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Cartilage degeneration is associated with activation of the PI3K/AKT signaling pathway in a growing rat experimental model of developmental trochlear dysplasia

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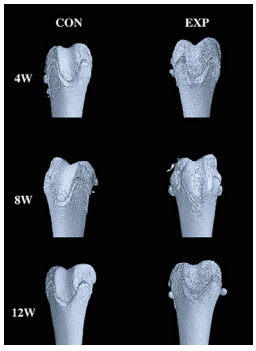
HIGHLIGHTS

- Established a new experimental rat model of the developmental trochlear dysplasia;
- Using the macroscopic morphological and micro-CT to assess trochlear dysplasia;
- Using Histological staining to investigate the cartilage degradation of the model;
- Investigated the relationship of the PI3K/AKT signaling pathway with trochlear dysplasia cartilage degeneration;
- Using immunohistochemistry and qPCR to investigate the PI3K/AKT and the marker of the cartilage degeneration.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Trochlear dysplasia is a commonly encountered lower extremity deformity in humans. However, the molecular mechanism of cartilage degeneration in trochlear dysplasia is unclear thus far.
Objectives: The PI3K/AKT signaling pathway is known to be important for regulating the pathophysiology of cartilage degeneration. The aim of this study was to investigate the relationship of the PI3K/AKT signaling pathway with trochlear dysplasia cartilage degeneration.
Methods: In total, 120 female Sprague-Dawley rats (4 weeks of age) were randomly separated into control and experimental groups. Distal femurs were isolated from the experimental group at 4, 8, and 12 weeks after surgery; they were isolated from the control group at the same time points. Micro-computed tomography and histological examination were performed to investigate trochlear anatomy and changes in trochlear cartilage. Subsequently, expression patterns of PI3K/AKT, TGFβ1, and ADAMTS-4 in cartilage were investigated by immunohistochemistry and quantitative polymerase chain reaction.
Results: In the experimental group, the trochlear dysplasia model was successfully established at 8 weeks after surgery. Moreover, cartilage degeneration was observed beginning at 8 weeks after surgery, with

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higher protein and mRNA expression levels of PI3K/AKT, TGFβ1, and ADAMTS-4, relative to the control group.

Conclusion: Patellar instability might lead to trochlear dysplasia in growing rats. Moreover, trochlear dysplasia may cause patellofemoral osteoarthritis; cartilage degeneration in trochlear dysplasia might be associated with activation of the PI3K/AKT signaling pathway. These results provide insights regarding the high incidence of osteoarthritis in patients with trochlear dysplasia. However, more research is needed to clarify the underlying mechanisms.

Introduction

Trochlear dysplasia (TD) of the femur is a common deformity of the lower extremities; it is regarded as an abnormal anatomical morphology of the depth or angle of the medial or lateral groove facets [1]. In 1964, Brattström first proposed a relationship between TD and patellar instability [2]. Approximately 85–90% of patients with patellar instability reportedly exhibit TD [3,4]. Consequently, many scholars thought that TD was a pathogenic factor of patellar instability [5,6]. Recent studies have shown that the development of TD might be related to excessive femur anteversion, patella alta, and greater tibial tubercle to trochlear groove distance [7–12]. In addition, TD can lead to patellar instability, patellar maltracking, and abnormal pressure load distribution; the most common long-term complication is osteoarthritis (OA) [13–14]. Some scholars consider TD to be a pathogenic factor of early patellofemoral OA [15]. To the best of our knowledge, there have been few studies regarding TD-induced cartilage degeneration at the molecular level.

Articular cartilage has a very low matrix and cell turnover rate, because of its permanent nature [16,17]. Because chondrocytes comprise the only cell type in articular cartilage, an imbalance between chondrocyte proliferation and apoptosis is an important factor associated with onset of OA [18]. Consequently, maintenance of the balance between chondrocyte proliferation and apoptosis can reduce cartilage degeneration. Many signaling pathways are known to be involved in cartilage degeneration; regulation of the PI3K/AKT signaling pathway is an important contributor to the pathogenesis of cartilage degeneration, because of its key roles in several characteristic changes in cartilage (e.g., expression of aggrecanases [ADAMTS-4 and ADAMTS-5] and matrix metalloproteinases) [19]. Furthermore, some studies have shown that activation of the PI3K/AKT signaling pathway can promote osteogenic differentiation of pre-osteoblasts and mesenchymal stem cells; targeted inhibition of the PI3K/AKT signaling pathway can induce bone loss and reduce bone formation [20–22]. Transforming growth factor β1 (TGFβ1) is known to be an important contributor to chondrocyte development; it can regulate extracellular matrix biosynthesis and has been extensively studied as a regulator of cartilage metabolic activity [23]. Previous studies have shown that TD can cause articular cartilage degeneration [24,25]. However, the molecular mechanism of TD-induced cartilage degeneration has been uncertain thus far. Moreover, the effects of the PI3K/AKT signaling pathway in TD-induced cartilage degeneration have not been reported.

