

# Expression of hypoxia-inducible factor-1 $\alpha$ in synovial fluid and articular cartilage is associated with disease severity in knee osteoarthritis

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**Abstract.** The aim of the present study was to examine hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) levels in the synovial fluid and articular cartilage of patients with primary knee osteoarthritis (OA) and to investigate their association with the severity of disease. A total of 36 patients with knee OA and ten healthy controls were enrolled. Anteroposterior knee radiographs and/or Mankin scores were assessed to determine the disease severity of the affected knee. Radiographic grading of OA in the knee was performed according to Kellgren-Lawrence criteria. HIF-1 $\alpha$  levels in synovial fluid were measured using enzyme-linked immunosorbent assay, whereas HIF-1 $\alpha$  levels in articular cartilage were assessed with immunohistochemical methods. Compared with healthy controls, OA patients exhibited an increased HIF-1 $\alpha$  concentration in synovial fluid (218.17 $\pm$ 25.12 vs. 156.66 $\pm$ 7.74 pg/ml; P<0.001) and articular cartilage (P<0.05). Furthermore, synovial fluid HIF-1 $\alpha$  levels demonstrated a positive correlation with articular cartilage HIF-1 $\alpha$  levels (Pearson's P=0.815; P<0.001). Subsequent analysis showed that synovial fluid HIF-1 $\alpha$  levels were significantly correlated with the severity of disease (Spearman's  $\rho$ =0.933; P<0.001). Furthermore, articular cartilage levels of HIF-1 $\alpha$  also correlated with disease severity (Spearman's  $\rho$ =-0.967; P<0.001). The findings of the present study suggested that HIF-1 $\alpha$  in synovial fluid and articular cartilage is associated with progressive joint damage and is likely to be a useful biomarker for determining disease severity and progression in knee OA.

## Introduction

Osteoarthritis (OA), which is an age-related condition, is the leading cause of pain, disability and shortening of adult working life (1). Incidence of OA increases with age, with 25% of individuals aged >50 years old exhibiting OA of the knee. Worldwide, ~10% of men, 18% of women, and 60–65% of individuals aged over 60 years have symptomatic OA, and 80% of these patients suffer from limitations in motion (2,3). Clinical symptoms of OA include stiffness, pain, limited motion, crepitus, swelling and deformity (4,5). Despite promising preclinical data covering various molecule classes, the etiology of OA remains poorly elucidated (4). Several biochemical and biomechanical factors have been reported and are considered to be potential lines of investigation for the pathogenesis of OA (5,6).

Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is considered to be a key regulator of cellular adaptation to hypoxic conditions and catabolic stress through the activation of hypoxic response elements (7-9). O<sub>2</sub>-dependent binding of the von Hippel-Lindau tumor suppressor protein ensures it is able to associate with HIF-1 $\alpha$  for proteasomal degradation and ubiquitination. The activity of the HIF-1 $\alpha$  transactivation domains also depends on O<sub>2</sub> regulation through a previously undefined mechanism. Articular cartilage is known to physiologically lack blood vessels, which results in a significantly decreased oxygen level within the tissue; thus, the resident cells require well-adapted mechanisms to ensure survival. Previous studies have demonstrated that HIF-1 $\alpha$  is associated with the regulation of anaerobic energy generation, glucose transport and matrix synthesis by articular chondrocytes (10-12). Furthermore, proinflammatory mediators and mechanical load have also been suggested to increase HIF-1 $\alpha$  activity in articular chondrocytes (13-15). These factors are known to be associated with the pathogenesis of OA. Therefore, it is a reasonable assumption that osteoarthritis chondrocytes depend on HIF-1 $\alpha$  to survive and function properly.

HIF-1 $\alpha$  transcription factor also has an important role in maintaining proper cellular functions under hypoxic conditions (16,17). Grimmer *et al* (18) have revealed that HIF-1 $\alpha$  is associated with the upregulation of microsomal

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prostaglandin E synthase-1 and has an important role in the metabolism of OA cartilage.

