Int J Clin Exp Med 2017;10(10):14660-14666
www.ijcem.com /ISSN:1940-5901/IJCEM0062833

Original Article

Correlations between Traditional Chinese Medicine syndromes and IL-1, MMP and TIMP-1 in knee osteoarthritis

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Received August 2, 2017; Accepted September 8, 2017; Epub October 15, 2017; Published October 30, 2017

Abstract: Objective: To investigate the correlations of the concentrations of cytokines IL-1, MMP and TIMP-1 in synovial fluid to disease course and age in patients with different Traditional Chinese Medicine (TCM) syndromes of knee osteoarthritis (KOR). Methods: A total of 120 patients with KOR admitted to the Department of TCM Orthopedics & Traumatology from January 2014 to December 2016 were recruited into this study. The patients were assigned to the Group of shenxusuikui (patients with syndrome of marrow depletion due to kidney deficiency), the Group of yangxuhanning (those with syndrome of yang deficiency and coagulated cold) or the Group of yuxuezuzhi (those with syndrome of stagnation of blood stasis) in terms of diagnostic criteria for TCM syndrome differentiation. The enzyme-linked immunosorbent assay (ELISA) was utilized for measuring cytokines IL-1, MMP and TIMP-1 levels in the synovial fluid from patients with KOR in all the groups. The ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 were also calculated. Pearson's correlation analysis was applied for assessing the correlations of cytokines IL-1, MMP, TIMP-1 levels, the ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 to age and disease course of the patients. Results: The levels of MMP-1, MMP-3, TIMP-1 and IL-1, the ratios of MMP-1/TIMP-1 and of MMP-3/TIMP-1 were significantly higher in the synovia fluid among the patients in the Group of yangxuhanning than those in the Group of shenxusuikui and the Group of yuxuezuzhi, respectively (All $P<0.05$). The correlation analysis indicated the concentrations of MMP-1 ($r_1=0.638$, $P=0.002$, $r_2=0.754$, $P=0.004$), MMP-3 ($r_1=0.793$, $P=0.001$; $r_2=0.719$, $P=0.002$), and of IL-1 ($r_1=0.587$, $P=0.001$; $r_2=0.668$, $P=0.001$), the ratios of MMP-1/TIMP-1 ($r_1=0.471$, $P=0.005$; $r_2=0.621$, $P=0.000$) and of MMP-3/TIMP-1 ($r_1=0.652$, $P=0.007$; $r_2=0.619$, $P=0.000$) were in direct proportion to disease course (r_1) and age (r_2) of the patients whereas the TIMP-1 centration was inversely indirectly proportional to disease course ($r=-0.569$, $P=0.003$) and age ($r=-0.714$, $P=0.002$) of the patients. Conclusion: The levels of inflammatory cytokines were the highest among the KOR patients with syndromes of yang deficiency and coagulated cold, suggesting they had the most serious inflammatory injury. The KOR patients with long disease course and old age had elevated levels of inflammatory cytokines, indicating that early treatment can reduce the inflammatory injury in knee joints.

Keywords: Knee osteoarthritis, differentiation of Traditional Chinese Medicine syndrome, IL-1, MMP, TIMP-1

Introduction

Knee osteoarthritis (KOR) is a common joint disease among the elderly population, characterized by progressive degeneration in the articular cartilage, which severely threatens the activity, life and treatment of the patients. However, the pathogenesis of KOR remains unclear. Cytokines are present in a wide range of tissues, which are strongly associated with damage to articular cartilage, synovium and subchondral bone. Cytokines play an essential role in the presence and progression of osteoarthritis [1, 2]. MMPs are a proteolytic enzyme degrading extracellular matrix. Under normal circumstances, MMPs is rarely expressed in chondrocytes, but its gene expression upregulates significantly in pathological conditions [3, 4]. Tissue inhibitor of metalloproteinase (TIMP), a protein encoded by multigene family, inhibits the activity of MMPs. Recent studies have demonstrated that the imbalance in gene expres-

sion between matrix metalloproteinases and their inhibitors is an essential factor for degradation of cartilage in KOR patients [5, 6]. Interleukin -1 (IL-1), as a factor for cartilage damage, can upregulate the expression levels of MMP in articular cartilage via various signaling pathways, promote chondrocyte degeneration and degradation of cartilage matrix [7, 8].

