (*TNFSF11*, rs2277438). Hardy-Weinberg equilibrium test for control: $\chi^2=0.168$, Fisher's $p=0.682$ (*TNFRSF11B*, rs2073618), $\chi^2=0.014$, Fisher's $p=0.907$ (*TNFRSF11B*, rs3102735), $\chi^2=1.020$, Fisher's $p=0.312$ (*TNFSF11*, rs2277438). No significant differences in the distribution frequency of the alleles and genotypes were observed between the case group and control group ($p>0.05$) in the three SNPs (Table II).

RA patients had a higher incidence of osteoporosis (36/113, 31.9%) than that in healthy controls (18/120, 15.0%) ($\chi^2=9.290$, $p=0.002$) when all subjects were divided into normal, osteopenic,

and osteoporotic groups according to WHO criteria (15). *Ridit* analyses showed that the distribution frequencies of bone metabolic state between RA patients and controls were notably different ($z=3.947$, $p<0.0001$). The results also showed that RA patients with *TNFSF11*-rs2277438 AG genotype (n=39) had significantly lower BMD values compared to those with AA or GG genotype (n=62) at L3 ($t=2.314$, $p=0.023$), L4 ($t=2.207$, $p=0.030$), L2-L4 ($t=2.788$, $p=0.007$) (Table III). No any associations were found between SNPs (rs2073618 and rs3102735) of *TNFRSF11B* and BMD in RA. Sharp score of RA with *TNFSF11*-rs2277438 heterozygous genotype was accordingly higher than that with homozygous genotype (43.00 [108.75] *vs*. 15.50 [46.75], $z=2.419$, $p=0.016$) SNPs (rs2073618 and rs3102735) of *TNFRSF11B* failed to show any associations with Sharp score in RA ($p=0.866$, $p=0.242$) (Fig. 1).

Serum concentrations of OPG and RANKL in RA patients and healthy individuals are shown in Table IV. Patients with RA showed obviously high-

er plasma levels of RANKL and lower plasma levels of OPG compared with controls, with a consequently significantly higher RANKL/OPG ratio. More interesting findings showed that the concentration of serum RANKL in patients with RANKL gene (rs2277438) AG genotype (heterozygous genotype) was higher than that in patients with AA or GG genotype (homozygous genotype) (84.51 [73.09] *vs*. 66.18 [29.05], $z=2.491$, $p=0.013$), while the serum OPG level between heterozygous genotype and homozygous genotype of the OPG gene (rs2073618 and rs3102735) in RA patients did not differ from each other ($p=0.588$, $p=0.834$) (Fig. 2).

As far as the possible correlations between evaluated parameters in RA patients are concerned, multivariate linear regression analysis was performed to evaluate the contribution of age, disease duration, DAS28, HAQ, RF, anti-CCP, serum RANKL/OPG ratio to the Sharp score in RA patients. The results revealed that age (B=-0.931, 95%CI -1.746 to -0.115, $t=2.274$, $p=0.026$), disease duration (B=5.785, 95%CI 4.515–7.054, $t=9.078$, $p<0.0001$) and ratio of RANKL to OPG in peripheral blood (B=5.012, 95%CI 3.986–9.548, $t=2.104$, $p=0.039$) were the contributors for the Sharp score ($R^2=0.570$, $F=33.137$, $p<0.0001$).

Analysis of binary logistic regression (backward: LR) was executed to investigate the potential risk factor for RA-induced osteoporosis. A new target, accounting for whether one patient with RA accompanied with osteoporosis or not, was defined as a dependent

Table II. Allele and genotype distribution in the RA group and the control group.

