

Detection of Urine C2C and Trace Element Level in Patients with Knee Osteoarthritis

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Abstract The objective is to determine the relation between severity of knee osteoarthritis (KOA) and levels of Collagen type II metabolite (C2C) and trace elements in the urine. The urine sample and knee joint films (anteroposterior and lateral) from the KOA patients and control subjects were collected. The KOA patients were divided into five groups (controls and grades I–IV) according to the Kellgren–Lawrence radiographic grading standards. Urine levels of C2C and trace elements were detected by enzyme-linked immunosorbent assay and inductively coupled plasma atomic emission spectrometry, respectively. Urine C2C levels in the KOA subjects (261.235 ± 39.944 pg/ml) were higher than those of the control group (218.341 ± 22.270 pg/ml). The Fe content in KOA groups was significantly lower than that of control group (group IV > group III > group II > group I or controls). The contents of Cu and Zn were also significantly higher in the KOA patients than in the control group ($p < 0.05$). However, Cr, Al, Cd, Ni, and Se levels of KOA patients were not significantly different from those of the controls ($p > 0.05$). Determination of the urine levels of C2C and trace elements may prove to be informative for an early diagnosis of KOA. It can also assist in the prognosis judgment of the disease and selecting an appropriate therapeutic regimen.

Keywords Osteoarthritis · C2C · Collagen type II · Trace elements

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Introduction

Knee osteoarthritis (KAO) is a degenerative disease of the knee joint that seriously affects everyday life of elderly people causing even disability in severe cases [1, 2]. The clinical symptoms and X-ray imaging are not found to be adequate in diagnosing the disease at an early stage. Additionally, X-rays can cause a severe damage to articular cartilage which is already affected by the disease [3–5]. Several studies have been conducted to investigate various biomarkers for the diagnosis of KOA [6–8]. Collagen type II metabolite, C2C is produced as a result of cartilage degeneration by the local enzymes and excreted in the urine. Hence, an elevated concentration of C2C in the urine has often been used as an indicator and important biomarker of KOA [9, 10]. However, changes in the urine levels of C2C in relation to varying severity grades of KOA have not been studied in detail before. The urine levels of trace elements have also been suggested to be critical in diagnosing and evaluating the prognosis of KOA [11]. In the current study, we determined the levels of C2C and trace elements in the urine and explored the possibility of their use for the diagnosis of KOA at an early stage.

Methods

Research Objects

Inclusion Criteria

(1) Postmenopausal women in whom C2C levels are changed due to an estrogen deficiency [12]; (2) Patients who had no liver and kidney dysfunction; (3) Those who had no other disease which could affect cartilage

Table 1 General distribution of the subjects in different groups (n/%)

Index	Control group	Grade I group	Grade II group	Grade III group	Grade IV group	χ^2 value/ F value	p value
Number of cases	20	31	14	23	22		
Gender							
Male	8	11	5	6	3	1.197	>0.05
Female	12	20	9	17	19		
Age (years)	56.5 ± 10.4	57.6 ± 8.3	57.0 ± 9.2	56.9 ± 9.4	57.3 ± 10.2	0.851	>0.05

metabolism, such as Ankylosing Spondylitis and rheumatoid arthritis; (4) Patients who were not taking drugs that affect hormone and cartilage metabolism; (5) Subjects in the control group (non-KOA group) belonged to the same age group as KOA patients, and they had no knee osteoarthritis reflected in radiographic images; (6) The research was approved by the medical ethics committee, the subjects consented for participation in research, and they were provided with the relevant information; (7) The radiographic imaging data were discussed and graded by 3 doctors.

Grading Standard

The patients were diagnosed according to KOA diagnostic criteria defined by the American Rheumatism Association in 1995, and KOA severity was graded according to the Kellgren–Lawrence radiographic grading standards. Thus, Grade 0: Normal; Grade I: doubtful narrowing of joint space and possible osteophytic lipping; Grade II: definitive osteophytes, and narrowing of joint space; Grade III: moderate multiple osteophytes, definitive narrowing of joints space, some sclerosis and possible deformity of bone contour; Grade IV: large osteophytes, marked narrowing of joint space, severe sclerosis and definitive deformity of bone contour.

Grouping Method

Ninety patients from outpatient clinic and inpatient department visiting during March 2013–June 2013 were enrolled in the study. They included 61 females, mean age 57.2 ± 9.1 , and 29 males, mean age 54. All patients were divided into 5 groups, including control, grade I, grade II, grade III, and grade IV. Grade 0: 20 cases including 8 males and 12 females; Grade I: 31 cases including 11 males and 20 females; Grade II: 14 cases including 5 males and 9 females; Grade III: 23 cases including 6 males and 17 females; Grade IV: 22 cases including 3 males and 19 females. Demographic data of the subjects in different groups are shown in Table 1.