As far as Traditional Chinese Medicine (TCM) is concerned, OR is a subtype of bone bi-disease, the major mechanisms of which include deficiency, stasis and Xie. In recent years, most studies on differentiation of TCM syndrome types in KOR have been implicated in analyses on the correlation of single factors in KOR [9, 10]. That is to say, analyses have been made on the correlations among various TCM syndromes of KOR from the perspective of certain cytokine. The changes in the levels of the cytokines are frequently impacted by many factors, which leads to the decrease in the specificity of the cytokines. Different from previous studies, in this study, we explored the correlations of various TCM syndrome types in KOR to the objective markers from diverse perspectives by detecting the concentrations of MMP-1, MMP-3 and TIMP-1, and the MMP-1/TIMP-1 and MMP-3/TIMP-1 ratios in synovial fluid from KOR patients. We expected the findings of this study werehelfulto find the relevant objective markers for differentiation of TCM syndrome types in KOR, as as to provide experimental evidence for the diagnosis and treatment of KOR.

Materials and methods

Participants

The subjects in this study were 120 patients selected from those who met diagnostic criteria of KOR and were outpatients or inpatients in the Department of TCM Orthopedics & Traumatology in our hospital from January 2014 to December 2016. In terms of the criteria for differentiating TCM syndrome types, the included patients were classified into those with syndrome of marrow depletion due to kidney deficiency (the Group of shenxusuikui), those with syndrome of yang deficiency and coagulated cold (the Group of yangxuhanning) and those with syndrome of stagnation of blood stasis (the Group of yuxuezuzhi). In this study, the patients were included if they had an age of 18 years or older, and the syndrome meeting the criteria for differentiating TCM syndrome types in KOR according to *Criteria of diagnosis and therapeutic effect of diseases and syndromes in traditional Chinese medicine*. The patients who were pregnant, had secondary KOR, cardio-cerebrovascular disease and severe hepatonephric impairment, took analgesic drugs and were not suitable for knee joint puncture were excluded from this study. This study was in line with the hospital ethics criteria and qualified for ethics review. Each eligible patient provided written informed content.

Concentrations of MMP-1, MMP-3, TIMP-1 and IL-1 in synovial fluidfrom KOR patients detected by ELISA

With the patient in the supine position and stretching out the knee joint with OR, 2 ml of synovial fluid was drawn from the site between the superior border of patella and the lateral border of quadriceps femoris by the puncture method. The synovial fluid was infused into a heparinized tube, and centrifuged for 15 min at 2500 r/min. Then the supernatant was moved to an Eppendorf tube and stored at -80°C for measure.

The concentrations of MMP-1, MMP-3, TIMP-1 and IL-1 in synovial fluid from KOR patients in each group were detected by the enzyme-linked immunosorbent assay (ELISA) kit (R&D, US). Following the instructions on the kit, the standard solutions and the samples to be tested were added into the ELISA plates pre-coated with anti-MMP-1, anti-MMP-3, anti-TIMP-1 or anti-IL-1 monoclonal antibodies. After the plate was sealed by the plate sealer, the mixture was incubated for 30 min at 37°C. The supernatants were pipetted and discarded, and then the wells were rinsed three times with wash solution, into which HRP-conjugated secondary antibodies were added and incubated for 30 min at 37°C, followed by 3 times of well-rewashing with wash solution. Following the addition of chromogenic agent, the samples were gently mixed. After color development for 10 min, the blank holes were adjusted to zero, and the absorbance of each hole was measured at wave length of 450 nm with the use of a microplate reader. Standard curves were drawn for calculation of the concentrations of the samples.

