Potential value of combining CTX-II with C2C in evaluating and diagnosing early rat osteoarthritis

Meilun Shen

Northeast Agricultural University <https://orcid.org/0000-0002-2503-1095>

Zhiheng Zhang

Northeast Agricultural University

Xiangying Ma

Northeast Agricultural University

Rouqian Li

Northeast Agricultural University

Xiaodi Jin

Northeast Agricultural University

Tianwen Ma

Northeast Agricultural University

Li Gao (✉ gaoli43450@163.com)

College of Veterinary Medicine, Northeast Agriculture University

Zhi Wang (✉ wangzhi19750227@163.com)

Inner Mongolia Agricultural University

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Abstract

Background

This article aims to study the changes of CTX-II and C2C biomarker concentrations during the pathogenesis of osteoarthritis with SD rats as the research object. Through the longitudinal and joint detection of the content of CTX-II and C2C markers in the serum of anterior cruciate ligament resection (ACLT) rats, the evaluation and early diagnosis of OA disease can be realized, and the theoretical basis for the rapid diagnosis technology of OA can be provided.

Methods

SD rats were randomly divided into two groups: Sham operation group and OA model group. The OA model was established by anterior cruciate ligament excision (ACLT) for 2, 4, 6, 8, and 10 weeks, respectively. The cartilage gross score and Mankin score were used to evaluate the model. The tibia of the right hind limb was sampled for pathological histology and HE staining. The collected blood samples are used to detect changes in the concentration of CTX-II and C2C in serum. The receiver operating characteristic curve (ROC) was used to compare the area under the curve (AUC) of CTX-II, C2C and combined biomarkers.

Results

In the model group, the content of CTX-II and C2C in SD rat serum increased significantly, and the content of CTX-II and C2C increased with the prolonged modeling time, which was time dependent; The levels of CTX-II and C2C in the serum of rats in the sham operation group did not change significantly; both macroscopic observations and Mankin scores increased significantly. The AUC of the combined biomarker is higher than that of CTX-II and C2C. Finally, CTX-II and C2C levels were positively correlated with Mankin score.

Conclusion

Early combined detection of serum CTX-II and C2C concentrations has potential value for the evaluation and diagnosis of osteoarthritis.

Background

Osteoarthritis (OA) is a group of degenerative joint diseases characterized by articular cartilage degeneration, synovitis, periarticular and subchondral bone changes [1]. OA is considered to be a disease of the whole joint, involving all joint tissues [2]. Cartilage, synovium, ligament, bone marrow and muscle are involved in the complex occurrence and development of osteoarthritis. OA is not only a wear process,

but also an abnormal remodeling and joint injury [3]. There are many reasons for the occurrence of OA. Mechanism, metabolism and inflammation are the main factors leading to the occurrence of OA, while decreased muscle strength, joint injury and obesity are recognized as risk factors [4]. With the gradual discovery of OA-related inflammatory mediators (such as cytokines, chemokines, adipokines and lipids, etc.), the role of inflammation in OA is becoming more and more clear. In animals with OA, the levels of some cytokines increased. The mechanism by which these cytokines affect OA is still unclear, but it is generally believed that they can induce catabolism and inhibit intra-articular anabolism [5].

In general, the diagnosis of osteoarthritis can only be carried out after the occurrence of clinical symptoms in diseased animals. Osteoarthritis can only show obvious clinical symptoms if it develops to a certain extent or is irreversible. This stage may be accompanied by changes in the subclinical structure of some cartilage and its related tissues. Early intervention in the occurrence of OA may be helpful to the treatment, but the value of early diagnosis of OA according to clinical symptoms is limited [6]. When the animals do not show OA clinical symptoms or the clinical symptoms are mild, the changes of clinical symptoms may not be related to the changes of cartilage structure, so it is difficult to carry out intervention treatment. Therefore, the treatment of early OA structural lesions should also be considered whether to show clinical symptoms [7]. Structural OA can be detected by rapid biomarkers, which is convenient and rapid, and can be used for early diagnosis of OA. According to the test results, a reasonable treatment plan can be made and the treatment effect can be evaluated [8].

