Circulating Dickkopf-1 Is Correlated with Bone Erosion and Inflammation in Rheumatoid Arthritis

SHI-YAO WANG, YAN-YING LIU, HUA YE, JIAN-PING GUO, RU LI, XIA LIU, and ZHAN-GUO LI

ABSTRACT. ***Objective.*** To explore the potential role of Dickkopf-1 (DKK-1) in rheumatoid arthritis (RA) and to evaluate the effect of a tumor necrosis factor-α (TNF-α) inhibitor (infliximab) and an interleukin 1 receptor antagonist (IL-1Ra; anakinra) on DKK-1 secretion in patients with RA.

Methods. Serum samples were collected from 100 patients with RA, 100 patients with other rheumatic diseases (e.g., osteoarthritis and ankylosing spondylitis), and 40 healthy controls. DKK-1 and osteoprotegerin (OPG) levels in serum were detected by ELISA. Serum C-reactive protein (CRP) levels, erythrocyte sedimentation rates (ESR), rheumatoid factor (RF) titers, and anti-cyclic citrullinated peptide antibody were also measured in patients with RA.

Results. The serum level of DKK-1 was significantly higher in patients with RA than in healthy controls and those with other rheumatic diseases ($p < 0.01$); the serum DKK-1 level was correlated with levels of CRP ($r = 0.488$, $p = 0.003$) and ESR ($r = 0.458$, $p = 2.4 \times 10^{-4}$) and the Sharp score of radiologic change ($r = 0.449$, $p = 0.001$) in RA. In contrast to the increasing level of OPG, DKK-1 was significantly decreased in RA patients treated with TNF-α inhibitor ($p < 0.01$). DKK-1 was significantly decreased in RA patients treated with IL-1Ra ($p < 0.01$).

Conclusion. DKK-1, as an important mediator, was correlated with bone erosion and inflammation in RA. The change of DKK-1 level may serve as a biomarker of disease activity and bone erosion.
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Key Indexing Terms:
DICKKOPF-1 OSTEOPROTEGERIN TUMOR NECROSIS FACTOR–α INHIBITOR
INTERLEUKIN 1 RECEPTOR ANTAGONIST RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that primarily attacks synovial joints, leading to articular destruction and functional disability. It typically presents as symmetric peripheral polyarthritis and most commonly involves the small joints of the hands and feet[1,2]. One of the hallmarks of RA is progressive bone erosion. Research on the mechanisms by which RA induces osteolysis has focused on the osteoclast's roles in shifting the normal balance between bone formation and resorption. This imbalance is generated by key molecules that regulate osteoclast differentiation, such as cross-talk between receptor activators of nuclear factor-κB ligand (RANKL) and Wingless (Wnt) signaling pathway, which is important for the growth and differentiation of osteoblasts[3,4].

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Dickkopf-1 (DKK-1) is an endogenous, secreted inhibitory factor in the canonical Wnt signaling by binding the Wnt coreceptor LRP5/6[5]. Studies have demonstrated that activation of the Wnt signaling pathway in mature osteoblasts upregulates osteoprotegerin (OPG), which blocks RANKL-induced osteoclastogenesis and results in the inhibition of bone resorption[6,7]. The balance between RANKL and OPG plays an important role in bone erosion[8]. DKK-1 could increase the expression of the osteoclast differentiation factors and RANKL, and decrease the expression of OPG. Moreover, Fujita, *et al* found that DKK-1 facilitates osteoclastogenesis by enhancing RANKL/RANK interaction[9]. Notably, studies have demonstrated that DKK-1 plays an important role in the promotion of synovial angiogenesis[10]. Vascular proliferation in RA leads to the occurrence and invasion of membrane synovialis pannus, which is critical in joints.

Cytokines play a pivotal role in RA pathogenesis. Tumor necrosis factor-α (TNF-α) contributes substantially to the pathology of RA. Interestingly, studies have shown that upregulation of DKK-1 could activate TNF receptor-1 (TNFR1) in cultivated articular mesenchymal cells[11]. TNF-α inhibitor could downregulate DKK-1 in ankylosing spondylitis (AS)[12]. Moreover, interleukin 1 (IL-1) can modulate the RANKL/OPG pathway, and may also affect the ability of the osteoblast to repair bone at sites of articular erosion[13]. To date, the questions of whether DKK-1 is

involved in bone destruction and inflammation in RA, or whether there are correlations between DKK-1 and clinical and laboratory characteristics of human RA, have not been thoroughly clarified. In this study, we explored the potential role of DKK-1 in RA and evaluated the effects of the TNF-α inhibitor infliximab and the IL-1 receptor antagonist (IL-1Ra) anakinra on DKK-1 secretion in patients with RA.