Although articular cartilage and/or synovial fluid levels of several cytokines have previously been investigated in patients with knee OA (4-6); to the best of our knowledge, there have been no detailed studies on articular cartilage and synovial fluid levels of HIF-1 $\alpha$  in various clinical stages of primary knee OA. The association between HIF-1 $\alpha$  levels in articular cartilage and disease severity has not been previously reported in the literature. The purpose of the present study was to investigate the expression levels of HIF-1 $\alpha$  in the articular cartilage and synovial fluid of patients with primary knee OA, and evaluate the potential correlation with the osteoarthritic disease process via Mankin scoring and radiographic grading of knee OA, and to further elucidate the pathways associated with the progression of OA.

## Materials and methods

**Patients and sample preparation.** The present study was approved by the Ethical Committee of Xiangya Hospital Central South University (Changsha, China), and was conducted in accordance with the Declaration of Helsinki. A total of 36 patients with primary knee OA (11 males and 25 females; mean age 67.4 years), diagnosed according to the criteria of the American College of Rheumatology, and 10 normal healthy individuals (4 males and 6 females; mean age 58.9 years) were enrolled. Data were reviewed to exclude any forms of secondary OA and inflammatory-associated joint diseases, such as rheumatoid arthritis (RA). Table I shows the patient characteristics.

Disease severity was evaluated with radiographs of the affected knee according to the Kellgren and Lawrence (KL) classification (19). A total of 36 osteoarthritic cartilage and synovial tissue samples were harvested from 36 patients who underwent a total knee replacement due to primary knee OA. Normal cartilage and synovial tissue was collected from 10 human knees at the time of autopsy. Osteoarthritic changes were classified histomorphologically, using modified Mankin scoring (20) as follows: Normal, 0-1; mild lesions, 2-5; moderate lesions, 6-9; and severe lesions, 10-14. The following samples were included in the present study: 10 samples (Mankin score 0-1), 8 samples (Mankin score 2-5), 13 samples (Mankin score 6-9) and 15 samples (Mankin score 10-14).

**Immunohistochemistry.** Specimens were immediately fixed with 4% paraformaldehyde in PBS, decalcified with 0.2 M ethylenediaminetetraacetic acid, embedded in paraffin wax and cut into 5- $\mu$ m-thick sections. Primary monoclonal antibodies against HIF-1 $\alpha$  (1:10,000; cat. no. NB100-105; Novus Biologicals, LLC, Littleton, CO, USA) were incubated with the specimens overnight at 4°C for immunohistochemical analyses. A catalyzed signal amplification kit (DakoCytomation, Carpinteria, CA, USA) based on a streptavidin-biotin peroxidase reaction was employed for the visualization of HIF-1 $\alpha$ , with diaminobenzidine as a chromogen. Control sections were incubated with nonimmune goat antisera (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China). Sections were examined under a microscope at 100x magnification to evaluate the expression of HIF-1 $\alpha$ . Relative HIF-1 $\alpha$  expression

Table I. Patient characteristics.

| Variable           | Osteoarthritis | Normal |
|--------------------|----------------|--------|
| Number of patients | 36             | 10     |
| Age range (years)  | 54-85          | 54-63  |
| Mean age (years)   | 67.4           | 58.9   |
| Sex                |                |        |
| Male               | 11             | 4      |
| Female             | 25             | 6      |

levels in articular cartilage were quantified and visualized as gray values. MIAS-4400 ImageJ (National Institutes of Health, Bethesda, MA, USA) software was used to perform semiquantitative assessment of the mean gray values of HIF-1 $\alpha$  expression. All the sections were evaluated by a pathologist who was blinded to the clinical data. All densities were normalized to PBS and repeated in triplicate. A total of three sections per sample were measured and the mean was calculated to reduce the error arising from the variations in section thickness. The final data consisted of a mean of three independent measurements representing the mean levels of HIF-1 $\alpha$  in the articular cartilage samples.

**Enzyme-linked immunosorbent assay (ELISA).** During total knee replacement surgery, synovial fluid was aspirated from the affected knee, and immediately centrifuged at 1,000 x g for 25 min at 4°C to remove joint debris and cells. According to the manufacturers' instructions, the expression of HIF-1 $\alpha$  in synovial fluid was detected using a commercial ELISA kit (Uscn Life Science, Inc., Wuhan, China). The assays had inter-assay coefficients of variation that were <6% and intra-assay coefficients of variation of <5%. Two independent experiments were performed.

