

INCREASED CHITOTRIOSIDASE ACTIVITIES IN PATIENTS WITH RHEUMATOID ARTHRITIS: A POSSIBLE NOVEL MARKER?**POVEĆANJE NIVOA HITOTRIOZIDAZE KOD BOLESNIKA SA REUMATOIDNIM ARTRITISOM: MOŽE LI TO BITI NOVI INDIKATOR?***Banu Isbilen Basok¹, Mine Kucur², Muhammed Kizilgul³, Ibrahim Yilmaz¹, Ozlem Balci Ekmekci², Mehmet Uzunlulu³, Ferruh Kemal Isman¹*¹*Istanbul Medeniyet University Goztepe Teaching and Research Hospital, Medical Biochemistry Department, Istanbul, Turkey*²*Istanbul University Cerrahpasa Medicine Faculty, Biochemistry Department, Istanbul, Turkey*³*Istanbul Medeniyet University Goztepe Teaching and Research Hospital, Internal Medicine Department, Istanbul, Turkey***Present address: Nizip Ministry of Health Hospital, Internal Medicine Department, Gaziantep, Turkey.***Summary**

Background: Chitotriosidase and YKL-40 are well-known in humans as Glyco₁₈ domain-containing proteins that are the common feature of mammalian chitinases and chitinase-like proteins. Previously, increased levels of YKL-40 were found correlated with the disease activity of rheumatoid arthritis. However, serum chitotriosidase activity in rheumatoid arthritis is not known yet. The aim of this study was to determine YKL-40 and chitotriosidase in patients with rheumatoid arthritis and to compare these markers with traditional ones such as C-reactive protein and erythrocyte sedimentation rate.

Methods: Chitotriosidase, YKL-40 and C-reactive protein were measured in serum samples from 27 patients with rheumatoid arthritis and 27 healthy people. Chitotriosidase, YKL-40, C-reactive protein, and erythrocyte sedimentation rate were determined by a fluorometer, ELISA, nephelometer, and Western Green method, respectively.

Results: Serum chitotriosidase activities and YKL-40 levels were higher in rheumatoid arthritis group than in control. A significant positive correlation was found between chitotriosidase and YKL-40. In ROC analysis, the areas under curves for chitotriosidase, C-reactive protein, erythrocyte sedimen-

Kratak sadržaj

Uvod: Hitotriozidaza i YKL-40 su dobro poznati kao proteini koji sadrže domen Gliko₁₈, koji je zajednička karakteristika hitinaze sisara i proteina sličnih hitinazi. Ranije se pokazalo da su povećani nivoi YKL-40 povezani sa delovanjem reumatoidnog artritisa. Svrha ove studije je određivanje nivoa YKL-40 i hitotriozidaza kod bolesnika sa reumatoidnim artritisom i upoređivanje sa uobičajenim indikatorima kao što su C-reaktivni protein i brzina sedimentacije eritrocita.

Metode: Nivoi hitotriozidaza, YKL-40, C-reaktivni protein i brzina sedimentacije eritrocita u uzorcima seruma 27 bolesnika sa reumatoidnim artritisom i 27 zdravih osoba izmereni su redom fluorometrijskom, ELISA, nefelometrijskom i Western-Green metodom.

Rezultati: U serumu grupe bolesnika sa reumatoidnim artritisom je zabeležena veća aktivnost hitotriozidaza i nivo YKL-40 u poređenju sa zdravim ispitanicima. Između hitotriozidaza i YKL-40 postoji statistički značajna pozitivna korelacija. U analizi ROC, za hitotriozidaze, C-reaktivni protein, brzine sedimentacije eritrocita i YKL-40, područja ispod krive su redom zauzela 0,96, 0,84, 0,76 i 0,65. Vrednost područja ispod krive testa za hitotriozidaze je bila znatno veća od vrednosti područja ispod krive brzine se-

Address for correspondence:

Banu Isbilen Basok
Istanbul Medeniyet University Goztepe Teaching and Research Hospital,
Medical Biochemistry Department
34720 Goztepe, Kadikoy, Istanbul
Phone: + 90 216 570 9022
Fax: + 90 216 570 9021
e-mail: drisbilen@yahoo.com

List of Abbreviations: CRP, C-reactive protein; CHIT, chitotriosidase; DMARDs, disease modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; JIA, juvenile idiopathic arthritis; NSAIDs, non-steroidal antiinflammatory drugs; PMNs, polymorphonuclear neutrophils; RA, rheumatoid arthritis; ROC, receiver operating characteristic; SF, synovial fluid.

tation rate, and YKL-40 were 0.96, 0.84, 0.76, and 0.65, respectively. Area under the curve for chitotriosidase was significantly higher than the area for erythrocyte sedimentation rate ($p=0.005$) and for YKL-40 ($p=0.0001$), but not for C-reactive protein ($p=0.055$).

Conclusions: Serum chitotriosidase was significantly increased in patients with rheumatoid arthritis. Among all the parameters evaluated, chitotriosidase was the most sensitive and specific one. Comprehensive studies covering larger populations are needed to elucidate the relationship between chitinases, in particular chitotriosidase and rheumatoid arthritis.

Keywords: chitotriosidase, C-reactive protein, inflammation, rheumatoid arthritis, YKL-40

Introduction

Chitotriosidase (CHIT) and YKL-40 are well-known in humans as Glyco_18 domain-containing proteins that are the common feature of mammalian chitinases and chitinase-like proteins (1). CHIT is mainly produced by active macrophages and partly polymorphonuclear neutrophils (PMNs), and is a major marker for Gaucher disease (2). Other than Gaucher disease, CHIT activity is also related to inherited lysosomal storage disorders (3) including Nieman-Pick disease and Fabry, and noninherited disorders such as nonalcoholic steatohepatitis, Alzheimer disease, cerebral adrenoleukodystrophy, chronic obstructive pulmonary disease, sarcoidosis, acute appendicitis, and atherosclerosis (4–10).

YKL-40, also called human cartilage glycoprotein-39 (HC gp-39) or chondrex, is a 40 kDA glycoprotein, secreted by macrophages, PMNs, fibroblast-like synovial cells, articular chondrocytes, hepatocytes, and smooth muscle cells, as well as tumor cells (1). Elevated serum levels of YKL-40 have been described in various diseases associated with inflammation such as rheumatoid arthritis (RA), osteoarthritis, systemic lupus erythematosus, inflammatory bowel diseases with joint involvement, acute appendicitis, preeclampsia, and also in the group of cardiovascular diseases and cancer (11–17).

RA is a chronic, systemic autoimmune disease characterized by synovial inflammation in multiple joints. The chronic inflammation and cellular infiltration of the synovial membrane of affected joints induce destruction of cartilage and bone, and hence this severe process results in progressive joint destruction, osteoporosis, and severe disability (18). Although the laboratory markers used routinely such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are rapid, objective, and inexpensive, they lack sensitivity and specificity for disease activity as well as disease prediction (19). Still, these tests have been included in the 2010 RA classification criteria (20) possibly due to the lack of more sensitive and specific markers of disease activity. Nevertheless, the search for other noninvasive biomarkers for RA is still ongoing.

dimentacije eritrocita ($p=0,005$) i YKL-40 ($p=0,0001$), ali identična sa područjem za C-reaktivni protein ($p=0,055$).

Zaključak: Aktivnost hitotrioizidaze u serumu bolesnika sa reumatoidnim artritismom je u vidljivom porastu. Između svih procenjenih parametara utvrđeno je da je hitotrioizidaza najosetljivija i najspecifičnija. Postoji potreba za opsežnim studijama na polju hitinaze, koje obuhvataju veće populacije i koje će istražiti odnos između hitotrioizidaza i reumatoidnog artritisa.