We found that the serum level of DKK-1 was significantly higher in patients with RA than that in controls, and DKK-1 was correlated with levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) and Sharp scores of radiologic change in RA. In contrast to the upregulation of OPG, the serum concentration of DKK-1 was significantly downregulated in RA patients treated with the TNF-α inhibitor and IL-1Ra. A change of level of DKK-1 may serve as a biomarker of disease activity and bone erosion, as well as a marker of response to anti-TNF and IL-1Ra therapy for RA.

MATERIALS AND METHODS

Patients. A total of 100 RA patients who met the American College of Rheumatology criteria for RA[14] were included in this study. Patients were a randomized subset. Among these 100 patients, 40 were treated with the TNF-α inhibitor infliximab. The daily dosage, 15–25 mg, was sustained for 6 months. Thirty RA patients were treated with the IL-1Ra. The daily dosage was 75–100 mg, sustained for 6 months.

The control groups included 140 individuals [30 patients with osteoarthritis (OA), 30 with AS, 30 with systemic lupus erythematosus (SLE), and 10 with systemic sclerosis (SSc) and 40 healthy controls].

The study was approved by the Research Ethics Committee at the Beijing University People's Hospital.

Clinical and laboratory profiles. Levels of ESR, CRP, anti-cyclic citrullinated peptide antibody (anti-CCP), and rheumatoid factor (RF) in patients with RA were recorded. ESR was evaluated by the Westergren method, CRP was examined by immunonephelometry, and anti-CCP was tested by ELISA. Radiographs of the hands and wrists were obtained at baseline. Radiographs were digitized and scored for erosions (ERO) and joint space narrowing (JSN) by radiologists and rheumatologists using the Sharp scoring method (total Sharp score = JSN + ERO). The physicians were blind to patient's treatment assignment and chronological sequence of examinations.

Measurement of serum protein levels of DKK-1 and OPG. Serum samples were taken from each subject and stored at –70˚C until the assays were performed. Measurements of cytokines in patients' sera were carried out simultaneously. Serum DKK-1 and OPG levels were examined by commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well plates (Corning, Schiphol, Netherlands) were coated overnight at room temperature with monoclonal mouse anti-human DKK-1 capture antibodies (R&D Systems) in phosphate buffered saline (PBS). All the following steps were performed at room temperature. The plates were washed with PBS-Tween and then blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Five-fold diluted serum samples were added to the plates and incubated for 2 h. Goat anti-human detection antibodies (R&D Systems) were added, and the plates were incubated another 2 h. Streptavidin-horseradish peroxidase was added and incubated for 20 min. After the plates were washed with PBS, the substrate reagent (R&D Systems) was added for another 20 min. The substrate reaction was stopped upon addition of 2 N sulfuric acid, and extinction was measured at 450 nm wavelength using a multiplate ELISA reader (Anthos Microsystems, Krefeld, Germany). All measurements were performed in triplicate for each sample, and the mean values were calculated.

Statistics. Baseline characteristics were calculated for the treated patient group and controls. Data are presented as the percentage or mean ± SD. A paired t test was used to analyze the data; p values < 0.05 were considered to be significant.

RESULTS

Patients. Study patients with RA consisted of 90% women, 90% RF-positive, 60% had CRP elevations above the normal range, 78% had ESR elevations above the normal range, 35% were current corticosteroid users; they had a mean age of 50 ± 28 years and mean disease duration of 12 ± SD 7.2 years.

The mean age of 40 healthy controls was 43 ± 15 years and 83% of them were female. There were no clinically relevant changes in routine hematology and biochemistry findings, including hemoglobin estimation, hematocrit, erythrocyte counts, total and differential leukocyte counts, cytochemical staining, and serum albumin concentration, etc.