Statistical analysis

The data analysis was made using the statistical software SPSS, version 19.0. Measurement

Table 1. Comparison of general data of patients among the groups

	Gender (male/female, n)	Age (year)	BMI (Kg/m^2)	KOR (left/right, n)	Disease course (year)
Group of shenxusuikui (n=45)	27/18	61.7±3.72	23.4±2.35	24/21	6.4±1.38
Group of yangxuhanning (n=40)	23/17	62.5±4.12	23.2±2.19	22/18	6.7±1.49
Group of yuxuezuzhi (n=35)	20/15	63.4±4.47	23.9±2.27	21/14	6.9±1.58
F/X^2	1.049	0.482	2.137	0.339	0.568
t	0.297	0.514	0.151	0.562	0.491

Table 2. Comparison of the concentrations of MMP-1, MMP-3 and TIMP-1 in synovial fluid among all the TCM syndromes (ng/mL)

Group	Case	MMP-1	MMP-3	TIMP-1
Group of yuxuezuzhi (A)	35	265.86±54.76	697.84±70.54	321.39±47.87
Group of shenxusuiku (B)	45	279.03±58.21	724.48±73.67	339.57±50.12
Group of yangxuhanning (C)	40	392.34±63.72*	2287.39±112.28*	383.64±56.18*
P (A/B)		0.576	0.125	0.373
P (A/C)		0.000	0.000	0.000
P (B/C)		0.000	0.000	0.000

Note: In comparison with the Group of yuxuezuzhi and the Group of shenxusuiku, *P=0.000.

data were represented as mean ± sd; comparisons among the groups were performed with the use of one-way ANOVA followed by post-hoc Bonferroni test. Count data were expressed as percentages, and comparisons among the groups were made using a chi-square test based on chi-square partition. Pearson correlation analysis was utilized for detecting the correlations between the levels of MMP-1, MMP-3, TIMP-1, IL-1, the ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 to age and disease course, respectively. P<0.05 was deemed to be statistically significant.

Results

General data of patients

All the eligible patients had OR in a single knee. Seventy patients were male and 50 were female. KOR developed in the left knee joint of 67 patients and KOR in the right knee joint in 53 patients. The patients had a mean age of 62.3±4.87 years, with the body mass index (BMI) of 23.6±2.78 kg/m^2 and a mean disease course of 6.8±1.53 years.

The Group of shenxusuikui (n=45) consisted of 27 male and 18 female patients, with a mean age of 61.7±3.72 years old, the BMI of 23.4±2.35 kg/m^2 and a mean disease course of 6.4±1.38 years. KORwas presentin the left knee joint of 28 patients and KOR in the right knee joint in 17 patients. The Group of yangxuhanning (n=40) had 23 male and 17 female patients, with a mean age of 62.5±4.12 years old, the BMI of 23.2±2.19 kg/m^2 and a mean disease course of 6.7±1.49 years. KOR was presentin the left knee joint of 24 patients and KOR in the right knee joint in 16 patients. The Group of yuxuezuzhi included 35 patients, with 20 male and 15 female patients, a mean age of 63.4±4.47 years old, the BMI of 23.9±2.27 kg/m^2 and a mean disease course of 6.9±1.58 years. The KOR developed in the left knee joint in 22 patients and KOR in the right knee joint in 13 patients. The differences in general data including age, sex ratio, BMI, lesion site in the knee joint, and disease course were not statistically significant across the groups (All P>0.05, **Table 1**).

Concentrations of MMP-1, MMP-3 and TIMP-1 in synovial fluid of KOR patients with different TCM syndrome types

Table 2 shows the differences in the concentrations of MMP-1, MMP-3 and TIMP-1 in synovial fluid from KOR patients were insignificant between the Group of shenxusuikui and the Group of yuxuezuzhi, respectively. However, the concentrations of MMP-1, MMP-3 and TIMP-1 were upregulated significantly in the Group of yangxuhanning, as compared with the Group of