SNP	Group	n	Allele*		Genotype#		
			C	G	CC	CG	GG
TNFRSF11B rs2073618	RA	200	109 (27.3%)	291 (72.8%)	16 (8.0%)	77 (38.5%)	107 (53.5%)
	control	201	96 (25.9%)	274 (74.1%)	13 (7.0%)	70 (37.8%)	102 (55.1%)
			A	G	AA	AG	GG
TNFRSF11B rs3102735	RA	200	342 (85.5%)	58 (14.5%)	146 (73.0%)	50 (25.0%)	4 (2.0%)
	control	201	313 (83.2%)	63 (16.8%)	131 (69.7%)	51 (27.1%)	6 (3.2%)
			A	G	AA	AG	GG
TNFSF11 rs2277438	RA	200	280 (70.0%)	120 (30.0%)	95 (47.5%)	90 (45.0%)	15 (7.5%)
	control	201	275 (73.1%)	101 (26.9%)	99 (52.7%)	77 (41.0%)	12 (6.4%)

*Chi-square test: $\chi^2=0.167$, $p=0.683$ (TNFRSF11B, rs2073618), $\chi^2=0.749$, $p=0.387$ (TNFRSF11B, rs3102735), $\chi^2=0.937$, $p=0.333$ (TNFSF11, rs2277438).
#Chi-square test: $\chi^2=0.179$, $p=0.914$ (TNFRSF11B, rs2073618), $\chi^2=0.852$, $p=0.653$ (TNFRSF11B, rs3102735), $\chi^2=1.058$, $p=0.589$ (TNFSF11, rs2277438).

Table III. Comparison of BMD between different genotypes of TNFSF11 (rs2277438) in RA patients.

BMD (g/cm²)	Genotype		t-test	p-value
	AA or GG (n=62)	AG (n=39)		
Femoral neck	0.88 ± 0.27	0.81 ± 0.16	1.602	0.113
Ward's triangle	0.74 ± 0.27	0.65 ± 0.20	1.643	0.104
Greater trochanter	0.73 ± 0.22	0.69 ± 0.18	0.815	0.417
Total hip	0.91 ± 0.20	0.86 ± 0.18	1.104	0.273
L2	0.97 ± 0.21	0.90 ± 0.20	1.405	0.164
L3	1.05 ± 0.22	0.93 ± 0.26	2.314	0.023
L4	1.06 ± 0.24	0.94 ± 0.28	2.207	0.030
L2-4	1.04 ± 0.21	0.89 ± 0.28	2.788	0.007

Values are mean ± standard deviation and analysed by two-tailed independent sample t-test.

Table IV. Serum levels of OPG, RANKL and the RANKL/OPG ratio in RA patients and controls.

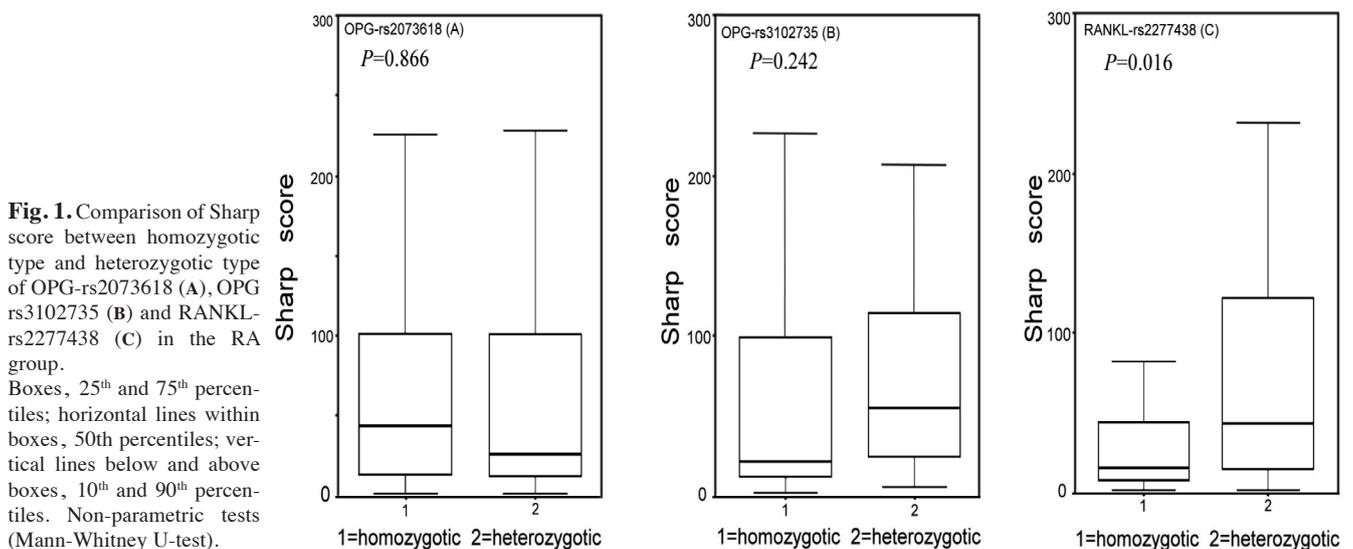| Indicator	Group		z	p-value*
	RA (n=113)	Control (n=100)		
OPG (pg/ml) M (Q)	202.86 (123.44)	285.14 (318.61)	4.029	<0.0001
RANKL (pg/ml) M (Q)	79.69 (43.59)	70.13 (30.61)	2.368	0.018
RANKL/OPG ratio M (Q)	0.41 (0.20)	0.27 (0.26)	5.259	<0.0001