Serum DKK-1 concentration was significantly increased in RA. As shown in Figure 1, the serum concentration of DKK-1 in patients with RA (5952.6 ± 3019.5 pg/ml) was significantly higher than that in healthy controls (3198.9 ± 2283.6 pg/ml; $p = 2.3 \times 10^{-4}$) and patients with OA (1715.22 ± 1098.4 pg/ml; $p = 1.8 \times 10^{-4}$), AS (4042.9 ± 2283.6 pg/ml; $p = 4.7 \times 10^{-4}$), SLE (1781.34 ± 752.02 pg/ml; $p = 1 \times 10^{-4}$), and SSc (2003.17 ± 418.35 pg/ml; $p = 4.1 \times 10^{-5}$). In addition, the results showed that the serum concentration of DKK-1 in OA patients was significantly lower than that in controls (p = 0.009). The level of DKK-1 in AS patients tended to be higher than that of controls (p = 0.134).

DKK-1 was associated with bone erosion in RA. Increasing DKK-1 levels were associated with bone erosion (Figure 2). There were positive correlations between the serum DKK-1 level and JSN (r = 0.538, $p = 1.4 \times 10^{-4}$), ERO (r = 0.337,

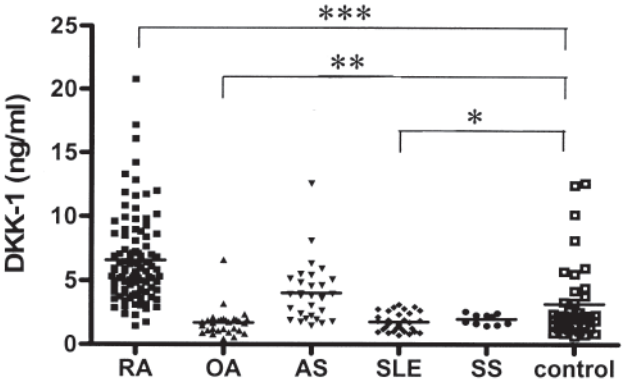

Figure 1. Compared with healthy controls, serum concentration of DKK-1 was significantly higher in patients with RA ($p = 2.3 \times 10^{-4}$); DKK-1 was significantly lower in osteoarthritis (OA) samples (p = 0.009), higher in ankylosing spondylitis (AS) samples (p = 0.134), and significantly lower in systemic lupus erythematosus (SLE) samples (p = 0.013), and there was no significant difference between systemic sclerosis (SS) and healthy controls (p = 0.147). Bars show the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.0001.