Cljučne reči: hitotrioizidaza, C-reaktivni proteini, inflamacija, reumatoidni artritis, YKL-40

Previously, YKL-40 mRNA expression was determined in the joint cartilage affected by RA (21). Interestingly, in normal human cartilage there was no expression of YKL-40. Later, several studies specified that not only synovial YKL-40 levels, but also serum levels were found correlated with the disease activity (22, 23). Although CHIT activity in serum was evaluated in several inflammatory diseases, besides lysosomal storage diseases, there is only one study which evaluated the activity of CHIT in the context of rheumatologic disease. In that study, authors suggested that increased activity of CHIT in synovial fluid might be a predictor of the course of juvenile idiopathic arthritis (24). Overall, serum CHIT activity in RA is still not known.

The objective of the present study was primarily to investigate the serum levels of CHIT and YKL-40 and whether these markers are superior to traditional markers such as CRP and ESR in a cohort of patients with RA.

Material and Methods

Patients

Between January 2011 and May 2011, 27 (22 female, 5 male) patients who applied to the Internal Medicine Department and had been diagnosed with RA according to the »American College of Rheumatology (ACR)-1987 Criteria for the Classification of Acute Arthritis of Rheumatoid Arthritis« (25) were included in the study. The patients were under standard treatment for their disease including corticosteroids, assorted nonsteroidal antiinflammatory drugs (NSAIDs), disease modifying antirheumatic drugs (DMARDs) before and during the study. All patients were in clinical remission, which was defined as clinician-determined remission, meaning stable, controlled disease, and requiring no need of change in medication. The exclusion criteria for the patients were: any collagen tissue disorder, any type of infection, presence of chronic diseases causing low-grade inflammation such as diabetes mellitus, dyslipidemia, hypertension, thyroid dysfunction, malignancy, liver or renal disease or smoking. Age- and gender-matched

27 healthy people (20 female, 7 male) who were eligible in terms of inclusion and exclusion criteria were accepted as a control group for the comparison of serum biochemical analyses. These volunteers were not given any medical and/or physical therapy.

The study was conducted in accordance with the Helsinki Declaration of 1975 and approved by the hospital's ethics committee (Date: 17.05.2011; Decision no: 12/B). All participants provided written informed consent.

Biochemical analyses

After an overnight fast, blood samples were taken from the subjects. The samples were left to clot, and serum was separated from cellular fragments by centrifugation within one hour after blood sampling. All serum samples were stored at -80°C until analysis. Serum CHIT activity was based on the method described by Hollak et al. (2), with minor modifications. In summary, 5 μL of serum samples were incubated with 100 μL of 22 $\mu\text{mol/L}$ 4-methylumbelliferyl β -D-N, N', N''-triacetylchitotriose (Sigma Chemical, St. Louis, MO, USA) as substrate in McIlvain phosphate-citrate buffer, pH 5.2, for 1 h at 37°C . The reaction was terminated by adding 120 μL of 0.5 mol/L Na_2CO_3 - NaHCO_3 buffer, pH 10.7, and the fluorescence of 4-methylumbelliferone was measured with a fluorometer with excitation set at 355 nm and emission at 460 nm (Titertek, Huntsville, AL, USA). According to the methods of Artieda et al. (26), CHIT activity was expressed as nanomoles of substrate hydrolyzed per hour per milliliter of incubated serum. Serum CHIT activity was measured by duplication and coefficient variation was less than 5% in all studies.

Serum YKL-40 concentrations were determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quidel Corporation, Santa Clara, USA) (27) according to instructions by manufacturers. The intra-, and inter-assay coefficient variations

(CVs) of the kit were 3.6% and 5.3%, respectively. The assay sensitivity was 20 ng/mL.

Serum CRP levels were measured by a nephelometric method (Image, Beckman-Coulter Inc., USA). ESR levels were determined by the Western Green method (Berkhun SDM-100, Turkey).