*Non-parametric tests (Mann-Whitney U-test).

variable (0=no, 1=yes). Age, gender, duration of disease, DAS28, Sharp score, serum RANKL/OPG ratio and BMI were defined as independent variables. The results showed that age (OR=1.058, p=0.014, 95%CI 1.012–1.106) and Sharp score (OR=1.021, p=0.001, 95%CI 1.008–1.033) were the risk factors for the occurrence of osteoporosis in patients with RA (R^2=0.262, χ^2=17.692, p=0.001).

Discussion

The RANK/RANKL/OPG system plays an important role in the immune system cross-linking it to bone in what is known as osteoimmunology, which is a new interdisciplinary field of study integrating the disciplines of immunology and bone biology, thus providing a new perspective on the pathogenesis of RA (17-20). RANKL, known as the OPG ligand, is a member of the tumour

necrosis factor (TNF) ligand superfamily of cytokines, and has been shown to play a major role in regulating bone metabolism. RANKL induces osteoclastic bone destruction, and OPG protects against bone destruction by preventing the binding of RANKL with its receptor RANK (21). The molecular triad composed of RANKL/OPG/RANK has been described as a key cytokine system for controlling the differentiation and function of osteoclasts, and hence osteoporosis and other bone diseases (22), especially participating in the mechanism of bone and articular injury in RA (23).

To the best of our knowledge, this is the first study of the association between the SNP of the RANKL gene (*TNFSF11*), the OPG gene (*TNFRSF11B*) and RA in the Han Chinese population. In our study, we did not find any associations between these SNPs and susceptibility to RA. However, Assmann *et al.* reported a definite association between the SNP in the RANKL gene and susceptibility to RA in the white population (10). The study, a cohort of 534 patients with RA and 516 healthy controls, showed that the RANKL gene SNP (rs2277438) was significantly associated with RA (p=0.039), resulting in a three times higher susceptibility to RA for the homozygous genotype of the minor allele (AA *vs.* GG: OR 0.35, 95%CI 0.15–0.80; AA *vs.* AG: OR 1.10, 95%CI 0.78–1.55). In addition,
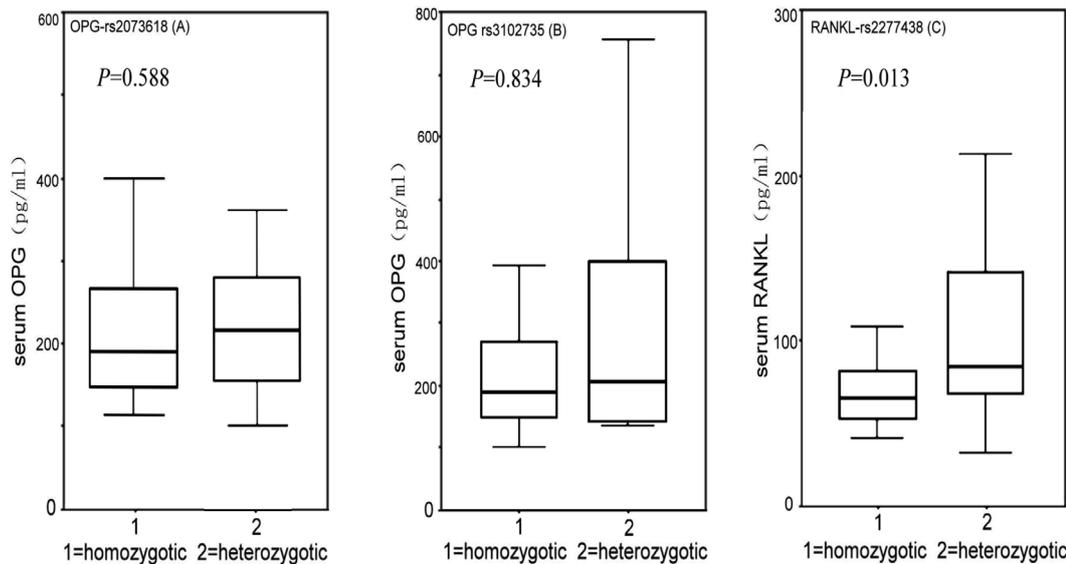