Here, to study the association of PI3K/AKT signaling with TD-induced cartilage degeneration, we investigated the expression pattern of the PI3K/AKT pathway in cartilage at different stages in a growing rat model of experimental TD by immunohistochemistry and quantitative real-time polymerase chain reaction (qPCR). We speculated that TD might lead to overexpression of PI3K/AKT, thus resulting in cartilage degeneration. To the best of our knowledge, this is the first study regarding the association between PI3K/AKT and cartilage degeneration in a growing rat model of experimental TD.

Materials and methods

Study design

Four-week-old female Sprague–Dawley rats were obtained from the Laboratory Animal Center of Hebei Medical University. Rats have been shown to require 12 weeks of bone development to reach a mature state [26]. In this study, 120 rats were randomly separated into control and experimental groups (n = 60 per group). In the control group, the rats' left knees did not undergo any surgery. In the experimental group, the rats' left knees underwent surgery to induce patellar instability. Rats were sacrificed by overdose anesthesia at 4, 8, and 12 weeks after surgery. Left distal femur tissues were collected after surgery (n = 20 knees/time point in each group).

Ethics statement

All experiments involving animals were conducted according to the ethical policies and procedures approved by the ethics committee of the Third Hospital of Hebei Medical University. (Approval no. Z2019-005-1).

Surgical technique

In this study, pentobarbital sodium (30 mg/kg, intraperitoneal injection) was used to induce anesthesia in all rats; they were then shaved and disinfected. An incision was then made along the midline of the skin; the skin and subcutaneous tissue were separated, exposing the joint capsule through the medial approach to the knee. The procedure to induce patellar instability was performed as described in our previous studies [24,25]. Specifically, a 0.5-cm longitudinal incision was made along the patella at the medial capsule and retinaculum. In this manner, patellar instability could be observed during surgery. All procedures were carefully performed to avoid cartilage damage. The incision was carefully flushed, then sutured. Finally, the wound was bandaged. To control postoperative pain, rats were administered acetaminophen (30 mg/kg, daily) for 5 days.

Macroscopic morphological and micro-computed tomography (CT) assessment

All distal femurs were carefully harvested and scanned via micro-CT (SkyScan model 1076, SkyScan, Kontich, Belgium; parameters: 10 μm voxel size at 50 kV and 800 μA) at 4, 8, 12 week after surgery, respectively. As described previously [12,24], axial slices of the trochlea were identified; trochlear depth and sulcus angle were then calculated (Fig. 1). Using the diagnostic criteria defined by Dejour et al. [4], TD was diagnosed in micro-CT images.

For analysis of microstructural parameters, the region of interest was located transversely below the lateral and medial facets of the trochlea with two red cylinders of 3-mm diameter (Fig. 1); micro-CT scanning data were transformed into a three-dimensional model by using Mimics software, version 19.0 (Mate-

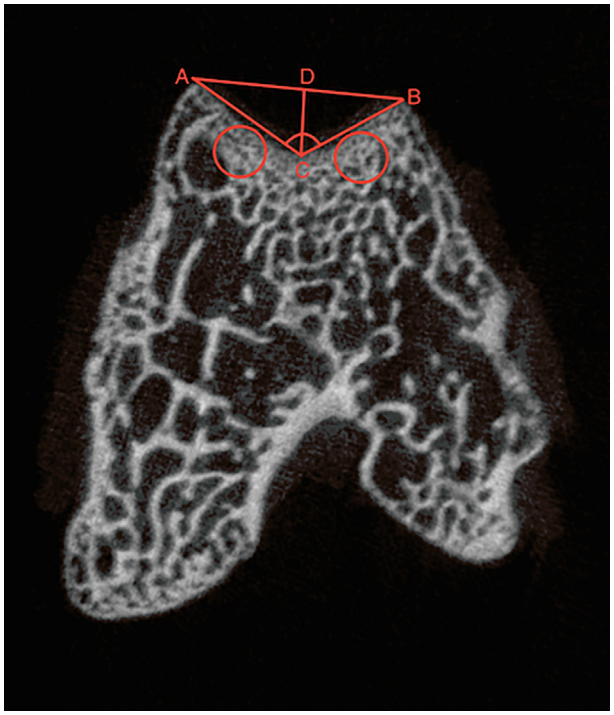

Fig. 1. Measurement diagram. DC, trochlear depth; ACB, sulcus angle.

rialise, Leuven, Belgium). Microstructural parameters analyzed in this study included bone volume to total volume fraction (%), trabecular number (1/mm), trabecular thickness (mm), trabecular separation (mm), and bone mineral density (mg/cm^3).