**Statistical analysis.** SPSS for Windows (version 17.0; SPSS, Inc., Waltham, MA, USA) was used for statistical analyses. Student's t-test was used to compare the means of two independent groups and one way analysis of variance was used to compare the means of >2 independent groups, followed by Student-Newman-Keul (SNK) test when comparing among the groups. Spearman's correlation was used to evaluate the correlation between synovial fluid levels of HIF-1 $\alpha$  and the severity of OA. Pearson's correlation and linear regression analysis were employed to determine the correlation between synovial fluid HIF-1 $\alpha$  expression levels and the gray values of HIF-1 $\alpha$  in articular cartilage. Spearman's correlation and linear regression analysis were applied to evaluate the correlation between the gray values of HIF-1 $\alpha$  in articular cartilage and the Mankin score of OA. All data were expressed as the mean  $\pm$  standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

**HIF-1 $\alpha$  expression levels in articular cartilage.** A total of 46 knees were evaluated from 10 controls and 36 patients. HIF-1 $\alpha$  expression levels were detected in the tissues of all the

four groups, which were categorized according to the severity of the lesions in the cartilage (Fig. 1). The average gray value of HIF-1 $\alpha$  expression was 205.49 $\pm$ 4.95 in normal cartilage, 185.34 $\pm$ 9.09 in cartilage with mild lesions, 171.26 $\pm$ 3.40 in cartilage with moderate lesions and 155.48 $\pm$ 10.41 in cartilage with severe lesions, respectively (Table II). Significant differences in the average gray values of HIF-1 $\alpha$  expression were detected among the groups ( $P < 0.05$ ). The average gray value of HIF-1 $\alpha$  expression in each group was demonstrated to be correlated with disease severity according to the modified Mankin score (Spearman's  $\rho = -0.967$ ,  $P < 0.001$ ) (Fig. 2).

**HIF-1 $\alpha$  expression levels in synovial fluid.** The concentrations of HIF-1 $\alpha$  in the synovial fluid of patients with knee OA are demonstrated in Fig. 3. OA patients exhibited higher HIF-1 $\alpha$  concentrations compared with the healthy controls (218.17 $\pm$ 25.12 vs. 156.66 $\pm$ 7.74 pg/ml,  $P < 0.001$ ). Synovial fluid concentrations of HIF-1 $\alpha$  were compared and analyzed in relation to the radiological KL grading values of OA. The concentrations of HIF-1 $\alpha$  in the synovial fluid of KL grade 2 were 179.91 $\pm$ 12.49 pg/ml [95% confidence interval (CI), 169.47-190.36], those from KL grade 3 were 216.37 $\pm$ 9.51 pg/ml (95% CI, 210.62-222.12), whereas those from KL grade 4 were 240.14 $\pm$ 8.16 pg/ml (95% CI, 235.62-244.66). The data indicated that synovial fluid levels of HIF-1 $\alpha$  in cartilage graded as KL grade 4 were significantly increased compared with those of KL grade 2 and 3 ( $P < 0.01$ ). The levels of HIF-1 $\alpha$  also correlated with the severity of disease (Spearman's  $\rho = 0.933$ ,  $P < 0.001$ ; Fig. 4). Notably, synovial fluid HIF-1 $\alpha$  concentrations exhibited a significant correlation with articular cartilage HIF-1 $\alpha$  expression levels (Pearson's  $\rho = -0.815$ ;  $P < 0.001$ ; Fig. 5).

## Discussion

To the best of our knowledge, the present study is the first to evaluate the levels of HIF-1 $\alpha$  in articular cartilage and synovial fluid and its correlation with the severity of knee OA disease. The present findings demonstrated a marked increase in HIF-1 $\alpha$  levels in the articular cartilage and synovial fluid of patients with knee OA compared with the controls. Previous studies have indicated that human and bovine articular chondrocytes and murine epiphyseal chondrocytes express HIF-1 $\alpha$  (21-23). HIF-1 $\alpha$  in the synovial fluid is thought to have originated from local tissues, such as the synovial membrane and articular cartilage. Numerous studies have demonstrated that HIF-1 $\alpha$  is a key factor that influences articular chondrocyte behavior during cartilage homeostasis and osteoarthritis (22-24). HIF-1 $\alpha$  is a highly conserved transcription factor that has important functions in the control of energy generation, matrix synthesis and cell survival by articular and growth-plate chondrocytes (25,26). Previous studies have revealed that the stabilization of HIF-1 $\alpha$  may be a potential tool for increasing cell vitality, matrix synthesis and cartilage integrity in patients with osteoarthritis (24-26). Notably, synovial fluid HIF-1 $\alpha$  concentrations exhibited a correlation with articular cartilage HIF-1 $\alpha$  expression levels in the present study. Therefore, detecting the levels of HIF-1 $\alpha$  in synovial fluid may be used as a marker to predict the degree of cartilaginous damage and disease severity.

