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Correlation of Bone Morphogenetic Protein-2 Levels in Serum and Synovial Fluid with Disease Severity of Knee Osteoarthritis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: This study aimed to investigate the bone morphogenetic protein-2 (BMP-2) levels in serum and synovial fluid (SF) of patients with primary knee osteoarthritis (OA) and to exam its correlation with radiographic and symptomatic severity of the disease.

Material/Methods: A total of 37 knee OA patients and 20 healthy controls were enrolled in this study. Knee OA radiographic grading was performed according to the Kellgren-Lawrence (KL) grading system by evaluating X-ray changes observed in anteroposterior knee radiography. Symptomatic severity of the disease was evaluated according to the Western Ontario McMaster University Osteoarthritis Index (WOMAC) scores. BMP-2 levels in serum and SF were determined using enzyme-linked immunosorbent assay.

Results: Serum BMP-2 level in patients with knee OA was higher than that in healthy controls. Knee OA patients with KL grade 4 showed significantly elevated BMP-2 levels in the serum and SF compared with those with KL grade 2 and 3. Knee OA patients with KL grade 3 had significant higher SF levels of BMP-2 than those with KL grade 2. BMP-2 levels in the serum and SF of knee OA patients were both positively correlated with KL grades and WOMAC scores.

Conclusions: BMP2 levels in serum and SF were closely related to the radiographic and symptomatic severity of knee OA and may serve as an alternative biochemical parameter to determine disease severity of primary knee OA.

MeSH Keywords: Biological Markers • Bone Morphogenetic Protein-2 • Osteoarthritis • Serum • Synovial Fluid

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Background

Osteoarthritis (OA) is a chronic and progressive joint disorder characterized by degradation of cartilage, menisci and ligaments, synovial inflammation and changes to the subchondral bone [1]. The clinical features of OA are manifested as severe pain, joint stiffness, reduced motion, swelling, and crepitation and are associated with reduced quality of life and impaired mobility in the aging population [1]. Among the various joints in the human body, the knee is the most common clinically significant site of primary OA involvement. It is now well accepted that sex, age, mass body index, and joint injury are all risk factors for OA, but the exact causes of OA are still poorly understood. The best-established methods to assess the progression of OA are plain radiography by measuring the changes in joint-space width (JSW); however, these changes in JSW are relatively small compared with the precision error of radiographic measurements, and it always requires 1–3 years to obtain reliable information [2]. A promising development is the use of quantitative MRI, which is more sensitive than plain radiography, to assess changes in cartilage volume or thickness, but this method is limited due to its high financial cost and controversial standards [3]. Arthroscopy provides a more direct and magnified view of the cartilage surface, but this is an invasive technique that cannot be routinely used in all patients. Therefore, it is attractive to have a biological marker that can precisely show quantitative and dynamic alternations in joint remodeling and OA progression.

Loss of matrix molecules in cartilage is the primary characterization of OA. However, the cartilage chondrocytes involved in these degenerative events are still thought to have anabolic actions, such as proliferation and synthesis of cartilage matrix components, and regain the chondrogenic phenotype and are committed to the regenerative pathway [4]. This regenerating action of chondrocytes in OA can be attributed to various factors. The bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β (TGF-β) superfamily, have long been known to be implicated in a diverse set of biological activities, including regulation of cell proliferation, differentiation, migration and apoptosis, embryonic development, and maintenance of tissue homeostasis [5] and are strong bone/cartilage inductive molecules [6]. BMP-2, 1 of the 30 currently known BMPs, has been shown to have the capacity to stimulate the synthesis of proteoglycan and promote endochondral osteogenesis, and has anabolic effects on chondrocyte metabolism [7].