Histological staining

Samples were isolated at each time point, fixed with 4% paraformaldehyde, decalcified in 10% ethylenediaminetetraacetic acid until completely demineralized, and embedded in paraffin. Five-micrometer slices were cut along the femoral axis to obtain axial images of the trochlear groove. Glycosaminoglycans in cartilage were assessed using Safranin O; evaluation of cartilage degradation was performed by fast green counterstaining of protein. The modified Mankin scale was used to determine the grade of cartilage degeneration [27].

Immunohistochemistry

Slices were deparaffinized in xylene and rehydrated. At room temperature, slices were washed three times with phosphate-buffered saline (5 min per wash). Endogenous peroxidase activity was blocked by incubation of slices in 3% hydrogen peroxide for 10 min. Antigen retrieval was performed by microwave treatment of slices for 10 min in 10 mm sodium citrate (pH 6.0). Slices were incubated overnight at 4℃ with anti-PI3K (BoAoSen, Beijing, China), anti-AKT (Servicebio, Wuhan, China), anti-TGFβ1 (BoAoSen), or anti-ADAMTS-4 (BoAoSen) primary antibodies at a dilution of 1:50. For the negative control, the primary antibody step was omitted. Subsequently, the objective magnification was adjusted to 20 × 100, five regions were randomly selected for all slices in each group, and the entire area of each region was imaged. During microscopy, the tissue covered the entire field of view; the background light was maintained at a consistent level. Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used for analysis of all microscopy images. All images were analyzed to acquire data regarding cumulative optical density and tissue area. The areal density was defined as cumulative optical density divided by tissue area. The areal density value is positively correlated with positive protein expression.

qPCR

Samples were analyzed by qPCR to determine mRNA expression levels at 4, 8, and 12 weeks after surgery. Trizol reagent (Servicebio) was used to extract RNA from chondrocytes and cartilage. The RevertAid™ first-strand cDNA synthesis kit (Cat. No. K1622, Thermo Fisher Scientific, Waltham, MA, USA) was used for reverse transcription of mRNA into cDNA. Primers for PI3K, AKT, TGFβ1, and ADAMTS-4 were used, with a sequence detection system for gene analysis. mRNA expression of target genes was determined with reference to *GAPDH* and calculated using the formula $2^{-\Delta\Delta Ct}$ (cycle threshold method). All primers used in this study are listed in Table 1. Each experiment was performed three times and mean values were used for further analyses.

Statistical analysis

Mean and standard deviation were used for descriptive statistical analysis. The Shapiro–Wilk test was used to determine normality for each variable, while Levene's test was used to assess homogeneity of variance. Student's *t*-test was used for comparisons between two groups; one-way analysis of variance was used for comparisons among ≥ 3 groups. SPSS Statistics, version 19.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Differences with $p < 0.05$ were considered statistically significant. Preliminary analysis suggested that at least six rats were needed at each time point and in each group, to achieve 80% efficacy (1-β) and 90% confidence [28].

Results

Macroscopic morphology and micro-CT assessment of patellar instability model of TD

The TD model was successfully established, characterized by a larger groove angle and flatter trochlear groove (Tables 2 and 3). We regard the trochlear depth and sulcus angle of the control group at different time points as normal values. The trochlear depth and sulcus angle did not exhibit significant differences between the control and experimental groups ($P > 0.05$) at 4 weeks after surgery; however, they exhibited significant differences between the two groups ($P < 0.05$) at 8 and 12 weeks after surgery. Over time, the trochlear groove became significantly flatter and sulcus angle became significantly larger, compared with the control group (Fig. 2). In the experimental group, the trochlear depth was 0.6 ± 0.2 mm, which was lower than the trochlear depth of the control group (0.9 ± 0.2 mm, $P = 0.039$) at 8 weeks after surgery. Similarly, in the experimental group, the trochlear depth was 0.9 ± 0.2 mm, which was lower than the trochlear depth of the control group (1.5 ± 0.2 mm, $P = 0.029$) at 12 weeks after surgery. In the experimental group, the trochlear sulcus angle was

Table 1
Primers for amplification of target genes and *GAPDH*.

	Forward primer sequence	Reverse primer sequence
PI3K	ACAGGCACAACGACAACATCAT	AGGTAAGCCCTAACGCAGACAT
AKT	TGAGACCGACACCAGGTATTTTG	GCTGAGTAGGAGAACTGGGGAAA
TGFβ1	CTAATGGTGGACCGCAACAAC	GTAACGCCAGGAATTGTTGCTAT
ADAMTS-4	GTACCTACCTGACTGGCACCATC	TGCTGCCATCTTGTCATCTGC
GAPDH	CTGGAGAAACCTGCCAAGTATG	GGTGGAAGAATGGGAGTTGCT

Table 2
Sulcus angle measurements compared between the two groups.

	Control group	Experimental group	*P* value
4 weeks	120.0° ± 3.0°	118.0° ± 2.6°	0.739
8 weeks	126.0° ± 3.1°	131.0° ± 2.9°	0.038
12 weeks	132.0° ± 3.4°	152.0° ± 3.0°	0.026

Mean ± standard deviation.

