CARTILAGE
2021, Vol. 13(Suppl 1) 818S–828S

DOI: 10.1177/1947603520932199
journals.sagepub.com/home/CAR
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# Altered Expression of Aggrecan, FAM20B, B3GALT6, and EXTL2 in Patients with Osteoarthritis and Kashin-Beck Disease

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**Abstract**

*Objective*. The objective of this study was to investigate the expression of enzymes involved in synthesis and modification of chondroitin sulfate (CS) in knee cartilage tissue of patients with osteoarthritis (OA) and Kashin-Beck disease (KBD). *Methods*. The knee articular cartilage samples were obtained from 18 age- and gender-matched donors with 6 each in KBD, OA, and control groups. Hematoxylin and eosin (HE) staining, toluidine blue (TB) staining, and immunohistochemical (IHC) staining were performed to estimate the expression level and localization of aggrecan, along with FAM20B, GalT-II, and EXTL2, which are associated with CS synthesis and modification. Rank-based analyses of variance test was used for the multiple comparisons of discrepancy in the positive staining rate among the 3 groups. *Results*. In HE and TB staining results, damaged morphology, decreased chondrocyte numbers and proteoglycans were observed in OA and KBD groups compared with the control group. In line with these trends, the positive staining rates of aggrecan were lower in KBD and OA groups than in the control group. Meanwhile, the positive staining rates of CS chain modifying enzymes FAM20B, GalT-II, and EXTL2 decreased in OA and KBD groups. *Conclusions*. In conclusion, it was demonstrated that altered expression of CS chain modifying enzymes in OA and KBD groups influenced the synthesis procession of CS and could contribute to the damage of cartilage. Further investigation of these enzymes can provide new theoretical and experimental targets for OA and KBD pathogenesis studies.

**Keywords**

Kashin-Beck disease, osteoarthritis, chondroitin sulfate, immunohistochemistry, cartilage

## Introduction

Osteoarticular disease is one of the most prevalent chronic joint diseases in the world.[1,2] Osteoarthritis (OA) is the major osteoarticular disease and causes joint pain and stiffness.[3] The pathologic changes in OA include progressive degradation of articular cartilage, formation of osteophytes, variable degrees of inflammation of the synovium, and subchondral bone remodeling.[4,5] Unlike OA, Kashin-Beck disease (KBD) is an endemic osteoarticular disease with high prevalence and morbidity in the Eastern Siberia of Russia, North Korea, and northeastern to southwestern belt in China.[6] KBD is characterized by degeneration and necrosis in the deep layer of hyaline cartilage, dyschondroplasia, inflammation, and metabolic disorders related to cartilage extracellular matrix (ECM) involving chondroitin sulfate (CS) modifying enzymes.[6-10]

The ECM of cartilage contributes to the cell structure and functional maintenance of articular cartilage.[11] The degeneration of cartilage ECM has been demonstrated in OA and KBD pathology, particularly in necrotic areas.[10-13]

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The cartilage ECM consists of large aggregating and small leucine-rich proteoglycans (PGs), hyaluronan, glycoproteins, and collagens.[14,15] PGs are important biomacromolecules composed of core protein and glycosaminoglycan (GAG) chains,[14,16,17] and participate in ECM deposition, cell differentiation, adhesion, and migration.[5] Aggrecan is the principal PG present in the ECM of articular cartilage. As a part of aggrecan, CS is the major component of cartilage and provides resistance against compression.[17] The negative groups of the GAGs can adsorb water and act as hydrogel and lubricant in the joints.[18] Furthermore, altered expression of CS and decreased content of CS have also been observed in both OA and KBD.[10,11,13]

CS sulfation is an essential process for the completion of PG function. A series of glycosyl transferase and sulfonyl transferase are involved in the synthesis and sulfation modification process of CS, including enzymes such as the family with sequence similarity 20, member B (Gene ID: FAM20B, Protein ID: XylK), β-1,3-galactosyltransferase 6 (GalT-II, B3GALT6), and exostosin-like 2 (EXTL2, EXTL2). Fam20B can phosphorylate the xylose residue in the GAG-protein linkage region of PGs, and regulate the rate of GAG chains synthesis.[16,19-21] GalT-II is an important enzyme participating in the biosynthesis of GAG tetrasaccharide linkage region,[18,22] while EXTL2 functions as a suppressor of Fam20B and induces CS biosynthesis.[23]

Subsequently, we speculate that the dysfunction of CS synthesis and sulfation modifying enzymes may be involved in the cartilage destruction of OA and KBD patients. However, studies related to those enzymes are still limited, and the mechanisms need to be further investigated. In this study, hematoxylin and eosin (HE) staining was used to observe the number and morphology of chondrocytes; toluidine blue (TB) staining was used to evaluate the change of matrix and the sulfated GAGs of articular cartilage in different layers of the KBD, OA, and control groups; immunohistochemical (IHC) staining was performed to investigate the expression levels of 3 CS metabolic enzymes (FAM20B, GalT-II, EXTL2) and aggrecan in the articular cartilage of KBD, OA, and control groups.

