Increased Concentration Of Indian Hedgehog (ihh) In Synovial Fluid (sf) Is Associated With Joint Injury And Early Cartilage Degradation In Human Knee Joint

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Disclosures:  C. Zhang: None. Y. Zhang: None. R. Jiang: None. J. Wang: None. Q. Pei: None. Q. Chen: None. L. Wei: None.

Introduction: Osteoarthritis (OA) is the most common joint disorder characterized by progressive articular cartilage degeneration. Since the progression of OA is a slow degenerative process, early diagnosis is critical for the prevention and treatment of OA. Unfortunately, radiography, as a primary method to diagnose and assess OA progression, has the relatively large errors and poor sensitivity of the method limits it use for early diagnosis and assessment of OA. Thus, There remains a need to identify patients in the early stages of disease before OA can be detected on radiographs. Identification of specific biological markers of articular cartilage metabolism to predict patients at risk for OA has received considerable attention. However, to date, there has not been a reliable biomarker which could detect early articular cartilage degeneration during OA development. Some previous papers have reported chondrocytes can recapitulate some of the differentiation processes that occur in embryogenesis during OA development, although normal articular cartilage chondrocytes maintains a “maturational arrested state”. Ihh is one of three Hh ligands, specifically expressed by flattened prehypertrophic chondrocytes during embryonic development. Previous study found that Ihh was induced in human OA cartilage and synovial fluid (SF), and that the concentration of Ihh in the OA cartilage was associated with the extent of OA cartilage damage, but no quantitative data was performed in SF. The objective of this study was to determine: 1) if the concentration of Ihh in SF is associated with the cartilage lesions determined by arthroscopy in human knee; 2) if Ihh is a potential novel marker for early cartilage damage.

Methods: The study enrolled 160 patients (78 males and 82 females) with an average age of 40.1 years (39.5±15). Among these patients, 60 received total knee replacement because of OA, and 100 underwent arthroscopy because of meniscus injury (n=35), anterior or posterior cruciate ligament injury (n=35), arthroscopic debridement due to pain and disability from OA (n=23), loose body removal (n=1), and amputation due to severe trauma (N=6). The SF was aspirated from the knee joint just before total knee replacement or arthroscopy and also the contralateral knee of ACL injury (N=40). OA diagnosis was made by clinician’s assessment using American College of Rheumatology (ACR) criteria. OA cartilage and Age-matched normal cartilage samples were obtained from knee joints immediately following total knee arthroplasty and amputation due to severe trauma, respectively. Absence of cartilage degeneration was confirmed in the normal cartilage samples using Safranin-O staining. The severity of cartilage damage in the human knee joint were classified using the Modified Outerbridge scoring system and graded using the Modified Mankin score. The normal group included 46 patients with Outerbridge grades of 0 who demonstrated normal cartilage. The early-stage OA group included 59 patients with Outerbridge grades of 1 and 2 who demonstrated slight cartilage erosion. The late-stage OA group included 54 patients with
Outerbridge grades 3 and 4 who demonstrated extensive cartilage erosion. Expression of Ihh in cartilage and SF samples were analyzed with immunohistochemistry (IHC), western blot and semi-quantified by densitometry.

**Results:** The increase of Ihh expression in human OA cartilage was associated with severity of cartilage degeneration (Fig.1). Furthermore, we found that the level of Ihh was increased in the synovial fluid of the joint injured knee in comparison to the contralateral or health control knees but the level in the injured knee was still lower than the OA synovial fluid (Fig.2). There was a significant correlation (P<0.001) between the Ihh concentration in synovial fluid and the Outerbridge score (cartilage damage) (r=0.660) (Fig.3). Thus, the elevated Ihh concentration in the synovial fluid is correlates with the early human knee cartilage lesions.

**Discussion:** Our study firstly illustrated that Ihh content in cartilage and SF was positively associated with the severity of cartilage damage (Fig.1) in human cartilage and synovial fluid samples. Our previous study has found Ihh is produced by the cartilage chondrocytes but not by the synovial membrane cells in the knee joint (1). Thus, this finding may suggest that Ihh expression in SF may be a potential biomarker for diagnosis of early OA and monitoring of OA progression. Furthermore, our semi-quantitative data (Fig.3) established a positive correlation between the concentration of Ihh in SF and cartilage damage determined by the Outerbridge grading system that is considered the “gold standard’’ for assessment of articular cartilage lesions and is repeatable between surgeons. Thus, detection of the Ihh concentration in SF appears to be a promising method to diagnose early cartilage damage in clinical patients.

**Significance:** Our findings suggest that the increased concentration of Ihh in SF is a novel biomarker to predict the early cartilage lesion in human knee.
Figure 1. Increased Ihh expression in OA cartilage determined by IHC. Cartilage damage is determined by Mankin score using Safranin O staining. A strong Ihh staining is seen in the upper layer of OA cartilage. In contrast, Ihh staining was minimal in normal cartilage. Grade I: Mankin score 0-2; Grade II: Mankin score 3-10; Grade III: Mankin score 11-18.

Fig 2. Ihh was increased after ACL injury and in OA SF by Western blot (left) and semi-quantitatively by densitometry (right). Ihh increased by 14.5% after ACL injury as compared to the contralateral knee (p<0.004). Ihh increased by 57.2% in the OA group relative to the control group (p<0.0002). Ihh in OA was significantly greater than that of ACL group (p=0.0048).
Figure 3. The increase of Ihh concentration found in OA SF was correlated to the early stage of OA cartilage lesions. (A) The level of the Ihh protein was increased in SF determined by western blot. (B-a) Representative radiographs confirmed cartilage damage and joint space narrowing in the OA patients and no joint changes in the normal controls. (B-b) Representative Western blot demonstrates a high level of Ihh protein in human OA SF compared to normal control. (B-c and B-d) A1AT and Coomassie Blue stain were used to confirm equal loading. (C) Gray value of Ihh band from Western blot was semi-quantified by Image Analysis Software (Image Lab 3.0, Bio-Rad, USA). Bar graphs show the average with SD, N=3, *: p=0.008. (D) There was a significant correlation (r=0.660; p<0.001) between the Ihh concentration in SF and the articular cartilage Outbridge score. There is a significant difference between the early stage OA and the late stage OA group compared to the normal control. Spearman test was used for statistic analysis. P values of less than 0.05 were considered statistically significant.