Table 3
Trochlear depth measurements compared between the two groups.

	Control group	Experimental group	*P* value
4 weeks	0.5 ± 0.1 mm	0.4 ± 0.2 mm	0.913
8 weeks	0.9 ± 0.2 mm	0.6 ± 0.2 mm	0.039
12 weeks	1.5 ± 0.2 mm	0.9 ± 0.2 mm	0.029

mean ± standard deviation.

131.0° ± 2.9°, which was higher than the trochlear sulcus angle of the control group (126.0° ± 3.1°, $P = 0.038$) at 8 weeks after surgery. Similarly, in the experimental group, the trochlear sulcus angle was 152.0° ± 3.0°, which was higher than the trochlear sulcus angle of the control group (132.0° ± 3.4°, $P = 0.026$) at 12 weeks after surgery.

Detection of subchondral bone loss by micro-CT

In the experimental group, micro-CT displayed obvious loss of subchondral bone at 8 weeks after surgery, and became more and more serious with time. Micro-CT scans of the two groups revealed that the bone volume to total volume fraction, trabecular number, and trabecular thickness decreased markedly at 8 and 12 weeks after surgery ($P < 0.05$); moreover, trabecular separation significantly increased at 8 and 12 weeks after surgery ($P < 0.05$). However, bone mineral density did not remarkably differ ($P > 0.05$) (Fig. 2).

Loss of cartilage proteoglycans and higher Mankin score in the TD model

At 4 weeks after surgery, no significant differences were present in cartilage appearance between the two groups. However, the amount of matrix proteoglycans was significantly reduced, the cartilage surface was damaged (accompanied by rougher and thinner appearance), and chondrocytes were disordered in the experimental group at 8 and 12 weeks after surgery, and the number of chondrocytes was significantly reduced in the superficial and middle zone, which was consistent with OA (Fig. 3). Moreover, Mankin scores were higher in the TD model cartilage at different stages and worsened over time (Fig. 3) ($P < 0.05$).

Increased expression of PI3K/AKT, TGFβ1, and ADAMTS-4 in the TD model cartilage

Immunohistochemistry analysis revealed that the expression of PI3K/AKT was slightly elevated at 4 weeks after surgery ($P < 0.05$), compared with the control group; however, the expression patterns of TGFβ1 and ADAMTS-4 showed no remarkable changes

