

ADAMTS4 or ADAMTS5: Which is the Key Enzyme in the Cartilage Degradation of Osteoarthritis and Kashin-Beck Disease?

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Research Article

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Abstract

Background: This study aims to investigate the altered expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4) and a disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS5) in the human articular cartilage between osteoarthritis (OA) and Kashin-Beck disease (KBD) and compare their roles in the cartilage injury.

Methods: Articular samples were collected from confirmed OA patients and KBD patients then divided into three groups, and the articular cartilages from the normal donors were used as controls. The morphology, location and expression of ADAMTS4 and ADAMTS5 as well as aggrecan were detected by histochemical staining and immunohistochemical staining.

Results: Compared to the control, the number of living cells in OA and KBD groups declined at three zones. Meanwhile, the results of toluidine blue staining showed that there was a loss of aggrecan in extracellular matrix of KBD and OA cartilages. The amounts of chondrocytes positively stained for ADAMTS4 were lower in the middle and deep zones of OA and KBD cartilages than those in the control samples. On the contrary, the immunostaining for ADAMTS5 significantly increased in OA and KBD group compared with control group at all three zones. Notably, although there was no statistical significance, the expression of ADAMTS5 in KBD was slightly higher than the ones in OA in superficial, middle and deep zones.

Conclusions: The role ADAMTS4 acted in aggrecan degradation was seemingly minor. It could be inferred that ADAMTS5 is the key enzyme in the cartilage destruction, particularly in KBD cartilage, which could explain the complex pathogenesis of KBD and provide a potential therapeutic target for KBD patients.

Background

Osteoarthritis (OA) is the most prevalent degenerative joint disease in the world. As the main cause of chronic pain and disability in the senior citizens, it is clinically characterized by stiffness, arthralgia, and limitation of movement (1). Kashin-Beck disease (KBD) is an endemic, chronic osteochondropathy, which can directly result in the joint deformity, dwarfism, depression and even disability (2), posing an increasing burden and challenge on society and individual. In China, this disease is widespread from northeast to the southwest, it can also be found in some regions of Eastern Siberia in Russia and in North Korea. Three hypotheses have been formulated to explain the occurrence of KBD: 1) an endemic deficiency of Selenium, 2) a cereal contamination by mycotoxins and 3) high humic acid levels in drinking water (3–5). Despite extensive investigations, none of these has been verified by epidemiological and experimental evidences. Thus, KBD is still regarded to have a multi-factorial etiology and complex pathogenesis.

Through reviewing previous studies, a conclusion can be drawn that KBD shares its clinical symptoms and pathology with OA, particularly at earlier stages of the disease, although gene expression and pathogenesis of KBD have been indicated to differ from OA (6, 7). For example, painful joints, narrowed joint space, osteophytes, and movement restrictions of joints can be observed in the cartilage from both

OA and KBD patients. Some pathological changes, such as predominant degradation of extracellular matrix (ECM) in affected articular cartilage, apoptosis and necrosis of chondrocytes overlap in OA and KBD. Given this, KBD should be undoubtedly viewed as a specific type of OA.

The imbalance of the synthesis and degradation of ECM is one of the important causes in the cartilage destruction. The chondrocytes maintain the cartilage structure and the homeostasis of the cellular environment by synthesizing and catabolizing ECM macromolecules. The amount of cartilage matrix will decline, if the efficiency of degradation of ECM surpasses their synthesis, and eventually leads to the destruction of cartilage(8). Aggrecan is the important component of ECM, imparting the compressive ability for cartilage(9). The depletion of aggrecan is a hallmark event happening in the advanced stage of OA, which is mediated by aggrecanases. Aggrecanases are the principal proteinases, present in the articular cartilage and growth plate cartilage, which belong to the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family. As the indispensable members of ADAMTS, recent studies have indicated that ADAMTS4 and ADAMTS5 are responsible for the degradation of aggrecan in human OA models(10–12). It is plausible that ADAMTS4 and ADAMTS5 make major contributions to the loss of proteoglycans during the progression of KBD.

In this study, the presences of ADAMTS4, ADAMTS5 and aggrecan were tested by immunohistochemical stainings of cartilage from the patients of KBD and OA, as well as the normal group. The comparison between OA, KBD and the normal groups could not only clarify which one of ADAMTS4 or ADAMTS5 is the key aggrecanase in cartilage depletion, but also explore the roles of these aggrecanases in the pathogenesis of KBD, which will be helpful to illustrate the etiology of KBD and put forward new potential therapeutic targets for KBD.

Materials And Methods

Samples and materials

OA patients (n=9, age: 45-65 years old, sex ratio: M/F=4/5) and KBD patient(n=11, age: 50-63years, sex ratio: M/F=8/3) were primarily enrolled from those who underwent surgical treatment. All KBD patients were diagnosed and confirmed according to Chinese diagnosis criterion(13). The normal cartilage samples were collected from the patients who suffered from traffic accidents or polydactyly treatment surgically (n=12, age: 37-70years, sex ratio: M/F=5/7). All normal samples are from donors living in non-KBD endemic areas. The detailed information about subjects is shown in the Table 1.