## Methods

### Sample Collection and Grouping

Knee articular cartilage samples were obtained from 18 patients during joint replacements or joint debridement operations. The donors were age and gender matched (KBD $n = 6$, OA $n = 6$, control $n = 6$).[9]

The chi-square test ($\chi^2_{gender} = 0.560$) and Student $t$-test ($F_{age} = 2.485$) analyses of the samples showed that subjects in the 3 groups did not differ significantly ($P > 0.05$) in gender and age distribution. The KBD cartilage samples were collected from patients with KBD assessed by clinical classification of grades II/III, and diagnosed according to the diagnostic criteria for KBD (WS/T207-2010)[24] diagnosed by the X-ray films of the right hand, knee, and hip. The OA samples were collected from donors living in non-KBD areas of Shanxi province and who had been OA diagnosed based on the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC)[25] and their samples graded based on histopathology.[8,26]

The normal control samples were collected from donors living in non-KBD areas of Shanxi province who suffered from accident injury and received total knee arthroplasty or amputations surgery. In addition, these donors were diagnosed with X-ray films, biochemical and histopathological examination to exclude OA, KBD, or other osteoarticular diseases. This investigation was approved by the Human Ethics Committees of Xi'an Jiaotong University and conducted according to the World Medical Association's Declaration of Helsinki. All the donors provided a written informed consent for the study participation and publication of their individual clinical details and images.

### HE Staining and TB Staining

Once collected, the articular cartilage tissues were fixed with 4% (w/v) paraformaldehyde for 24 hours, and decalcified in 10% (w/v) ethylenediaminetetraacetic acid disodium salt (EDTA-$Na_2$) for 4 weeks with regular agitation to eliminate calcium. Then, the samples were embedded in paraffin and cut into serial 6-μm-thick sections in the coronal direction. The sections were deparaffinized in xylene, followed by decreasing concentrations of ethanol solution (100%-80%). After deparaffinization, the sections were stained with HE and 0.1% (w/v) TB solution. After rinsing with running distilled water for 10 minutes, the sections were dehydrated by increasing concentrations of ethanol solution (80%-100%). Finally, the sections were dehydrated in xylene before covering with coverslips and then observed with microscope.

### IHC Staining

IHC staining was performed to investigate the expression levels of 3 CS metabolic enzymes and aggrecan in the articular cartilage of KBD, OA, and control groups. The protocols of IHC staining were based on the method used in our previous study.[9] The primary antibodies used were as following: Polyclonal rabbit primary antibodies against human FAM20B (ab121261, Abcam, Cambridge, MA, USA), human GalT-II (ab103375, Abcam), human ACAN (13880-1-AP, Proteintech, Rosemont, IL, USA), and monoclonal rabbit primary antibody against human EXTL2 (ab168391, Abcam). The primary antibodies were diluted in phosphate buffered saline (PBS) for IHC staining. To ensure the specificity and comparability of IHC results, negative controls

were performed. For negative controls, the primary antibody was replaced by PBS, and the other protocol was consistent with the IHC staining protocol.

### Image Capture

Representative regions of the sections were photographed using Olympus BX51 microscope (Olympus, Tokyo, Japan). For different staining, the sections were observed at 100× magnification and six images from superficial, middle, and deep layers were captured. The resolution of all images was 1200 × 1600 pixels and they were saved in TIFF format.

### Statistical Analysis

For HE staining, the total cell numbers in each image were calculated. For TB staining, the rates of staining area were obtained using Image J software (National Institutes of Health, USA) with threshold of 180. For IHC staining, the rates of positive staining cells in superficial, middle, and deep layers of FAM20B, EXTL2, and GalT-II (only cells with integrity morphology were counted) were calculated from 6 images per one sample. The positive staining rates were analyzed with SAS 9.3 software. The data were evaluated using normality test and homogeneity of variance analysis. Median (P25, P75) was calculated and rank-based analysis of variance (ANOVA) test was used for the multiple comparisons of discrepancy in the positive staining rate among KBD, OA, and control groups. The results are represented by $T$ value. Statistical significance was accepted when $P < 0.05$.

## Results

### HE Staining Analysis

The integrity and continuity of superficial layer in OA and KBD groups were interrupted compared with the control group (**Fig. 1**). The chondrocyte density in superficial layer decreased and chondrocytes were in a loose arrangement. Unlike in the OA or control group, the chondrocytes in the deep layer of KBD were circular or ovoid and loosely arranged, and some cells appeared to be in clustered aggregates. The numbers of chondrocytes in superficial layer of OA and KBD groups were lower than in the control group. The chondrocytes in the deep layer were long spindle- or polygon-shaped and in dense arrangement, which suggested that the morphology of chondrocytes had changed, and necrosis or apoptosis had occurred in the damaged cartilage.

### TB Staining Analysis

TB staining was performed to investigate the sulfated GAGs of articular cartilage. In the control group, the chondrocyte nuclei had deep staining intensity, and ECM was homogenously stained (**Fig. 2**). Compared with the control group, the depth and area of ECM staining obviously decreased in OA and KBD groups (**Fig. 2**). In addition, the stained areas had uneven distribution, and some parts of the superficial articular cartilage were unstained. The deficient or uneven staining indicated the decreased PG and/or GAG sulfation expression level in OA and KBD groups compared with the control group.

### IHC Staining of Aggrecan, FAM20B, EXTL2, and GalT-II

In IHC staining of aggrecan, the positive staining zones of aggrecan were distributed in the ECM around the chondrocytes (**Fig. 3**). Therefore, we used the positive staining areas to estimate the expression level of aggrecan. The positive staining areas were highest in the middle, then superficial and deep layers of the 3 groups. Statistical analysis indicated that the positive staining areas decreased significantly in the deep layers of KBD and OA groups compared with the control group.