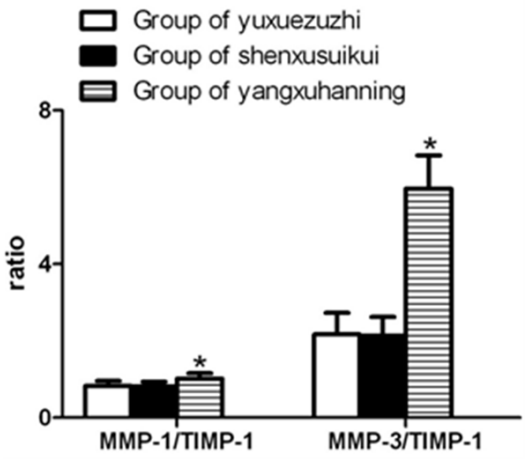

Figure 1. Comparison of the ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 in synovial fluid from KOR patients with different TCM syndromes The data were represented as mean ± standard deviation. * In comparison with the Group of yuxuezuzhi and the Group of shenxusuiku, P=0.000.

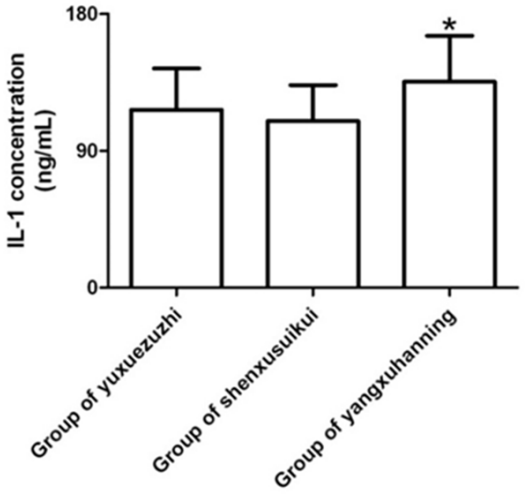

Figure 2. Comparison of IL-1 concentrations in synovial fluid of different TCM syndromes. * In comparison with the Group of yuxuezuzhi and the Group of shenxusuiku, P=0.000.

shenxusuikui and the Group of yuxuezuzhi, respectively (All *P*=0.000).

The ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 in synovial fluid were significantly higher in the Group of yangxuhanning than in the Group of yuxuezuzhi and the Group of shenxusuiku, respectively (All *P*=0.000), but the ratios did not differ significantly between the Group of yuxuezuzhi and the Group of shenxusuiku (**Figure 1**).

Comparison of the IL-1 concentrations in synovial fluid of different TCM syndromes

Figure 2 shows the IL-1 concentrations in synovial fluid differed insignificantly between the Group of yuxuezuzhi (109.52+23.64 ng/mL) and the Group of shenxusuiku (116.95+27.09 ng/mL); but the IL-1 concentration was upregulated significantly in the Group of yangxuhanning (135.41+30.15 ng/mL) than in the Group of yuxuezuzhi and the Group of shenxusuiku, respectively (All *P*=0.000).

Correlatesof MMP-1, MMP-3, TIMP-1 and IL-1 concentrations in synovial fluid with disease course and age among the patients

Pearson correlation analysis revealed that MMP-1, MMP-3 and IL-1 concentrations were in direct proportion to disease course and age of the patients, respectively (All P<0.05), but the TIMP-1 concentration was inversely indirectly proportional to disease course and age, respectively (All P<0.05, **Tables 3** and **4**).

Discussion

Syndrome differentiation treatment is a basic principle for understanding and diagnosing disease based onthe theories of traditional Chinese medicine. The prevention and treatment of KOR by TCM has been the focus of the study on TCM orthopedics and traumatology. However, in the treatment of disease by TCM, the priority is to differentiate the symptoms accurately, and then we can select appropriate medication. Therefore, correct differentiation is abasic stepin clinical treatment of KOR in TCM. The pathophysiology of KOR is degeneration of knee joints caused by abnormal proteoglycan synthesis in articular cartilage. Under normal circumstances, the synthesis and catabolism of articular cartilage matrix is well-balanced under the control of a seas of cytokines [11, 12]. In the above process, cytokines MMP-1, MMP3 and TIMP-1 seem to be more crucial. MMP-1 is mainly involved in the decomposition of type II collagen. MMP-3, the most important enzyme in the degradation of articular cartilage matrix, canlead to virtually complete breakdown of proteoglycan. TIMP-1 can bind MMP-1 and MMP-3 to produce a compound inhibiting their activity. The gene expression of MMP-1 and TIMP-1 in the pathological state has shown to differ significantly from those in normal syno-

