Research article

Association of synovial expression of growth and differentiation factor 5 (GDF5) with radiographic severity of knee osteoarthritis

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ABSTRACT

Objectives: The growth and differentiation factor 5 (GDF5) gene plays a significant role in facilitating chondrogenesis, and GDF5 polymorphism is a genetic factor contributing to osteoarthritis (OA). However, the role of GDF5 expression in the synovial membrane remains unclear. The aim of this study was to determine the expression of GDF5 in the synovium in patients with primary knee OA.

Materials and methods: Thirty patients scheduled for total knee arthroplasty were enrolled. Patients were grouped according to the Kellgren and Lawrence classification (KL) as grade 3 (15 patients) and grade 4 (15 patients). Synovial tissue was collected, and the GDF5 expression level was determined by real-time PCR. Ten synovial samples were randomly selected to evaluate the degree of synovitis.

Results: Baseline characteristics did not differ between the two groups. The expression of GDF5 was significantly higher in the KL4 group (median expression 3.50, range 1.45–13.62) than in the KL3 group (median expression 1.81, range 0–9.46) (p value = 0.02). Histological staining of the synovium indicated low-grade synovitis in both groups.

Conclusions: GDF5 expression in the synovium was positively associated with the radiographic severity of knee OA. The difference in GDF5 expression between the KL3 and KL4 groups supports the hypothesis that, through GDF5, the synovium may have important roles in cartilage maintenance and homeostasis in primary knee OA.

1. Introduction

Osteoarthritis (OA) is characterized by articular cartilage degradation associated with deterioration of other joint tissues, including the synovium, subchondral bone, joint capsules, ligaments and muscles [1]. OA greatly impacts patient morbidity, and emerging evidence suggests an association with increased mortality, as those suffering symptoms of hip or knee OA have a standardized mortality ratio of 1.55 compared with the general population [2], reflecting a high disease burden. A better understanding of the molecular mechanisms underlying OA would facilitate the development of biological approaches to slow disease progression. An imbalance of homeostasis between the synthesis and degradation of cartilage has been proposed as a central etiological mechanism underlying OA [1]. According to the most recent evidence, the synovial membrane, the joint capsule, ligaments, muscles and the infrapatellar fat pad contribute to the progression of early OA [3, 4]. Identifying specific molecules that are expressed in the synovial membrane or other tissues around the joint would provide greater insight on the pathophysiology of OA [5, 6], and such biological markers could be useful for predicting and treating OA in its early stages.

Several studies have identified strong genetic factors that contribute to OA [7, 8, 9, 10, 11, 12, 13, 14] and inspired other researchers to explore OA risk alleles via candidate gene analysis, linkage analysis and genome-wide association studies. Single nucleotide polymorphisms (SNPs) of the identified alleles may provide a better understanding of the disease mechanisms and molecular characteristics of OA. The SNP rs143383, a common cytosine allele (C) to thymine allele (T) transition in the 5\textsuperscript{0} untranslated region (5\textsuperscript{0}UTR) of growth differentiation factor 5 (GDF5; also known as cartilage-derived morphogenetic protein 1, CDMP1), is consistently reported to be associated with knee OA in European, Asian and Thai populations [8, 9, 10]. A number of meta-analyses have confirmed the association of this SNP with OA [11, 12, 13, 14]. GDF5 is an extracellular signaling molecule in the transforming growth factor \( \beta \) superfamily that participates in the development, maintenance, and repair of bone, cartilage, and other tissues in synovial joints [15, 16, 17, 18, 19, 20]. This broad

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role of GDF5 suggests that the effect of rs143383 might not be restricted to cartilage tissue. GDF5 expression is upregulated in the cartilage of patients with OA compared with control patients [20]. There is limited evidence of GDF5 expression in the synovial membrane, which has an important role in maintaining normal joint function. In this study, it was hypothesized that GDF5 expression is associated with the severity of OA.

The purpose of the present study was to determine the levels of GDF5 expression in synovial membrane patients with primary knee OA and to investigate whether the level of GDF5 expression is associated with the radiographic severity of knee OA.

