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ARTICLE

Association of Cathepsin K and Tartrate-Resistant Acid Phosphatase-5b in Different Stages of Rheumatoid Arthritis Patients in South Indian Population

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ABSTRACT

The aim of the study was to identify effective biomarkers of changes in bone mineral density (BMD) at different stages of rheumatoid arthritis patients from South Indian population, using disease activity score in 28 joints (DAS28) which is being used as a measurement for assessing disease activity in patients with rheumatoid arthritis. The study was carried out in 92 rheumatoid arthritis patients. Serum level of tartrate-resistant acid phosphatase-5b (TRAP-5b) and cathepsin K was measured using ELISA. Serum rheumatoid factor (RF) and C-reactive protein (CRP) was recorded by the attending rheumatologists. The C-reactive protein (CRP) was quantified using a latex immunoturbidimetric method. The rheumatoid arthritis (RA) was measured by turbidimetric immunoassay method. The bone mineral density (BMD) T-score was calculated according to WHO guidelines. Reverse transcription PCR (RT-PCR) was used to determine the expression of cathepsin K. The rheumatoid arthritis (RA) patients were categorized into three groups based on the DAS28 score as inactive (DAS ≤ 3.2), moderately active (DAS > 3.2 ≤ 5.1) and very active (DAS > 5.1) at the time of admission. Out of 92 patients, 16 (17.4%) patients had inactive disease condition, 42 (45.6%) patients had moderately active disease condition and 34 (37%) patients had very active disease condition. The mean BMD was significantly lower in very active (0.28 ± 0.04; $p < 0.001$) as compared with moderately active (0.71 ± 0.13; $p < 0.001$) and inactive (1.21 ± 0.14). TRAP5b and cathepsin K showed significant increases in very active group ($p < 0.001$) as compared with moderately active ($p < 0.001$) and inactive groups. In conclusion, The biomarker TRAP-5b and cathepsin K identified in this study may become a new and highly specific biomarker for rheumatoid arthritis.

KEYWORDS

Rheumatoid arthritis, Cathepsin K, TRAP5b, Osteoclast, Bone mineral density.

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INTRODUCTION

Rheumatoid arthritis (RA) is a common systemic autoimmune disorder affecting approximately 0.5% to 1% of the population worldwide [1]. It has a female preponderance affecting women 3 to 4 times more commonly than men [1]. The aetiology of this condition is still indefinable and no single test defines rheumatoid arthritis. The progression of rheum-

atoid arthritis is highly variable. Sometimes rheumatoid arthritis can be very mild and thus remain undiagnosed or it can be rapidly progressive. However, the majority of patients present with an intermediate form of rheumatoid arthritis, in which events of exacerbation occur [2]. Tight control of disease activity prevents disease progression and improves physical function and quality of life [3].

Degradation of cartilage matrix is an important pathologic feature of rheumatoid arthritis and osteoarthritis. In rheumatoid arthritis, several major cell types have been implicated in joint degradation, including synovial fibroblast-like cells [4,5], chondrocytes [6] and osteoclasts [7,8]. These cells destroy cartilage and sub-chondral bone by secreting proteolytic enzymes and/or phagocytizing extracellular matrix components. Several lines of evidence suggest that collagenolytic and proteoglycanolytic metalloproteinases and cathepsins are pivotal proteases in the degradation of the main protein components in cartilage and bone [9]. These proteases have thus become a major focus of research as potential therapeutic targets for the treatment of joint diseases especially cathepsin K in arthritis research [10-13]. Bone metabolism markers have been studied for many years and have been used for the assessment of fracture risk and to select treatment. Of the known biomarkers, tartrate-resistant acid phosphatase 5b (TRAP-5b), have been shown to be useful for bone density and are reported to be excellent specific biomarkers for bone quality and have been demonstrated to be useful for predicting bone mineral density (BMD) [14]. Thus, the aim of the study was to identify effective biomarkers of changes in BMD at different stages of South Indian rheumatoid arthritis patients, using disease activity score in 28 joints (DAS28), which is being used as a measurement for assessing disease activity in patients with rheumatoid arthritis for the past several years.