This study was permitted by medical research permission granted by the Human Ethical Committee in Xi'an Jiaotong University. All the participants were informed of this studies, after which they provided a written informed consent.

Antibodies

The primary antibodies applied were as follows: ADAMTS4(catalogue no. ab185722, Abcam, Cambridge, England), ADAMTS5(catalogue no. ab182795, Abcam, Cambridge, England). The specificities of immunohistochemical method of these antibodies have been widely validated(14-16).

Immunohistochemical staining

These cartilage tissues fixed in the 4% paraformaldehyde were decalcified, embedded in paraffin within 8h after surgery, then cut in coronary direction into 5µm thickness after dehydration, and prepared for the applications described below. For deparaffinization, 5µm sections were immersed in xylene and rehydrated with a series of gradually decreasing concentrations of ethanol(100%-80%), then incubated with 3% hydrogen peroxide H₂O₂ for 15 min at room temperature. After rinsing with phosphate-buffered saline (PBS) for three times, the sections were incubated 2 mg/ml hyaluronidase for 20 min. After incubation in blocking buffer (the normal goat serum, from Zhong Shan Gold Bridge Rabbit SP reagent, Beijing Zhong Gold Bridge Biological Technology Co., 18112A11) for 20 min, the samples were incubated with the primary antibodies of ADAMTS4 at 1:200 dilutions and ADAMTS5 at 1:50 dilutions in 1% bovine serum albumin in PBS at 4 °C overnight. The primary antibody of negative control was replaced by PBS. Then the sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG at 37°C for 20 min. After rinsing in PBS, hematoxylin counterstaining was performed. For visualization, a diaminobenzidine (DAB) kit (ZLI-9017, ZSGB-BIO, China) was applied. Finally, dehydration, clearing and sealing coverslips were performed.

Hematoxylin and eosin (H&E) and toluidine blue (TB) staining

For hematoxylin-eosin (H&E) and toluidine blue (TB) stainings the deparaffinization followed the procedure used for immunohistochemical samples. Then sections were stained with H&E or 0.1% (w/v) TB dye solution. After washing with running water, dehydrated by a series of gradually increasing concentrations of ethanol solution (80–100%) and xylene, then samples were sealed by coverslips.

Image capture and quantification

The stained section images were acquired by a Leica SCN400 slide scanner (Leica Microsystems GmbH, Wetzlar, Germany) under a 100× magnification field, then three representative regions were randomly selected from superficial, middle and deep zones were quantified by Image J software (NIH, USA). The positive staining rate was calculated as follows:

$$\frac{\text{Positive staining cells}}{\text{Total cells}} \times 100\%$$

The positive staining cell rate =

Statistical analysis

SPSS 22.0 software (SPSS Inc, USA) was applied for the data entry and statistical analysis. If the data from OA, KBD and control groups were in accordance with normal distribution by the test for normality and equal variance, the rank-based ANOVA test was used to examine the statistical discrepancy of

positive staining cell rate among OA, KBD and control groups. If not, nonparametric rank sum test was used, and all statistical tests were bilateral tests. $P \leq 0.05$ was regarded for statistically significant difference.

Results

H&E staining

H&E staining manifested that the quantity of living cells of OA (superficial zone, $P \leq 0.001$, middle zone, $P = 0.001$, deep zones, $P = 0.003$) and KBD (superficial zone, $P \leq 0.001$, middle zone, $P = 0.004$, deep zones, $P \leq 0.001$) declined evidently among three zones compared to the control group. This suggested that the cartilage may undergo a focal chondrocyte death (necrosis), which is associated with proteoglycans depletion. In the superficial zones, the number of living cells of OA were less than KBD ($P = 0.036$) (Fig.1.).

TB staining

TB staining was applied to examine the content of ECM in cartilage. The results of TB staining showed a significant decrease in superficial ($P = 0.021$), middle ($P = 0.021$), deep zones ($P = 0.027$) of KBD cartilage in comparison to healthy cartilage, whereas the percentage of intensive staining of OA cartilage was distinctly lower compared to the control only in superficial zone ($P = 0.034$) (Fig2.).

IHC staining

Theoretically, the expression of aggrecanases should increase in OA and KBD cartilage. However, there were no difference ADAMTS4 stainings in the superficial zone among the three groups. In the middle zone, the positive staining rate of ADAMTS4 in OA cartilage reduced compared to control one ($P = 0.001$). Similarly, this kind of decrease also happened in the deep zone in both OA and KBD cartilage ($P = 0.008$, $P < 0.001$). As for the comparison of expression of ADAMTS4 between KBD and OA cartilage, the positive staining rates in KBD group had a rise when compared to OA in both superficial and middle zones, wherein the statistically difference were observed in the middle zones ($P = 0.009$). In the deep zone, condition changed: as the number of positive staining cells in KBD were lower than in OA (Fig3.).