2. Materials and Methods

2.1. Study design and patients

This study was approved by the Ethics Committee of the Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand (MURA2014/735/April, 2014). Thirty patients with knee OA scheduled for total knee replacement in Ramathibodi Hospital were recruited during 2014. All patients were of Thai nationality and had ancestors who had resided in Thailand for at least 3 generations. All patients were diagnosed with primary knee OA according to the American College of Rheumatology criteria [21]. Patients with underlying diseases affecting synovial tissue or stability of the knee joint, e.g., rheumatoid arthritis, malignancy, genetic conditions, history of regular steroid usage and patients with a history of osteoporotic fracture, were excluded. Informed consent was gained prior to enrollment. Patient demographics, including sex, age, weight, height, BMI, profession, underlying diseases, excessive kneeling activity and smoking history, were collected. Patients completed the Western Ontario and McMaster Universities Arthritis Index (WOMAC) questionnaire to evaluate their clinical condition, including pain, stiffness, and physical functioning of the joints [22]. Standard weight-bearing knee radiographs were taken, and the severity of knee OA was classified according to Kellgren and Lawrence criteria [23]. Radiograph grading was performed by two surgeons (TT and BW). Intraobserver validity of the severity grading was assessed for substantial agreement.

2.2. Synovial tissues collection

Synovial samples were collected at the time of total knee arthroplasty. Each sample was divided into subsamples for mRNA analysis and histological study. The subsamples for mRNA analysis were stored at -20 °C. The remaining subsamples were fixed in 4% PFA for 12 h and stored at 4 °C until histological study.

2.3. mRNA extraction and quantification using real-time PCR

RNA from synovial tissue was extracted using the RNeasy® Plus Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript VILO cDNA Synthesis Kit (Life Technologies, Grand Island, NY, USA). For real-time PCR, KAPA SYBR® FastqPCR Master Mix (2x) Universal (KAPA Biosystem, Woburn, MA USA) was used. The GDF-5 primers were designed using a web-based tool: GACAAAGCCAAAGATCACG (forward) and TCTCTGGCAAGATCGAGC (reverse). The 20-μL PCR included 10 μL of Master Mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, 1 μL of template DNA and 9.2 μL of PCR-grade water in a 200-μL PCR tube (Axygen, Corning, Tewksbury, MA, USA). The real-time PCR program protocol was as follows: Step 1, enzyme activation (95 °C for 3 min); Step 2, 35 cycles of 95 °C for 5 s (denaturation) and 60 °C for 20 s (annealing and data acquisition). PCR melt curves were used to validate the PCR product from the nonspecific product. The melt setting temperature was increased from 72 °C to 99 °C in step intervals of 1 °C, waiting 15 s at the first step and 4 s at each subsequent step. The PCR platform was a Rotor gene 6000 (Qiagen, Hilden, Germany). Data were analyzed using Rotor gene software 6.1 (Qiagen, Hilden, Germany). Relative quantification was performed using functional comparative quantification in Rotorgene 6.1 software using GAPDH as the housekeeping gene. The results are reported as the n-fold difference between samples.

2.4. Histological study of the synovium

Ten synovial samples were randomly selected (n = 5 for KL3 and n = 5 for KL4) for histological analysis. Samples were embedded in paraffin and sectioned. After standard hematoxylin and eosin staining, the degree of synovitis was determined using a light microscope according to the synovitis score described by Krenn et al. [24]. The synovial lining cell layer, density of the resident cells and inflammatory infiltrate of synovial tissues were scored (no, low-grade and high-grade synovitis). The histological score was determined by a musculoskeletal pathologist (AJ) in a blinded fashion.

2.5. Statistical analyses

Statistical analysis was performed using GraphPad version 5.0 software. The normality of the data was assessed using the Kolmogorov–Smirnov test. For the analysis of baseline characteristics, Fisher’s exact test and t-tests were used for categorical and continuous data, respectively. For the analysis of GDF5 expression and radiographic severity of knee OA, the Mann–Whitney U test was used, as the level of GDF5 expression between KL3 and KL4 was not normally distributed. Fisher’s exact test was used to assess the degree of synovitis and radiographic severity of knee OA. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Demographic data

A total of 30 patients with knee OA undergoing total knee arthroplasty were included in this study. Fifteen patients had a KL score of 3, and patients had a KL score of 4. There were no significant differences in demographics or baseline characteristics (including sex, age, onset of the disease, BMI, smoking, history of labor work, Onset of disease, History of labor work, Excessive kneeling activity, Smoking, Womac score, range, Mean (SD), Table 1. Baseline characteristics between KL groups.