EXPERIMENTAL

Reagents: TRAP5b and cathepsin K kit was purchased from Immunodiagnostic Systems (Gaithersburg, MD, USA). TRIzol reagent, Primer Sequences and iScript™ cDNA synthesis Kit were purchased Invitrogen Inc., (Carlsbad, CA, USA), Ocimum Biosolutions (India) and Bio-Rad laboratories, Inc., (CA, USA) respectively. The 24 well and 96 well cell culture plates were purchased from SPL Life Sciences (Korea).

Samples collection: During the year January 2019 to December 2019, a total of 152 patients were recruited for the study who were attending outpatient department of orthopaedics for the management of rheumatoid arthritis. Among them, 92 patients were enrolled for the study based on the inclusion criteria. All the patients were enrolled in the study after taking informed consent. The patients with a clinical fragility fractures including hip, femur and vertebral fracture and the patients having a history of malignancy particularly bone metastases were excluded from the study. The study was approved by institutional ethics and research advisory committee. The demographic and anthropometric details were obtained from the medical records of the study subjects. Disease activity was recorded as the disease activity score in 28 joints-erythrocyte sedimentation rate (DAS28-ESR). In addition, the uses of medications for rheumatoid arthritis were noted.

Blood and serum biochemistry: A peripheral (10 mL) whole blood was drawn from the participants. About 3 mL of blood was used to carry out biochemical assays. Serum level of tartrate-resistant acid phosphatase-5b (TRAP-5b) and cathepsin K were measured using ELISA. Serum rheumatoid factor (RF) and serum C-reactive protein (CRP) were recorded. The CRP was quantified using a latex immunoturbidimetric method. The RF was measured by turbidimetric immunoassay method.

Dual energy X-ray absorptiometry (DEXA) measurements: A real bone mineral density was assessed at lumbar spine, hip and total body by discovery dual energy X-ray absorptiometry scanner (Hologic Inc, Bedford, MA). The BMD T-score was calculated (the number of standard deviations below the average for a young adult at peak bone density) according to WHO guidelines [14]. In accordance with these criteria, patients with T-score at either skeleton area between -2.5 and -1.0 were classified as osteopenic and those with a value higher than -1.0 as normal.

Isolation of mononuclear cells from whole blood: Whole blood was diluted in 1:1 with phosphate buffered saline (PBS, without Ca^{2+} and Mg^{2+}). Two parts of diluted blood were layered on top of one part ficoll and centrifuged at 1800 rpm for 30 min at room temperature. The cell layer on top of the Ficoll-Paque was collected, resuspended in phosphate buffer saline and centrifuged at 1500 rpm for 10 min for three times to get the purified cells. Subsequently, the cells were counted in a hemocytometer using tryphan blue method.

RNA extraction: Isolation of RNA was done from the osteoclast cells using TRIzol reagent (Invitrogen Inc., USA) following the manufacturer's instructions. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm in Biophotometer (Eppendorf, Germany). First-strand cDNA was synthesized from the total amount of RNA (0.5-1 µg) using the iScript cDNA synthesis kit (Bio-Rad, USA), following the manufacturer's instructions.

Reverse transcription PCR (RT-PCR)

Polymerase chain reaction (PCR) amplifications were performed as follows: Thirty cycles for cathepsin K (94 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min), with primer 5′-CAG CAA AGG TGT GTA TTA TGA TGA AAG C-3′ and antisense 5′-ATG GGT GGA GAG AAG CAA AGT AGG AAG G-3′. Then, the PCR product 10 µL was electrophoresed in 2% agarose gel and analyzed in gel doc XRS plus (Bio-Rad, USA). The densitometric analyses were carried out with image lab software (Bio Rad, USA). The expression of each target gene was normalized with internal control and represented as a ratio.

Statistical analysis: Data were expressed as mean ± SEM and percentage wherever appropriate. Student's t test, correlations and multivariate linear regression were performed for evaluating the baseline characteristics among the groups. Differences between two variables were considered statistically significant when $p < 0.05$. The analyses were performed with SPSS 20 version software.

