Original Article
Increased serum fibroblast growth factor-23 (FGF-23) and bone turnover in patients with osteoarthritis of knee

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Abstract: Objective: This study aimed to investigate the serum fibroblast growth factor-23 (FGF-23) of patients with knee osteoarthritis (OA) and to elaborate its correlation with radiographic and symptomatic severity of OA. Methods: Sixty-three subjects were divided into three groups, including healthy controls group (n = 15), patients with rheumatoid arthritis (RA, n = 23) and OA (n = 25). Serum FGF-23 levels were examined by ELISA, and other clinical biochemical parameters were tested based on standard methods. Results: First, we found a significant increase in FGF-23 level in the serum of patients with knee OA compared to healthy controls. Next, an increased trabecular bone formation in subchondral bone was confirmed in the patients with knee OA. In addition, FGF-23 levels were positively correlated with radiographic grading and symptomatic severity of knee OA, and this observation indicated a significant elevation in the systemic levels of FGF-23 correlation with WOMAC scores in patients with knee OA. Furthermore, the results showed that the mRNA and protein expression of FGF-23 were significantly increased in synovial fluid from OA patients as compared to those in healthy control, and the upregulation of FGF-23 could inhibit the apoptosis of mononuclear cell in synovial fluid from OA patients. Conclusions: Serum FGF-23 level was significantly higher and was a significant determinant of increased bone turnover in knee OA patients. FGF-23 might be a potential biomarker for diagnosing and evaluating the onset and development of knee OA.

Keywords: Fibroblast growth factor-23, osteoarthritis, osteoporosis, bone turnover

Introduction
Osteoporosis or osteopenia is common in patients with inflammatory arthritis [1]. In inflammatory arthritis, osteoporosis is mediated by a chronic inflammatory state, in which is an increase in osteoclast activity leading to accelerated bone resorption and suppressed bone formation in the focus [2]. The prevalence of low BMD is reported, including both osteopenic and osteoporotic classifications, in patients with early ankylosing spondylitis (AS) [3]. In rheumatoid arthritis (RA), a reduction in BMD in all measured sites, including the lumbar spine, femoral neck and total hip, is confirmed, and high frequency of glucocorticoid use may be a primary cause to low BMD in RA patients [4]. However, increased risk of bone deteriorations in patients with osteoarthritis (OA) is controversial. Epidemiological surveys suggest that OA and osteoporosis are rarely present together in the same patient. Cross-sectional studies have demonstrated that OA is related to increased bone mineral density (BMD) [5, 6]. These diverse arguments lead to assumptions that increased BMD is rather protective for OA progression. Intriguingly, this postulated has now proven in the OA with postmenopausal women undergoing total hip arthroplasty [7]. Consistent with a previous report, the similar results are found by Zupan et al [8]. In contrast, a growing body of evidences shows a detrimental effect of osteoporosis on articular cartilage in OA animal models [9, 10]. These results indicate that the positive relationship between osteoporosis and OA has been noted in a small fraction of studies. Therefore, the relationship between osteoporosis and OA warrants further study.

Fibroblast growth factor-23 (FGF-23) is a novel member of the FGF family and is an important circulating phosphaturic factor in serum, which is essential in chondrocyte differentiation and
Recent research finds that FGF-23 is localized in the hypertrophic chondrocytes and may play a vital role in chondrocyte maturation and acts as a negative regulator of chondrogenesis, which may induce premature exit of proliferating chondrocytes into hypertrophy leading to shorter bone growth [12, 13]. Interestingly, the mRNA and protein expression of FGF-23 are significantly upregulated in osteoarthritic cartilage, and FGF-23 contributes to hypertrophy and mineralization in osteoarthritic chondrocytes [14, 15]. Moreover, circulating FGF-23 can serve as a biomarker for disease diagnosis. For example, clinical and experimental studies are validated the use of circulating FGF-23 as a biomarker for the early identification of acute kidney injury (AKI) and prediction of short- and long-term adverse outcomes post-AKI [16, 17]. However, for all we know, no literature has been reported regarding the serum FGF-23 levels for early screening of OA.

In the present study, we investigated the mineral metabolism and FGF-23 in patients with OA, and FGF-23 as a biomarker for OA detection were evaluated.

