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Original Article

Study on effect of GGCX in knee osteoarthritis pathogenesis

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Abstract: Objective: To explore the effect of γ-glutamyl carboxylase (GGCX) in the pathogenesis of knee osteoarthritis. Methods: 40 cases of patients with knee osteoarthritis treated in our hospital from January 2014 to January 2015 were selected as the observation group, and another 40 cases of patients with normal knee during the same period were selected as the control group. The joint cartilage and synovial fluid were collected for further study. The expression of GGCX in the cartilage tissues of both groups was determined using immunohistochemical assay, the expressions of GGCX protein and mRNA in both groups were evaluated by Western blot and Real-time PCR, respectively; And the expression level of GGCX in synovial fluid was measured by using Magnetic chemiluminescence immunoassay. SPSS 21.0 software was used for the statistical analysis. Results: Immunohistochemical assay showed that the integrated optical density (IOD) in observation group was (0.578 ± 0.221), which was significantly lower than that of the control group (1.339 ± 0.118), the difference was statistically significant (t = 17.14, P = 0.00); Western blot showed the relative gray value of GGCX in observation group was (0.296 ± 0.194), which was significantly lower than that of the control group (0.898 ± 0.049), the difference was statistically significant (t = 19.34, P = 0.00); Real-time PCR results showed that the mRNA expression of GGCX in observation group was (2.992 ± 1.316), which was significantly lower than that of the control group (10.978 ± 2.112), the difference was statistically significant (t = 18.01, P = 0.00). Chemiluminescence immunoassay showed that the content of GGCX in synovial fluid of observation group was (111.215 ± 25.106 nmol/L), which was significantly lower than that of the control group (578.546 ± 121.622 nmoL/L), indicating a statistically significant difference (t = 23.01, P = 0.00). The correlation analysis showed that the expression of GGCX decreased with the degeneration of joint cartilage. Conclusion: The expression of GGCX significantly reduced in the cartilage and the synovial fluid in patients with knee osteoarthritis; and with the worsening of cartilage degeneration, expression of GGCX decreased too.

Keywords: γ-glutamyl carboxylase, knee, osteoarthritis

Introduction

Knee osteoarthritis is one of the joint diseases characterized with knee cartilage degeneration at different degrees, osteophytes formation at the edge of joint, appearance of joint loose bodies that affect the joint activities, and other pathological changes. Knee osteoarthritis usually appears in the elderly, causing a great impact to the body and mind of the patients. In recent years, studies have shown [1-3] that vitamin K has a protective effect to the osteoarthritis, and associated with osteoarthritis of the knee [4, 5]. Due to the lack of GGCX in arthritis cartilage, the carboxylated matrix GLA protein is much less in arthritis cartilage, by comparing with the cartilage of normal knee. GGCX is a vitamin K-dependent carboxylase, which is a key enzyme for vitamin K-dependent proteins to play their physiological roles [6, 7]. However, at present, it's still not clear about whether GGCX is associated with osteoarthritis. In this study, by using molecular biology techniques, we detected the expression of GGCX in normal knee cartilage and knee osteoarthritis cartilage, and preliminarily discussed the role of GGCX in the pathogenesis of osteoarthritis, to provide new ideas for in-depth study of the pathogenesis of osteoarthritis and clinical prevention and treatment of the disease.

Material and methods

General information

40 cases of patients who received joint debridement or total knee arthroplasty due to knee osteoarthritis in our hospital from January 2014 to January 2015 were selected, as obser-