RESULTS AND DISCUSSION

Rheumatoid arthritis is the chronic autoimmune disease in developing countries like India, especially associated with

disease-related complication, physical disability and early mortality because of lack of awareness of patients regarding the disease or non-compliance to the therapy, which could be due to the high cost of management or temporary improvement of illness. Therefore, it is important to understand the magnitude of the problem of the disease especially in countries like India. The present study analyzed the demographic, clinical, comorbid, serological and therapeutic data on the patients with rheumatoid arthritis in the south region of India.

The demographical characteristic of the patients are given in Table-1. The mean age of the study population was found to be 52.18 ± 18.73 years and gender wise distribution showed that majority of them was female 66 (71.73%). Early morning stiffness is one the most important clinical characteristics of rheumatoid arthritis patients. In present study, population the mean morning stiffness duration was found to be 103.62 ± 43.0 min. The RF positive patients had a longer duration of morning stiffness 126.6 ± 44.20 min than RF negative patients 98.5 ± 30.30 min but a significant association was not found between duration of morning stiffness and RF status.

TABLE-1
DEMOGRAPHIC CHARACTERISTICS OF RA PATIENTS (N = 92)

Patient's demographic characteristics	
Mean age ± SD in years	52.18 ± 18.73
Female sex, n (%)	66 (71.73%)
Male sex, n (%)	26 (28.27%)
Disease duration, Mean ± SD in Years	4.12 ± 3.15 years
Patient's clinical symptoms	
Morning stiffness, Mean ± SD in min	103.62 ± 43.0 min
Multiple joint pain	73 (79.34%)
Fatigue	64 (69.56%)
Severity assessment	
ESR, Mean ± SD	62.10 ± 26.77 mm/h
CRP, Mean ± SD	27.7 ± 34.3 mg/dL
RF, Positive (n %)	82 (89.13)
Comorbidities	
Diabetes, n (%)	52 (56.52)
Hypertension, n (%)	33 (35.87)
Others (infection), n (%)	7 (7.61)
Treatment	
DMARDs n (%)	49 (53.26)
NSAIDs n (%)	33 (34.79)
Others n (%)	11 (11.95)

This study revealed that prevalence of rheumatoid arthritis was more in female patients than male patients (73% *vs.* 28%, respectively) which were almost similar to studies conducted by Al-Bishri (78% *vs.* 22%, respectively) [15]. (76.8% *vs.* 23.2%, respectively) [16]. Among most common symptoms of rheumatoid arthritis in present study multiple joint pains (79%) and fatigue (69%) was observed considerably. It was found in present study that diabetes was 52% and hypertension was 33% which was higher to the study conducted by [15]. The present study also showed a higher RF positive than other reported studies which were found to be (69.2%) [17-19] (78.9% and 77.3% respectively) [18,19].

The ESR and CRP are one of the most important acute phase markers of inflammation in rheumatoid arthritis. The mean erythrocyte sedimentation rate (ESR) of rheumatoid arthritis patients values were found to be on higher side 62.10 ± 26.77 mm/h. The mean CRP value was 27.7 ± 34.3 mg/dL. The most common comorbidity was diabetes mellitus (DM), 52 (56.52%) patients were having DM, followed by hypertension (HTN) in 33 (35.87%) patients and other infection was found to be 7 (7.69). Drug utilization among all the 92 rheumatoid arthritis patients was analyzed. Disease modifying anti-rheumatic drugs (DMARDs) were the most commonly prescribed medication. Among the study population 49 (53.26%) patients received DMARDs. Non-steroidal anti-inflammatory drugs (NSAIDS) was 33 (34.79%) and other analgesics were prescribed in 11 (11.95%) patients for symptomatic pain management. Patients exhibited prominent symptoms like multiple joint pains 73 (79.34%) and fatigue 64 (69.56%). In present study, it is observed that DMARDs (57%) was prescribed most commonly followed by NSAIDs (34%), which are similar to other studies [20-22].

Categorizing of patients according to severity: The patients were categorized into 3 groups based on the DAS28 score as inactive (DAS ≤ 3.2), moderately active (DAS > 3.2 ≤ 5.1) and very active (DAS > 5.1) at the time of admission. Out of 92 patients, 16 (17.4%) patients had inactive disease condition, 42 (45.6%) patients had moderately active disease condition and 34 (37%) patients had very active disease condition (Table-2). Mean DAS28 score was found to be 5.84 ± 1.22.