The immunostaining of ADAMTS5 were much stronger in OA and KBD group than those in control group in the superficial zone ($P \leq 0.001$, $P \leq 0.001$). Meanwhile, the positive staining cells in the middle zones of OA and KBD also had an obviously increase compared to the healthy cartilage ($P = 0.003$, $P \leq 0.001$). In the deep zone, the distribution of positive staining cells in OA and KBD were more intensive than in the control ($P \leq 0.001$, $P \leq 0.001$). Totally, there was a tendency that the expression of ADAMTS5 in KBD was slightly higher than OA among all three zones in cartilage, although without statistical difference (Fig4.).

Discussion

Apoptosis, necrosis, chondrocytes aging and loss of proteoglycans were universally acknowledged as the primary features of cartilage injury(17–21), As direct consequences of these, the density of the living cells in the cartilage decreases. The results of H&E staining verified that such a phenomenon really was obvious in the cartilage of OA and KBD.

Proteoglycans are complex macromolecules distributing in the ECM of articular cartilage, having a protective effect on joints. Dominant proteoglycans in cartilage are aggrecan (22). Increasing number of research has demonstrated that there is a close association between loss of aggrecan and cartilage diseases(23–25).It could be observed in the TB staining that the staining intensity of proteoglycans in KBD was quite low compared to OA group and control group, which showed that the depletion of aggrecan is more serious in cartilage of KBD.

It is generally accepted that two families of metalloproteases are the major contributors in the progression of degradation of ECM: matrix metalloproteinase (MMP) family and ADAMTS family(26). However, in comparison to the MMPs, aggrecanases seemingly have more association with the depletion of aggrecan, because it was the greatest in areas of cartilage adjacent to sites of cartilage erosion(25).

When referring to the roles of ADAMTS family played in the degradation of aggrecan, researchers often pay more attention to the ADAMTS4 and ADAMTS5, since other members of family related to degradation of aggrecan, such as ADAMTS1, ADAMTS8, ADAMTS9, has been shown to have a rather low aggrecan-degrading activity(27, 28). ADAMTS4 and ADAMTS5 leave the aggrecan core protein at the aggrecanase-specific Glu373- Ala374 bond in the interglobular domain (IGD) region(29, 30), therefore, they have been regarded as the key aggrecanase. ADAMTS4 and ADAMTS5 have similar structure, containing a disintegrin domain, a thrombospondin domain, a cysteine-rich domain, and a spacer domain, but ADAMTS5 has an additional TS domain after the spacer domain. A study reported that in the IGD region the aggrecanolytic activity of ADAMTS5 are fourfold compared to ADAMTS4, in the CS-2 region of aggrecan 2.5 fold, and under physiological conditions the aggrecanase activity of ADAMTS5 was at least 1000-fold greater than that of ADAMTS4(31, 32).

Based on the research of activity and molecular basis of ADAMTS4 and ADAMTS5, advanced works from lots of laboratories have further illustrated the impact of aggrecanase on increase of aggrecan loss related to cartilage diseases in different ways(33–36). Although there are some reviews comprehensively summarizing the regulation of ADAMTS4 and ADAMTS5 in OA(26, 37–39), it is still controversial that which of them is the main aggrecanase in the destruction of cartilage. Some reports argue that ADAMTS4 is primarily expressed in an active form in osteoarthritic cartilage, while ADAMTS5 would be constitutively expressed in both normal and OA cartilage(40).

Others suggested that owing to the high affinity of its non-catalytic domains for glycosaminoglycan chains, ADAMTS5 is the most active aggrecanase to increase the loss of aggrecan(32). It has also been proposed that ADAMTS4 is the most inducible aggrecanase upon cytokine stimulation, whereas ADAMTS5 is the most abundant aggrecanase(41), which manifests that ADAMTS5 is constitutive. In the murine models of OA, ADAMTS5 deficiency could protect mice from OA, but deletion of ADAMTS4 didn't

have any influence on the normal growth and physiology of mice(42, 43). As discussed above, ADAMTS5 appears to play the key role in the cartilage depletion, though these conclusions are limited to animal model, and the discrepancy between human and murine should not be ignored. Therefore, aims of this study not only investigated the main aggrecanases involving the cartilage degradation, but also explored whether ADAMTS4 and ADAMTS5 play the equivalent roles in the progression of KBD.

In our study, the expression of ADAMTS4 is not in accordance with the points of some previous studies: the percentage of positive staining of the control was more intensive than OA and KBD in the middle and the deep zones. Generally speaking, ADAMTS4 should be overexpressed in the damaged cartilage. However, the mRNA expression of ADAMTS4 was significantly repressed at the early stage of OA(44). Besides, in the superficial zone, the expressions of ADAMTS4 of OA and KBD had a similar proportion with the control. These results indicated that ADAMTS4-induced cartilage degradation maybe not happened at the early stage of cartilage diseases, and the role of ADAMTS4 is less significant, especially in the superficial zone.