Table 1. Comparison of basic clinical data between the two groups

Group	Cases	Gender (n) (males/females)	Age (years old)	Side (n)	
				Left	Right
Observation group	40	27/13	51.2 ± 1.4	19	21
Control group	40	26/14	52.3 ± 1.2	18	22

vation group, another 40 cases of patients who received post-traumatic amputation or loose knee cartilage removal under arthroscopy during the same period were selected as control group; both groups of patients were willing to donate their articular cartilage. There were 27 males and 13 females in observation group, aged from 40-65 years with a mean age of (51.2 ± 1.4) years old; there were 19 cases of left knee cartilage lesions and 21 cases of the right side. The control group consisted of 26 males and 14 females, aged from 41-64 years old with a mean age of (52.3 ± 1.2) years old; there were 18 cases of left knee cartilage and 22 cases of the right side. By comparing the general information between the two groups of patients, there was no significant difference ($P > 0.05$) and the two groups were comparable, see **Table 1**. This study was approved by the hospital ethics committee, and all patients voluntarily participated in the study and signed informed consent.

The inclusion and exclusion criteria

Inclusion criteria for observation group: (1) Recurrent pain on knee joint lesion that lasted for nearly one month; (2) the knee joint X-ray examination showed relative images of osteoarthritis with erect position or weight-bearing position; (3) knee joint fluid showed viscous appearance and white blood cell count < 2000/ml under microscope; to be accurate, the observation should be done at least for twice; (4) age ≥ 40 years; (5) time of morning stiffness ≤ 30 min; (6) bone fricative could be heard during knee activities, or could be felt by the patients [5]. Patients could be diagnosed if the symptoms and signs meet the first two criteria, if the imaging result didn't match item 2, the patients could be diagnosed only when the symptoms meet item 3 or 4 and the rest. Exclusion criteria: patients were formally treated before, or patients with traumatic arthritis, rheumatoid arthritis, septic arthritis and other types of arthritis.

Inclusion criteria for control group: imaging examinations showed no knee osteoarthritis disease, patients without joint pain, joint stiffness, joint swelling or other symptoms.

Research methods

Safranin O-fast green staining: The knee cartilage of both groups were embedded in paraffin, sectioned and de-waxed, after staining for 3 min with hematoxylin, the cartilage sections were rinsed with PBS three times, each time of 3 min; then differentiated with 1% hydrochloric acid for 15 sec, and rinsed with PBS; later, stained with 0.02% aqueous solution of fast green dye for 3 min, and rinsed with 1% acetic acid, and then stained with 0.1% Safranin O for 3 min, followed with 95% alcohol immersion and dehydration, and sealed at the end. The sections were placed under light microscope to observe the staining of cartilage cells and matrix, and the integrity of calcified layer and tide line as well as the other pathological status. We evaluated and classified the knee cartilage specimens according to modified ManKin score system [8, 9]. The modified ManKin score system evaluatesd the articular cartilage from 4 aspects: soft structure (0-6 points), chondrocytes (0-3 points), safranin O staining (0-4 points) and the integrity of the tide line (0-1 point), with a total score of 0-1 as normal, 2-5 points as mild degeneration, 6-9 points as moderate degeneration and 10-14 as severe degeneration.

Immunohistochemistry staining: The paraffin embedded cartilage sections of both groups were de-waxed and rehydrated through gradient ethanol into water, and then antigen repair was performed by using high-pressure repair method. The sections were incubated with 3% H_2O_2 at room temperature for 10 min in order to eliminate the endogenous peroxidase activity. After washing with PBS, the sections were incubated with 10% normal goat serum to seal at room temperature for 10 min. Gently throw off the serum, then add mouse anti human GGCX monoclonal antibody (Santa company, USA) and kept in a 4°C wet box overnight; later the sections were flushed with PBS for 3 times with 5 min of each time, then add HRP labeled goat anti mouse GGCX IgG antibody and repeat the flush again; DAB was used to show color, light microscope was used to observe and end the color showing, then re-stained with hematoxylin before mounting.