TABLE-2
CATEGORIZING THE PATIENTS ACCORDING TO DISEASE ACTIVITY SCORE (DAS28)

DAS	DAS28 (ESR)	
	N (%)	Mean ± SD
Inactive (DAS ≤ 3.2)	16 (17.4)	3.8 ± 0.40
Moderate active (DAS > 3.2 ≤ 5.1)	42 (45.6)	4.71 ± 1.32
Very active (DAS > 5.1)	34 (37)	6.2 ± 27.48

A study conducted by Inoue *et al*. [19] showed that DAS28-ESR and DAS28-CRP were generally well correlated. The NRSA, U.K. has concisely summarized the role of DAS28 score for evaluation of rheumatoid arthritis. The rheumatoid arthritis patients were categorized into 3 groups based on the DAS28 score as inactive (DAS ≤ 3.2), moderate active (DAS > 3.2 ≤ 5.1) and very active (DAS > 5.1). Out of 92 patients, 16 (17.4%) patients had inactive disease condition, 42 (45.6%) patients had moderately active disease condition and 34 (37%) patients had very active disease condition (Table-2). Majority of the patients are in moderately active state in present study.

Assessment of fracture risk using dual energy X-ray absorptiometry (DEXA): DEXA is the preferred technique for the evaluation of bone mineral density (BMD) because of its low radiation dose, accuracy and rapid performance. Scan was performed in three different areas (spine, hip and femur); the average value for BMD (g/cm^2) was taken. Based on the Das 28 category, the BMD was compared between the groups. The mean BMD was significantly lower in very active (0.28 ± 0.04; $p < 0.001$) as compared with moderately active (0.71 ± 0.13; $p < 0.001$) and inactive (1.21 ± 0.14) (Fig. 1).

Serum markers of bone resorption TRAP5b: Fig. 2 shows the level of serum markers of bone resorption. TRAP5b showed a significant increase in very active group (3.42 ± 0.40; $p < 0.001$) as compared with moderately active (2.48 ± 0.32; $p < 0.001$) and inactive group (1.64 ± 0.16).