**Materials and methods**

**Patients and specimens**

This study included a total of sixty-three subjects contained those with healthy controls group (n = 15), patients with RA (n = 23) and OA (n = 25), and they were recruited from the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. Knee OA were diagnosed according to the clinical symptomatic criteria of the American College of Rheumatology and Radiographic criteria for OA of at least 1 knee. The study was approved by the local ethical committee of the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology and informed consent was obtained from every subject. Serum samples were collected, centrifuged, aliquoted and stored at -80°C until various routine laboratory test and quantification by ELISA.

The mononuclear cells were separated from synovial fluid by density centrifugation in the patients with OA and healthy control, and maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO2, 95% air atmosphere.

**Physiological and biochemical parameters**

Calcium (Ca), phosphorus (P) and magnesium (Mg) concentrations in serum were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, U.S.A.).

Serum levels of fibroblast growth factor-23 (FGF-23), C-reactive protein (CRP), interleukin-
FGF-23 as a predictive biomarker in knee OA

Table 2. Mineral metabolism in serum of healthy controls and patients with rheumatoid arthritis and osteoarthritis

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 15)</th>
<th>RA (n = 23)</th>
<th>OA (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca (mg/dl)</td>
<td>10.05 ± 0.51</td>
<td>10.36 ± 0.58</td>
<td>9.87 ± 0.66</td>
</tr>
<tr>
<td>P (mg/dl)</td>
<td>3.94 ± 0.33</td>
<td>3.44 ± 0.32</td>
<td>4.52 ± 0.50</td>
</tr>
<tr>
<td>Mg (mg/dl)</td>
<td>2.34 ± 0.28</td>
<td>2.56 ± 0.35</td>
<td>2.47 ± 0.30</td>
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Ca, calcium; P, phosphate; Mg, magnesium. Values are expressed as mean ± SD. *P < 0.05, versus control group; *P < 0.05, versus RA group.

1β (IL-1β) and tumor necrosis factor-α (TNF-α) were detected using human bioactive ELISA assay (Immutopics, Inc., San Clemente, CA, USA) with ELISA reader (MD SpectraMax M5, USA).

Radiographic assessment and symptomatic severity in 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 [18]. 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 [19].

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which were exposed on the cell surface of apoptotic cells. Apoptosis Detection Kit (Beyotime, China) by a flow cytometer (Becton Dickinson, USA) according to the guidelines.

Quantitative RT-PCR (qRT-PCR) and western blotting

Total RNA was extracted from synovial fluid using TRIzol (Invitrogen, USA) and reverse transcribed into cDNA using SuperScriptIII reverse transcriptase kit (Invitrogen, USA), following the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) for FGF-23 mRNA level was performed using the SYBR Green Master (Invitrogen, USA) on a Stratagene MX3005P system (Agilent, USA). β-actin served as an internal standard. Relative gene expression was calculated using 2-ΔΔCt method (Livak and Schmittgen, 2001).

Protein extracted from synovial fluid was separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Germany). Membranes were blocked and then incubated with primary antibodies. β-actin was used as protein loading control. The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with the detected by chemiluminescence (Thermo, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard deviation for each group. All statistical analyses were performed by using PRISM version 5.0 (GraphPad). The Kolmogorov-Smirnov test was performed to analyze data normality, while the unpaired t-test, Mann-Whitney U test, or chi-square test was used to assess significance in clinical char-
characteristics between patients with knee OA and healthy controls where appropriate. FGF-23 levels in the serum from OA patients with different KL grades were compared using one-way analy-

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**Figure 3.** Quantitative analysis of microstructure and mineralization in subchondral bone of healthy subjects and patients with osteoarthritis (OA). BV/TV, bone volume over total volume (A); Tb. N, trabecula number (B); BMD, bone mineral density (C); Tb. Sp, trabecular separation (D); Tb. Th, trabecula thickness (E); Conn. D, connectivity density (F).
FGF-23 as a predictive biomarker in knee OA

The FGF-23 concentrations and the correlation between phosphorus and FGF-23 in serum