Nevertheless, the expression of ADAMTS5 in OA and KBD were greatly increased compared to the control, particularly in the cartilages from KBD patients. In addition, there was gradual upregulation of ADAMTS5 in the OA group from superficial to deep zone, while the expression in KBD group down-regulated in the deep zone. It confirmed that ADAMTS5 was the crucial aggrecanase, which led to the cartilage damage. This adverse effect is evidence in the deep zone of OA cartilage. What's more, the expression of ADAMTS5 in KBD group was higher than the one in OA group. Based on the results of TB staining of the aggrecan positive cell rates among three groups, it could be inferred that the increased expression of ADAMTS5 is more likely to cause the decrease of aggrecan in OA and KBD.

However, mainly owing to the ethical issue, it was hard to collect enough cartilage from KBD patients. It couldn't manifest adequately the casual relationships between cartilage degradation and ADAMTS5. In order to investigate the role of ADAMTS5 in the cartilage disease, increasing amount of research is required, which could further explain the complex pathogenesis of KBD and provide a potential therapeutic target for KBD patients.

In conclusion, we found that the function of ADAMTS4 in degradation aggrecan is seemingly slight. Hence, we prefer to favor of the opinion that ADAMTS5 is the key enzyme in the cartilage destruction, in particularly in KBD cartilage. Most of previous studies have focused on the roles of ADAMTS4 and ADAMTS5 in OA, while it is rare to investigate the importance of ADAMTS4 and ADAMTS5 in KBD. The test of the expression of ADAMTS4 and ADAMTS5 in OA and KBD cartilage further affirmed our standpoint that ADAMTS5 was more significant in the loss of aggrecan and cartilage damage.

Declarations

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Conflicts of interest/Competing interests The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Ethics approval and consent to participate The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Xi'an Jiaotong University. Written informed consent was obtained from individual or guardian participants.

Consent for publication Not applicable.

Availability of data and material All data generated or analysed during this study are included in this published article

Code availability Not applicable

Authors' contributions All authors have made substantial contributions to the conception or design of the work, or the acquisition, analysis, or interpretation of data for the work, M.P.L and W.S. have drafted the work or revised it critically for important intellectual content, M.P.L, Z.F.E and L.P.L. have performed the staining, M.P.L., Y.L.L. and T.S.J have organized and analyzed the data, Mikko J. Lammi has edited this manuscript, S.W. and G.X. have approved the final version to be published, S.W. and G.X. agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tables

Table1. Characteristics of experiment subjects($\chi^2_{\text{gender}} = 2.602$, $F_{\text{age}} = 2.940$, both $P > 0.05$)

OA			KBD			Control		
Sample set	Gender	Age	Sample set	Gender	Age	Sample set	Gender	Age
1	F	59	1	M	62	1	F	45
2	F	62	2	M	56	2	F	59
3	M	45	3	M	51	3	M	37
4	F	63	4	F	63	4	M	46
5	F	52	5	M	63	5	F	56
6	M	56	6	F	59	6	F	62
7	M	54	7	M	54	7	M	39
8	M	60	8	M	50	8	M	56
9	F	65	9	M	60	9	F	70
			10	M	57	10	F	42
			11	F	53	11	F	50
						12	M	44
Mean	-	57.3	Mean	-	57.1	Mean	-	50.5

Abbreviation. OA Osteoarthritis, KBD Kashin-beck disease, Control Normal people, F Female, M male