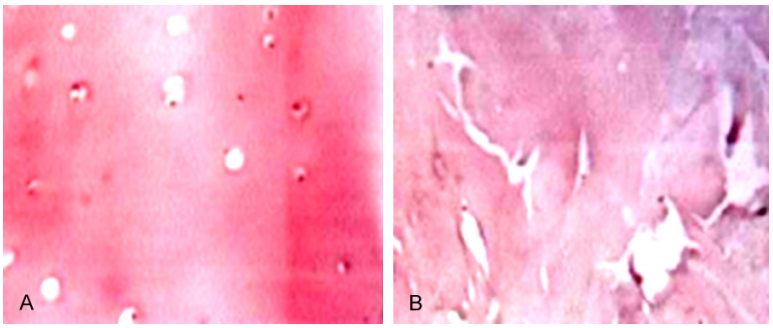

Figure 1. The safranin O-fast green staining (× 100) of the knee joint cartilage; A: Normal articular cartilage; B: Osteoarthritis of articular cartilage.

Table 2. Comparison of the scores on knee joint cartilage between the two groups according to Mankin score system (mean ± standard deviation)

Group	Cases	Mankin score
Control group	40	0.578 ± 0.412
Observation group		
Mild degeneration	10	3.194 ± 0.867
Moderate degeneration	11	7.526 ± 0.528
Severe degeneration	19	12.682 ± 1.141

Western blot determination

Put the broken knee articular cartilage tissues into homogenate device, and add the lysate to extract the total protein. After SDS-PAGE electrophoresis for immunoblotting trans-membrane, the membranes were rinsed with TBS, 5 min × 3 times, and incubated with GGCX mouse anti human monoclonal antibody at 4°C overnight, then rinse the membrane with TBS again, 10 min × 3 times; add HRP labeled goat anti mouse IgG antibody and incubated at room temperature for 1 h, then rinsed with TBS, 10 min × 3 times. Chemiluminescence was used to develop the image and gel documentation system was used to analyze the optical density of the bands.

Real time PCR determination

Total RNA was extracted by Trizol reagent after full grinding of articular cartilage by homogenizer. The total RNA was used to synthesize cDNA under the action of reverse transcriptase. β-actin was used for internal control. Upstream primer of β-actin: 5'-CATTAAGGAGAAGCTGTGCT-3', the downstream primer: 5'-GTTGAAGGTAGTTTCGTGGA-3', Upstream primer of GGCX: 5'-AACTGGACAAATGGGCTGT-3', the downstream primer: 5'-ATCTGGGGCTCAGTGACAT-3', Reaction system 20 uL: cDNA 2 uL, up and down primer 0.8 uL for both, SYBR Green PCR Master Mix (2 ×) 10 uL, ddH_2O 7.2 uL. After centrifugation, the cDNA were put into Applied Biosystems 7500 quantitative PCR instruments for amplification. Reaction conditions: pre-degenerated at 95°C for 8 min, degenerated at 94°C for 20 sec, refold at 56°C for 20 sec, and extend at 72°C for 23 sec, 30 cycles. β-actin was used as control gene, $2^{-\Delta\Delta Ct}$ method was used to calculate relative expression of GGCX mRNA of both group.

Magnetic separation chemiluminescence immunoassay

The expression level of GGCX in synovial fluid of both groups was detected by magnetic separation chemiluminescence immunoassay. Synovial fluid, FITC, and alkaline phosphatase markers were add into the tube and mixed well. After incubated at 37°C in water for 15 min, add the magnetic separation reagent, then incubated at 37°C in water for 5 min again, then precipitate 3 min in magnetic separator machine; the supernatant fluid was obtained and repeated the magnetic separation method again, then add luminescent substrate and mix well for detection by luminescence detector.

Statistical method

SPSS 21.0 statistical software was used for statistical analysis of all data, chi-square test was used to compare the count data between the two groups, and t test was used to compare measurement data between the two groups, single factor analysis for multi group comparison, $P < 0.05$ was considered statistically significant.