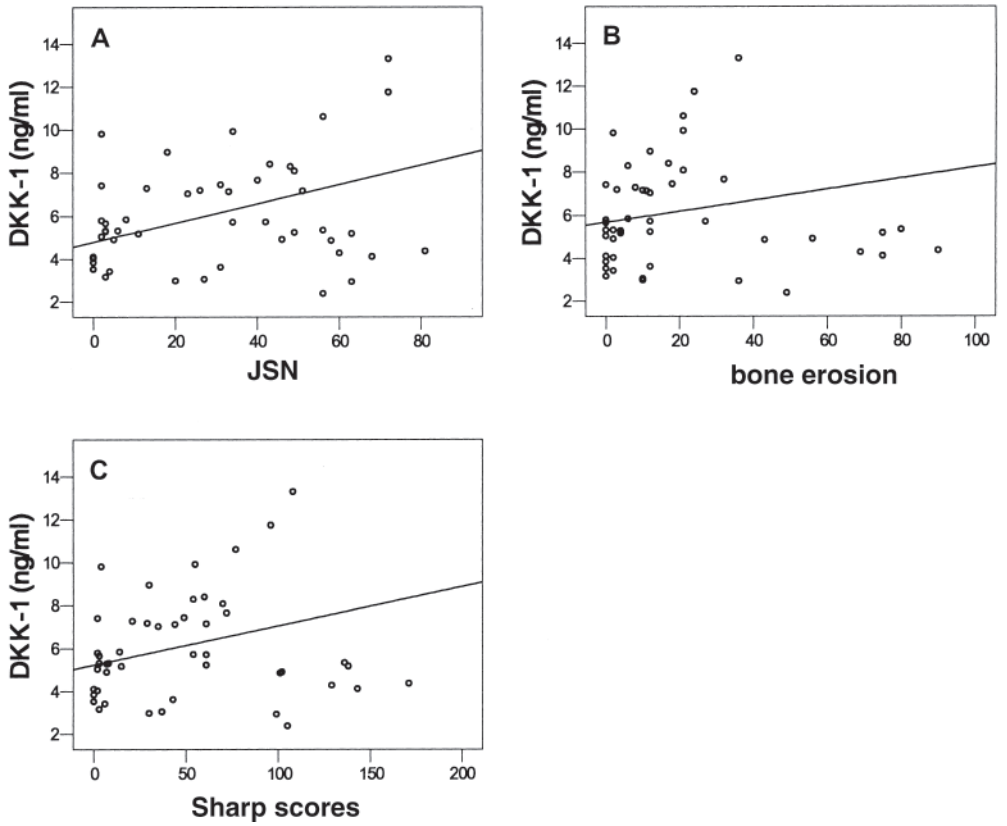

Figure 2. Correlations of serum DKK-1 with joint space narrowing (JSN) (A), erosions (B), and Sharp scores (C) in patients with RA. There were positive correlations between serum DKK-1 and JSN ($r = 0.538$, $p = 1.4 \times 10^{-4}$), erosions ($r = 0.337$, $p = 0.018$), and Sharp scores ($r = 0.449$, $p = 0.001$).

$p = 0.018$), and Sharp score ($r = 0.449$, $p = 0.001$). There were no correlations between the serum OPG and the JSN ($r = 0.272$, $p = 0.058$), ERO ($r = 0.098$, $p = 0.503$), and Sharp score ($r = 0.189$, $p = 0.193$).

Serum DKK-1 is correlated with ESR, CRP, and RF in RA. In RA patients, DKK-1 levels were positively correlated with the levels of ESR ($r = 0.458$, $p = 2.4 \times 10^{-4}$) and CRP ($r = 0.488$, $p = 3.37 \times 10^{-4}$). However, no correlation was found between serum DKK-1 levels and RF ($r = 0.105$, $p = 0.311$) or anti-CCP ($r = 0.263$, $p = 0.108$) (Figure 3). OPG was negatively correlated with the CRP, ESR, anti-CCP, and RF.

Levels of DKK-1 and OPG were regulated by TNF-α inhibitor treatment. We found that 10 of 40 RA patients were nonresponders to anti-TNF-α therapy after 6 months of treatment. The circulating levels of DKK-1 and OPG also changed in the 2 groups before and after treatment. As shown in Table 1 and Figure 4, serum DKK-1 levels were significantly downregulated (6653.1 ± 3336 pg/ml vs 3424.6 ± 1918 pg/ml, respectively; $p = 3.6 \times 10^{-4}$), while the serum OPG levels were significantly upregulated (1060.6 ± 961 pg/ml vs 4237.8 ± 2648 pg/ml; $p = 0.01$) in patients who were anti-TNF-α therapy responders. However, there were no significant differences in the levels of DKK-1 and OPG in patients who were nonresponders to anti-TNF-α therapy before and after the treatment (4965.2 ± 3336 pg/ml vs 4851.6 ± 1918 pg/ml, $p = 0.776$; and 820.4 ± 538 pg/ml vs 1870.5 ± 511 pg/ml, $p = 0.516$).

Further, a significant clinical-serological improvement was recorded at the 6-month reevaluation. Mean values of anti-TNF-α therapy responders' assessments of general health and anti-CCP antibody titers were not significantly different: 63.6 ± 43 vs 75 ± 57 ($p = 0.13$); RF decreased from 21.6 ± 13.9 to 10.1 ± 3.7 ($p < 0.0001$), ESR from 56.3 ± 24.5 to 32.8 ± 21 ($p = 5 \times 10^{-5}$), and CRP from 34.6 ± 27 to 10.5 ± 15 ($p = 0.001$). In addition, there were no significant differences in any clinical-serological markers in anti-TNF-α nonresponder patients before and after the treatment (Table 1).

Levels of DKK-1 and OPG were regulated by IL-1Ra treatment. Levels of DKK-1 and OPG were affected by IL-1Ra in patients with RA. Serum DKK-1 and OPG levels were detected before and after administration of IL-1 inhibitor. As shown in Figure 5, the mean serum DKK-1 level was significantly lower after treatment (5679.77 ± 1292.12 pg/ml before vs 3778.25 ± 1451.5 pg/ml after treatment; $p = 1.72 \times 10^{-5}$). There was no significant difference of the level of OPG in RA patients before and after the treatment (1218.4 ± 879 pg/ml before vs 1055.22 ± 533.2 pg/ml after treatment; $p = 0.464$). Data are expressed as mean ± SD.