Table II. Mean gray value of HIF-1 $\alpha$  expression.

| Group            | No. of samples | Average gray value of HIF-1 $\alpha$ expression |
|------------------|----------------|---|
| Normal           | 10             | 205.49 $\pm$ 4.95                               |
| Mild lesions     | 8              | 185.34 $\pm$ 9.09                               |
| Moderate lesions | 13             | 171.26 $\pm$ 3.40                               |
| Severe lesions   | 15             | 155.48 $\pm$ 10.41                              |

Data are presented as the mean  $\pm$  standard deviation. Difference in the average gray value of HIF-1 $\alpha$  expression levels in the cartilage of the different groups indicated statistically significance when compared between each group ( $P < 0.05$ ). HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ .

HIF-1 $\alpha$  is degraded by an O<sub>2</sub>-dependent mechanism. Under normoxic conditions, HIF-1 $\alpha$  is hydroxylated to allow binding of the von Hippel-Lindau tumor suppressor protein to HIF-1 $\alpha$ , and this binding triggers subsequent enzymatic degradation. Under hypoxic conditions (<5% O<sub>2</sub>), HIF-1 $\alpha$  is stable and can be detected (8). HIF-1 $\alpha$  localizes into the nucleus to associate with HIF-1 $\beta$  to form a heterodimer and activate hypoxia-inducible target genes (13). Hypoxia has previously been demonstrated to increase matrix synthesis of epiphyseal chondrocytes (27), and to induce vascular endothelial growth factor (VEGF) expression in normal chondrocytes via HIF-1 $\alpha$  activity (28,29). VEGF is a key angiogenesis factors, which is able to thicken the synovial membrane, deposit new extracellular matrix and increase the proliferation of synovial fibroblasts. All those factors are essential for the development of OA. The importance of angiogenesis in OA has been further elucidated by several authors in recent publications that demonstrated that new blood vessels are not only formed in synovium but also in other tissues (30,31). The articular cartilage of patients with OA is invaded at the osteochondral junction by blood vessels from the subchondral bone, which subsequently induces an increase in blood vessel density in the non-calcified cartilage (30).

HIF-1 $\alpha$  also is a potent transactivator of numerous genes, including various MMPs (MMP2, 3, 9 and 13) (15) and heat shock protein (28), which are known to act as cellular chaperones for proteins that are misfolded under conditions of cellular stress. Therefore, we hypothesize that HIF-1 $\alpha$  has an important role in O<sub>2</sub>-dependent signaling pathways in the articular chondrocytes, as supported by previous research (32). Therefore, elevated HIF-1 $\alpha$  levels may be associated with the development of osteoarthritis.

The role of oxygen as an important modulator of gene expression is well-recognized and extensive evidence has indicated that HIF is the primary gene and regulatory factor that responds to key variations in O<sub>2</sub> levels (7,8). Genes regulated at the transcription level by HIF-1 $\alpha$  are associated with numerous cellular functional events, including angiogenesis, vascular reactivity and remodeling (9), vasomotor control, glucose and energy metabolism (26), erythropoiesis, iron homeostasis, pH regulation, cell proliferation and