Fig. 1. Comparison of Sharp score between homozygotic type and heterozygotic type of OPG-rs2073618 (**A**), OPG rs3102735 (**B**) and RANKL-rs2277438 (**C**) in the RA group.
Boxes，25th and 75th percentiles; horizontal lines within boxes，50th percentiles; vertical lines below and above boxes，10th and 90th percentiles. Non-parametric tests (Mann-Whitney U-test).

Fig. 2. Comparison of serum levels of OPG, RANKL between homozygotic type and heterozygotic type of OPG rs2073618 (**A**), OPG rs3102735 (**B**) and RANKL rs2277438 (**C**) in the RA group.
Boxes, 25th and 75th percentiles; horizontal lines within boxes, 50th percentiles; vertical lines below and above boxes, 10th and 90th percentiles. Non-parametric tests (Mann-Whitney U-test).

the RANKL gene SNP (rs9533156) also correlated with RA and there was an obviously higher frequency of the minor allele in the RA patients than that in controls (OR 0.84, 95%CI 0.71–0.99, p=0.047), while the genotype distribution did not display any significant dissimilarities in this SNP (p=0.144). This research failed to show any effect of the other SNP (rs1054016) of the RANKL gene, and no differences were shown in the OPG gene SNPs (rs3102735, rs2073618), which is the same result we have found in our investigation. Another observation reported by Wu *et al*. also revealed that SNP rs922996 of the RANKL gene was significantly associated with age at onset of symptoms in RA (24). Patients who represented as CC genotypes of rs922996 had younger ages at onset of disease than those with CT or TT genotypes, suggesting a role for RANKL variants in the etiology of RA. Six years later, findings that a single promoter SNP rs7984870 of the RANKL gene was consistently significantly associated with earlier age of RA onset in three independent seropositive (RF or anti-cyclic citrullinated peptide antibody positive) RA cohorts but not in seronegative RA patients were reported by the same researchers (25). The risk C allele of rs7984870 of the RANKL gene conferred 2-fold higher plasma RANKL levels in RF-positive RA patients. Although the above studies concerning the SNP of the RANKL

gene in RA were always reported with positive outcomes, discrepancies about the susceptibility to RA still exist, and perhaps this is due to racial differences between western and eastern patients. Further in-depth studies that enroll more participants and select patients with a more involved locus of the OPG and RANKL genes are required to confirm this.