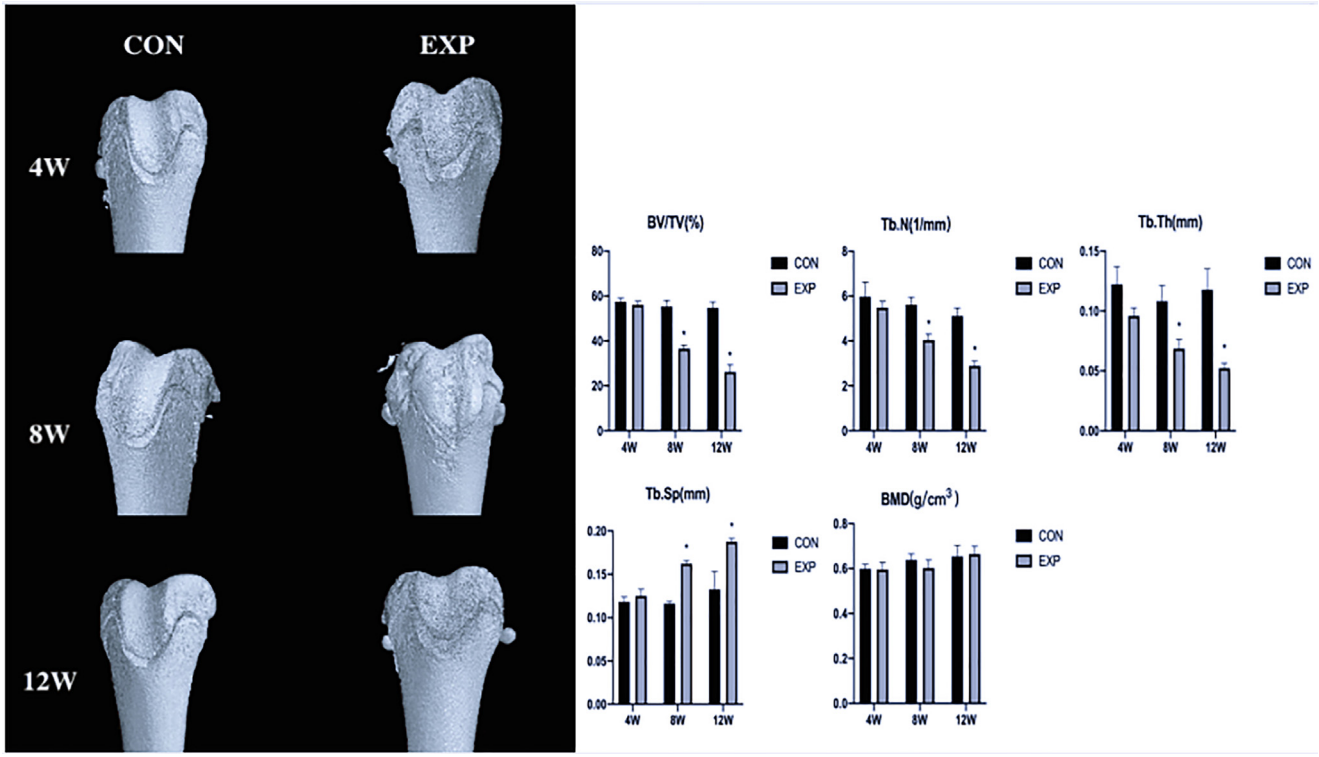

Fig. 2. Three-dimensional reconstruction of distal femur and Histomorphometric comparison by micro-computed tomography scans at 4, 8, and 12 weeks between CON and EXP groups. We regard the trochlear depth and sulcus angle of the control group at different time points as normal values. Over time, the trochlear groove became significantly flatter and sulcus angle became significantly larger, compared with the control group at 8, 12 weeks after surgery. In the experimental group, micro-CT displayed obvious loss of subchondral bone at 8 weeks after surgery, and became more and more serious with time. *$P < 0.05$ (BV/TV: bone volume to total volume; TB.N: trabecular number; TB.Th: trabecular thickness; TB.Sp: trabecular separation; BMD: bone mineral density; CON, control; EXP, experimental).

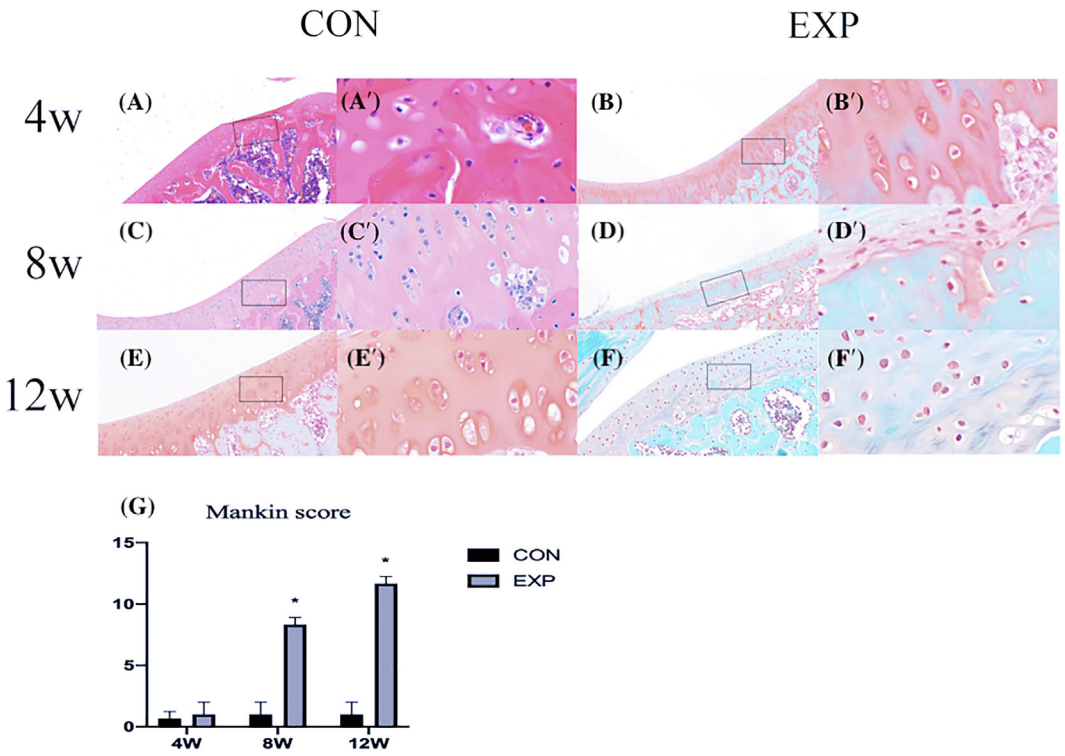

Fig. 3. Safranin O staining in rat cartilage. CON and EXP groups, respectively: (A, B) 4 weeks; (C, D) 8 weeks; (E, F) 12 weeks. A′–F′ show higher magnifications of boxed areas in A–F. The safranin O and fast green histochemical staining showed that the amount of matrix proteoglycans was significantly reduced, the cartilage surface was rougher and thinner, and the chondrocytes were disordered in the experimental group at 8 and 12 weeks after surgery. In D′, F′, we observed the number of chondrocytes in the superficial and middle zone was significantly reduced, which was consistent with OA. (G): Mankin score for trochlear cartilage: higher Mankin scores in the cartilage at different stages and worsened over time. CON, control; EXP, experimental. *$P < 0.05$.