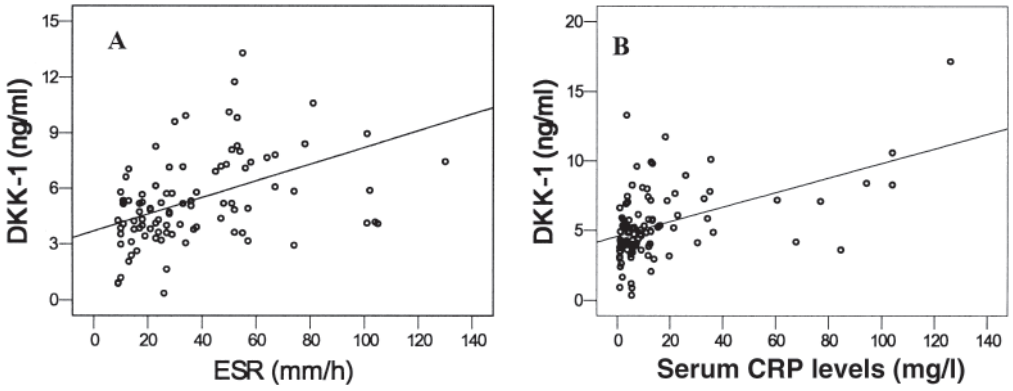

Figure 3. Correlation of serum DKK-1 with erythrocyte sedimentation rate (ESR; A) and C-reactive protein (CRP; B) in RA patients. There were positive correlations between serum DKK-1 and ESR ($r = 0.458$, $p = 2.4 \times 10^{-4}$) and CRP ($r = 0.488$, $p = 3.37 \times 10^{-4}$).

Table 1. Clinical characteristics of TNF-α inhibitor treatment in patients with RA.

	Therapy Responders			Therapy Nonresponders		
	Before Treatment	After Treatment	p	Before Treatment	After Treatment	p
DKK-1, pg/ml	6653.1	3424.6	0.00036	4965.2	4851.6	0.776
OPG, pg/ml	1060.66	4237.8	0.01	820.4	1870.5	0.516
ESR, mm/h	56.3	32.8	0.00005	29.3	33.3	0.231
RF, IU/ml	173.8	99.9	0.006	121.5	113.1	0.805
CRP, mg/h	34.6	10.5	0.001	19.6	14.3	0.233
CCP, U/ml	63.6	70.5	0.131	66.4	81.5	0.303

OPG: osteoprotegerin; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; CRP: C-reactive protein; CCP: citric citrullinated peptide.

DISCUSSION

Inflammatory joint disease can lead to bone erosion and joint deformation. Immune-mediated inflammation leads to joint destruction but virtually no signs of bone repair in RA. Interactions between the receptor activator of RANKL and RANK, or OPG, have a dominant role in the activation and survival of osteoclasts. Elevated serum levels of RANKL are associated with bone resorption, osteolytic lesions, and reduced survival in multiple myeloma. However, accurate measurement of circulating RANKL is very difficult because of uncertain factors about which forms are the most biologically relevant and the limited sensitivity of available assays. Therefore, in our study we did not detect level of RANKL. There are 4 DKK proteins: DKK-1, DKK-2, DKK-3, and DKK-4, of which DKK-1 has the strongest inhibitory effect on the Wnt pathway[15,16,17]. Previous studies have demonstrated that DKK-1 can inhibit the secretion of OPG[9] and promote osteolytic lesions *in vivo* by enhancing RANKL-mediated osteoclastogenesis.

RA and OA are the most common human articular diseases. In our study, DKK-1 levels in patients with RA were significantly higher than levels in healthy controls and subjects with other rheumatic diseases. In contrast, the serum concentration of DKK-1 in patients with OA was significantly lower than in controls. The infiltration of immunocompetent cells and proliferation of synovial fibroblasts in synovial lining leads to the formation of pannus tissue, which invades the articular cartilage and subchondral bone as a characteristic of RA. The above features were all associated with DKK-1. In comparison, the major pathological features of OA are osteophyte formation, progressive narrowing of the joint space between bone endings, subchondral sclerosis, and bone deformity. DKK-1 impairs local bone formation, thus there was a lower level of DKK-1 in patients with OA.