Genetic variation involved in the RANKL/RANK/OPG bone remodeling pathway are strongly associated with BMD at different skeletal sites in normal subjects (26-27), which was explored by Hsu *et al*., who reported a significantly positive association for rs3102735 polymorphisms of the OPG gene, rs9594782 SNP of the RANKL gene with BMD in adult men only, but not in women (26). They also found men with TC/CC genotypes of the rs9594782 SNP had a 2.1 times higher risk of extremely low hip BMD (p=0.004), and lower whole body BMD (p<0.001); subjects with the GG genotype of the rs3102735 polymorphism had a 70% reduced risk of having extremely low hip BMD (p<0.05), and higher whole body BMD (p<0.01). These observations clearly showed SNP of some gene were correlated with reduced BMD in specific site of bone. Furthermore, it was well-known to us that osteoporosis in RA at difference position had distinct mechanism and influence factors. An association between the RANKL gene SNP (rs2277438)

and reduced BMD only at site of lumbar might be due to this reason. These results preliminarily revealed associations between SNP of RANKL or OPG and bone loss in normal subjects, and prompted a series of similar research studies in RA, with some significant discoveries. Furuya *et al*. found that RA patients in Japan with the G allele in SNP rs2277438 of the RANKL gene led to more severe joint damage evaluated using Larsen's methods at 2 years than those who did not possess this allele (17). Their results were duplicated in our study showing that RA patients with AG genotype in the RANKL gene (rs2277438) had more severe bone erosion (higher Sharp score) and bone loss (lower BMD). In the subgroup of RA with BMD in our paper, there was only 4 cases with GG genotype (rs2277438), so we combined groups of AA and GG into group of homozygous genotype. That meant GG genotype just accounted for 6.5% (4/62) in the group of homozygous genotype, so differences between groups of homozygous genotype (AA or GG) and heterozygous genotype (AG) actually reflected differences between AA and AG. Therefore, our findings about association between RANKL gene SNP (rs2277438) with osteoporosis and bone damage in RA patients were in consistent with reports of Furuya *et al*.

In spite of the discrepancy in conclusions from the different investigations, these results generally suggest that

SNPs of the RANKL and OPG genes might have certain effects on bone erosion and bone loss in RA patients. However, there are currently not enough programmes to explore the exact relationship between SNPs of the RANKL or OPG genes and bone and joint injury in RA patients, especially to investigate whether SNPs of the RANKL and OPG genes influence bone destruction or bone loss via alteration of serum RANKL and OPG as a result of SNPs of the RANKL and OPG genes. In our study, we demonstrated that there existed an association between the SNP of the RANKL gene and BMD as well as Sharp score in RA patients. Our discovery showed that patients with AG genotypes of the rs2277438 polymorphism had apparently lower BMD values at lumbar spine 3, lumbar spine 4, lumbar spine 2-4 and obviously higher Sharp scores compared with subjects with AA or GG genotypes. What is more important and more interesting is that we also found patients with AG genotypes of the RANKL gene (rs2277438) had higher serum RANKL levels than that in patients with AA or GG genotypes. Thus, we can draw a probable conclusion that SNP (rs2277438) of the RANKL gene contributes to the elevated circulating RANKL levels in RA. The inference was also made in a previous investigation that the C allele of rs7984870 of the RANKL gene conferred 2-fold higher plasma RANKL levels in RF-positive RA patients (25). We did not find any notable difference between the two SNPs of the OPG gene and BMD or Sharp score in RA, which was in accordance with the data of Masi *et al.*, who did not observe any distinct difference in BMD at lumbar spine between the groups of different genotypes of OPG gene either (28).

Bone erosion, along with bone loss and the resulting osteoporosis, have become a major problem in RA. This phenomenon implies an inherent connection between bone erosion and bone loss in RA. RANKL and OPG acting as both immune modulators and regulators of bone homeostasis have been shown to mediate an imbalance in bone resorption and bone formation resulting in joint degeneration and bone loss (29-