Figures

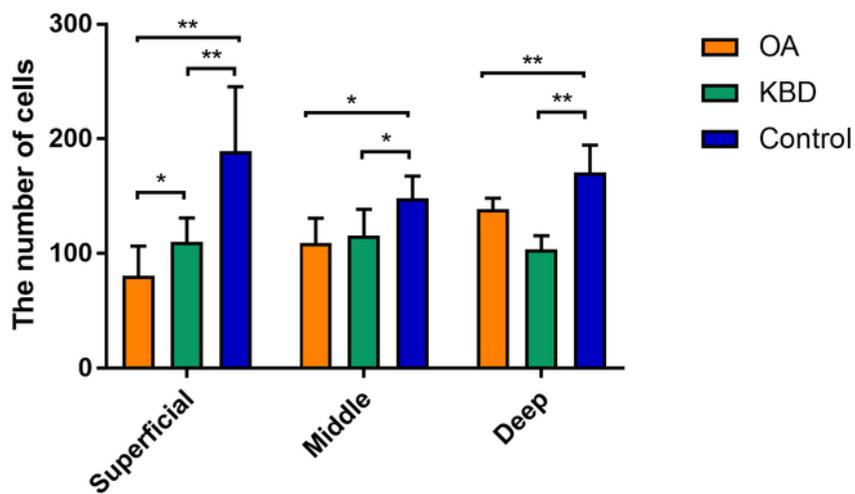
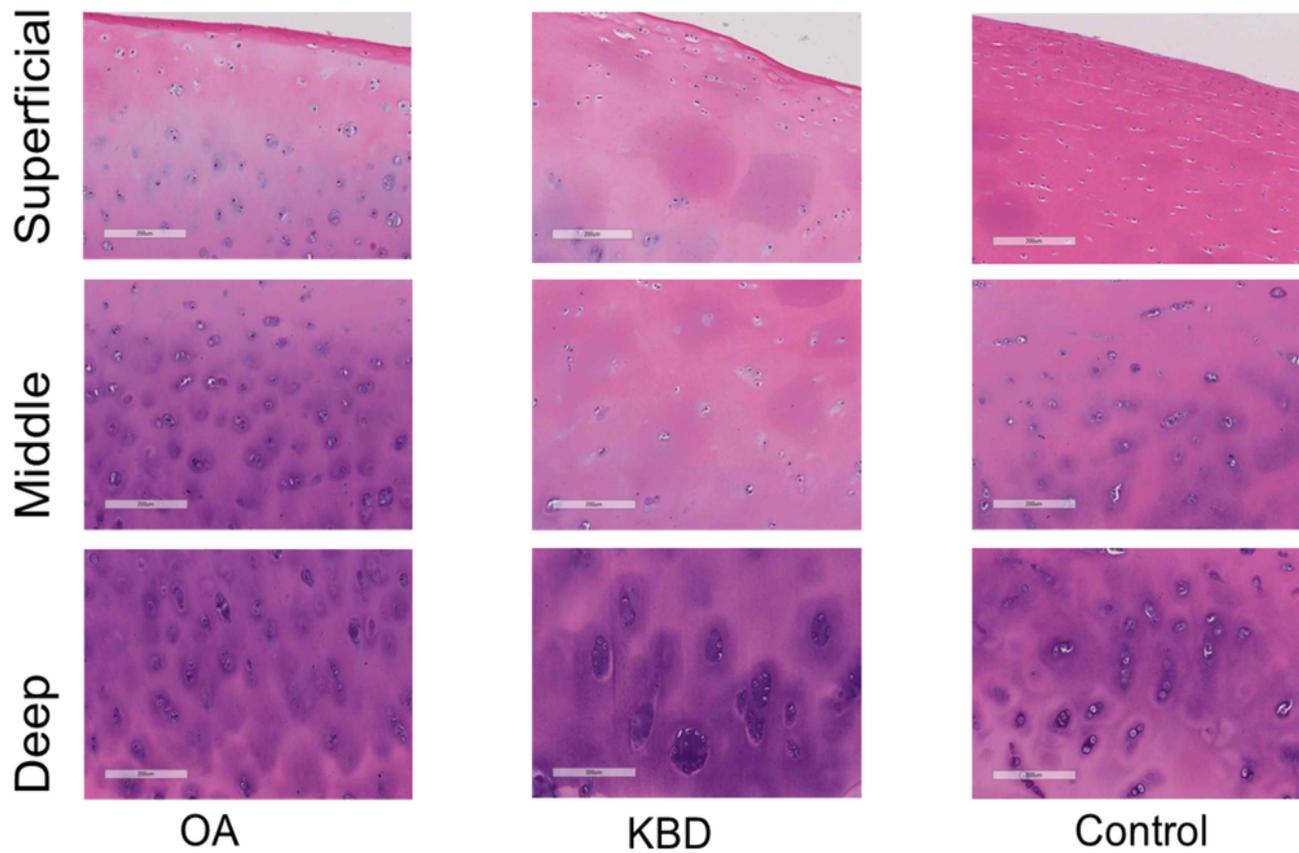


Figure 1

Representative H&E staining of articular cartilage from an OA patient, a KBD patient, a normal (control). Note. The number of living chondrocytes in OA and KBD groups were decreased among three zones compared with the control group. (* $P < 0.05$, ** $P < 0.01$, scale bar 200 μm .)