($P > 0.05$). Additionally, there were remarkable differences in PI3K/AKT, TGFβ1, and ADAMTS-4 expression at 8 and 12 weeks after surgery ($P < 0.05$) (Figs. 4 and 5). Furthermore, qPCR analysis showed expression patterns similar to those of immunohistochemical analysis ($P < 0.05$) (Fig. 6).

Discussion

In this study, we successfully established an experimental model of TD in growing rats after induction of patellar instability; the model was characterized by subchondral bone loss and cartilage degeneration, which worsened over time. Importantly, we observed remarkably elevated expression of PI3K/AKT at the protein and mRNA levels in the cartilage degeneration model, which revealed that TD-induced cartilage degeneration might be associated with activation of the PI3K/AKT signaling pathway. These results provide new insights regarding the high incidence of OA in patients with TD. To the best of our knowledge, this is the first report regarding the relationship between PI3K/AKT signaling and cartilage degeneration in the patellar instability model of TD.

Mechanical stress is known as a major factor in the development of bone and cartilage [29]. Changes in stress distribution in the patellofemoral joint will affect cartilage metabolism; positional mismatches between the patella and femoral trochlea may lead to OA [30–32]. When patellofemoral joint pressure increases due to abnormal patellar biomechanics, cartilage is damaged more easily [33–34]. The present results support the hypothesis that TD may be induced by mechanical stimuli, because pressure changes in the patellofemoral joint due to patellar instability; such changes worsen over time during TD progression. However, mechanical factors may not be the only means by which TD develops into OA. Because TD is a developmental disease, abnormal joint development may lead to early articular cartilage degeneration.

The PI3K/AKT signaling pathway is a key component of cell development, including cell metabolism, apoptosis, transcription, and cell cycle [35]. Additionally, abnormal expression levels of PI3K/AKT have been found in many cancers [36]. Previous studies have shown that PI3K/AKT signaling contributes to the pathogenesis of OA [37,38]; inhibition of PI3K/AKT expression in rat chondrocytes can improve autophagy and delay the progression of OA [39,40]. The progression of OA is known to be affected by inflammatory cytokines, and the PI3K/AKT signaling pathway can mediate NF-κB activation and the mRNA expression of TNFα in osteoblasts; inhibition of PI3K/AKT signaling can protect chondrocytes from inflammatory disruption in OA [41–44]. Thus, we presumed that the PI3K/AKT signaling pathway plays an important role in TD pathology. To test this hypothesis, we examined PI3K/AKT expression in rat trochlear cartilage. Immunohistochemistry and mRNA expression analyses revealed that the levels of PI3K/AKT increased over time in the patellar instability model of TD. Thus, persistent high expression of PI3K/AKT in the TD model may explain the continued existence of developmental factors in articular cartilage. However, further studies are needed to clarify how PI3K/AKT contributes to TD pathology.

TGFβ1 is an important research focus, with potential for clinical use; it may aid in diagnosis and treatment of OA [45]. Abnormally elevated levels of TGFβ1 in cartilage can promote production of proteoglycans and lead to abnormal growth of osteophytes and synovium [46]. TGFβ1 accelerates the condensation of mesenchymal stem cells, and improves early chondrocyte differentiation, while inhibiting terminal hypertrophic differentiation; thus, it influences regular bone morphology [47]. In the present study, persistent high expression of TGFβ1 in the TD model increased in an age-dependent manner. Over-