In 2003, 2 independent research groups demonstrated BMP-2 mRNA and protein expression by articular chondrocytes *in vivo* and confirmed that the expression level of BMP-2 was upregulated in OA chondrocytes and cartilage with the severity of OA [8,9]. The localization of BMP-2 also varied with the severity of cartilage damage. In control cartilage, BMP-2 was expressed mainly in the middle cartilage layer, with a few in the superficial and deep layers. In moderately damaged OA cartilage, BMP-2 was localized in both upper and middle zone chondrocytes, but was not detected in deep layer chondrocytes. In severely damaged OA cartilage, cellular localization of BMP-2 was extended to the deep zone [8,9]. In the area of osteophyte formation, BMP-2 was intensely localized in fibrous tissue matrix, as well as in osteoblasts in newly formed osteophytic tissue [9,10]. Moreover, Fukui et al. demonstrated that expression of BMP-2 can be stimulated by the pro-inflammatory cytokines IL-1β and TNF-α [8]. Recent studies have indicated that the presence of intra-articular low-grade inflammation contributes to the development and progression of OA [11], and that pro-inflammatory factors are present in synovium and cartilage of OA patients [12]. All of these results suggest that chondrocytes depended on the cartilage remodeling or repair functions of BMP-2 to maintain anabolic metabolism during the course of OA, raising the possibility that BMP-2 plays a critical role in the pathogenesis of knee OA.

Although extensive investigations in plasma, urinary, and/or synovial fluid (SF) biochemical markers for OA have been carried out in patients with knee OA, thus far there have been no detailed data on the association of circulating and SF levels of BMP-2 with disease severity of primary knee OA. Thus, the present study aimed to determine the BMP-2 levels in serum and SF of patients with knee OA and the correlation of BMP-2 levels with radiographic and symptomatic severity of the disease.

Material and Methods

Study population

This study was approved by the Ethics Review Committee of China-Japan Union Hospital of Jilin University and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all subjects. A total of 37 patients who complained of symptomatic OA (joint pain, swelling, crepitation, and early-morning stiffness and fatigue) and who were diagnosed with knee OA according to the clinical symptomatic criteria of the American College of Rheumatology and radiographic criteria for OA of at least 1 knee, were enrolled in the present study. Twenty sex- and age-matched healthy individuals with normal knee radiographs were recruited as controls. Participants were excluded on the basis of having arthropathy due to gout, pseudogout, rheumatoid arthritis, systemic lupus erythematosus, psoriasis, hemochromatosis, previous knee injury, and previous joint infection, or with histories of corticosteroid medication, bilateral knee replacements, cancer, or other chronic inflammation diseases.

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Table 1. Basline clinical characteristics.

Characteristics	Controls (n=20)	OA patients total (n=37)	KL grade 2 (n=10)	KL grade 3 (n=14)	KL grade 4 (n=13)
Age (years)	68.95±5.67	69.58±10.80	68.73±11.25	70.56±9.12	69.93±10.78
Gender (M/F)	7/13	13/24	3/7	5/9	3/10
BMI (kg/m^2)	24.65±2.26	24.56±1.74	23.83±2.25	25.59±1.88	24.63±1.97

All values are expressed as mean ±SD or *n*. OA – osteoarthritis; KL – Kellgren-Lawrence; BMI – body mass index.

Radiographic assessment of OA

OA severity was determined using weight-bearing anteroposterior radiographs of the affected knee. Radiographic severity was evaluated according to the Kellgren and Lawrence grading system [13]: grade 0, no radiological changes; grade 1, doubtful narrowing of joint space and possible osteophytic lipping; grade 2, definite osteophytes and possible narrowing of joint space; grade 3, moderate multiple osteophytes, definite narrowing of joint space, some sclerosis, and possible deformity of bone contour; grade 4, large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity of bone contour. Subjects who had radiographic knee OA of KL grade 2 or higher in at least 1 knee were defined as OA patients and those who had KL grades of 0 for both knees were defined as healthy controls. The grading scale used for analysis was the higher of the 2 knees.