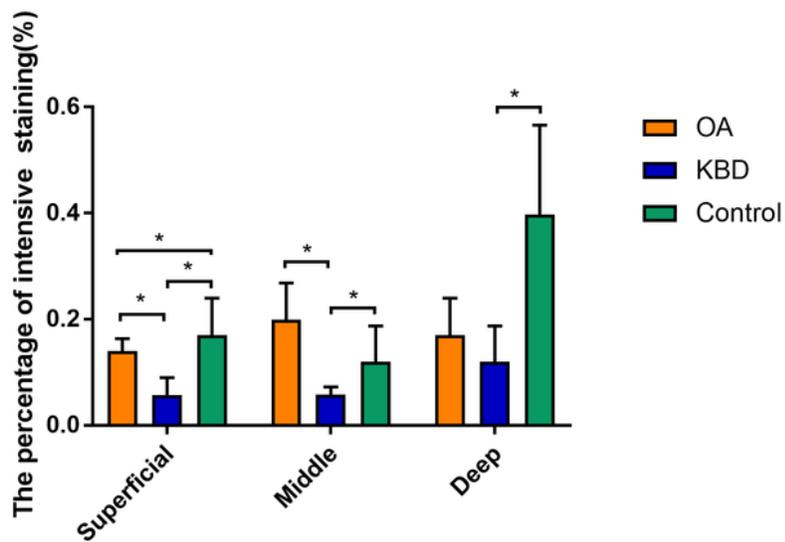
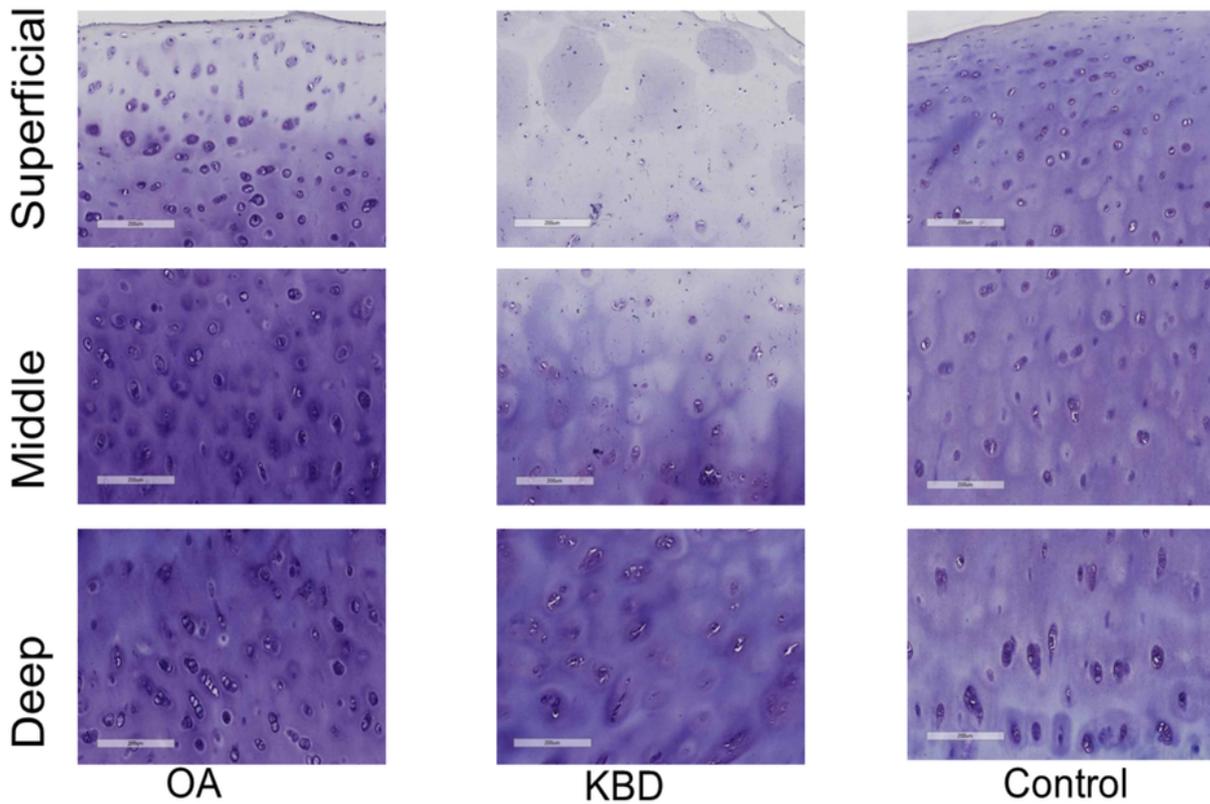


Figure 2

Representative TB staining of articular cartilage from an OA patient, a KBD patient, a normal (control). Note. The average percentage of intensive staining areas in KBD group was significantly decreased compared with the control group. (* $P < 0.05$, ** $P < 0.01$, scale bar 200 μm .)