Methods

Sample Collection

The morning urine specimens (25 ml) of the patients were collected and centrifuged at 2,500 rpm for 20 min. Supernatants were collected and stored in a -80°C freezer for future analysis. Anteroposterior and lateral films of the knee joint were taken.

Detection of C2C Level

ELISA was performed to detect C2C levels in the urine by a commercial kit purchased from Tian Jin Hao Yang Biological Manufacture CO., LTD, and the method was followed as instructed by the manufacturer. The urine samples stored at -80°C were allowed to thaw at room temperature and 5 ml were then centrifuged at 2,500 rpm for 20 min. In the antibody-coated ELISA plate, 10 wells were allocated for standards and 0 blank. The samples were added in the remaining wells after diluting them 5 times. The plate was incubated at 37°C for 1 h and then washed 5 times. Then 50 μl of ELISA reagent was added in each well except the blank, after that, the color developing agents A and B were added and incubated in the dark for 15 min. Stopping buffer was added, and OD values were read at 450 nm within 15 min. The C2C content in pg/ml was calculated using the curvilinear equation derived from the standard curve.

Detection of Trace Elements

The levels of trace elements, Cr, Fe, Mn, Al, Cr, Cu, Zn, Ni, and Se in the urine of KOA patients and control subjects were determined. Ten milliliter of the urine was added to a conical flask containing HNO_3 , HClO_4 , and H_2O_2 (10:6:1), and mixed. After 24 h, the flask was placed on an electric hot plate and heated to 140°C to allow a complete dissolution. The deionized water was then added to obtain a clear solution, and 1 % nitric acid was added to dilute it for detection of trace elements by the ICP-AES with AFS photomultiplier. Standard curves of different trace

Table 2 The C2C levels in controls and KOA subgroups

Group	C2C levels	<i>F</i> value	<i>p</i> value
Control group	218.341 ± 22.270	104.060	<0.05
Grade I group	219.098 ± 21.986		
Grade II group	257.319 ± 24.056		
Grade III group	268.209 ± 28.761		
Grade IV group	311.918 ± 31.618		

elements were plotted and concentrations in the urine samples were calculated. Each sample was run in five replicates and measured twice to calculate a mean value by a professional technician.

Observational Index

The radiographic images were discussed and graded by 3 doctors, and urine samples were analyzed by 2 professional technicians.

Statistical Analysis

The data were analyzed with SPSS13.0 software. Enumeration data were analyzed by Chi square test, and measurement data were analyzed by *t* test. The test level was set as $\alpha = 0.05$. The difference was considered as statistically significant when $p < 0.05$.

Results

The Urine C2C Levels in KOA and Control Groups, a Comparison

The levels of C2C in urine were higher in KOA group (261.235 ± 39.944 pg/ml) than those in the controls *p* (218.341 ± 22.270 pg/ml), $t = 11.174$, $p = 0.000$.

The C2C Levels in Controls and KOA Subgroups

Concentrations of C2C in the urine were the highest in grade IV patients and decreased gradually in the grades III, II to negligible amounts in grade I and controls: Grade IV group > Grade III group > Grade II group > Grade I group or control group ($F = 104.060$, $p = 0.000$). As shown in Table 2, the concentrations of C2C in grade I were not significantly different from those of the controls ($p > 0.05$).

Trace Element Levels in Urine, Comparison Between KOA and Control Groups

Iron (Fe) content was significantly lower whereas Cu and Zn were significantly higher in the KOA group compared

Table 3 Trace element levels in urine, a comparison between KOA and control groups (g/ml, $\Sigma x \pm s$)

Trace element	Control group (<i>n</i> = 20)	KOA group (<i>n</i> = 90)	<i>T</i> value	<i>p</i> value
Cr	0.0219 ± 0.0174	0.0224 ± 0.0159	0.154	>0.05
Fe	0.3595 ± 0.2655	0.2118 ± 0.1904	7.208	<0.05
Al	0.2508 ± 0.1446	0.2497 ± 0.1350	0.521	>0.05
Cd	0.0069 ± 0.0048	0.0071 ± 0.0036	1.033	>0.05
Cu	0.0329 ± 0.0258	0.0544 ± 0.0319	9.250	<0.05
Zn	0.5115 ± 0.4950	0.7268 ± 0.5797	13.169	<0.05
Ni	0.0216 ± 0.0147	0.0225 ± 0.0150	0.119	>0.05
Se	0.0055 ± 0.0031	0.0061 ± 0.0025	1.241	>0.05

to those in the control group ($p < 0.05$). The levels of Cr, Al, Cd, Ni, and Se in the two groups were not significantly different ($p > 0.05$) (Table 3).