Evaluation of symptomatic severity

The symptomatic severity of the disease was evaluated according to the Western Ontario McMaster University Osteoarthritis Index (WOMAC), which consists of 3 subscales: pain, stiffness, and physical function [14]. Total scores range from 0 to 100, where a higher score on the WOMAC scale represents poorer function or greater pain. The psychometric properties of the WOMAC score, including reliability, validity, and responsiveness, are all well established in OA populations.

Samples collection and BMP-2 levels measurement

SF was obtained from the affected higher KL grading knee of OA patients using sterile knee puncture just prior to surgery for total knee replacement, centrifuged to remove cells and joint debris, and the supernatants were aspirated and stored at –80°C. No SF was extracted from controls due to ethics concerns. Venous blood samples were collected from all subjects after overnight fasting on the morning of surgery and were centrifuged and stored at –80°C immediately until utilized. Once all patients were recruited, serum samples were thawed at room temperature for 20 min and SF were dissolved in Guanidine-HCl. Quantitative measurement of serum and SF BMP-2 were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine, R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. The manufacturer-reported precision was 2.4–2.8% for intra-assay and 5.3–7.3% for inter-assay. Sensitivity was <29 pg/ml. The ELISA was conducted using an Infinite M200 PRO automated microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) software, version 16.0 for Windows. Data are presented as mean ±SD, median (interquartile range), or *n* when appropriate. Kolmogorov-Smirnov test was performed to analyze the data normality and unpaired t test, Mann-Whitney U test, or chi-square test was used to assess significance in clinical characteristics between patients with knee OA and healthy controls, as appropriate. Comparison of BMP-2 levels in serum and SF between OA patients with different KL grades were performed using one-way analysis of variance (ANOVA), followed by Tukey post hoc analysis if ANOVA showed significance, Kruskal-Wallis analysis or chi-square test, as indicated. Differences between BMP-2 levels in serum and SF were analyzed using the Wilcoxon signed rank test for paired samples. Spearman's rank correlation was used to determine the correlation among the level of BMP-2 in serum and SF and KL grades or WOMAC scores. Multinomial logistic/multivariate linear analysis was used to assess the independent predictors of KL grades/WOMAC scores. Because the serum/SF BMP-2 levels and WOMAC scores were not normally distributed; log transformations values were used for multivariate linear analysis. *P* values less than 0.05 were considered statistically significant for differences and correlations.

Results

Baseline characteristics of the study groups

The baseline clinical parameters of OA patients and healthy controls are shown in Table 1. No significant differences in

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Table 2. BMP-2 levels in serum and SF.

BMP-2 levels	Controls (n=20)	OA patients total (n=37)	KL grade 2 (n=10)	KL grade 3 (n=14)	KL grade 4 (n=13)
Serum (pg/mL)	37.46 (30.17–49.35)	59.54 (52.61–77.43)	48.76 (45.62–54.35)	58.83 (53.85–63.10)	79.95 (74.32–92.95)
SF (pg/mL)		83.33 (71.82–97.45)	66.55 (61.02–70.66)	79.65 (76.45–84.55)	105.86 (95.90–122.39)

All values are expressed as median (interquartile range) or *n*. SF – synovial fluid; OA – osteoarthritis; KL – Kellgren-Lawrence; BMI – body mass index.

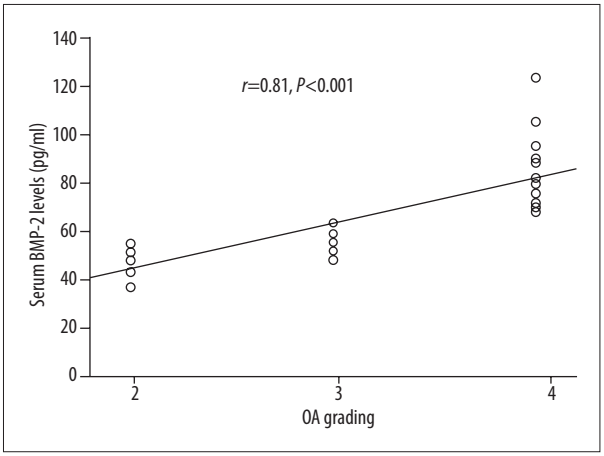

Figure 1. Correlation between serum BMP-2 levels of knee OA patients and disease severity classified according to KL grading system (r=0.81, P<0.001). BMP-2: morphogenetic protein 2; OA: osteoarthritis.