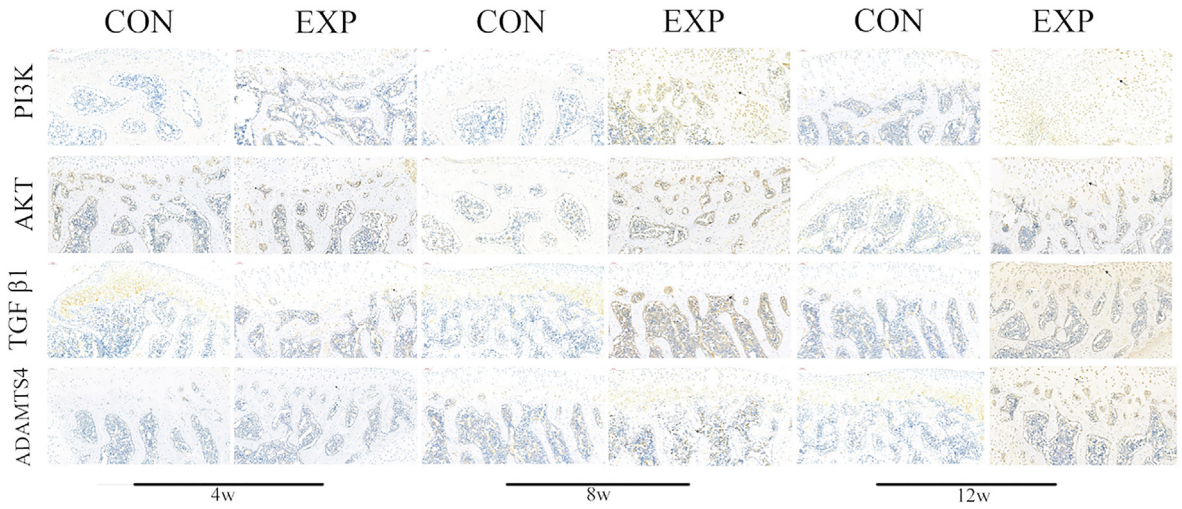

Fig. 4. Immunohistochemical comparison between CON and EXP groups: PI3K/AKT, TGFβ1, and ADAMTS-4 at 4, 8, and 12 weeks. Arrows in EXP group indicate positive expression. Brown to black color indicates positive staining. Scale bar: 50 μm. CON, control; EXP, experimental.

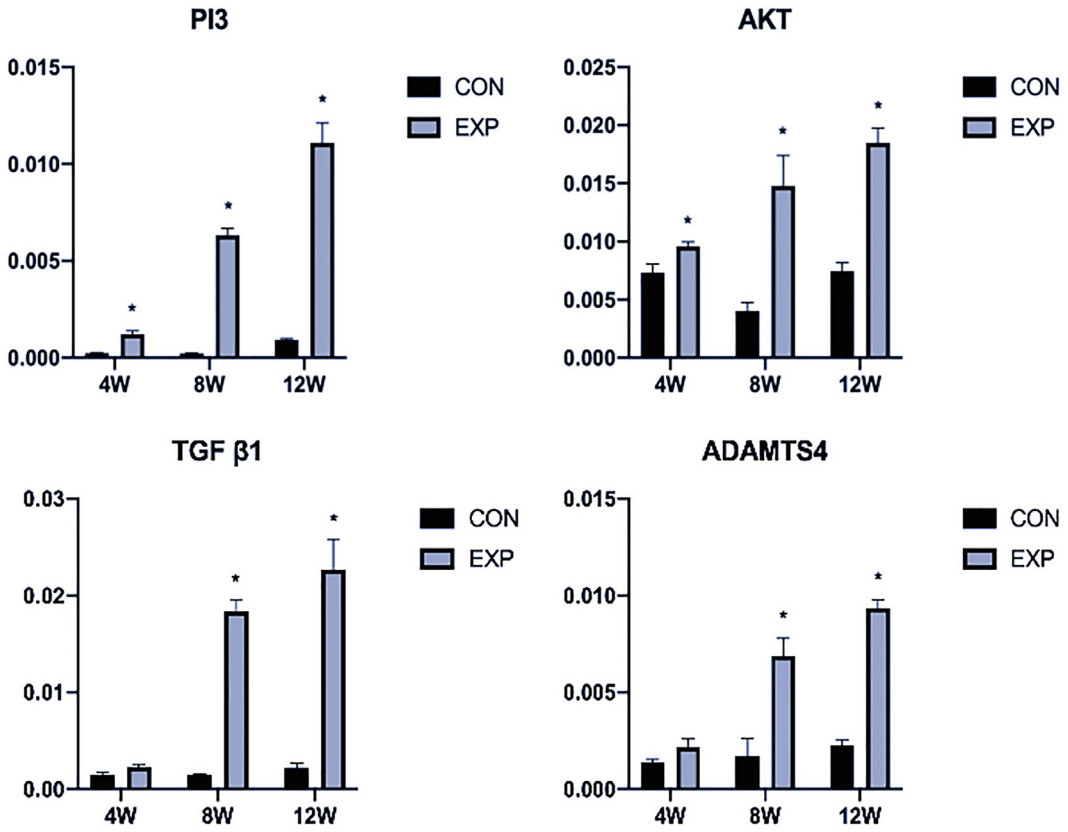

Fig. 5. The result of analyzing the staining intensity of PI3K/AKT, TGFβ1, and ADAMTS-4 in Fig. 4. There were remarkable differences in PI3K/AKT, TGFβ1, and ADAMTS-4 expression at 8 and 12 weeks after surgery. CON, control; EXP, experimental. *$P < 0.05$.

all, these findings suggested that articular cartilage in the rat TD model exhibited early molecular expression of OA.

Notably, our previous study confirmed that TD can lead to cartilage degeneration [24,25]. In the present study, we found that, with increasing age, cartilage morphology changed in the rat TD model: its surface became rough, vertical cracks appeared, and articular chondrocytes aggregated. Furthermore, qPCR analysis revealed that ADAMTS-4 [48], a marker of cartilage degeneration, was overexpressed in cartilage of the TD model. Thus, this study confirmed the presence of cartilage degeneration in a model of TD. These findings were consistent with the manifestation of OA, suggesting that TD may contribute to onset of patellofemoral OA.