Twenty-three serum samples from RA patients, twenty-five serum samples from OA patients and fifteen serum samples from healthy controls were collected for FGF-23 concentrations measurement. As shown in Figure 1, the levels of serum FGF-23 were significantly elevated in OA patients as compared to those in healthy controls ($P < 0.001$), and serum levels of FGF-23 in patients with RA were significantly decreased compared with those in healthy controls ($P < 0.001$). FGF-23, an important regulatory cytokine, is a hormone found in the blood that controls phosphate metabolism [21]. Intriguingly, the levels of serum phosphorus were significantly increased in OA patients as compared to healthy control (Table 2). To test whether there was a relationship between serum FGF-23 and phosphorus in OA patients. As shown in Figure 2A, the correlation analysis of serum FGF-23 levels indicated a positive correlation with serum phosphorus levels in OA patients. The Pearson product-moment correlation coefficient was 0.809, and the difference was statistically significant ($P < 0.001$). In RA patients, the serum phosphorus was downregulated as compared to healthy control (Table 2). The Pearson product-moment correlation coefficient was 0.295, and the difference was statistically significant (Figure 2B, $P < 0.01$). These results indicated that the correlation between phosphorus and FGF-23 in OA patients was more closely than in RA patients.

Physiological and biochemical parameters

C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) have been reported to correlate with clinical findings and radiographic severity in knee osteoarthritis [20], but there were no obvious difference between healthy subjects and OA patients in our work (Table 1). However, CRP and ESR levels were higher in patients with RA than that of the healthy control (Table 1). Moreover, the serum inflammatory factors, IL-1$\beta$ and TNF-$\alpha$, were significantly elevated in RA and OA patients as compared to those in healthy controls (Table 1). Additionally, serum levels of IL-1$\beta$ and TNF-$\alpha$ in RA group were substantially higher than that in OA group (Table 1).

Microstructure and mineralization in subchondral bone

Quantitative analysis revealed that subchondral bone within the OA group had significantly higher BV/TV (Figure 3A), Tb. N (Figure 3B), BMD (Figure 3C), Tb. Th (Figure 3E), conn. D (Figure 3F) and lower Tb. Sp (Figure 3D) compared with the healthy control group. These results suggested that an increased trabecular bone formation in subchondral bone might account for the progression and development of OA.

Serum FGF-23 correlation with OA grading and WOMAC scores

OA was divided into 3 subgroups according to the KL grading system. The association of FGF-23 levels in serum with radiographic severity

Results

Physiological and biochemical parameters

C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) have been reported to correlate with clinical findings and radiographic severity in knee osteoarthritis [20], but there were no obvious difference between healthy subjects and OA patients in our work (Table 1). However, CRP and ESR levels were higher in patients with RA than that of the healthy control (Table 1). Moreover, the serum inflammatory factors, IL-1$\beta$ and TNF-$\alpha$, were significantly elevated in RA and OA patients as compared to those in healthy controls (Table 1). Additionally, serum levels of IL-1$\beta$ and TNF-$\alpha$ in RA group were substantially higher than that in OA group (Table 1).
FGF-23 as a predictive biomarker in knee OA

and WOMAC scores was illustrated. As shown in Figure 4A and 4B, spearman’s rank correlation analysis showed that FGF-23 levels in serum were both positively correlated with radiographic severity and WOMAC scores in OA patients ($r = 0.771, P < 0.001$ and $r = 0.869, P < 0.001$, respectively).

**Discussion**

FGF-23 has been shown to be highly expressed in osteoarthritic chondrocytes and have low expression levels in normal chondrocytes [14]. In osteoarthritic chondrocytes, FGF-23 binds to FGFR1c with greater ability than in normal chondrocytes, confirming thus the enhanced activation of FGF23 signaling in OA [15]. Moreover, the link between chondrocyte hypertrophy and matrix mineralization is well established in endochondral ossification at late-stage OA, which is associated with FGF-23 overexpression [15]. These results demonstrate that endogenous FGF-23 is up-regulated during joint injury, which may be a risk factor for OA. So far, emerging data strongly implicates that FGF-23 in plasma or serum may be utilized as a tool for chronic kidney disease (CKD) diagnosis [17, 24]. Specifically, in children with predialysis CKD, high plasma FGF-23 is the earliest detectable abnormality in mineral metabolism, and levels are highest in glomerular diseases [17]. Moreover, serum FGF-23 level is significantly higher and is a significant determinant of increased bone turnover at early periods in postmenopausal osteoporosis patients [25]. However, we found that the effect of FGF-23 in the early diagnosis and prediction of OA had not been investigated. Therefore, we tried to investigate the role of FGF-23 in the progression and development of OA.