Statistical analyses

The SPSS 15.0 (SPSS Inc. Chicago, IL, USA) was used for all analyses. The normality of the variables was tested with the Shapiro-Wilk test. Continuous variables are presented as median with interquartile range (IQR). Mann-Whitney U-test with Bonferroni correction was used for groups' comparisons. Correlation analyses were performed by the Spearman rho correlation test. The clinical performances of all parameters were measured using receiver operating characteristic (ROC) curves. ROC analysis and also comparisons of ROC curves were performed with MedCalc® for Windows software (MedCalc, Belgium). A p value of less than 0.05 was considered statistically significant for all analyses.

Results

The characteristics and the laboratory findings of the groups are shown in the Table I. Mean ages of the patients with RA and healthy subjects were 46.3 ± 13.1 and 46.2 ± 9.0 , respectively. Both groups did not show any differences in terms of age and gender (Table I). As expected, significant differences in serum CRP and ESR values were found in patients with RA compared to the values of the healthy subjects ($p=0.0001$ and $p=0.001$, respectively) (Table I). In particular, serum CHIT activities were almost two times higher in the RA group when compared to control (178.0 ± 72.0 vs. 89.0 ± 21.0 ; $p=0.0001$). Serum YKL-40 levels were also higher in the patient group than in the control group (66.95 ± 87.27 vs. 48.7 ± 29.3 ; $p=0.026$).

Table I Age, gender ratio, and laboratory findings in patients with rheumatoid RA compared to control group.

	RA (n=27)	Control (n=27)	p value
Age (years)	46.3 ± 13.1	46.2 ± 9.0	0.735
Gender M/F	5/22	7/20	0.513
Chitotriosidase (nmol/ml/h)	178.0 ± 72.0	89.0 ± 21.0	=0.0001
C-reactive protein (mg/L)	5.6 ± 6.2	2.3 ± 1.8	=0.0001
Erythrocyte sedimentation rate (mm/h)	28.0 ± 21.0	18.0 ± 17.0	=0.001
YKL-40 (ng/mL)	66.95 ± 87.27	48.7 ± 29.3	=0.026

Values expressed as median $\pm$ IQR
M/F: Male/Female

Table II Correlation analysis of serum YKL-40 and chitotriosidase (CHIT) with C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR).

Variable	CHIT (nmol/mL/h)	YKL-40 (ng/mL)
CRP (mg/L)	$r=0.528$ ($p=0.0001$)	$r=0.440$ ($p=0.001$)
ESR (mm/h)	$r=0.364$ ($p=0.007$)	$r=0.370$ ($p=0.006$)
YKL-40 (ng/mL)	$r=0.349$ ($p=0.011$)	–

r: Spearman rho correlation coefficient.

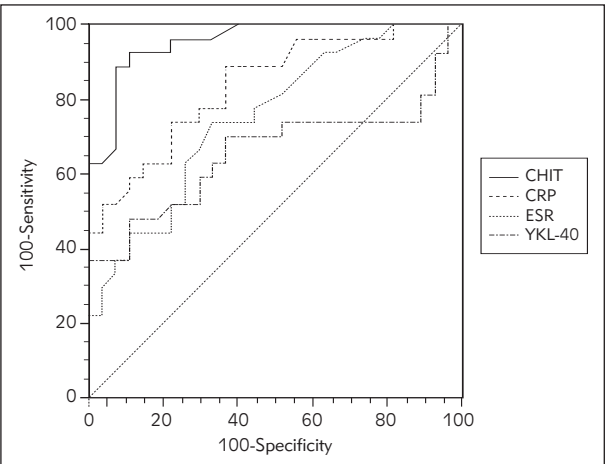


Figure 1 Receiver operating characteristic (ROC) curves comparing chitotriosidase (CHIT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and YKL-40 for prediction of rheumatoid arthritis. The area under the curve was 0.96 for CHIT, 0.84 for CRP, 0.76 for ESR, and 0.65 for YKL-40.