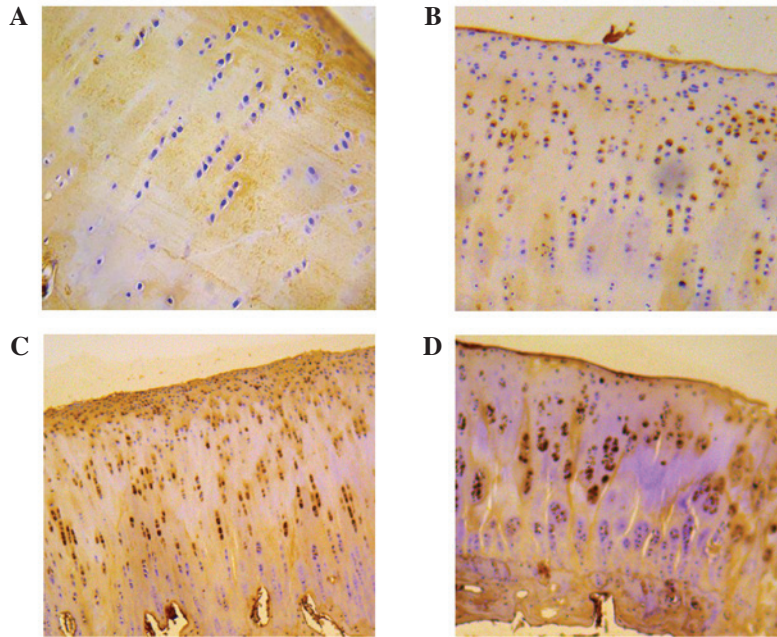


Figure 1. Hypoxia-inducible factor 1α immunohistochemical staining in the articular cartilage of knee osteoarthritis patients and controls demonstrated (A) normal cartilage, (B) mildly leisoned cartilage, (C) moderately leisoned cartilage (D) and severely leisoned cartilage. Magnification, x200.

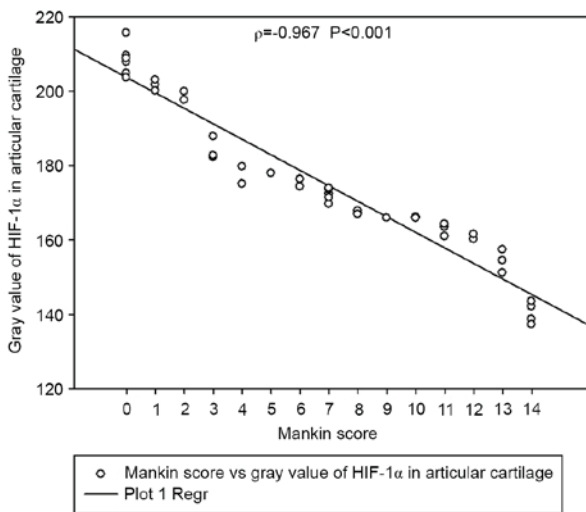


Figure 2. Gray value of HIF-1α in articular cartilage is correlated with Mankin score of osteoarthritis (Spearman's  $\rho=-0.967$ ;  $P<0.001$ ). Osteoarthritis changes were classified histomorphologically, using the Mankin grading system. Spearman's correlation and linear regression analyses were used to determine whether the gray value of HIF-1α in articular cartilage correlated with the Mankin score of osteoarthritis. HIF-1α, hypoxia-inducible factor 1α.

viability, nucleotide metabolism, matrix metabolism, and metal transport (29,30). Furthermore, HIF-1α is essential for chondrogenesis, as it is associated with chondrocyte growth arrest, survival, maturation, and apoptosis (33-35). HIF-1α also regulates the configuration and maintenance of articular cartilage via the induction of anabolic factors and the suppression of key catabolic factors (36). The migration and invasion of fibroblast-like synoviocytes (FLSs) have also been demonstrated to be critical for the pathogenesis of OA (37,38). Li *et al* (15) showed that HIF-1α levels contribute to the migration and invasion of FLSs by upregulating the

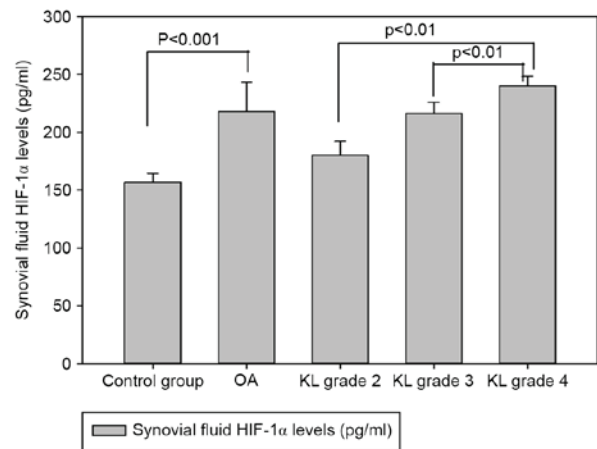


Figure 3. Synovial fluid levels of HIF-1α in healthy controls (n=10), osteoarthritis patients (n=36) and osteoarthritis patients with KL grade 2 (n=8), KL grade 3 (n=13) and KL grade 4 (n=15). HIF-1α, hypoxia-inducible factor 1α; KL, Kellgren and Lawrence; OA, osteoarthritis.

expression of MMP2 and MMP9 via the activation of the NF-κB/HIF-1α signalling pathway. These findings indicated that levels of HIF-1α in both synovial fluid and cartilage may play an important role in the pathogenesis of OA.