Table 3. Coefficients for the concentrations of MMP-1, MMP-3, TIMP-1 and IL-1 and the MMP-1/TIMP-1 and MMP-3/TIMP-1 ratios in synovial fluid and the disease course, respectively

	MMP-1	MMP-3	TIMP-1	IL-1	MMP-1/TIMP-1	MMP-3/TIMP-1
Disease course	0.638	0.793	-0.569	0.587	0.471	0.652
P value	0.002	0.001	0.003	0.001	0.005	0.007

Table 4. Coefficients for the concentrations of MMP-1, MMP-3, TIMP-1 and IL-1 and the MMP-1/TIMP-1 and MMP-3/TIMP-1 ratios in synovial fluid and age, respectively

	MMP-1	MMP-3	TIMP-1	IL-1	MMP-1/TIMP-1	MMP-3/TIMP-1
Age	0.754	0.719	-0.714	0.668	0.621	0.619
P value	0.004	0.002	0.002	0.001	0.000	0.000

vial folds [13]. The gene expression of protein MMP-1 was upregulated significantly, but that of protein TIMP-1 insignificantly in the chondrocytes of KOR patients [14]. In other studies, the gene expression of MMP-3 in synovial fluid and serum from patients with KOR were upregulated significantly as compared with healthy individuals, and the magnitude of upregulation was consistent with that of cartilage destruction [15, 16]. Similar to the results of the abovementioned studies, in the present study, we found different levels of MMP-1, MMP-3 and TIMP-1 in diverse TCM syndromes of KOR, with the highest levels among the patients in the Group of yangxuhanning.

The imbalanced MMPs/TIMP ratio is associated with an upregulation of proteolytic enzyme in cartilage matrix, leading to degradation of articular cartilage and induction and aggravation of the pathologic process of KOR [17, 18]. In addition, protein IL-1 is a key factor in KOR and an initial factor in the inflammatory response. IL-1 has been reported to reduce the activity of chondrocytes, induce apoptosis of chondrocytes, promote the secretion of MMPs by chondrocytes, and aggravate the cartilage degradation [19, 20]. Anincremental upregulation of IL-1 level suggests aggravation of KOR [21]. Notably, the inflammatory response in the pathogenesis of KOR is associated with damage to inflammatory cytokines -mediated cartilage. When compared with previous studies, the IL-1 levels and the ratios of MMP-1/TIMP-1 and of MMP-3/TIMP-1 in the currentstudy varied in KOR patients with different TCM syndromes [22, 23].

In our currentstudy, the various expression levels of inflammatory cytokines in different TCM syndromes in KOR were examined. We found that the concentrations of MMP-1, MMP-3, TIMP-1 and IL-1 and the ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 in synovial fluid from KOR patients were significantly higher in the Group of yangxuhanning than in the Group of yuxuezuzhi and the Group of shenxusuiku, respectively. In correlation analysis, positive correlations were shown between the concentrations of MMP-1, MMP-3 and IL-1 and disease course and age, respectively, whereas negative correlations were found between the concentrations of MMP-1 and disease course and age, respectively. This demonstrates that the inflammatory response of cartilage tissues among patients with syndrome of yang deficiency and coagulated cold were higher. Besides, the levels of inflammatory cytokines in patients with longer disease course and older age were also higher, which indicates that KOR should be treated as early as possible to reduce the damage of inflammatory response to articular cartilage. This has not been reported in previous studies.

In summary, cytokines MMP-1, MMP-3, TIMP-1, IL-1, and the ratios of MMP-1/TIMP-1 and of MMP-3/TIMP-1 can be applied to early diagnosis of KOR, and are associated with efficacy evaluation and severity of KOR. However, there are some limitations in this study, such as a small sample size. Future studies are required for further validation.

Acknowledgements

This work was funded by Hebei province administration of traditional Chinese medicine scientific research projects (2015136).

Disclosure of conflict of interest

None.

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