In recent years, more and more molecular markers have been used in the diagnosis of diseases, and the application of molecular markers makes the diagnosis of diseases more accurate and convenient. The diagnosis of OA is also expected to be better analyzed by molecular markers. Molecular biomarkers refer to any substance or structure that can be measured in the body or secretion. It can objectively reflect the disease state of the body and predict the development trend of the disease [9]. More and more molecular biomarkers of osteoarthritis have been widely studied, but no biomarker can be used for clinical diagnosis. CTX-II (C-terminal peptide of type II collagen) belongs to the marker of tissue degradation and is the most widely studied and promising molecular biomarker of all BIPED categories [10]. In the study of steady-state destruction of cartilage, its degradation product C2C is also a highly sensitive targeted molecular biomarker [11]. CTX-II is highly correlated with the occurrence, development, injury degree, bone marrow lesion and osteophyte formation of OA [12, 13]. For patients with (ACLR) undergoing anterior cruciate ligament reconstruction, their level of CTX-II decreased with the relief of pain and improvement of physical function, and the decrease of CTX-II content indicated a decrease in the severity of the disease [14]. Based on this, in order to verify the hypothesis that CTX-II and C2C can be used as biomarkers of OA joint of knee joint. In this study, through the establishment of OA model in vivo (using ACLT model), we conducted a longitudinal study on the joint determination of serum CTX-II and C2C levels, and evaluated the relationship between CTX-II and C2C levels and articular cartilage degeneration. The area under the curve (AUC) of CTX-II, C2C and combined biomarkers in the evaluation and diagnosis of osteoarthritis was analyzed by ROC curve. The purpose of this study is to realize the evaluation and early diagnosis of OA, which provides a theoretical basis for the rapid diagnosis of OA.

Materials And Methods

Animals

Sixty SD rats (10 weeks old, male, weighing 240 g-270 g) were purchased from the Experimental Animal Management Center of Harbin Medical University. All the test animals were individually housed in the animal room. The room was well ventilated and clean and comfortable: temperature 21 ± 3 °C, humidity $50\% \pm 15\%$, light time 12 hours/day, adequate food and water supply, and regular replacement of litter. The ACLT model was established and tested, and all animals were adapted to the new environment one week prior to the test to reduce the stress response. All trials were conducted in accordance with the guidelines published by the China Animal Testing Ethics Committee. Fasting for 8 hours before operation and water could not be avoided.

First, isoflurane was introduced into the anesthesia box (concentration: about 3.5%) to anesthetize SD rats. After the rats were anesthetized, a respiratory mask circuit was used to maintain anesthesia. Shave the hair on the right hind limb with an electric razor and scrub the operation area; sterilize iodophor twice for 3 min each time. After laying a sterile wound, make a 0.5 cm skin incision in the right hind limb parallel to the inside of the knee joint. Cut the subcutaneous tissue, cut and open the nodular sac, push away from the knee ligament and patella and flex the knee joint to reveal the anterior cruciate ligament. The anterior cruciate ligament was cut under the operating microscope, and the joint capsule was washed with warm saline (0.9%) to remove blood clots. Use 6 – 0 PGA absorbable suture nodules to sew the joint capsule, and sew the subcutaneous tissue and skin layer by layer. After the operation, the skin incision was disinfected regularly, and drinking and eating were free for 8 hours. SD rats were sacrificed after eyeball blood collection at 0, 2, 4, 6, 8, and 10 weeks postoperatively. All right hind limbs of the rats were completely peeled off, and 10% formaldehyde solution was fixed after taking pictures. The collected blood samples were centrifuged (1500 rpm, 15 min) and the serum was collected and stored in a sterile EP tube.