30). Evidence from the literature shows that serum RANKL in RA significantly increases, and is often accompanied with decreased serum OPG, and bone resorption is regulated by the relative levels of expression of RANKL and OPG (31). A study on patients with early untreated RA found that radiographic progression of the bone component of joint destruction was dependent on both inflammation (ESR) and osteoclast activation (the OPG:RANKL ratio) (32). In our previous and current studies, the RA group had significantly higher serum levels of RANKL, lower serum levels of OPG and a higher ratio of serum RANKL to serum OPG (5). Furthermore, linear regression analysis indicated that the ratio of serum RANKL to OPG positively correlated with Sharp score, the risk factor for the occurrence of osteoporosis in RA, which was confirmed by logistic regression simultaneously in our study. On the basis of these combined results, we believe it may be a rational explanation that the SNP of the RANKL gene (rs2277438) resulted in the increase of serum RANKL and accordingly the elevated ratio RANKL/OPG in RA, eventually leading to osteoporosis, as well as bone erosion in RA. This information is likely to become increasingly important as more bone-directed treatments become part of RA management paradigms.

Conclusion

Finally, our data do not provide definite evidence of associations between SNPs of *TNFRSF11B* (rs2073618 and rs3102735) or *TNFSF11* (rs2277438) and susceptibility to RA in the Chinese Han population, perhaps due to an inadequate sample size and racial difference of *TNFRSF11B* and *TNFSF11*. Nevertheless, we can conclude that SNP (rs2277438) of the RANKL gene may be a predictor of bone and joint damage in patients with RA.

Acknowledgments

References

1. HAUGEBERG G, UHLIG T, FALCH JA, HALSE JI, KVIEN TK: Bone mineral density and frequency of osteoporosis in female patients with rheumatoid arthritis: results from 394 patients in the Oslo County Rheumatoid Arthritis. *Arthritis Rheum* 2000; 43: 522-30.
2. TETLOW LC, WOOLLEY DE: Mast cells, cytokines, and metalloproteinases at the rheumatoid lesion: dual immunolocalisation studies. *Ann Rheum Dis* 1995; 54: 896-903.
3. PETTIT AR, JI H, VON STECHOW D *et al.*: TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. *Am J Pathol* 2001; 159: 1689-99.
4. HOFBAUER LC, HEUFELDER AE: The role of osteoprotegerin and receptor activator of nuclear factor kappa B ligand in the pathogenesis and treatment of rheumatoid arthritis. *Arthritis Rheum* 2001; 44: 253-9.
5. XU S, WANG Y, LU J, XU J: Osteoprotegerin and RANKL in the pathogenesis of rheumatoid arthritis-induced osteoporosis. *Rheumatol Int* 2012; 32: 3397-403.
6. HAYNES D, CROTTI T, WEEDON H *et al.*: Modulation of RANKL and osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis in response to disease-modifying antirheumatic drug treatment and correlation with radiologic outcome. *Arthritis Rheum* 2008; 59: 911-20.
7. AINOLA M, MANDELIN J, LILJESTRÖM M, KONTTINEN YT, SALO J: Imbalanced expression of RANKL and osteoprotegerin mRNA in pannus tissue of rheumatoid arthritis. *Clin Exp Rheumatol* 2008; 26: 240-6.
8. HUGHES LB, REYNOLDS RJ, BROWN EE *et al.*: Most common single-nucleotide polymorphisms associated with rheumatoid arthritis in persons of European ancestry confer risk of rheumatoid arthritis in African Americans. *Arthritis Rheum* 2010; 62: 3547-53.
9. KIM JG, KIM JH, KIM JY *et al.*: Association between osteoprotegerin (OPG), receptor activator of nuclear factor-kappaB (RANK), and RANK ligand (RANKL) gene polymorphisms and circulating OPG, soluble RANKL levels, and bone mineral density in Korean postmenopausal women. *Menopause* 2007; 14: 913-8.
10. ASSMANN G, KOENIG J, PFREUNDSCHUH M *et al.*: Genetic variations in genes encoding RANK, RANKL, and OPG in rheumatoid arthritis: a case-control study. *J Rheumatol* 2010; 37: 900-4.
11. ARNETT FC, EDWORTHY SM, BLOCH DA *et al.*: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1998; 31: 315-24.
12. VANDER HEIJDE D: How to read radiographs according to the Sharp/van der Heijde method. *J Rheumatol* 1999; 26: 743-5.
13. PAN F, LIAO F, XIA G *et al.*: Association of IL-1F7 gene with susceptibility to human leukocyte antigen-B27 positive ankylosing spondylitis in Han Chinese population. *Clin Chim Acta* 2010; 411: 124-6.
14. LIU Z, PIAO J, PANG L *et al.*: The diagnostic criteria for primary osteoporosis and the

incidence of osteoporosis in China. *J Bone Miner Metab* 2002; 20: 181-9.