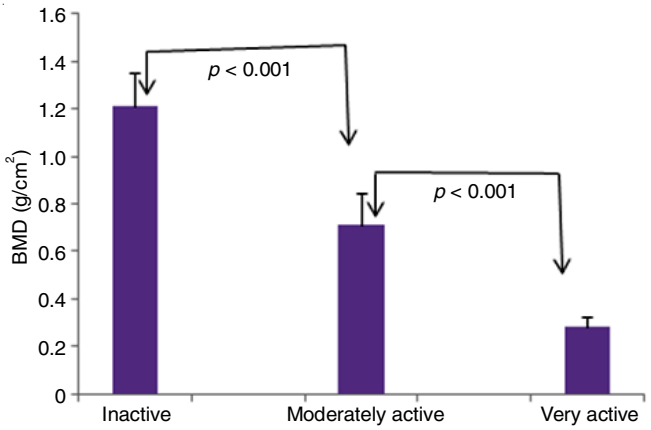

Fig. 1. Illustrate mean BMD (spine, hip and femur) between study subjects

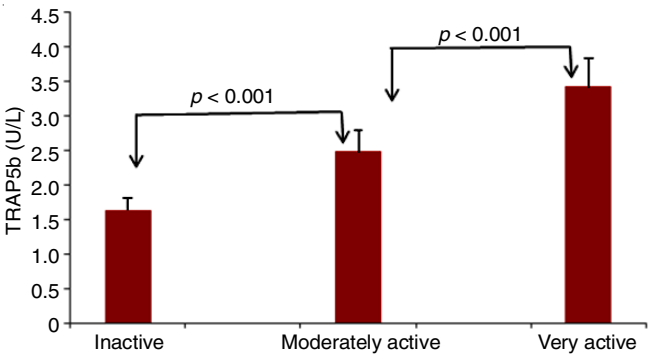

Fig. 2. Levels of serum marker of bone resorption (TRAP5b) in study subjects

Rheumatoid arthritis inhibits the expressions of osteoclast specific gene cathepsin K: To study the effect of rheumatoid arthritis on osteoclast specific gene cathepsin K, RT-PCR was used to analyze the mRNA level of gene. The level of cathepsin K gene expression was significantly increased in moderately active (2 fold) ($p < 0.001$) and very active group (1.1 fold) ($p < 0.01$) compared with inactive group (Fig. 3). Fig. 4 shows the levels of cathepsin K in the study subjects. Cathepsin K showed a significant increase in very active group (98.19 ± 42.13; $p < 0.001$) as compared with moderate active (63.34 ± 32.1; $p < 0.001$) and inactive group (46.12 ± 23.1).

Study has demonstrated an increased cathepsin K protein expression in osteoclasts adjacent to eroding cartilage in rheumatoid arthritis subjects [23,24]. Dodds *et al.* [25] described the expression of cathepsin K in multinucleated giant cells and in a population of phagocytic-like cells within synovial tissue of rheumatoid arthritis subjects. In present study, the level of Cathepsin K gene expression was significantly increased in moderate active (2 fold) ($p < 0.001$) and very active group (1.1 fold) ($p < 0.01$) compared with inactive group of rheumatoid arthritis.

The overexpression in moderate rheumatoid arthritis group could be a function of synovial fibroblast proliferation as well as an increase of enzyme production and activity by proinflammatory cytokines. Cytokines have been previously described to increase the secretion of Cathepsin, 2 to 3 fold in synovial fibroblasts from rheumatoid arthritis patients [26,27]. However, no comparative studies of cathepsin K expression in these diseases stages have been performed in South Indian

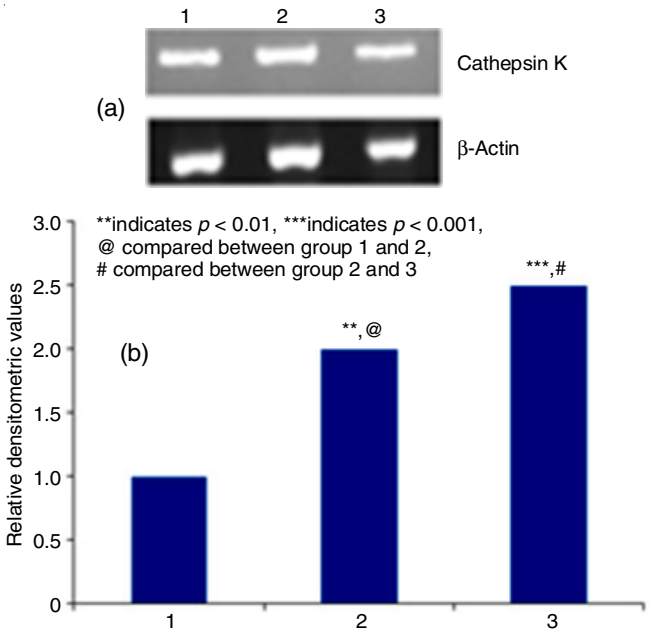

Fig. 3. RT-PCR analysis of osteoclasts specific markers, cathepsin K (a); Representative RT-PCR gel image (b)

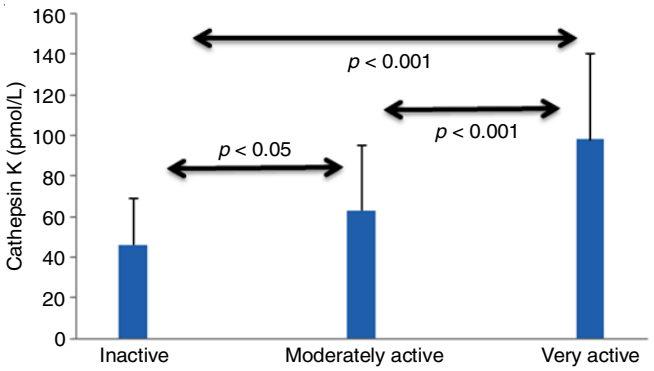

Fig. 4. Levels of serum cathepsin K in study subjects

population. The decrease expression of Cathepsin K in very active group (1.1 fold) ($p < 0.01$) may be due to intake of medicines of rheumatoid arthritis.