The positive staining zones of FAM20B were distributed in the cytoplasm of chondrocytes. The positive staining rates of FAM20B in different layers of KBD, OA, and control group were highest in the middle, then deep and superficial layers (**Fig. 4**). The positive staining rates of FAM20B decreased in the superficial, middle, and deep layers of OA and KBD groups compared with the control group (**Fig. 4**).

The positive staining zones of GalT-II were distributed in the cytoplasm of chondrocytes. In the control group, the positive staining rates were highest in the superficial, then middle and deep layers (**Fig. 5**). In OA and KBD groups, decreased positive staining rates were observed in the superficial layer, and in the middle layer of OA group compared with the control group (**Fig. 5**).

The positive staining zones of EXTL2 were distributed in the cytoplasm of chondrocytes. The positive staining cells of EXTL2 was abundant in the control group (**Fig. 6**) In OA group, the positive staining rates of EXTL2 decreased in the middle and deep layers compared with control, while in KBD group, the positive staining rates of EXTL2 decreased in all of the layers compared with the control group (**Fig. 6**).

## Discussion

The results of our study demonstrated a decreased expression of FAM20B, GalT-II and an increased expression of EXTL2 in OA and KBD, which were involved in CS synthesis and modification processes. Furthermore, the expression of aggrecan was lower in the deep layer of KBD and OA groups compared with the control group. Considering

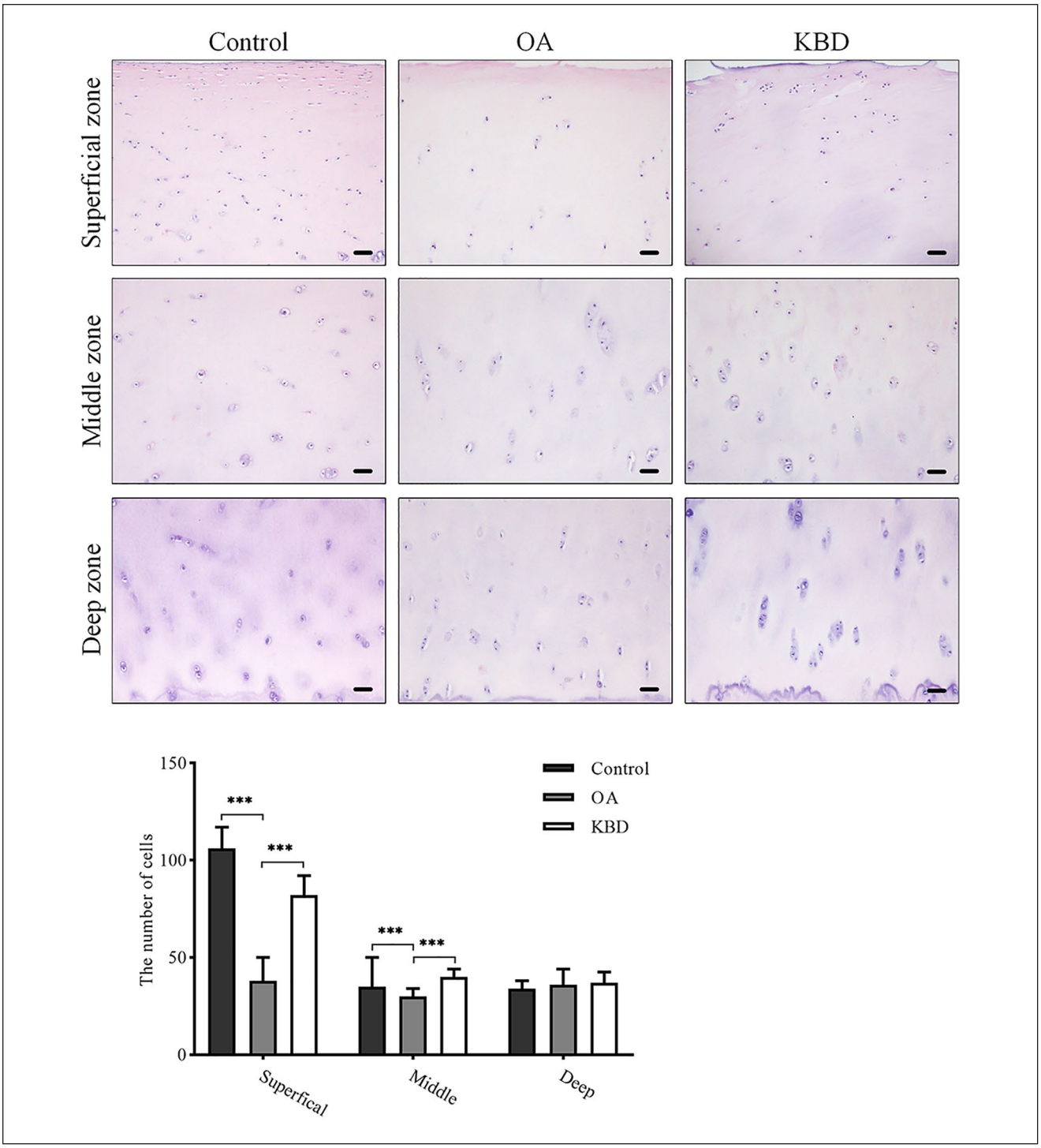


**Figure 1.** Hematoxylin and eosin (HE) staining of articular cartilage in control, Kashin-Beck disease (KBD), and osteoarthritis (OA) groups. In KBD and OA groups, the cell numbers were much lower in all 3 layers compared with the control group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$; scale bar 20 μm.

the function of the enzymes, we speculate that the altered expression of the aforementioned enzymes may explain the resulting decreased expression of aggrecan.