Results

Results of safranin O-fast green staining

Under light microscope, 6 different visual fields were randomly selected to observe the articular cartilage tissue, see **Figure 1**. According to the modified Mankin score system [8], the cartilage of knee joint in control group was normal without degeneration, and the average Mankin score was (0.578 ± 0.412); In the observation

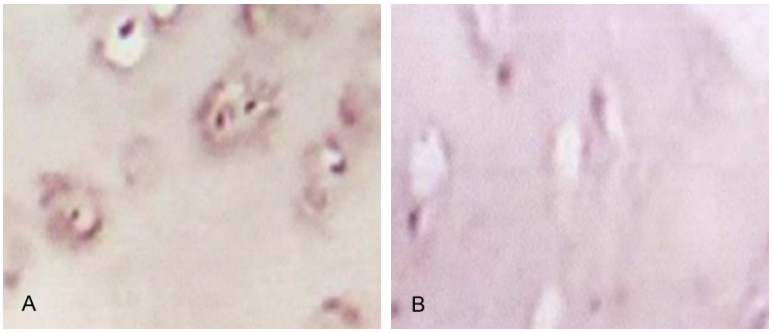

Figure 2. Immunohistochemical staining of knee joint cartilage (× 100), A: Normal articular cartilage; B: Osteoarthritis articular cartilage.

Table 3. Comparison of GGCX expression in cartilage between each group (mean ± standard deviation) by immunohistochemical staining assay

Group	Cases	Integral light density value of GGCX
Observation group	40	0.578 ± 0.221
Mild degeneration	10	0.984 ± 0.057
Moderate degeneration	11	0.586 ± 0.033
Severe degeneration	19	0.379 ± 0.041
Control group	40	1.339 ± 0.118

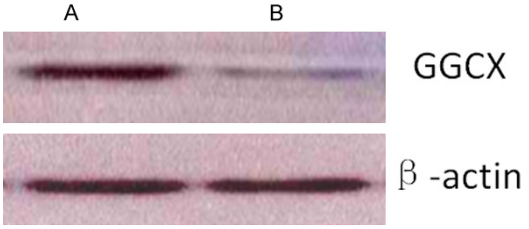

Figure 3. Expression of GGCX protein in articular cartilage of two groups; A: The control group; B: The observation group.

group, there were 10 cases of mild degeneration with an average score of (3.194 ± 0.867) points, 11 cases of moderate degeneration with an average score of (7.526 ± 0.528) points, and 19 cases of severe degeneration with an average score of (12.682 ± 1.141) points, the comparisons of scores between each group were statistically different, as shown in **Table 2**.

The expression of GGCX in cartilage by immunohistochemical assay

The results of immunohistochemical assay showed that GGCX expressed in all the articular cartilage tissues of both two groups, the expression was observed in the cytoplasm with positive results of brown or yellow staining, which had varying shades and scattered in the distribution. The integral optical density (IOD) of GGCX in the observation group and control group was (1.339 ± 0.118) and (0.578 ± 0.225), respectively, as shown in **Figure 2**. The optical density values in observation group were significantly higher than that in the control group, and the differences were statistically significant (t = 17.14, P = 0.00). Also, in observation group, the expressions of GGCX in patients at different degrees of degeneration had statistically significant difference, indicating that with the increase in severity of knee cartilage degeneration, the expression of GGCX in cartilage would decrease, as shown in **Table 3**.

The expression of GGCX protein in articular cartilage by western blot assay

In the observation group, the relative gray value of GGCX expression was (0.296 ± 0.194), while that of the control group was (0.898 ± 0.049); the difference was statistically significant (t = 19.34, P = 0.00), as shown in **Figure 3**. The relative gray values of GGCX in patients with mild degeneration, moderate degeneration and severe degeneration were (0.597 ± 0.057), (0.284 ± 0.026) and (0.138 ± 0.039), respectively, and there were statistically significant difference between the groups (all $P < 0.05$), as shown in **Figure 4**.