Pathological analysis of inflammatory lesions in RA and AS displays not only some similarities but also major differences. AS is characterized by 2 key pathological findings: sacroiliac joint and spinal inflammation and new bone formation with the possible consequence of bone fusion, usually in the axial skeleton. In AS the primary site of inflammation is located at the enthesis or subchondral bone marrow, with bone marrow edema, lymphocytic infiltrates, increased osteoclast density, and increased microvessel density as typical findings in acute inflammation. Although AS and RA exhibit similarities in bone destruction, AS is defined by

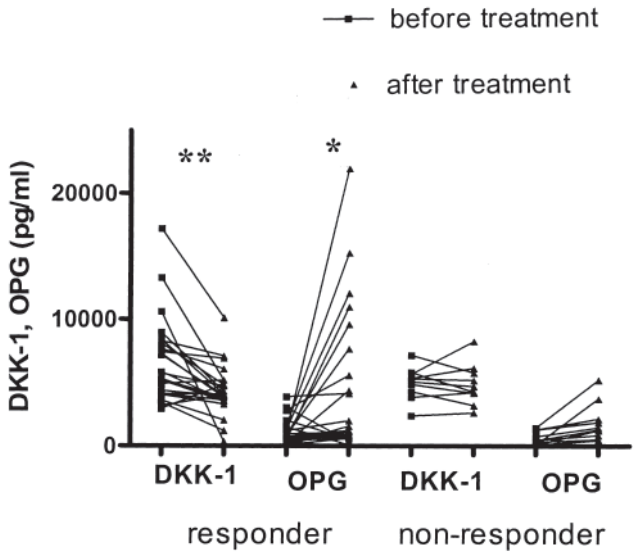

Figure 4. Circulating DKK-1 and osteoprotegerin (OPG) levels before and after TNF-α inhibitor treatment in RA. Serum DKK-1 levels were significantly downregulated (6653.1 ± 3336 pg/ml vs 3424.6 ± 1918 pg/ml; $p = 3.6 \times 10^{-4}$), whereas serum OPG levels were significantly upregulated (1060.6 ± 961 pg/ml vs 4237.8 ± 2648 pg/ml; p = 0.01) in patients who responded to anti-TNF-α therapy. There was no significant difference in levels of DKK-1 and OPG in anti-TNF-α nonresponder patients before and after the treatment (4965.2 ± 3336 pg/ml vs 4851.6 ± 1918 pg/ml, p = 0.776; 820.4 ± 538 pg/ml vs 1870.5 ± 511 pg/ml, p = 0.516). Bars show the mean ± SEM. *p < 0.05; **p < 0.0001.

new bone formation and RA by the destruction of cortical bone. Thus, the level of DKK-1 was lower in patients with AS than those with RA. These results are in accord with results from Diarra, *et al*[18]. Moreover, 7 subjects in our healthy control group were female (mean age 50 ± 8 yrs), with serum DKK-1 levels that were significantly higher than those of other healthy controls, which may be related to hormone disorder in older women[19].

Sharp scores were found to be positively correlated with DKK-1 levels, as either continuous or categorical variables. Further, increased circulating DKK-1 was associated with a greater risk for progression of bone erosions in patients with RA[20]. DKK-1 is produced by synoviocytes in patients with RA[18]. In addition, ESR and CRP levels were positively correlated with DKK-1 levels, which can be used to determine disease activity and assess drug efficacy in patients with RA. Therefore, DKK-1 may serve as a new clinical indicator for RA in the future.

Clinical trials in RA have demonstrated that TNF-α-blocking agents are highly beneficial for most patients refractory to classic treatment with disease-modifying antirheumatic drugs[21,22]. However, a significant proportion of patients are still relatively resistant to such therapy[23]. No reliable markers predictive for clinical response have been identified, although a recent report suggests that a decrease in RF and anti-CCP antibody titers might be a useful adjunct in assessing the efficacy of treatment[24], but inconsistent findings were reported. Atzeni, *et al* identified significant decreases in anti-CCP autoantibodies and RF titers after both 6 and 12 months of TNF-α inhibitor therapy[25]. It is possible that anti-CCP-positive patients with RA might display a more active disease associated with a higher response to therapy in comparison with patients negative for anti-CCP autoantibodies. An additional study with a larger series of anti-CCP-negative patients with RA would be necessary to evaluate such a hypothesis, because the number of anti-CCP-negative patients in our study was too small.