Supporting to present study, Svelander *et al.* [28] demonstrated high levels of cathepsin K expression in osteoclast at sites of extensive bone loss. In synovium of rheumatoid arthritis, the cathepsin K protein was localized in synovial fibroblast, CD68+ macrophage like synoviocytes, stromal multinucleated giant cells. This was around two to five times greater compared with osteoarthritic synovium. In normal synovium, the cathepsin K expression was restricted to fibroblast like cells. Increased expression of cathepsin K around lymphocytic infiltrates in synovial tissue seems to facilitate the movement of mononuclear cells through the perivascular matrix [29]. Pro-inflammatory cytokines such as IL-IB and tumor necrosis factor alpha facilitate the expression of cathepsin K, its over expression in rheumatoid synovium, induced by IL-IB and tumor necrosis factor alpha due to increase of cathepsin K expressing cells.

The serum level of cathepsin K in rheumatoid arthritis patients was found to be increased in many studies [29]. In present study based on DAS 28, the serum level of cathepsin K showed a significant increase in very active group (98.19 ± 42.13; $p < 0.001$) as compared with moderately active (63.34

± 32.1; $p < 0.001$) and inactive group (46.12 ± 23.1) (Fig. 4). Similar results were reported by Hou *et al*. [24] that there was significant correlation between cathepsin K and disease severity, which determined by the selective and the critical role of cathepsin K in articular cartilage and articular bone erosion, where bone and cartilage erosion derived by cathepsin K is irreversible degenerative process leading to loss of joint function.

Of the existing biomarkers, the tartrate-resistant acid phosphatase 5b (TRAP-5b) has been shown to be useful for bone density [14]. Tomizawa *et al*. [30] demonstrated that RA patients with higher TRAP-5b tended to lose BMD in the distal forearm in the 2-year period [30]. It is well known that TRAP-5b is predominantly expressed in bone by osteoclasts [31]. Serum TRACP 5b is secreted by osteoclasts and its activity can be used as a clinically relevant bone resorption marker in various diseases because it reflects osteoclast number [32-35].

In present study, the level of serum marker of bone resorption, TRAP5b showed significant increase in very active group (3.42 ± 0.40; $p < 0.001$) as compared with moderately active (2.48 ± 0.32; $p < 0.001$) and inactive group (1.64 ± 0.16). Janckila *et al.* [36] and Nenonen *et al*. [37] reported that the level of TRAP-5b protein was elevated in rheumatoid arthritis patients compared with healthy controls and other disease groups . They suggested that TRAP-5b activity is a marker of osteoclast number and local or systemic bone destruction, which suggests the hypothesis that osteoclast activity induced by local and/or systemic inflammation might strongly influence bone metabolism, particularly in the distal forearm of rheumatoid arthritis patients. Cheng *et al.* [38] reported that measurement of serum TRAP-5b in rheumatoid arthritis patients reflects clinical and radiological measures of disease activity, treatment with certain biologics and degree of response to therapy.

One of the significant results of this study is that the biomarkers identified were also potent than other known predictors of BMD changes such as age, diabetes mellitus and the use of steroids, possibly because this study was conducted relatively in a short term and because the participants were predominantly women with rheumatoid arthritis. The mean BMD was significantly lower in very active (0.28 ± 0.04; $p < 0.001$) as compared with moderately active (0.71 ± 0.13; $p < 0.001$) and inactive (1.21 ± 0.14). However, these biomarkers might become a powerful tool to predict the changes in BMD of patients with rheumatoid arthritis. The results showed that patients with rheumatoid arthritis in very active group a had lower BMD and increased levels of serum TRAP-5b and cathepsin K.

Conclusion

In conclusion, the early diagnosis and prompt treatment can improve outcomes in rheumatoid arthritis because significant joint damage occurs early in the course of the disease, when rheumatoid arthritis is most aggressive. Therefore, present results suggest that the biomarker TRAP-5b and cathepsin K identified in this study on south Indian subjects can be considered as a highly specific biomarker for rheumatoid arthritis.

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