| Patient characteristics | KL grading scale | P-value |
|-------------------------|-----------------|--------|
| Sex                     |                 |        |
| Male No.                | 3               | 1      |
| Female No.              | 12              | 14     |
| Age                     |                 |        |
| Mean (SD)               | 68.73 (10.03)   | 64.53 (7.53) |
| range                   | 53-83           | 50-77  |
| Onset of disease        |                 |        |
| Mean (SD)               | 62.07 (10.5)    | 55.87 (7.54) |
| range                   | 46-81           | 45-67  |
| BMI                     |                 |        |
| Mean (SD)               | 27.9 (6.38)     | 29.7 (5.58) |
| range                   | 21.2–41         | 19.6–39.2 |
| History of labor work   |                 |        |
| Yes                     | 2               | 1      |
| No                      | 13              | 14     |
| Excessive kneeling activity |             |        |
| Yes                     | 0               | 1      |
| No                      | 15              | 14     |
| Smoking                 |                 |        |
| Yes                     | 1               | 2      |
| No                      | 14              | 13     |
| Womac score             |                 |        |
| Mean (SD)               | 119.6 (43.29)   | 126.4 (42.47) |
| range                   | 55-184          | 46-189 |
history of labor work, excessive kneeling activity, smoking and WOMAC score) between the groups (Table 1). Representative radiographic severity images are presented from the KL3 (Figure 1 A,B) and the KL4 (Figure 1 C,D) groups.

### 3.2. Synovial GDF-5 gene expression correlates with radiographic OA severity

The level of GDF-5 gene expression was significantly higher in patients with KL4 OA (fold change 3.50, range 1.45–13.62) than in patients with KL3-graded disease (fold difference 1.81, range 0–9.46) \( (p = 0.02) \) (Table 2).

| GDF5 expression (folds) | KL grading scale | P-value |
|-------------------------|------------------|---------|
| KL3 (n = 15)            | KL4 (n = 15)     |         |
| Median                  | 1.81             | 3.50    | 0.02    |
| range                   | 0–9.46           | 1.45–13.62|

**Figure 1.** Radiographic representative images of KL3 knee OA (A,B) and KL4 knee (C,D).
Table 3. Synovitis grading between KL groups.

| Synovitis grading | KL grading scale |
|-------------------|------------------|
|                   | KL3 (n = 5)      | KL4 (n = 5)      |
| No synovitis      | -                | -                |
| Low-grade synovitis | 5               | 4                |
| High-grade synovitis | -              | -                |

3.3. No association between the degree of synovitis and radiographic OA severity

Synovial tissues from 10 patients with knee OA (n = 5 for the KL3 and KL4 groups) underwent histomorphological grading for identification of the synovitis degree. Synovial tissue samples from both the KL3 and KL4 groups showed low-grade synovitis upon hematoxylin and eosin staining (Table 3) in 2 or 3 layers of synovial lining cells and a minor increase in cellularity (Figure 2 A) with moderate infiltration of inflammatory cells (Figure 2 B). There was no significant difference in the synovitis score between the groups (p > 0.05).

4. Discussion

This study demonstrated that GDF5 is expressed in the synovial membrane of the knee joint and that the level of GDF5 expression is associated with the radiographic severity of knee OA. The level of GDF5 expression in the synovial membrane was significantly higher in patients with severe OA (KL4) than in patients with moderate-grade OA (KL3). The degree of synovitis was not associated with the radiographic severity of knee OA. Histomorphological analysis revealed relatively low levels of synovitis in the joints, regardless of radiographic severity. These findings suggest a role of GDF5 in the synovial membrane and pathogenesis of knee OA and confirm that OA should be considered a noninflammatory form of arthritis.