The results of correlation analyses were shown in *Table II*. There was a significant positive correlation between serum YKL-40 and both CRP and ESR. Whilst serum CHIT activity was positively correlated with CRP and ESR, the most robust correlation was found between CHIT and CRP ($r=0.528$, $p=0.0001$) (*Table II*). Also, a significant positive correlation was found between CHIT activities and YKL-40 levels ($r=0.349$; $p=0.011$).

The areas (Standard Error (SE)) under ROC curves (AUC) were 0.96 (0.03) for CHIT, 0.84 (0.06) for CRP, 0.76 (0.07) for ESR and 0.65 (0.08) for YKL-40 (*Figure 1*). AUC for CHIT was significantly higher than the area for ESR ($p=0.005$) and the area for YKL-40 ($p=0.0001$). Although AUC for CHIT was higher than the area for CRP, the difference was not statistically significant ($p=0.055$). Diagnostic cut-off levels with the optimum sensitivity and specificity derived from the ROC curve were found to be CHIT >112 nmol/ml/h and YKL-40 >92.6 ng/mL. Using these optimum cut-off levels, CHIT and YKL-40 had 93% and 37% sensitivity, and 89% and 100% specificity, respectively.

Discussion

The present study indicates that two chitinases, CHIT and YKL-40, significantly increased in the patients with RA. Moreover, in terms of the disease prediction, CHIT was superior to YKL-40. Previously, serum YKL-40 levels were determined to assess its usefulness as a marker in RA pathogenesis (22, 28). However, serum CHIT activity was not evaluated in any RA cohort and, to our knowledge, this is the first study in the literature to present elevated CHIT activities in patients with RA.

Despite their low sensitivity and specificity, the traditional acute phase response markers, CRP and ESR, are still used in clinical practice to evaluate the disease activity of RA. Furthermore, new classification criteria for RA published by the American College of Rheumatology/European League Against Rheumatism Collaborative Initiative in 2010 included these laboratory tests at classification of newly presenting patients (20). In the present study, we did not use the new criteria because all of our patients were diagnosed with RA by the »ACR 1987 Criteria« before 2010. However, we determined both CRP and ESR levels of the groups, and then we compared their clinical performances with each of the biomarkers tested in this study. The ROC analyses showed that CHIT was superior to both CRP and ESR, in contrast to YKL-40.

Unlike YKL-40, CHIT activity in patients with any rheumatoid disorder including RA is still unknown. In this respect, CHIT activity was evaluated only in one study that included children diagnosed with juvenile idiopathic arthritis (JIA), a childhood rheumatic disorder (24). In our study, both serum and synovial fluid (SF) CHIT activities in the patients with JIA were evaluated. While serum CHIT activities of all patients were within the normal range, SF CHIT activities of some patients were increased. Moreover, in the latter group, the clinical course of JIA was more complicated and the CRP and ESR levels of this group were also elevated. Thus, the authors speculated that, as a possible presenter of the role of macrophages in synovia, CHIT could be helpful for the prediction of disease course. In our study, there was a clear elevation in serum CHIT activity. Although we did not analyze the CHIT activities of patients in SF, we still believe that these elevated activities of serum CHIT might represent the role of macrophages in RA. The differences between the serum CHIT results might be due to patients' age and the nature of the two diseases. Nevertheless, it cannot be addressed whether CHIT activity is related directly to damage within the rheumatoid joints. To answer this question, there is a need for the determination of the activity of CHIT in the SF of affected joints.