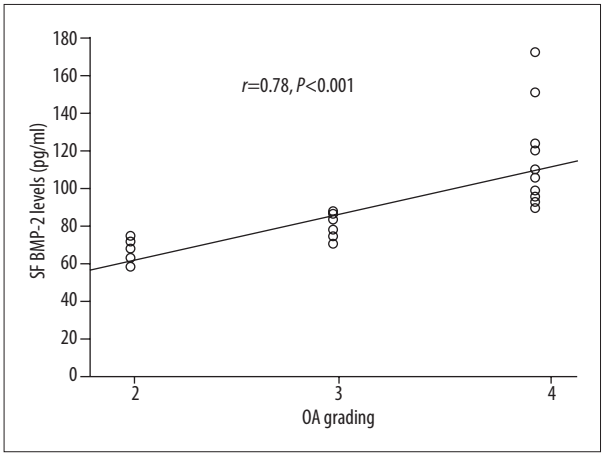

Figure 2. Correlation between SF BMP-2 levels of knee OA patients and disease severity classified according to KL grading system (r=0.78, P<0.001). BMP-2: morphogenetic protein-2; SF: synovial fluid; OA: osteoarthritis.

age, sex, or body mass index (BMI) were observed between OA patients and healthy controls (P>0.05). OA was divided into 3 subgroups according to the KL grading system. There were also no significant differences in baseline characteristics among the subgroups (P>0.05).

The BMP-2 concentrations in serum and SF

Thirty-seven serum and paired SF samples from knee OA patients and 20 serum samples from healthy controls were collected for BMP-2 concentrations measurement. The levels of BMP-2 in serum and SF of knee OA patients and serum levels of healthy controls are shown in Table 2. The levels of serum BMP-2 were significantly elevated in knee OA patients compared with those in healthy controls (P<0.001), and serum levels of BMP-2 in patients with KL grade 4 were significantly elevated compared with those with KL grade 2 and grade 3 (P<0.001). Although the average serum levels of BMP-2 in KL grade 3 were greater than KL grade 2, the differences were not statistically significant (P>0.05). Additionally, MBP-2 levels in SF were substantially higher than that in paired serum samples of knee OA patients (P<0.001), and BMP-2 levels in SF increased significantly with increased KL grades in knee OA patients (P<0.001).

Association of BMP-2 levels in serum and SF with KL grades

The association of BMP-2 levels in serum and SF with radiographic severity is illustrated in Figure 1 and Figure 2. Spearman's rank correlation analysis showed that BMP-2 levels in serum and SF were both positively correlated with radiographic severity of knee OA (r=0.81, P<0.001 and r=0.78, P<0.001, respectively). We also analyzed the association of BMP-2 levels in serum and SF; the results showed that serum BMP-2 levels had a positive correlation with SF BMP-2 levels (r=0.65, P<0.001) (Figure 3). Multinomial logistic regression analysis showed that BMP-2 levels in serum and SF were both positively correlated with KL grades after adjusting for other variables such as age, sex, and BMI (chi-square=11.56, P=0.003 and chi-square=12.32, P=0.002, respectively).