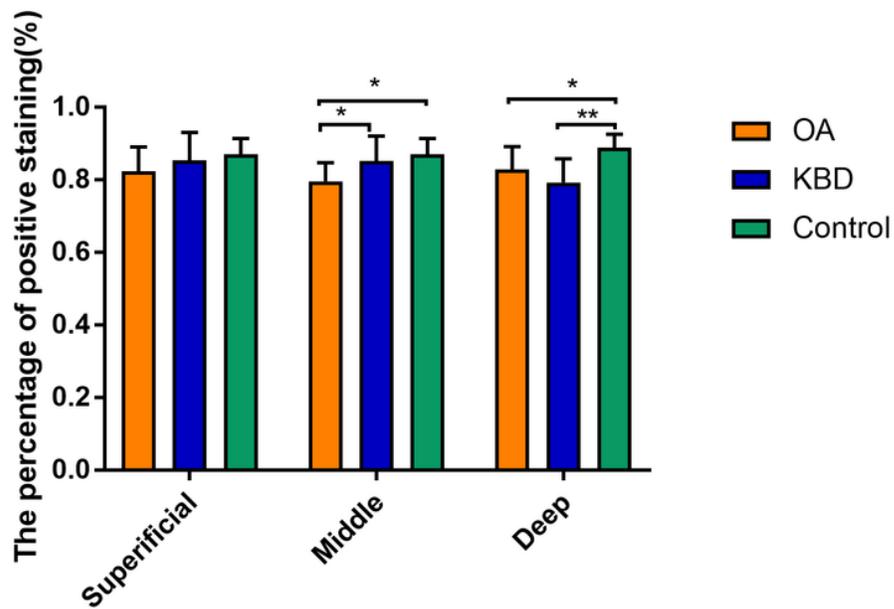
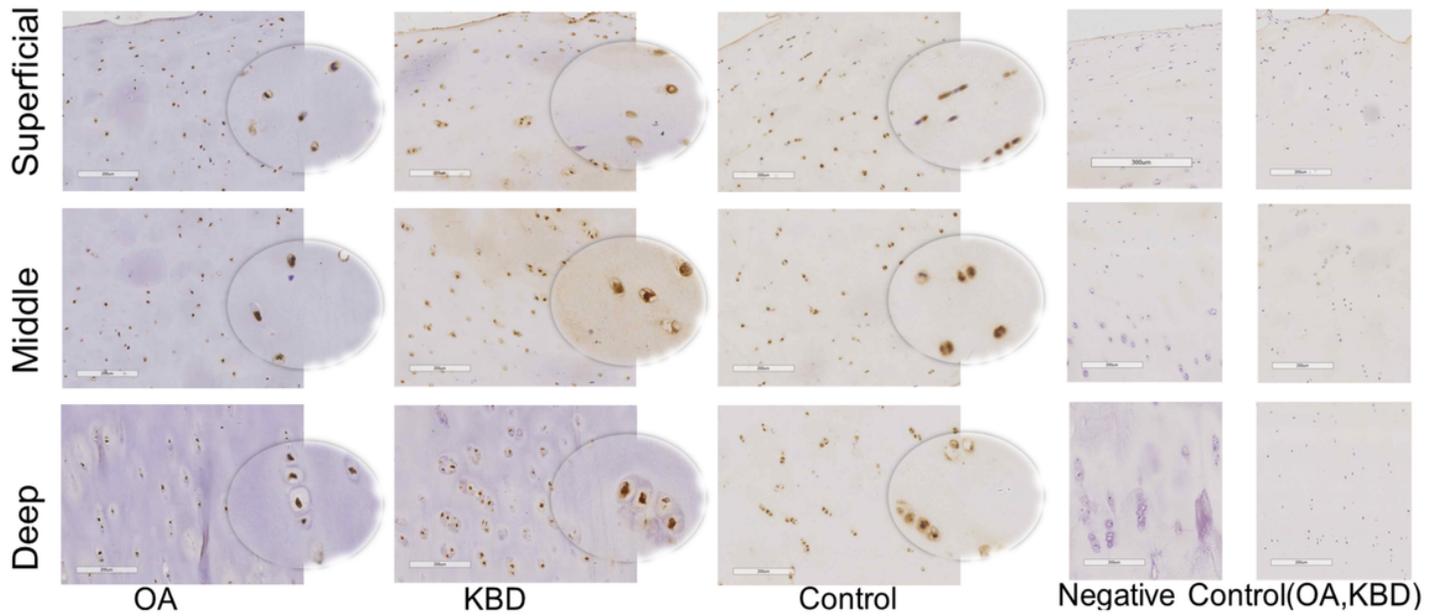


Figure 3

Representative IHC staining of ADAMTS4 (positive staining is in brown) of articular cartilage from OA group, KBD group, and control group among superficial, middle and deep zones. Note. The primary antibody was replaced with PBS in negative control. In superficial zone, there are no obvious difference among three groups. In middle and deep zones, OA and KBD groups increased as compared with control group. (* $P < 0.05$, ** $P < 0.01$, scale bar 200 μm , the round parts showed the staining more clearly with 200 \times magnification)