GDF-5, also known as bone morphogenetic protein 14 (BMP14), is a member of the TGF-β/BMP superfamily. There are 2 types of transmembrane serine/threonine kinase receptors, types I and II, which transduce GDF-5 signals via the downstream Smad pathway. This pathway is involved in the transcription of proteins involved in extracellular matrix synthesis, including COL2A1 and ACAN. GDF5 plays a major role in synovial joint formation and articular cartilage synthesis [15]. Several studies support a role of GDF5 in skeletal development. Mutations in this gene are associated with several skeletal disorders and developmental abnormalities, such as acromesomelic chondrodysplasia Grebe type, brachydactyly type C, proximal symphalangism [25, 26] and decreased human height in both males and females [27]. Moreover, there is growing epidemiological evidence to support an association between an SNP of the GDF5 gene (rs143383, located in the promoter region of GDF5) and an increased risk of knee OA [8, 9, 10]. We have previously demonstrated that the T allele of the SNP is a significant risk factor for knee OA with an odds ratio (OR) of 1.53 (95%C.I. = 1.01–2.31) [10]. A number of large genetic studies have further supported these findings, demonstrating that the progression of knee OA is associated with this SNP. After adjustment for major risk factors, rs143383 was significantly associated with the radiographic severity of knee OA, including an association of the T allele with a more severe tibiofemoral KL grading [28]. A recent meta-analysis also confirmed the association of this SNP with OA [16]. GDF5 was expressed within the synovial membrane, and large epigenetic studies suggest that there is an imbalance in allelic expression of rs143383 within the synovial membrane of patients with knee OA [29]. This is in keeping with the established role of GDF5 in joint homeostasis.

The pathophysiology of OA involves not only cartilage tissue but also the surrounding tissue around the joint, including the synovial membrane ([30]). The synovial membrane secretes important inflammatory molecules implicated in the progression of articular disease. The synovial membrane is composed of two layers, the intima and subintima. The synovial intima is an inner layer comprising fibroblasts and macrophage-like synoviocytes. The functions of fibroblast-like synoviocytes include the secretion of hyaluronan and lubricin. Loose fibrous and fatty tissue is found around the subintima. The synovial membrane may be a source of mesenchymal progenitor cells for cartilage repair [31, 32]. It is possible that the higher expression of GDF5 associated with advanced OA may lead to an increase in the synthesis of articular cartilage to maintain joint homeostasis. The human synovial membrane harbors mesenchymal progenitor cells that display multilineage potential [32]. Shirasawa et al. found that mesenchymal progenitor cells isolated from the synovial membrane have greater chondrogenic potential than those from other tissues [33]. Mesenchymal progenitor cells from the synovial membrane were shown to undergo proliferation and chondrogenic differentiation [34] after induction of cartilage injury in a mouse model. The findings from these previous studies imply that synovial
membrane and mesenchymal progenitor cells from this source might be involved in cartilage maintenance and repair processes and that progenitor cells isolated from the synovial membrane may be a suitable source for cartilage regeneration. Furthermore, GDF5 has been shown to stimulate mesenchymal progenitor aggregation and chondrogenic differentiation in vitro and the induction of cartilage and bone formation in vivo [35]. In rheumatoid arthritis patients, GDF5 has been shown to play a role in regulating the differentiation of fibroblast-like synoviocytes into chondrocytes [36]. Conversely, treatment of OA chondrocytes from cartilage tissue with exogenous GDF5 did not result in any consistent response [37]. Therefore, it is highly likely that GDF5 regulates the process of cartilage maintenance and repair in knee OA via the synovial membrane, which harbors mesenchymal progenitor cells or synovial fibroblast-like cells. The findings from this study support the hypothesis of homeostatic imbalance, in which GDF5 regulates the process of cartilage repair via the synovial membrane, with higher expression of GDF5 in more severely damaged knees.

There are several limitations of the present study, including its relatively small sample size. Furthermore, the associations of other potential confounding factors that may influence GDF5 gene expression, including BMI and age and pain intensity, were not analyzed. However, baseline characteristics and patient demographics were equivalent between the two groups. Further studies are needed to elucidate the potential contribution of the synovial membrane to the cartilage repair process. The identification of the specific location of expression will provide insights on the specific role of this gene in cartilage synthesis. In addition, the role of GDF5 in synovial fibroblasts or mesenchymal progenitor cells from the synovial membrane and the development of OA should be investigated to understand the linkages of GDF5 with pathophysiologic in the progression of OA. In conclusion, this study demonstrated that the level of GDF5 expression in the synovial membrane was associated with the radiographic severity of knee OA. This may imply that GDF5 expression in the synovial membrane plays an important role in cartilage maintenance and repair in primary knee OA. A greater understanding of the molecular basis of OA may lead to therapies that modify disease progression to benefit patients suffering from or at risk of developing OA.

Declarations

Author contribution statement
Bhee Witoonpanich: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Artit Jinawath; Tuempong Wongtawan: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Tulyapruk Tawonsawatruk, M.D., Ph.D.: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement
Data will be made available on request.

Declaration of interests statement
The authors declare no conflict of interest.

Additional information
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