Aggrecan is most crucial for the proper function of articular cartilage and chondrocytes,[17,27] and it functions as lubricant in the biomechanical properties of cartilage.[11,28]

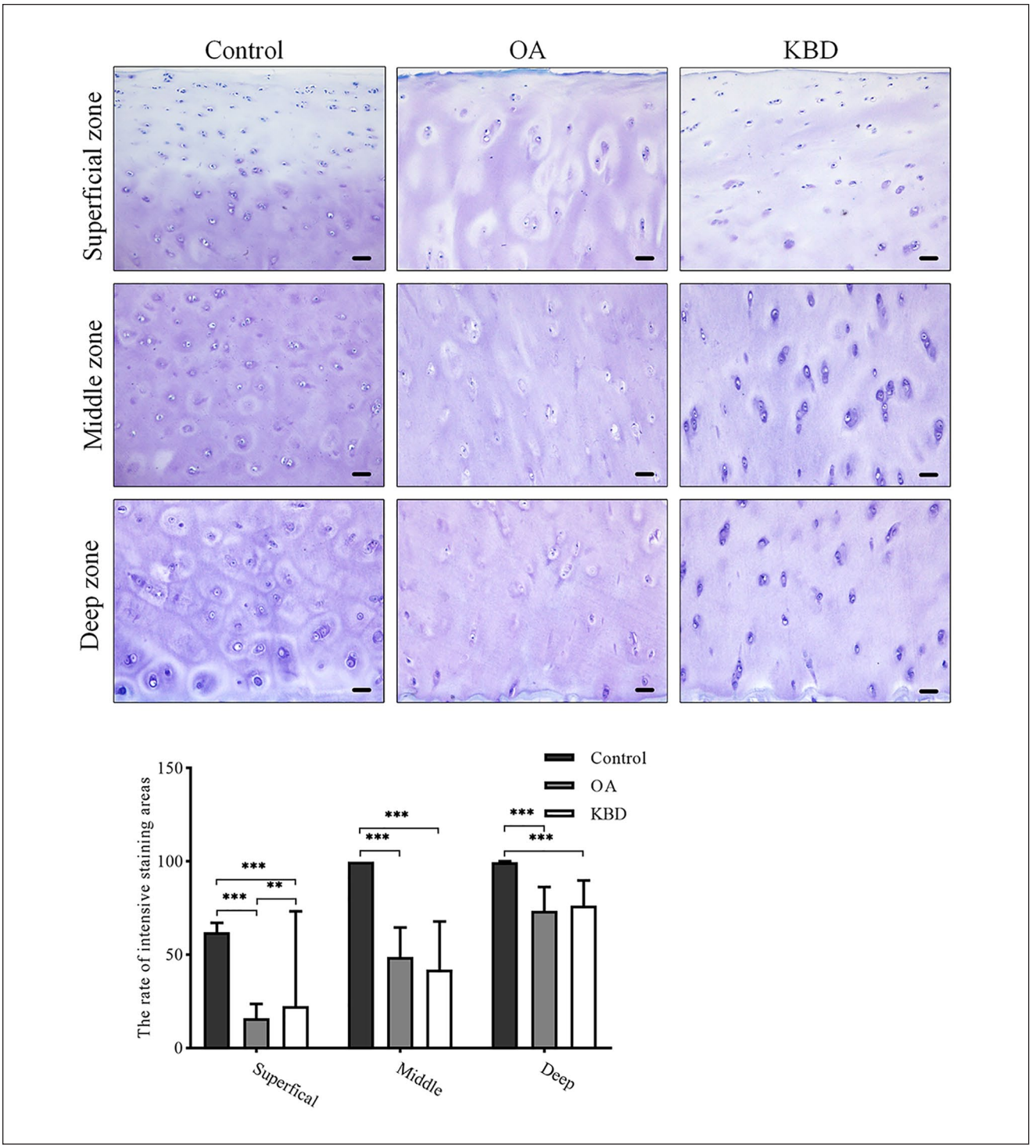


**Figure 2.** Toluidine blue (TB) staining of articular cartilage in control, Kashin-Beck disease (KBD), and osteoarthritis (OA) groups. In KBD and OA groups, the rates of intensive staining areas were much lower in all 3 layers compared with the control group. *$P <$ 0.05; **$P < 0.01$; ***$P < 0.01$; scale bar 20 μm.

Aggrecan can create a high osmotic activity and maintain collagen fiber assembly to create swelling pressure for the load-bearing capability of the cartilage.[29,30] We found that the immunostaining for aggrecan decreased in the deep layer of KBD and OA groups compared with the control group. Furthermore, TB staining loss also occurred in the