Association of BMP-2 levels in serum and SF with WOMAC scores

In OA patients, a significant correlation was found between serum BMP-2 levels and WOMAC scores (r=0.63, P<0.001) (Figure 4). Furthermore, BMP-2 levels in SF were also

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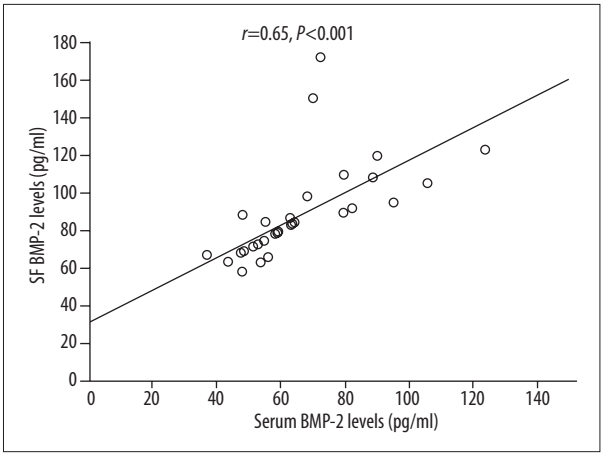

Figure 3. Correlation between serum and SF BMP-2 levels in knee OA patients (r=0.65, P<0.001). BMP-2: morphogenetic protein-2; SF: synovial fluid; OA: osteoarthritis.

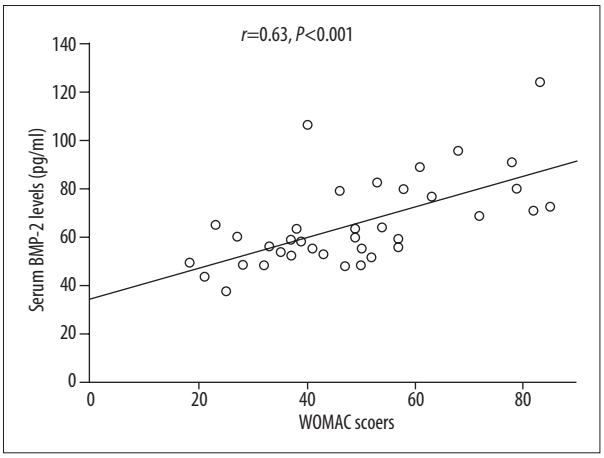

Figure 4. Correlation between serum BMP-2 levels in knee OA patients and WOMAC scores (r=0.63, P<0.001). BMP-2: morphogenetic protein-2; OA: osteoarthritis; WOMAC: Western Ontario McMaster University Osteoarthritis Index.

significantly correlated with WOMAC scores (r=0.77, P<0.001) (Figure 5). Multivariate linear regression analysis revealed that there were still positive correlations between serum and SF BMP-2 levels with WOMCA scores after controlling for the influence of potential confounders (t=2.35, P=0.032 and t=2.58, P=0.015, respectively).

Discussion

Identification of patients at risk for incident disease or disease progression in OA remains challenging, because radiography is an insensitive reflection of molecular changes that presage cartilage and bone abnormalities. Thus, there is a widely

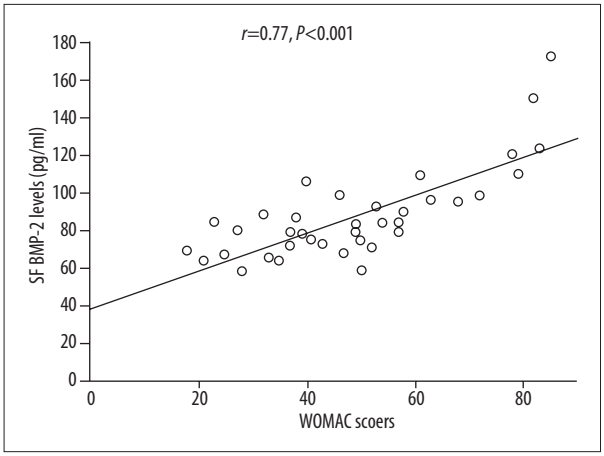

Figure 5. Correlation between SF BMP-2 levels in knee OA patients and WOMAC scores (r=0.77, P<0.001). BMP-2: morphogenetic protein-2; OA: osteoarthritis; SF: synovial fluid; WOMAC: Western Ontario McMaster University Osteoarthritis Index.