Discussion

Cartilage is comprised chondrocytes, the specialized cells that secrete large amounts of extracellular matrix which is composed of collagen, proteoglycan elastin fibers and water [13–15]. The major constituents of articular cartilage matrix are water and the collagen II (CII) [16–18]. Collagen II is constituted of a triple helix with two almost completely identical and one slightly different α chains [19–21]. The degeneration of cartilage matrix is marked by depolymerization of the collagen II triple helical structure followed by the production of C2C (3/4 fragment of CII carboxyl terminal, Col-3/4) [22–24].

The split product of CII, a 3/4 fragment of its carboxyl terminal, also named Col-3/4 or C2C is a small peptide that enters the urine for excretion. Thus, C2C concentrations in the urine can directly reflect decomposition of the articular cartilage [9, 10]. In fact, the presence of cartilage metabolites in the urine in different concentration has been positively related to the severity of knee joint [3, 25–27]. It has also been suggested that an estimation of cartilage metabolites in urine may be employed as a quick and reliable measure for KOA diagnosis [28]. In 2004, Poole et al. [29] made monoclonal antibody against C2C using a synthetic peptide CGGE787GPOGPQG794 (O represents hydroxyproline). In 2007, Cahue et al. [30] found that in 18 months follow up study, C2C levels were highly related to the patient surveillance and progress of disease. As the clinical research progressed further, in 2008 Conrozier et al. [3] detected more degradation products of CII, such as CTX-II, C2C, CI, C2 in the plasma of 56 KOA patients. They observed no change in the levels of CTX-II associated with KOA in contrast to the earlier reports cited in

their paper. They also found that C2C levels were related to the occurrence of single hip OA. El-Maadawy et al. [31] also reported that an increase in plasma C2C levels was correlated to the KOA diagnosed by X-ray imaging. However, thus far, the C2C levels in urine have not been correlated to the severity and grading of KOA determined by X-ray imaging. Furthermore, the C2C concentrations in the urine of KOA patients have not been compared with those of the control subjects before. We showed for the first time that C2C levels in the urine of KOA patients were significantly higher than those in the control group. Also, as C2C concentrations in the urine increased, the degree of disease severity was also elevated. Clearly, our results indicated a direct correlation between the severity of disease and increased cartilage catabolism that leads to an enhanced production of C2C. However, the C2C concentrations in grade I patients were not significantly different from controls. Thus, the detection of urine levels of C2C can assist in the diagnosis of KOA, however, not in the early stage of disease.

Cytokines are known to play a critical role in the occurrence and development of early stage OA. For instance, the levels of interleukin-1 (IL-1), interleukin -6 (IL-6), tumor necrosis factor (TNF) are found to be implicated in OA. They stimulate cartilage cells to secrete nitric oxide, prostaglandins and interleukin-6, and thereby further the pathological changes that cause synovitis and pain [32]. Since, at an early stage of KOA, surface of cartilage is not involved in the collagen II catabolism, the change is not detectable with X-ray and MRI imaging. At this stage, C II undergoes mainly the structure changes, such as the swelling of type II collagenous fibers. As the disease progresses, the cartilage degeneration is advanced gradually leading to roughness and cracking, to reduced thickness and finally to complete loss and exposure of bone. Thus, our results demonstrated that the urine C2C levels in grade I were not very different from the controls. However, in grades II–IV groups, the C2C level was significantly enhanced as compared to the control group.

In comparison to controls, the KOA patients showed a lower content of Fe and significantly higher levels of Cu and Zn ($p < 0.05$). However, the concentrations of Cr, Al, Cd, Ni and Se in the KOA patients were not significantly different from the controls ($p > 0.05$). The decreased Fe content may indicate an insufficient local blood supply aggravating the gradual degeneration of joints. On the other hand, increased concentrations of Cu and Zn can stimulate metalloprotease activation to increase the degradation rate of articular cartilage.

In conclusion, C2C concentrations in the urine of KOA patients were significantly higher than in controls. The C2C content increased as the severity of KOA progressed gradually. This study also evidenced an important role of

trace elements, Fe, Cu, and Zn in the progression of KOA. On the basis of these findings, we propose that measurement of C2C combined with determination of trace elements in the urine may assist in the early diagnosis, therapeutic regimen selection, and prognosis judgment of KOA.

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