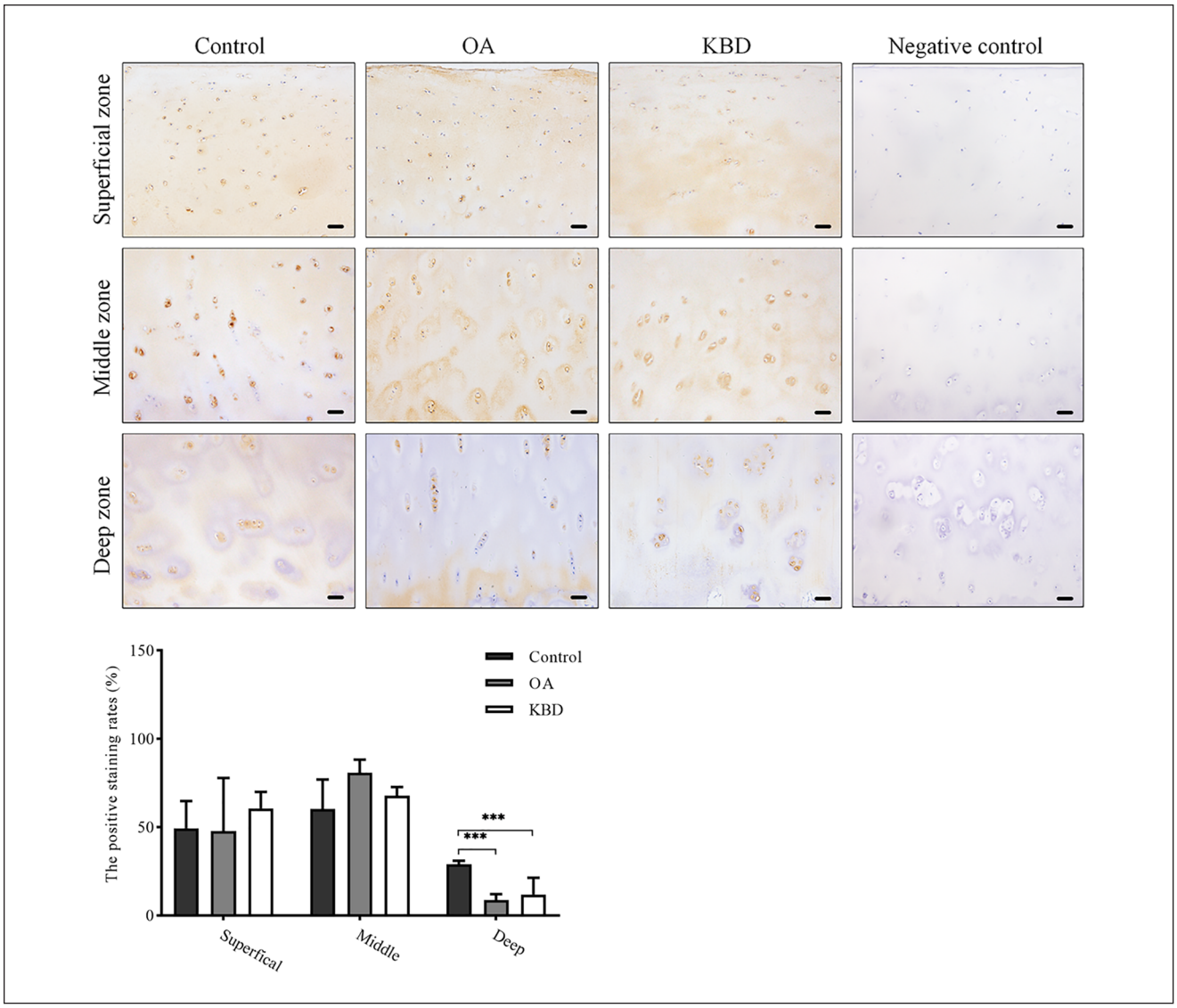


**Figure 3.** Immunohistochemical (IHC) staining of aggrecan in the articular cartilage of control, Kashin-Beck disease (KBD), and osteoarthritis (OA) groups. In deep layers of KBD and OA groups, the rates of intensive staining areas were much lower than the control group. In the negative control group, no nonspecific immunostaining or background reactivity was observed. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.01$; scale bar 20 μm.

KBD and OA groups. CS is an important part of aggrecan, and decreased aggrecan expression level can reflect the defective function of CS.

The CS synthesis starts by the formation of GAG-protein linkage region, and a series of glycosyl transferase and sulfonyl transferases were involved in the elongation of GAG chains.[18,31] The first step in the tetrasaccharide linker formation is the xylosylation of the core protein, which was catalyzed by xylosyltransferase.[19,22] Fam20B is a xylose kinase, which can phosphorylate the xylose residue of the tetrasaccharide linker. Fam20B is also an essential switch for the regulation of the PGs-GAGs formation and enhancing the synthesis of CS.[16,19,32] Animal experiments demonstrated that GAGs in the Fam20B-deficient cartilage remarkably decreased.[21,33] In our results, the expression levels of FAM20B decreased in all 3 layers of KBD and OA groups compared with the control group. These expression levels were also consistent with the TB staining results, suggesting that decreased expression level of FAM20B may have affected the CS synthesis procession.

The second step is the addition of 2 galactoses catalyzed by 2 distinct enzymes β1,4-galactosyltransferase 7 (GalT-I) and β1,3-galactosyltransferase 6 (GalT-II).[19] GalT-II is an essential enzyme involved in the biosynthesis of tetrasaccharide linkage region.[22] Previous study demonstrated that the increased expression of GalT-II was induced by