Grouping and sample processing

Sixty SD rats (10 weeks old, male, weighing 240 g-270 g) were randomly divided into two groups: sham operation group (n = 30) and OA model group (n = 30). The SD rats in the model group were anesthetized and the incisional sac (right hind limb knee joint) was cut and the anterior cruciate ligament was cut under the operating microscope, and the wound was closed layer by layer. The sham operation group used the same method, but did not cut the anterior cruciate ligament, and then closed the layered incision. The wounds of all animals were disinfected regularly. The OA model was established by anterior cruciate ligament transection ((ACLT)). The model was evaluated by gross cartilage score and Mankin score. Samples were taken at 0, 2, 4, 6, 8 and 10 weeks after operation, and the tibia of the right hindlimb was taken for histopathology. The collected blood samples were centrifuged (1500 rpm 15 min) and the serum was taken to detect the concentration changes of CTX-II and C2C in the serum.

Macroscopic observation

Observe and evaluate the degradation of cartilage on the surface of the tibial plateau through a dissecting microscope. The scoring criteria are as follows: smooth joint surface, normal color was scored as 0, joint surface was rough, cartilage defect with small fissures and gray color was marked as 1, articular cartilage defect to the middle layer of cartilage was scored as 2, joint surface ulcer and cartilage defect to the deep layer of cartilage was scored as 3, and exfoliation of articular cartilage and exposure of subchondral bone was scored as 4 [15]. Macroscopic observations were conducted by an observer who was blinded to the groups.

Histopathology Scoring

After the operation, the modified tibial plateau was placed in the embedding box and rinsed under running water for 24 h to remove excess fixative. Use different concentrations of ethanol for dehydration. Then, xylene was soaked twice to replace the ethanol in the tissue, and the tissue without ethanol can be embedded in wax. The sample was placed in a bath filled with a paraffin solution at 65 °C for 150 min, and after curing, it was repaired into a trapezoidal shape for later use. After fixing the wax block and slicing, cut a slice with a thickness of 5 µm, pick it up and unfold it in warm water, spread it on a glass slide, and oven overnight. After hematoxylin-eosin staining, neutral gum was dropped on the specimen of the slide glass, and a cover glass was added to cover the slide. The score of articular cartilage destruction was based on the improved Mankin score, mainly according to the degree of damage to the surface structure, the number of cells, the degree of staining and the integrity of the tidal line. The sum of the 4 items was the result of Mankin score [16]. 0–4 (mild), 5–9 (moderate), 10–14 (severe). The higher the score, the more serious the degree of cartilage degeneration.

Analysis of serum CTX-II and C2C concentrations

The serum of SD rats was thawed and the contents of CTX-II and C2C in each group were determined by ELISA kit. Before the test, the enzyme plate was equilibrated at room temperature for 30 min, and standard wells with different concentrations of each index were set according to the instructions, and 50 µL was added to each well. Set up blank wells (without adding test samples and enzyme-labeled reagents) and test sample wells (add 40 µL of test sample diluent first, then add 10 µL of test sample), and mix gently. After covering the sealing film, incubate in a 37 °C incubator for 30 min. Peel off the sealing film, wash with washing solution five times for 30 s each time, pat dry. In addition to blank wells, 50 µL of enzyme-labeled reagent was added to each well. After incubation, add developer A (50 µL) and developer B (50 µL) to each well, shake gently, and react at 37 °C in the dark for 15 min. Add 50 µL of stop solution to each well, measure the OD value of each well at 450 nm with a microplate reader, and finally draw a standard curve, and calculate the concentration of each sample according to the standard curve.

Data statistics and analysis

All data in this experiment were statistically analyzed by SPSS22.0 software and one-way ANOVA method, and the test results were expressed by mean ± standard deviation (SD). The differences between

groups were analyzed by Q test, $P < 0.05$. the correlation was analyzed by Spearman correlation analysis, and the sensitivity and specificity of marker detection were analyzed by ROC subjects' working characteristic curve.