Expression of GGCX mRNA in each group by Real-time PCR

The relative expressions of GGCX mRNA in the observation group and the control group were (2.992 ± 1.316) and (10.978 ± 2.112), respectively. The content of GGCX mRNA in knee articular cartilage of observation group was obviously lower than that of control group, and the difference was statistically significant (t = 18.01, P = 0.00). Real-time PCR showed that with the increasing severity of degeneration in osteoarthritis articular cartilage, the relative expression of GGCX mRNA gradually decreased, which had statistical difference, as shown in **Figure 5**.

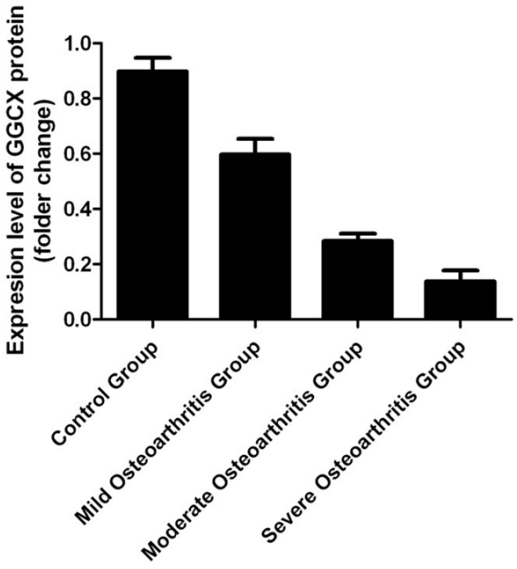

Figure 4. Expression level of GGCX protein in articular cartilage of each group.

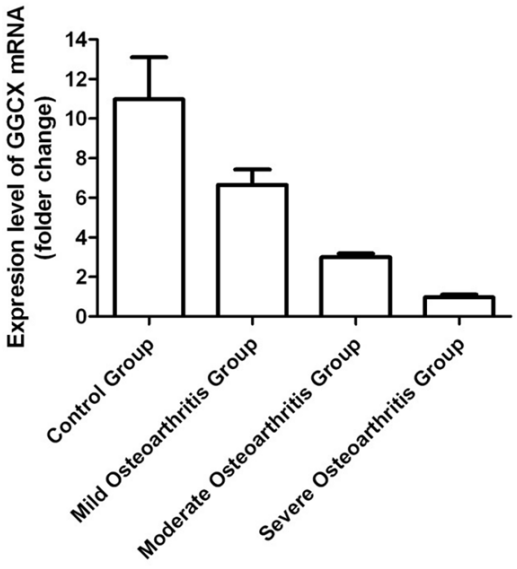

Figure 5. The expression of GGCX mRNA of each group by Real-time PCR.

The expression of GGCX of knee joint synovial fluid in two groups

Magnetic separation chemiluminescence immune method was used to detect the content of GGCX in synovial fluid, and the results showed that GGCX expressed in the synovial fluid of both groups. Compared with the control group (578.546 ± 121.622 nmol/L), the content of GGCX in the observation group (111.215 ± 25.106 nmol/L) significantly decreased, and the difference was statistically significant (t = 23.01, P = 0.00), as shown in **Figure 6**.

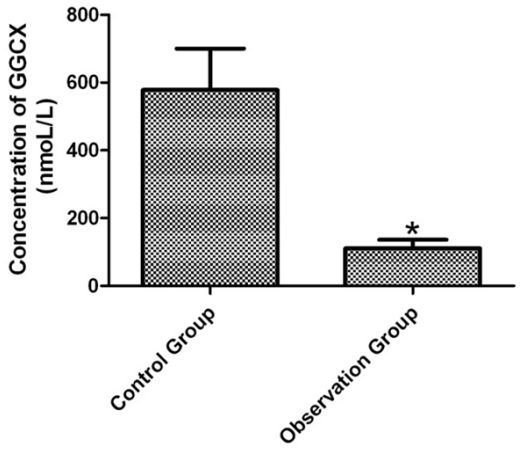

Figure 6. The content of GGCX in synovial fluid of knee joint in two groups. *Compared with the control group, P < 0.05.