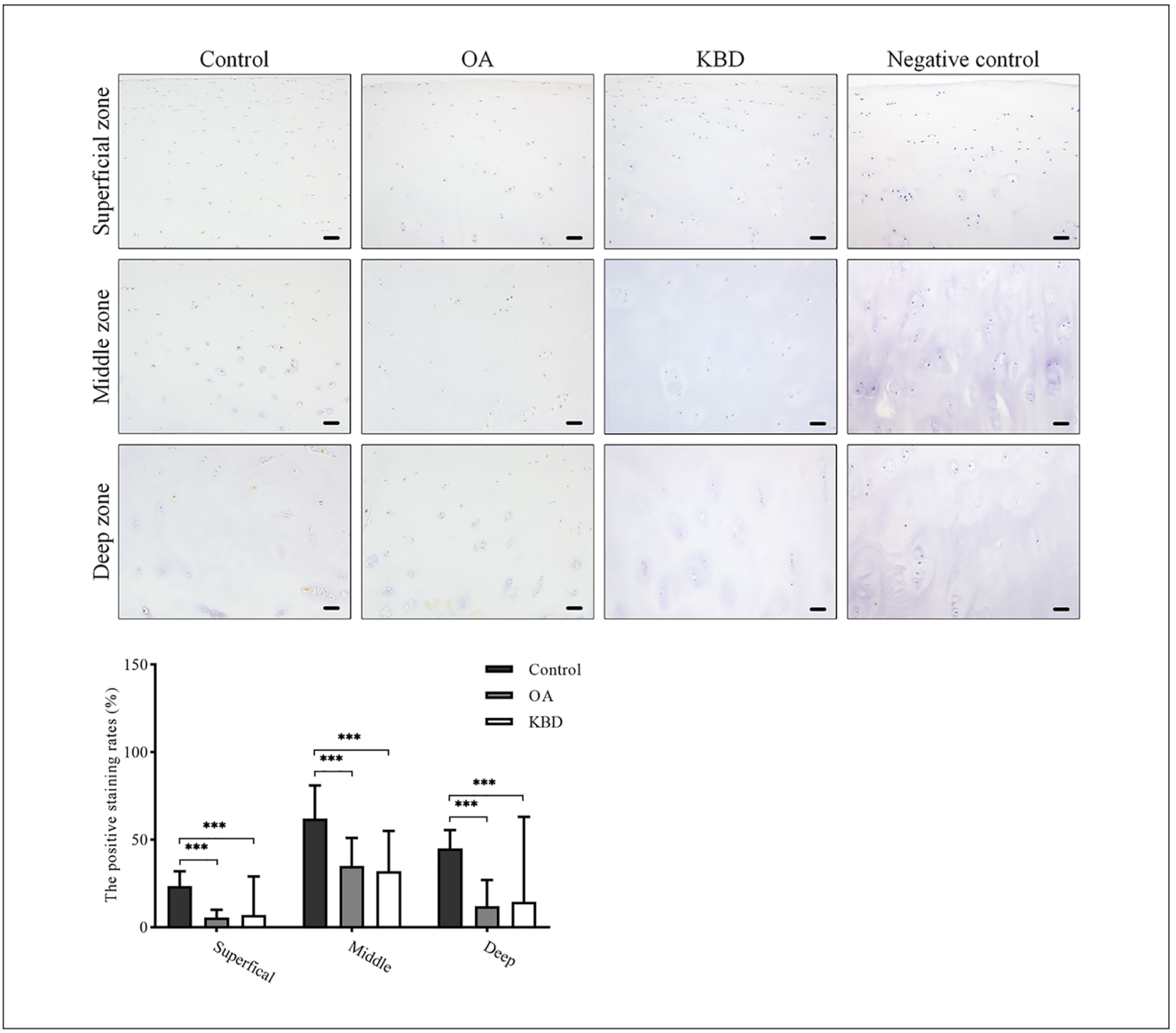


**Figure 4.** Immunohistochemical (IHC) staining of FAM20B in the articular cartilage of control, Kashin-Beck disease (KBD), and osteoarthritis (OA) groups. In all 3 layers of OA and KBD groups, the positive staining rates in KBD and OA group were significantly lower compared with the control group. In the negative control group, no nonspecific immunostaining or background reactivity was observed. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$; scale bar 20 μm.

Fam20B-dependent xylose phosphorylation, and the knockdown of Fam20B could suppress GalT-II expression level and reduce the rate of GAG synthesis.[32,34] GalT-II can enhance the CS extension on the tetrasaccharide linker.[22] Our results indicated that the expression levels of GalT-II declined in the superficial and the deep layers of OA and KBD groups compared with the control group. This finding is consistent with the TB staining results, suggesting that the decreased expression level of GalT-II in cartilage may cause the disfunction of GAGs synthesis procession.

Previous investigations have demonstrated that EXTL2 functioned as an inhibitor of CS synthesis by adding N-acetylglucosamine to the end of FAM20B–phosphorylated tetrasaccharide,[35,36] which means that extension of CS chain cannot be initiated by EXTL2. Moreover, the phosphorylated pentasaccharide produced by EXTL2 could terminate GAG elongation.[36] Animal experiments have indicated that EXTL2 knockout mice produced more GAGs compared to the wild-type mice.[17,37] The IHC staining of EXTL2 diminished in the middle and deep layers of OA and KBD groups compared with the control group. Besides, the EXTL2 expression levels in the superficial layer of KBD group decreased significantly compared with OA and control group.