There are several potential limitations to the present study. Firstly, the sample size was not large enough to achieve definitive conclusions. Secondly, only those patients who attended Xiangya Hospital for treatment of knee OA were investigated. Thirdly, the cross-sectional design of the study precluded addressing whether the analyzed level of HIF-1α predicted alterations in the severity of knee OA.

In conclusion, patients with knee OA exhibited elevated levels of HIF-1α compared with healthy controls in synovial fluid. Furthermore, the expression of HIF-1α in articular cartilage and synovial fluid was significantly correlated with

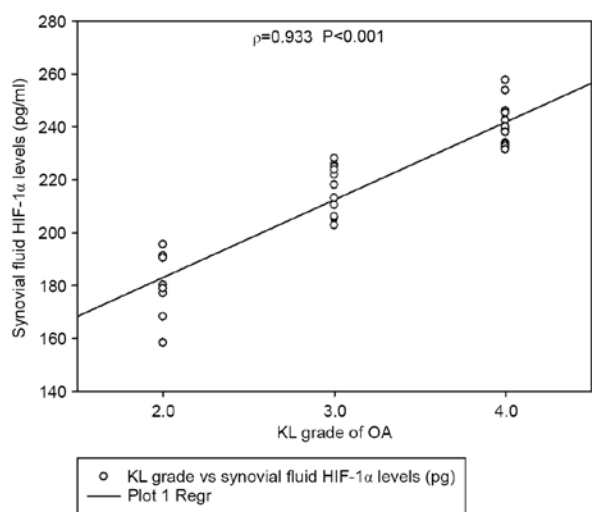


Figure 4. Synovial fluid levels of HIF-1 $\alpha$  are correlated with the KL grade of osteoarthritis (Spearman's  $\rho=0.933$ ;  $P<0.001$ ). Disease severity was determined via weight-bearing anteroposterior radiographs of the affected knee. Knee radiographs were evaluated according to the KL classification. Spearman's correlation was employed to determine the correlation between synovial fluid levels of HIF-1 $\alpha$  and severity of osteoarthritis. HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ ; KL, Kellgren and Lawrence; OA, osteoarthritis.

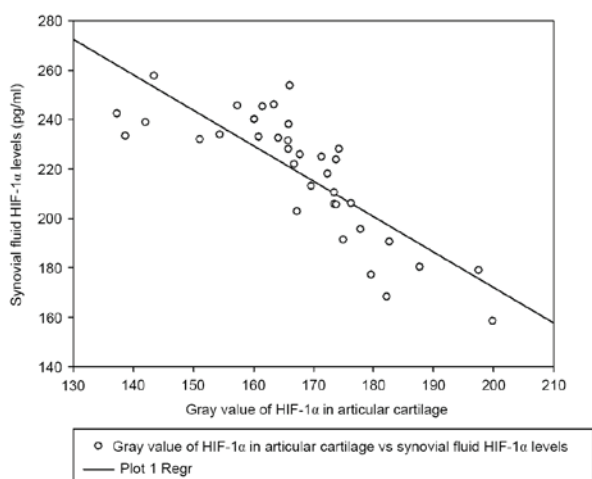


Figure 5. Synovial fluid HIF-1 $\alpha$  levels are correlated with gray value of HIF-1 $\alpha$  in articular cartilage (Pearson's  $\rho=-0.815$ ;  $P<0.001$ ). Pearson's correlation and linear regression analyses were applied to determine the correlation between synovial fluid HIF-1 $\alpha$  levels and the gray values of HIF-1 $\alpha$  in articular cartilage. HIF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ .

the severity disease in knee OA. Further studies are required to elucidate the contribution of HIF-1 $\alpha$  to the pathogenesis of OA.

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