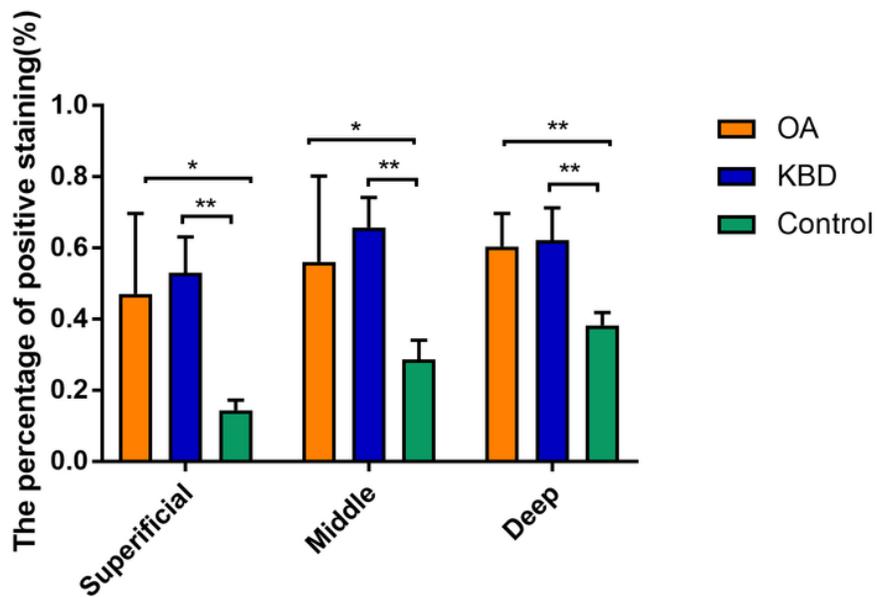
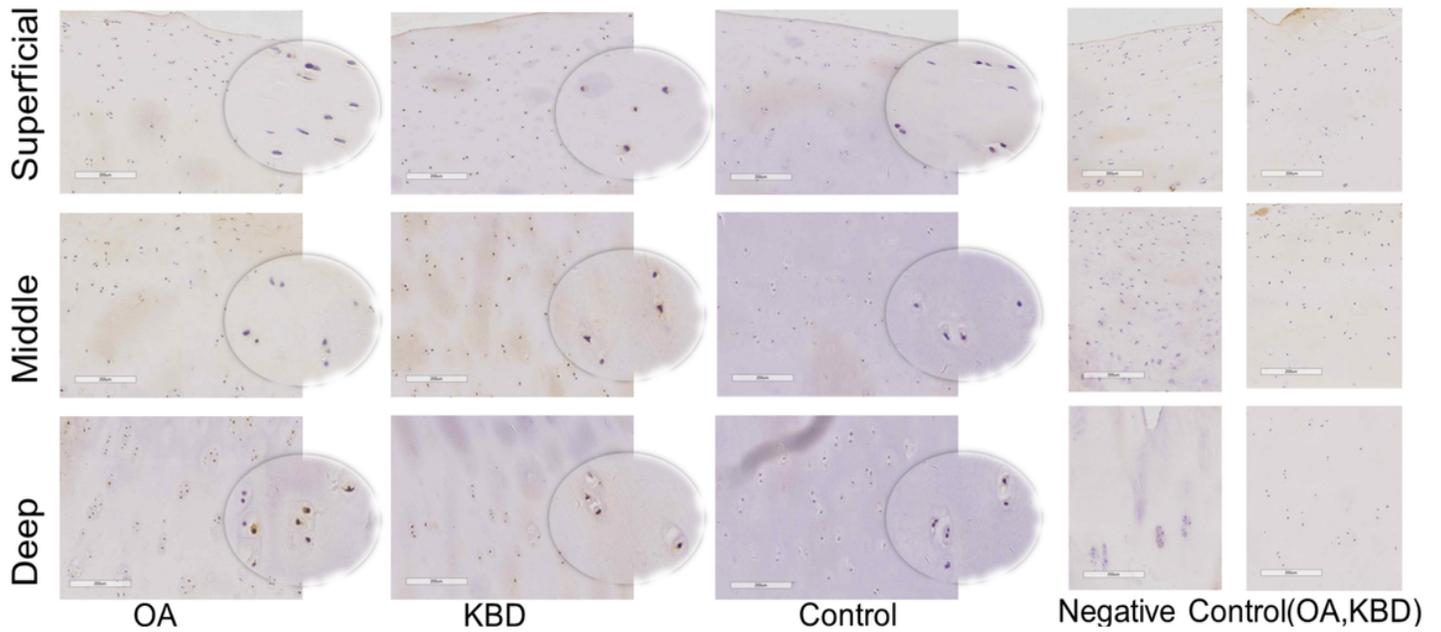


Figure 4

Representative IHC staining of ADAMTS5 (positive staining is in brown) of articular cartilage from OA group, KBD group, and control group among superficial, middle and deep zones. Note. The primary antibody was replaced with PBS in negative control. Among three zones, a significantly increased number of ADAMTS5 positive-stained chondrocytes was observed of OA and KBD cartilage compared to the control. There are also increasing trends in three groups from superficial to deep zones. (* $P < 0.05$, ** $P < 0.01$, scale bar 200 μm , the round parts showed the staining more clearly with 200 \times magnification)