Discussion

Osteoarthritis is a serious threat to the physical and mental health of elderly patients [10, 11]. A large number of studies at home and abroad showed that the deposition of calcium ion crystallite played an important role in the development of osteoarthritis [12, 13]. Some studies have reported that there was calcium ion crystal formation in joint fluid of the knee with different degrees of osteoarthritis [14, 15]. *In vitro* studies found that the large amount of calcium ion crystallite was produced by osteoarthritis chondrocytes instead of normal chondrocytes, which further confirmed that the calcium ion crystallite is related closely to osteoarthritis [16, 17]. In the recent years, studies have found that the matrix GLA protein (MGP) expressed in articular cartilage was closely related to the formation of calcium ions crystallite and the calcification of cartilage. MGP is a low molecular weight protein, mainly composed of chondrocytes, osteoblasts and vascular smooth muscle cells [18, 19]. MGP can be activated only by GGCX carboxylation, and the active MGP works mainly through inhibiting the deposition of calcium ions crystallite.

GGCX is the keystone enzyme of MGP and other vitamin K dependent protein molecules in the process of carboxyl group, and it is a single

chain glycoprotein [20] that composed of 758 amino acids. GGCX activity can directly affect the degree of the γ-carboxylation of glutamic acid residues in MGP, and further affect the activity of MGP inhibiting the formation of joint cartilage calcification and calcium ion crystal [21]. In the study of the rat model of ethylene glycol, it is found that the activity of GGCX was significantly increased, which may be a compensatory mechanism [22]. The expression of GGCX in renal tissues of patients with calcium oxalate stone is significantly decreased, indicating that GGCX is associated with the formation of calcium oxalate stones.

In this study, modified Mankin score system was used to evaluate the severity of osteoarthritis. According to Mankin score, patients in observation group was divided into mild degeneration, moderate degeneration and severe degeneration. And there was statistically significant difference between groups ($P < 0.05$). Immunohistochemical staining was used to compare the GGCX activity of articular cartilage in each group; the results showed that GGCX was expressed in both groups, but the GGCX activity in observation group was obviously lower than that of the control group with statistically significant difference. Magnetic separation chemiluminescence immunoassay was used for knee joint fluid detection, and the results showed that GGCX content in observation group was significantly lower than that of the control group. In addition, there were significant differences in GGCX activity between patients with different degree of degenerations, indicating that the more serious degeneration of articular cartilage was, the lower the GGCX activity would be, which further caused the reduction in MPG activation. Therefore, it cannot effectively inhibit cartilage calcification and calcium ion crystallite formation, and ultimately promotes the occurrence and development of osteoarthritis. To further elucidate the mechanism of GGCX activity decreasing, in this study, we used Western blot and real time PCR to detect the expression level of GGCX in articular cartilage, and the results showed that the expressions of GGCX protein and mRNA in osteoarthritic cartilage were significantly lower than those of normal cartilage, and the two groups had significant differences. At the same time, the results also showed that the expressions of GGCX protein and mRNA decreased as the severity of joint cartilage degeneration increased and the differences in the expression of GGCX among the patients with mild, moderate and severe cartilage degeneration were statistically significant (all $P < 0.05$).

In summary, the expressions of both GGCX protein and mRNA in osteoarthritis cartilage were significantly lower than those of normal cartilage, and the GGCX content in synovial fluid of observation group was significantly lower than that of normal group; GGCX expression was correlated with knee articular cartilage degeneration, the more serious degeneration of articular cartilage was, the lower the GGCX expression would be. The decrease in activity of GGXC may cause MGP carboxylation process block, reduce the activity of MGP, and further result in the ineffective inhibition of cartilage calcification and calcium ion crystal formation. But how does GGCX specifically participate in the occurrence and development of osteoarthritis is not clear yet. This study provides an experimental basis and new ideas for further research on the effect of GGCX in the process of osteoarthritis.

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Disclosure of conflict of interest

None.

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