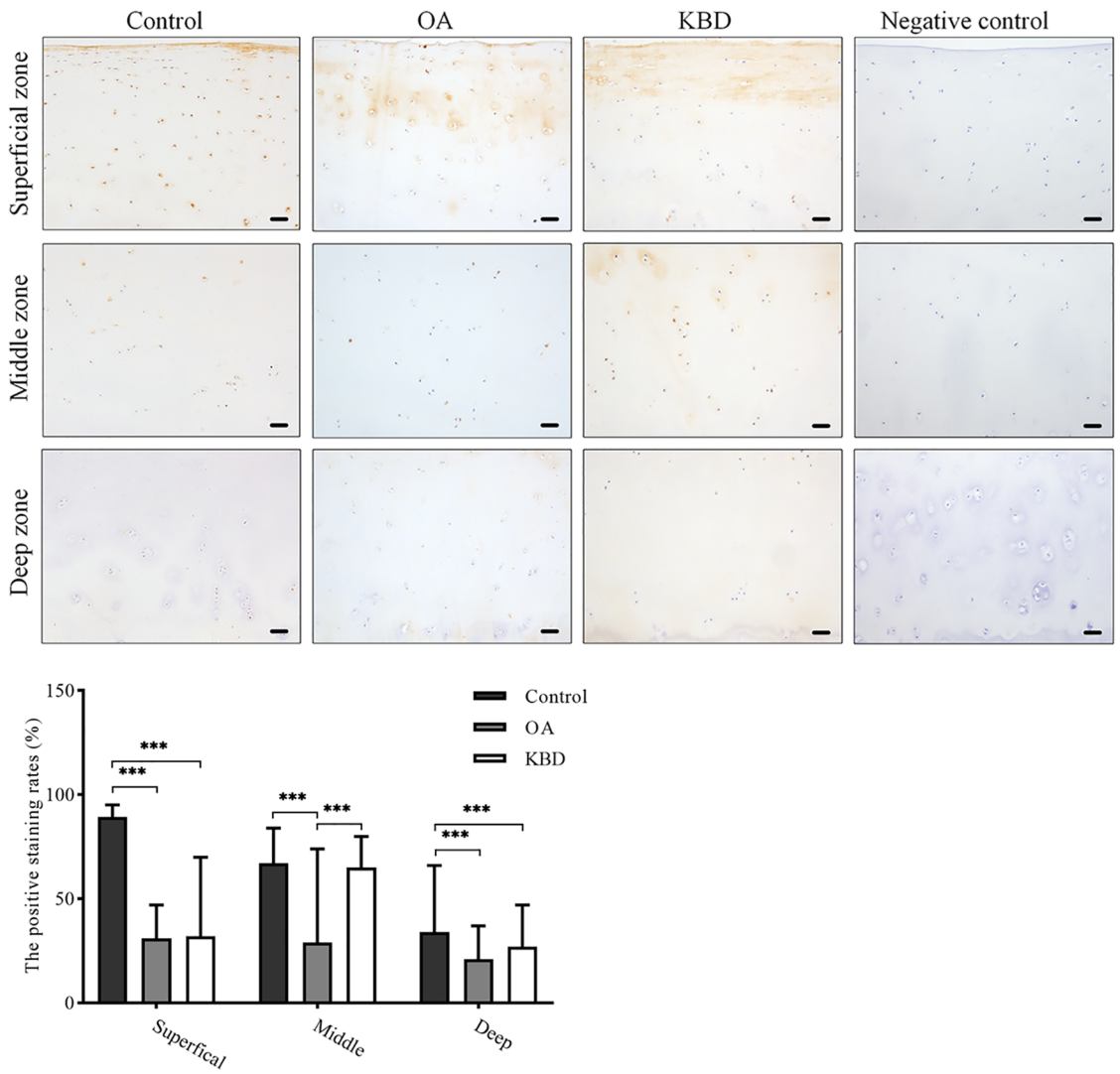


**Figure 5.** Immunohistochemical (IHC) staining of GalT-II in the articular cartilage of control, Kashin-Beck disease (KBD), and osteoarthritis (OA) groups. The positive staining rates in superficial and deep layers of KBD and OA groups were markedly lower than the control group, and also the middle layer of OA group. The middle layer of KBD group is higher than OA group. In the negative control group, no nonspecific immunostaining or background reactivity was observed. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$; scale bar 20 μm.

There are still limitations in this study due to the procedures in IHC staining, which might influence the results, such as specificity of antibody, tissue specimen fixation, antigen retrieval and pH of repair liquid. Furthermore, the limited number of the samples (in this research, $n = 18$) may lead to some deviations in the results, thus, an experiment with larger sample size would be needed to validate the results of this experiment. Another limitation is that the samples were obtained in 20th century and embedded in paraffin, and the samples are not suitable for Western blots or reverse transcription–polymerase chain reaction analysis.

In summary, our study demonstrated a decreased expression of FAM20B, GalT-II and an increased expression of EXTL2 in OA and KBD groups. The expression of aggrecan decreased in the deep layer of KBD and OA groups compared with the control group. The studied enzymes can regulate the modification processes, thus influence the