Results

Macroscopic observation

There was no significant change in the eye view of cartilage in the sham operation group, the surface of articular cartilage of tibial plateau was smooth, the color was normal, and no pathological changes or wear could be seen in the sham operation group (Fig. 1a). At week 2 to week 4 after surgery, the joint surface was worn, roughened, the color was dark, the transparency of cartilage decreased and the cartilage layer became thinner (Fig. 1b,c). At week 6 after surgery, the cartilage injury reached the middle layer of cartilage, the roughness of cartilage surface increased and accompanied by obvious wear, and articular surface ulcer appeared (Fig. 1d). At week 8 after surgery, the cartilage defect reached the deep layer of cartilage, and the subchondral bone could be seen in some areas. the surface of articular cartilage was worn seriously and the cartilage became thinner obviously (Fig. 1e). At week 10 after surgery, part of the cartilage was exfoliated, the subchondral bone was exposed, and the roughness of the joint surface continued to increase and obvious fissures appeared (Fig. 1f).

As shown in Fig. 2. The macroscopic score of the tibia plateau of the right hind limb of the SD rats in the model group after surgery gradually increased with the prolongation of the model weeks, which was time-dependent, which indicated that the degree of cartilage damage increased with time. There was no significant change in the macroscopic scores of the sham operation group at various time points. In the second week, there was significant difference between the model group and the sham operation group ($P < 0.05$), and there was significant difference in the fourth week ($P < 0.01$) (Fig. 2a).

Histology

In the control group, the perichondrium was intact, the cells were arranged in clusters, and the size was normal and regular (Fig. 3a). At week 2, the cartilage was slightly damaged and partially thickened. Irregular, thin surface of cartilage (Fig. 3b). At week 4, a large amount of perichondrial defect, increased number of chondrocytes, disordered arrangement, and surface cell hypertrophy (Fig. 3c). At week 6, cartilage was lost, surface cartilage cells were enlarged and decreased, and the boundary between cartilage calcification layer and deep cells was unclear (Fig. 3d). At week 8, the perichondrium was lost, and the surface layer of cartilage was greatly damaged and thinned, and the cartilage defected to the deep layer (Fig. 3e). At week 10, the cartilage defected to the deep layer, the damage was serious, and the chondrocytes were arranged irregularly (Fig. 3f).

As shown in Fig. 2. The Mankin score of the tibial plateau of the right hindlimb of the SD rats in the model group increased gradually with the extension of the model weeks, in a time-dependent manner,

indicating that the degree of cartilage injury gradually aggravated with time, while there was no significant difference in the tibial plateau score of the right hindlimb at each time point in the sham operation group. The Mankin score was different from the sham operation group at the second week ($P < 0.05$), and began to show a significant difference at the fourth week ($P < 0.01$) (Fig. 2b).

CTX-II and C2C concentration changes

As shown in Fig. 4. The concentration of CTX-II and C2C in the serum of SD rats in the model group increased significantly after the second week of surgery ($P < 0.01$), and the concentrations of CTX-II and C2C increased with the prolonged modeling time, showing Time dependence. The concentrations of CTX-II and C2C in the serum of rats in the sham operation group did not change significantly (Fig. 4a,b). In addition, a positive correlation was observed between serum CTX-II levels and serum C2C levels ($r = 0.928$, $P < 0.001$) (Fig. 5a).

Analysis of correlation between serum CTX-II, C2C and Mankin score

The Spearman correlation analysis of each molecular marker and Mankin score showed that the change of CTX-II concentration in the model group showed a significant positive correlation with its Mankin score ($r = 0.935$, $P < 0.001$) (Fig. 5b). The change of C2C concentration and its Mankin score also showed a significant positive correlation ($r = 0.847$, $P < 0.001$) (Fig. 5c).

ROC analysis of CTX-II and C2C and combined biomarkers

The results of ROC analysis of rat serum biomarkers C2C and CTX-II showed that the areas under the curve of C2C and CTX II and the combined detection were more than 0.5, indicating that the single detection and combined detection of mouse serum molecular markers have a certain diagnostic value for mouse OA (Fig. 6). The AUC values of CTX-II, C2C and combined detection were 0.882, 0.876 and 0.947 respectively. The data show that the ROC value of the combined biomarker is significantly higher than that of the single biomarker.