Diarra, *et al* identified that TNF-α is a key inducer of DKK-1, which is a member of a class of proteins involved in joint remodeling in RA[18]. Daoussis, *et al* indicated that serum DKK-1 levels were significantly increased in patients with AS compared with normal subjects[12]. Decreased serum DKK-1 is found after TNF-α inhibitor treatment in RA patients, which is consistent with our results. We observed that circulating DKK-1 was significantly downregulated in the responder group before and after treatment, while the

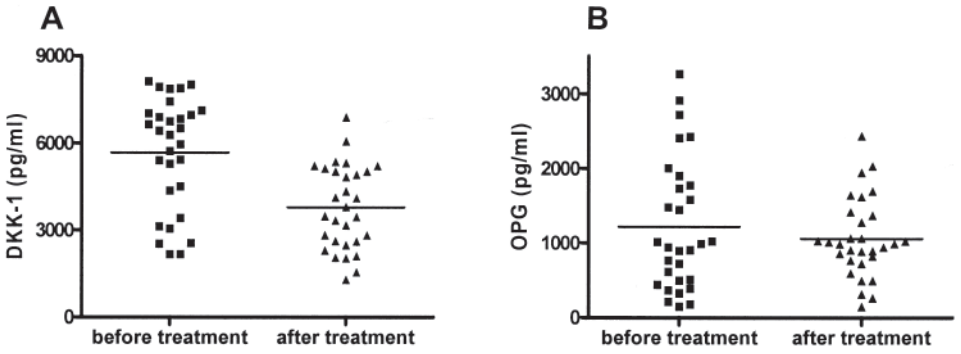

Figure 5. Effects of interleukin 1 (IL-1) inhibitor on DKK-1 and osteoprotegerin (OPG) levels in RA. Comparison of serum DKK-1 (A) and OPG (B) levels in RA patients before and after treatment with IL-1Ra for 6 months. The mean serum DKK-1 level was significantly decreased after treatment (5679.77 ± 1292.12 pg/ml vs 3778.25 ± 1451.5 pg/ml; $p = 1.72 \times 10^{-5}$). The mean serum OPG level showed no significant difference in RA patients following treatment (1218.4 ± 879 pg/ml vs 1055.22 ± 533.2 pg/ml; p = 0.464). Data are mean ± SD.

serum OPG levels were significantly upregulated. In addition, there was no significant difference of the level of DKK-1 in anti-TNF-α therapy nonresponder patients before and after the treatment. The distinct patterns of DKK-1 expression between TNF responders and nonresponders are intriguing. At the moment, we are unable to fully understand the mechanism underlying this observation. However, in RA, TNF-α has been shown to be a driving force for upregulation of DKK-1 by the evidence that DKK-1 is molecularly regulated in cultivated articular mesenchymal cells challenged by TNF-α. Thus, we speculate that in the TNF responder population, the anti-TNF treatment suppressed the production of TNF-α significantly. Suppression of TNF-α production led to the downregulation of DKK-1, as shown in an arthritic mouse model. It can be inferred from the data that serum DKK-1 can predict effective treatment compared with CCP.

Moreover, a large body of evidence supports a role for IL-1 in cartilage and bone erosion. *In vitro* studies suggest that IL-1 can increase bone resorption by stimulating osteoclast differentiation and activation[22,23,24,25,26]. Therefore, one purpose of our study was to develop an alternative targeted therapy for this subgroup of patients with RA. Our results also indicated that serum DKK-1 and OPG levels were detected before and after administration of IL-1 inhibitor. The mean serum DKK-1 level was significantly lower after treatment, whereas the mean serum OPG level did not change significantly in RA patients following treatment. We also found that after inhibiting the expression of DKK-1 of fibroblast-like synoviocytes by RNA interference, the secretion of inflammatory factors, including NF-κB, IRAK-1, ERK, and JNK, was significantly reduced (data not shown). Therefore, DKK-targeting therapy may be a promising method of treatment for RA.

In our study, we observed that increased levels of serum DKK-1 were significantly correlated with disease activity and more severe bone destruction in patients with RA. Thus, DKK-1 may serve as a biomarker of RA activity and bone erosion, and as a novel target for treatment of RA. It is necessary to clarify the causative relationship between DKK-1 and RA in further studies, and to define the role of DKK-1 underlying the effect on RA.

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