appreciated need for biochemical markers, and the validation of prognostic biomarkers is of particular importance. Using prognostic biomarkers to identify patients at high risk for progression could reduce the mortality of the disease and may also help facilitate the development of disease-modifying osteoarthritis drugs [15]. In 2006, Bauer et al. proposed a new classification scheme for OA biomarkers, which can be represented by the acronym BIPED to denote the 5 categories of markers: Burden of disease, Investigative, Prognostic, Efficacy of intervention, and Diagnostic, which assesses the severity or extent of disease, provides the need for more information to allow a biomarker inclusion into 1 of the existing categories, predicts the future onset of OA among those without OA at baseline or the progression of OA among those with existing disease, provides information about the efficacy of treatment among those with OA or those at high risk of developing OA, and classifies individuals as either diseased or non-diseased, respectively [16]. A recent review in *Annals of the Rheumatic Diseases* summarized the conclusions of the working meeting convened by the European Society for Clinical and Economic Aspects of Osteoporosis and Osteoarthritis (ESCEO) discussing the value of biomarkers in drug development for OA therapy in October 2012, concluding that there is a potential role for biomarkers in drug development for OA [2]. The identified biomarkers of OA can be classified into markers of bone, cartilage, and synovium metabolism (including synthesis and degradation), and systemic inflammation. Biomarkers of matrix degradation, specifically cartilage degradation, received much attention in comparison with other biochemical marker categories [17].

BMPs elicit a well-documented anabolic response in cartilage explants and regulate recruitment of chondroprogenitors,

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synthesis of cartilage matrix, and endochondral bone formation during embryonic skeletogenesis [18]. In addition, genetic evidence shows that the BMP pathway is needed for joint homeostasis in adulthood [19]. More recently, 2 bone devices consisting of a bovine collagen matrix soaked with BMP-2 (Infuse Bone Graft, a lumbar tapered fusion device) or BMP-7 (Osigraft) have been introduced to the market to deal with non-healing bone or complicated bone fractures [20,21] and a novel biocompatible carrier device, OSTEOGROW, required small amounts of BMP-6, which can stimulate differentiation of mesenchymal stem cells and accelerate healing of critical-size bone defects has been tested in rats and rabbits[22].

BMP-2 is a growth and differentiation factor that belongs to the TGF-β superfamily, which plays important roles in embryonic development and skeletal growth [23] and has been used in tissue-engineering experiments for the repair of defects in bone [20] and articular cartilage [24]. Exogenous BMP-2 is known to have potent anabolic actions on adult articular chondrocytes, increasing proteoglycan and collagen synthesis and maintaining the chondrocyte phenotype [7,25,26]. The expression of BMP-2 mRNA is always associated with the capacity of *in vitro* expanded adult human articular chondrocytes to form stable cartilage *in vivo*, resistant to vascular invasion and endochondral bone formation [27]. Katagiri et al. even demonstrated that implantation of BMP-2 into muscular tissues induced ectopic bone formation at the site of implantation [28]. Recent studies have demonstrated that BMP-2 is endogenously expressed in cartilage [8–10] and its mRNA expression level is up-regulated during mechanical injury [29], which itself is a risk factor for OA.