15. ORIMO H, HAYASHI Y, FUKUNAGA M *et al.*: Diagnostic criteria for primary osteoporosis: year 2000 revision. *J Bone Miner Metab* 2001; 19: 331-7.

16. SHI YY, HE L: SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005; 15: 97-8.

17. FURUYA T, HAKODA M, ICHIKAWA N *et al.*: Associations between HLA-DRB1, RANK, RANKL, OPG, and IL-17 genotypes and disease severity phenotypes in Japanese patients with early rheumatoid arthritis. *Clin Rheumatol* 2007; 26: 2137-41.

18. NAKASHIMA T, TAKAYANAGI H: RANKL signal and osteoimmunology. *Clin Calcium* 2011; 21: 1131-40.

19. ARRON JR, CHOI Y: Bone versus immune system. *Nature* 2000; 408: 535-6.

20. BAR-SHAVIT Z: The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell. *J Cell Biochem* 2007; 102: 1130-9.

21. ROMAS E, GILLESPIE MT, MARTIN TJ: Involvement of receptor activator of NF κ B ligand and tumor necrosis factor-α in bone destruction in rheumatoid arthritis. *Bone* 2002; 30: 340-6.

22. KEARNS AE, KHOSLA S, KOSTENUIK PJ: Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev* 2008; 29: 155-92.

23. HAYNES DR, CROTTI TN, LORIC M, BAIN GI, ATKINS GJ, FINDLAY DM: Osteoprotegerin and receptor activator of nuclear factor κB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint. *Rheumatology* (Oxford) 2001; 40: 623-30.

24. WU H, KHANNA D, PARK G *et al.*: Interaction between RANKL and HLA-DRB1 genotypes may contribute to younger age at onset of seropositive rheumatoid arthritis in an inception cohort. *Arthritis Rheum* 2004; 50: 3093-103.

25. TAN W, WU H, ZHAO J *et al.*: A functional RANKL polymorphism associated with younger age at onset of rheumatoid arthritis. *Arthritis Rheum* 2010; 62: 2864-75.

26. HSU YH, NIU T, TERWEDOW HA *et al.*: Variation in genes involved in the RANKL/RANK/OPG bone remodeling pathway are associated with bone mineral density at different skeletal sites in men. *Hum Genet* 2006; 118: 568-77.

27. GARCÍA-UNZUETA MT, RIANCHO JA, ZARRABEITIA MT *et al.*: Association of the 163A/G and 1181G/C osteoprotegerin polymorphism with bone mineral density. *Horm Metab Res* 2008; 40: 219-24.

28. MASI L, MADDALI BONGI S, ANGOTTI C *et al.*: The role of osteoprotegerin (OPG) and estrogen receptor (ER-α) gene polymorphisms in rheumatoid arthritis. *Clin Cases Miner Bone Metab* 2007; 4: 156-60.

29. KWAN TAT S, PADRINES M, THEOLEYRE S, HEYMANN D, FORTUN Y: IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev* 2004; 15: 49-60.

30. THEILL LE, BOYLE WJ, PENNINGER JM: RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol* 2002; 20: 795-823.

31. GRAVALLESE EM, MANNING C, TSAY A *et al.*: Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor. *Arthritis Rheum* 2002; 43: 250-8.

32. GEUSENS PP, LANDEWÉ RB, GARNERO P *et al.*: The ratio of circulating osteoprotegerin to RANKL in early rheumatoid arthritis predicts later joint destruction. *Arthritis Rheum* 2006; 54: 1772-7.