Discussion

Osteoarthritis is an important cause of dyskinesia in elderly animals and reduced production performance of in-service animals [17]. At present, late OA, can only be relieved by joint replacement, which is not only expensive, but also painful for patients. Therefore, it is very important to clarify the pathogenesis and early diagnosis of osteoarthritis for the prevention and treatment of OA. At present, the diagnostic methods of OA include X-ray, MRI and arthroscopy. These imaging techniques have different sensitivities for the lesions of cartilage, synovium and subchondral bone [18, 19]. How to make early diagnosis of OA is still a problem to be solved. As a kind of highly sensitive molecules, biomarkers have been widely studied in OA and have broad promising applications [20–23]. However, few scholars have systematically studied the concentration trend of serum molecular markers in different stages of OA. So

far, there is no reliable biomarker for veterinary clinical diagnosis. Therefore, in this study, the rat model of OA was induced by ACLT operation, and the pathological changes of OA cartilage and the concentration of CTX-II and C2C were detected longitudinally and jointly, so as to realize the evaluation of condition and early diagnosis of OA.

CTX-II, as a degradation product of type II collagen, reached the highest level of CTX-II in serum at 3 weeks after birth, decreased rapidly at 4 months after birth, and almost could not be detected in serum 7 months later. This study focused on the degradation of type II collagen to explore the biomarkers of OA. When OA occurs, type II collagen is destroyed by cartilage-degrading enzymes, produces CTX-II and then excreted into urine then [24]. Studies did by Reijman and Sowers et al have shown that urinary CTX-II (uCTX-II) is a reliable biomarker for early diagnosis of OA [25, 26]. In our study, two weeks after ACLT surgery, the concentration of serum CTX-II in the model group was significantly higher than that in the control group. And the concentration of CTX-II at each time point was higher than that of the control group. This is also the same result as previous studies [27]. The histological results in our study also have a corresponding experimental trend. This also shows that the articular cartilage has been obviously damaged from the second week in histology, and continued to aggravate with the passage of time, suggesting that CTX-II may also be used as a suitable biomarker for longitudinal study [28]. In addition, we used Mankin scoring system to explore the relationship between the concentration of CTX-II and the degree of articular cartilage degeneration. In our study, we found that the concentration of the model group was significantly higher than that of the control group. And the higher the score was, the more serious the degenerated of articular cartilage and the higher the concentration of CTX-II was. Therefore, the detection of CTX-II may become one of the effective biomarkers for the diagnosis of early OA.

Type II collagen is the main component of articular cartilage. In osteoarthritis, type II collagen is continuously degraded and cleaved into two length fragments by matrix metalloproteinases. The exposure of a new epitope in a fragment with a length of 3 to 4 is called C2C. Other studies have shown that type II collagen degradation fragment C2C in synovial fluid will also increases after knee injury [29]. Conrozier et al also confirmed that serum C2C was associated with joint space stenosis (JSN) in patients with OA, and the C2C level in OA group was higher than that in normal group [30, 31]. In our study, compared with the control group, the concentration of C2C in the model group also increased significantly. It was higher than that of the control group from the second week, and gradually higher than that of the control group at other time points. We have obtained consistent results from the eye changes and histological results. The relationship between Mankin scoring system and C2C also suggests that in our study, the higher the C2C concentration was, the higher the Mankin score was. It shows that there is a positive correlation between them. Therefore, we think that C2C may help us to detect the early diagnosis of OA.

In this study, both CTX-II and C2C may be used as biomarkers to evaluate OA. Therefore, the combined detection of CTX-II and C2C can more accurately diagnose the degree of degeneration of early osteoarthritis more accurately. Therefore, we carried out Spearman analysis on the change trend of serum CTX-II and C2C concentration and Mankin score in rat OA model. The results showed that there was a

significant correlation between CTX-II and C2C and the OA model Mankin score ($P < 0.001$). It indicates that the change trend of CTX-II and C2C content is closely related to the severity of OA, and the more serious OA is, the stronger the degradation of cartilage matrix and the higher the concentration will be. Some studies have shown that the metabolic cycle of type II collagen in normal cartilage is very long, and the concentration of its metabolic degradation product CTX-II can evaluate the degradation of type II collagen in a certain period of time, and can also reflect the degree of degradation of type II collagen in mineralized tissues [32, 33]. In a follow-up study, Cahue also found that C2C levels were closely related to the severity of the disease [34]. This is similar to our study, but also supports our research. After that, we analyzed the molecular markers C2C and CTX-II and their combined biomarkers by ROC, and evaluated the diagnostic value of C2C and CTX-II in the detection of rat OA. The results showed that the AUC value of combined biomarkers was significantly higher than that of CTX-II and C2C. It shows that the combined detection of serum CTX-II and C2C has a certain diagnostic value for the detection of rat OA.