We assessed mRNA and protein expression of FGF-23 in synovial fluid from OA patients. The results showed that the mRNA and protein expression of FGF-23 were significantly increased in OA patients as compared to those in healthy control (Figure 5A and 5B). Previous study shows that FGF-23-Klotho signaling stimulates proliferation and prevents vitamin D-induced apoptosis [22]. FGF-23 loss-of-function exhibits several pathophysiological processes consistent with premature aging including severe atrophy of tissues [23]. In the present study, the spontaneous apoptosis was assessed in synovial fluid mononuclear cell from OA patients and healthy subjects using annexin-V assay. The results demonstrated that rates of apoptosis were 4.8-fold higher in synovial fluid mononuclear cell from healthy subjects as compared to OA patients ($P < 0.001$) (Figure 5C and 5D). Moreover, the levels of FGF-23 in synovial fluid from OA patients correlated negatively with the rate of apoptosis in mononuclear cell ($r = -0.835$, Figure 5E).

We found a significant increase in FGF-23 levels in the serum of patients with knee OA compared to healthy controls. Our results suggested enhanced systemic production of FGF-23 in knee OA patients. Moreover, an increased trabecular bone formation in subchondral bone was confirmed in the patients with knee OA. Recent research has shown that the presence in FGF-23 gain-of-function animals of discrete skeletal phenotypic changes in both the metaphysis and the diaphysis, and the circulating concentrations of FGF-23 may contribute to the type of skeletal injury [26, 27]. These results indicated that the increased levels of FGF-23 in the serum might be responsible for increased BMD in OA joints. Numerous cross-sectional studies have indicated that OA is associated with increased BMD. Mean femoral BMD at the 3 proximal femur sites is 5-9% higher in men and women with knee OA compared with those with no knee OA [5]. Consistent with previous report, our results showed that the BMD of subchondral bone is 8% higher in the patients with knee OA as compared to those of the healthy subjects. In the present study, we were the first to show that FGF-23 was detected in serum obtained from patients with knee OA, and that FGF-23 levels were positively correlated with radiographic grading and symptomatic severity of knee OA. Additionally, this observation indicated a significant elevation in the systemic levels of FGF-23 correlation with WOMAC scores in patients with knee OA. These results indicated that the measurements of serum levels of FGF-23 might possibly serve as a biomarker for determining disease severity in OA.

Interestingly, we found that mRNA and protein expression of FGF-23 in synovial fluid from OA patients were significantly increased, which could regulate the apoptosis of mononuclear cell in synovial fluid. The FGF-23 in synovial fluid may originate from synovial cells and chondro-...
FGF-23 as a predictive biomarker in knee OA

A. Relative FGF-23 mRNA expression

B. Western blot analysis of FGF-23 and β-actin expression in Healthy control and OA patients. P < 0.001

C. Flow cytometry analysis of apoptosis in Healthy control and OA patients. Gating strategy and SSC vs Annexin V plots.

D. Apoptotic cell proportion in Healthy control and OA patients. P < 0.001

E. Scatter plot showing the correlation between synovial fluid FGF-23 levels and apoptotic cell proportion. P < 0.05, r = -0.835
cytes in the local tissues because there have been demonstrated that FGF-23 is endogenously expressed in synovial and articular cartilage [14, 15]. In proximal tubular epithelial cells, exposure to FGF-23 increases in proliferation and upregulates the expression of the cell cycle proteins cyclin D1 and c-myc [22]. Therefore, spontaneous apoptosis in synovial fluid mononuclear cell isolated from OA patients and its regulation by FGF-23 might be involved in the development of OA.

In conclusion, this study revealed a significant increase in serum FGF-23 levels of knee OA patients as compared to healthy controls and demonstrated a pronounced positive correlation of serum levels with the degree of radiographic changes of osteoarthritis of the knee: data from the Baltimore Longitudinal Study of Aging. J Rheumatol 1996; 23: 1943-1947.

Disclosure of conflict of interest

None.

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