It has been described that YKL-40 levels increase with age (29, 30). Still, the reason for this increase is unknown, but it might be explained by the

higher level of inflammation and apoptosis in the elderly. However, neither the RA group nor the control group in this study included any individual above 65 years of age. Therefore, increased YKL-40 levels are not related to age, but most likely to the inflammation caused by RA. Indeed, YKL-40 is expressed by chondrocytes and also macrophages in the synovial membrane as a feedback mechanism against inflammatory cytokines, such as TNF- α and IL-1 β (31). On the basis of correlation studies between serum and synovial fluid concentrations of YKL-40, serum YKL-40 might reflect articular cartilage degradation and the degree of synovial inflammation in RA (32). In this regard, serum YKL-40 levels increase even in early RA patients (22, 33). In a recent study, it has been proposed that a »multi-biomarker disease activity score« which was calculated using the concentrations of twelve biomarkers, including YKL-40, measured disease activity (34, 35). However, as seen in the present study, the low sensitivity of serum YKL-40 for RA might be an obstacle to its clinical use.

Although serum YKL-40 and CHIT were increased in patients with RA, CHIT activity was the more specific and sensitive one in our study. *In vivo*, both CHIT and YKL-40 are secreted by PMNs and macrophages. Whereas macrophages predominantly release CHIT, PMNs secrete YKL-40 (1). In fact, neutrophil-released YKL-40 might act as an autoantigen in RA (36). In our cohort, which included RA patients in »clinical« remission, the elevated activity of CHIT might signal that subclinical inflammation can proceed upon macrophages in RA. Despite clinical remission determined by the physician, imaging-detected (by MRI and musculoskeletal Doppler US) synovitis has been reported in RA patients who were receiving conventional DMARDs (37). According to these findings, authors speculated that the subclinical joint inflammation might account for the structural deterioration in this patient group. It is possible to speculate that increased CHIT activities found in a similar patient group – taking DMARDs and in remission defined by the clinician – might reflect a long-term subclinic inflammation induced by macrophages in synovitis, hence might also explain the deterioration of articular cartilage. Additionally, *in vitro* culturing studies of macrophages stated that while CHIT expression started after 7 days and dramatically increased with time, YKL-40 expression was detected after 2 days and reached the maximum at day 14 (38). Accordingly, elevated CHIT activity, but not YKL-40, could also reflect the prolonged effect of macrophages in the synovium of affected joints. The

absence of association between radiographic progression and serum YKL-40 levels in a 10-year follow-up study of patients with RA might support this hypothesis (39).

One of the limitations of this study might be the lack of a group composed of patients with active disease. Evaluation of CHIT in patients with active disease might be of value to understand its role in disease activity. Also, we did not measure rheumatoid factor and/or cyclic citrullinated peptide antibodies; hence, we have no information whether there is any influence of the serological status on CHIT and YKL-40. Additionally, a 24-base pair (bp) duplication in exon 10 of the CHIT gene is a common polymorphism that leads to a null allele, thus a defective CHIT protein (40). As a limitation, this polymorphism, which is responsible for the recessive inherited deficiency in CHIT, was not determined in the study. In a research performed in a Spanish population, there was no CHIT activity in subjects with a homozygous defective allele and approximately half of the normal allele activities in subjects with a heterozygous defective allele (26). In the literature search, we could not find any information regarding the frequency of this polymorphism in Turkish individuals. Interestingly, the distribution of CHIT activities in both patients and healthy subjects might be explained by the CHIT genotype of individuals. Thus, it would be reasonable to determine whether there are any differences in the allelic and genotype distributions between the groups we studied, and if there is a need to adjust the CHIT activity according to the CHIT genotype. Accordingly, it is possible to speculate that a genotype adjustment for CHIT activity may result in even better correlated results with other markers.

Conclusion

These results are consistent with those obtained in other studies, confirming that human chitinases – not only YKL-40 and also CHIT – may play significant roles in the pathogenesis of RA. Nevertheless, comprehensive studies covering larger populations are needed to elucidate the relationship between chitinases, in particular CHIT, and RA.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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Received: June 13, 2013

Accepted: July 24, 2013

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