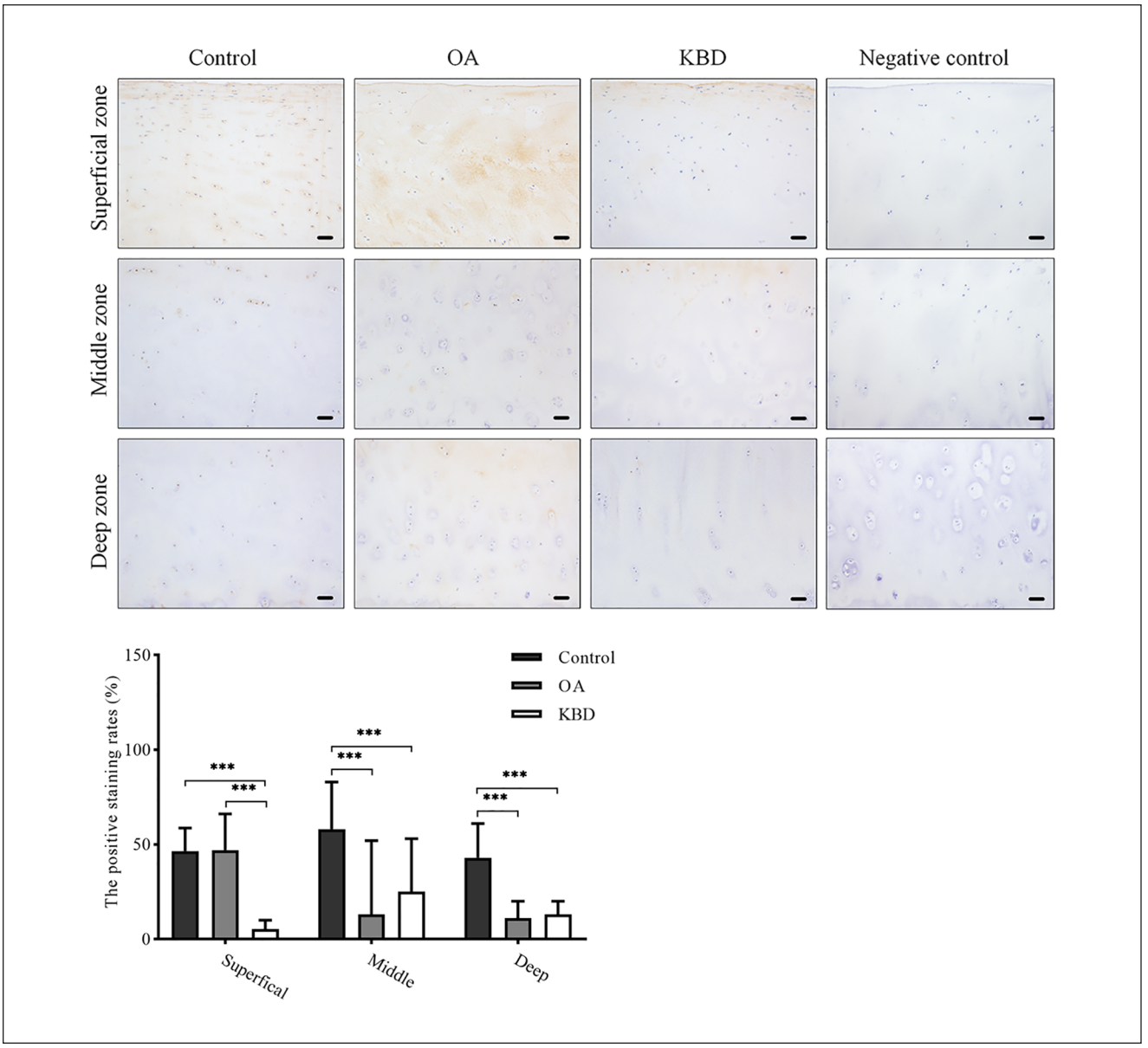


**Figure 6.** Immunohistochemical (IHC) staining of EXTL2 in the articular cartilage of control, Kashin-Beck disease (KBD), and osteoarthritis (OA) groups. The EXTL2 positive staining rates in the middle and deep layers of KBD and OA groups were lower than the control group. and the positive staining rates in OA and control groups were higher than the KBD group. In the negative control group, no nonspecific immunostaining or background reactivity was observed. *$P < 0.05$; **$P < 0.01$; ***$P < 0.01$; scale bar 20 μm.

expression level of aggrecan and CS synthesis. Further investigation of these enzymes can provide new theoretical and experimental targets for OA and KBD pathogenesis studies. At the same time, targeted therapies could be provided for the prevention and treatment of OA and KBD.

## Authors' Note

The detailed data in this research are available from the corresponding author on request.

## Author Contributions

All authors participated in drafting the article and critically modifying the important content of knowledge, with all authors endorsing the final version. Lei, Wang, Ran had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Study conception and design: Han. Acquisition of data: Lei, Deng, Ran, Feyissa, Lv, Wang. Analysis and interpretation of data: Lei, Deng, Lammi, Wang. Drafting the article or revising it critically for important intellectual content:

Lei, Deng, Ran, Lv, Feyissa, Wang, Lammi, Han. Final approval of the version of the article to be published: Lei, Deng, Ran, Lv, Feyissa, Wang, Guo, Han, Lammi.

## Acknowledgments and Funding

We thank Dr. Weizhuo Wang from the Second Affiliated Hospital of Xian Jiaotong University, Dr. Chao Xu from Linyou County Hospital, and all the staff from Linyou County Endemic Disease Control Center for their support and cooperation in the collection of cartilage specimens. The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by National Natural Science Foundation of China (81872567, 81402639) and Shenzhen Science and Technology Project (JCYJ20170816100755047). The study sponsors did not involve in the study design, data collection, analysis or interpretation, or in the writing of the manuscript; nor did they affect the decision to submit the manuscript for publication.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Ethical Approval

This investigation was approved by the Human Ethics Committees of Xi'an Jiaotong University.

## Informed Consent

All the donors provided a written informed consent for the study participation and publication of their individual clinical details and images.

## Trial Registration

Not applicable.

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