As we all know, human OA is characterized by pathological changes of most joint tissues, including cartilage degradation, subchondral bone structure changes and synovial inflammation, which eventually lead to joint space narrowing and osteophyte formation, leading to serious damage of knee joint function [49–51]. Our study found that in our model of TD in growing rates after

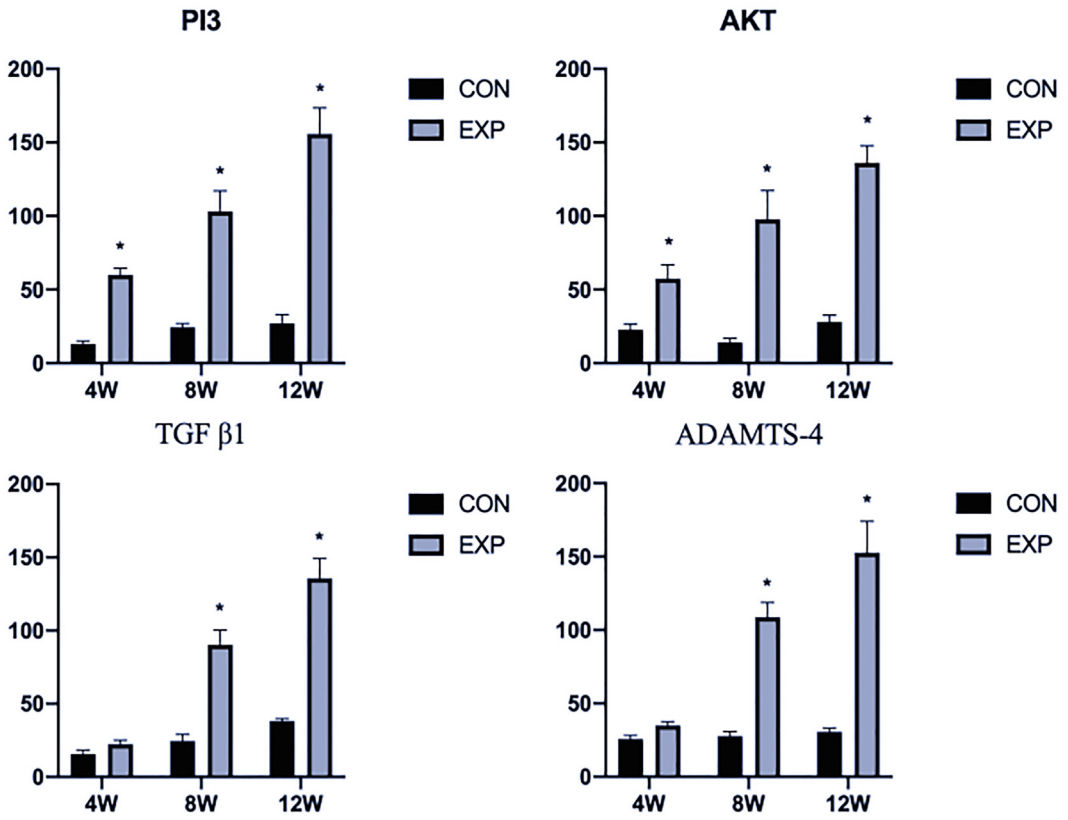

Fig. 6. qPCR evaluation of PI3K/AKT, TGFβ1, and ADAMTS-4 expression levels at 4, 8, and 12 weeks in CON and EXP groups: showed higher expression of PI3K/AKT, TGFβ1, and ADAMTS-4 in the EXP Group at 8, 12 weeks after surgery. The Y-axis represents the PCR amplification multiple of the target gene. CON, control; EXP, experimental. $^{*}P < 0.05$.

induction of patellar stability; the model was characterized by subchondral bone loss and cartilage degradation, which worsen over time. Importantly, we observed significantly elevated expression of PI3K/AKT at the protein and mRNA levels in the TD model, which were consistent with the characteristics of human OA. Through these studies, we speculate that the PI3K/AKT signaling pathway can be used as a potential therapeutic target for the treatment of human OA to prevent cartilage degradation and subchondral bone loss.

Our study had several limitations. First, findings in a rat model can not be readily translated to the clinic. Second, this study only investigated the early stage of patellofemoral OA at 12 weeks after induction of patellar instability; it did not generate long-term follow-up data. Third, further investigation is needed regarding the molecular mechanisms of PI3K/AKT signaling during TD-induced cartilage degeneration.

Conclusions

The findings in this study suggested that patellar instability may lead to TD in growing rats. Moreover, TD may cause patellofemoral OA; TD-induced cartilage degeneration might be associated with activation of the PI3K/AKT signaling pathway. These results provide new insights regarding the high incidence of OA in patients with TD. However, additional research is needed to clarify the underlying mechanisms.

Compliance with ethics requirements

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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