There have been studies showing that BMP-2 was detected in the serum of patients with degenerative joint disease requiring total arthroplasty [30] and SF of patients with arthrofibrosis after total knee arthroplasty [31,32]. However, to the best of our knowledge, thus far there are no published data on the relationship between BMP-2 levels in the serum and SF of knee OA patients and disease severity. This study is the first to show that BMP-2 was detected in both serum and SF obtained from patients with primary knee OA, and that BMP-2 level was positively correlated with radiographic grading and symptomatic severity of knee OA; this correlation remained significant after the adjustment of potential confounders such as age, sex, and BMI. The present study showed a significant increase of BMP-2 levels in both serum and SF of patients with knee OA compared to that of healthy controls. Our findings suggest enhanced local and systemic production of BMP-2 in primary knee OA, but it should be noted that the level of BMP-2 in SF was a great deal higher than that in paired serum samples. The mechanism of this increase may be attributed to either secretion of BMP-2 residing in extracellular matrix, increased BMP-2 synthesis, or both. The BMP-2 in SF may originate from synovial cells and chondrocytes in the local tissues (such as the synovial membrane and articular cartilage) because there have been studies demonstrating that BMP-2 is endogenously expressed in synovial cells and articular cartilage [33]. Additionally, this observation indicates a significant elevation in the systemic and local expression of BMP-2 in patients with advanced knee OA, but the mechanism for this increase in serum and SF of knee OA patients requires further investigation. We assume that the increased levels of BMP-2 in serum and SF may be a reparative response to degenerative changes in OA joints. A study by Honsawek et al. [34] showed that plasma and SF levels of BMP-7 were increased in knee OA patients and was positively related to the radiographic severity of the disease, which was consistent with the present study, because BMP-7 has also been showed to be implicated in the stimulation of cartilage repair and the maintenance of articular cartilage integrity [35]. However, the levels of BMP-7 they detected in serum were somewhat higher than in SF, without significant difference, which is in contrast to our results. This discrepancy may be due to different expression patterns of BMP-2 and BMP-7, ethnicity differences between the studied populations, and/or divergence originating from the relatively small numbers of enrolled patients in both studies.

Therefore, measurements of serum and/or SF levels of BMP-2 may possibly serve as a biomarker for determining disease severity and might be categorized into the *Diagnostic* or *Burden of disease* markers in the BIPED criteria of OA biomarkers. However, due to some inevitable limitations in this study, more research is needed. First, this study was based on a small sample size of enrolled patients, and further studies conducted on a random sample of a larger population sample should be validated. Second, we did not measure the BMP-2 levels in SF of healthy controls because of ethics concerns. Although there were several studies reporting BMP-2 levels in SF of control groups, these controls underwent a diagnostic arthroscopy for unspecific knee complains [36] or referred pain [31,32], who were excluded from the present study. Also, we did not find any published data regarding the baseline SF levels of BMP2 in non-diseased patients that we can refer to. Third, we just focused on BMP-2 and missed the other factors that may affect the status of articular cartilage. Many inflammation cytokines, such as IL-6, TNF-α, C-reactive protein, and CXCL12 [37,38], and other factors involved in the degradation of cartilage such as thymosin β4 [39] and leptin [40], have been detected in serum and/or SF of OA patients and were associated with the radiographic severity of the disease. Moreover, the oxygen levels were greatly reduced [41] and the oxidative stress levels increased significantly in OA joints [42]. This may be why elevated levels of BMP-2 are not sufficient to stop or reverse OA. Finally, as this was designed as a cross-sectional study, it is impossible to determine a definite cause-and-effect relationship and draw a strong conclusion. Further prospective longitudinal studies are warranted to substantiate our results.

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Conclusions

This study revealed a significant elevation in serum and SF BMP-2 levels of knee OA patients compared to healthy controls and illustrated a pronounced positive correlation of serum and SF BMP-2 levels with the degree of radiographic and symptomatic severity in patients with knee OA. This study is the first to show that serum and/or SF BMP-2 may be used as a new alternative biomarker to reflect the disease severity of primary knee OA and that BMP-2 levels in serum were directly correlated with those in SF. Further investigations are needed to define the mechanisms underlying this association, with the aim to develop effective pharmacological agents to delay the progression of OA.

Conflict of interest

The authors declare that they have no conflict of interest.

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