In this study, the OA model was evaluated from three aspects: gross observation and score, Mankin score and HE staining. The results showed that the degree of articular cartilage injury aggravated with the extension of modeling time, cartilage matrix defect aggravated and chondrocyte loss increased, which was similar to the previous research results [35]. It further indicates that when OA occurs, the matrix synthesis ability of articular cartilage decreases and the degree of degradation increases. In the sham operation group, only incision of the articular capsule without transection of the cruciate ligament would not cause osteoarthritis [36], so the changes of molecular markers in the sham operation group were not significant. We also verified the changes of serum type II collagen degradation products in SD rat OA model, and combined detection can further show that the degradation and metabolism of extracellular matrix of cartilage was enhanced during the occurrence of OA.

During OA, the change trend of CTX-II and C2C in SD rats is similar to that of OA, which has a certain value for the diagnosis of OA. It also shows that it may be used as a more potential combined biomarker for the diagnosis of OA, which is more stable and more sensitive than a single biomarker. However, this study also has some limitations, and the longitudinal research cycle is insufficient. There is no further study on the late changes of CTX-II and C2C. And only the tibial plateau was histologically analyzed. However, this study adopted scientific and effective methods within 10 weeks, so the results are reliable.

Conclusions

The concentration of CTX-II and C2C in serum of OA rats increased with the severity of cartilage injury, and there was a positive correlation between them. The degree of cartilage injury is positively correlated with the corresponding Mankin score. The ROC value of combined biomarker is higher than that of single biomarker. Combined detection of CTX-II and C2C molecular markers has more potential value in the diagnosis of OA.

Abbreviations

ACLR: Reconstruction of anterior cruciate ligament; ACLT: Anterior cruciate ligament transection; CTX-II: Crosslinked C-telopeptides of type II collagen; C2C: COL2-3/4C_{long mono}; AUC: Area under the curve; ROC: Receiver operating characteristic; OA: Osteoarthritis; SD: Sprague-Dawley

Declarations

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Availability of data and materials

Please contact the author for data requests.

Authors' contributions

All authors participated in the research and design of the experiment, or collected and analyzed data. The experiments were designed by MS and LG. The experiments were performed by MS, ZZ, XM, RL, XJ, TM and ZW. MS, ZZ, XM and LG collected and analyzed data. MS, ZZ, ZW and LG interpreted the data. MS wrote and edited the manuscript. All authors read it and finally approve the final version.

Ethics approval

The animal ethical treatment in this study was approved by the Animal Welfare Committee of Northeast Agricultural University (Harbin, China).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Macroscopic changes on tibial plateau.

Figure 2

a. Macroscopic score of the ACLT model. b. Mankin score of the ACLT model. Note: $P < 0.05$ were considered statistical significance. * is $P < 0.05$ ** is $P < 0.01$.

Figure 3

HE staining paraffin section of the ACLT model.

Figure 4

Changes of serum of CTX-II and C2C in SD rats with ACLT model. Note: $P < 0.05$ were considered statistical significance. * is $P < 0.05$ ** is $P < 0.01$.

Figure 5

a. Correlation between serum levels of CTX-II and C2C ($r = 0.928$). b. Correlation between serum levels of CTX-II and the Mankin score of the corresponding articular cartilage ($r = 0.935$). c. Correlation between serum levels of C2C and the Mankin score of the corresponding articular cartilage ($r = 0.847$).

Figure 6

ROC curve analysis of single and combined biomarkers in OA prediction. The diagonal segment is the reference line.