Evaluating the expression level of genes related to autophagy in rheumatoid arthritis patients

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ARTICLE INFO

Original paper

ABSTRACT

Rheumatoid arthritis or joint rheumatism is the most common systemic inflammatory disease of the joints and is considered one of the chronic autoimmune diseases. T cells and other immune cells are called to the synovial tissue and cause this disease to progress. Autophagy is a process that is associated with the breakdown of intracellular organelles. As a regulator of cell homeostasis, it can affect the activation of immune cells and participate in the pathogenesis of rheumatoid arthritis. This study aimed to evaluate the gene expression level of autophagy genes in two groups of rheumatoid arthritis patients and healthy individuals. For this purpose, peripheral blood was obtained from two groups of people, including 40 rheumatoid arthritis patients, and 40 healthy individuals. The expression of two genes related to autophagy, Atg5, and Beclin-1, was evaluated in peripheral blood cells using the real-time PCR method. The results showed that the expression of the Beclin-1 gene increased by 2.21 times in rheumatoid arthritis patients compared to healthy individuals ($P = 0.024$). The expression of the Atg5 gene in rheumatoid arthritis patients increased by 1.53 times compared to healthy subjects ($P = 0.041$). In general, this study showed that in rheumatoid arthritis patients, increased expression of autophagy genes could be involved in the pathogenesis of this disease. In other words, the findings showed that reducing autophagy can reduce the severity of the disease in people with rheumatoid arthritis.

Introduction

Rheumatoid arthritis is a common autoimmune disease associated with progressive disability and systemic and local conflicts (1). Rheumatoid arthritis is associated with synovial inflammation, joint swelling, and production of autoantibodies, cartilage and bone destruction, and systemic complications such as cardiac, vascular, and pulmonary disorders (2). Disturbance of the immune system balance plays a vital role in the development of rheumatoid arthritis. Many studies consider both arms of the immune system, i.e., humoral and cellular immunity, to be involved in the development of arthritis (3).

In addition, cells such as monocytes and T cells and their secreted cytokines such as TNF, IL-8, and IFN-γ play an essential role in the development of rheumatoid arthritis (4). Among the subtypes of T cells, Th1 and Th17 cells play the most crucial role in the development of RA (1). The activation of T cells and other immune cells more than expected and disruption of the internal homeostasis of these cells play the most critical role in the development of rheumatoid arthritis. On the other hand, as a cellular catabolic process, autophagy plays an essential role in maintaining the balance of cells and recycling damaged cellular components (5). Autophagy is a process during which auto-phagosomes, which contain cytoplasmic details, are merged with lysosomes, their internal contents are broken into their constituent subunits, and finally, they are used by the cell itself (6).

Autophagy has different stages, which include induction of autophagy, nucleation of auto-phagosome, maturation of auto-phagosome, integration of auto-phagosome with the lysosome, and breakdown of auto-phagosome contents (7). In each step, a series of specific genes are activated, two of which are studied in this study, Beclin-1, and Atg5. Beclin-1 binds other proteins related to autophagy at the site of autophagosome formation and promotes auto-phagosome nucleation. Atg5, in a complex form with Atg12 and Atg16L, plays a role in membrane curvature and auto-phagosome maturation. Autophagy can regulate immune responses against various antigens by influencing the development, survival, and proliferation of lymphocytes (2). Uncontrolled autophagy is associated with several autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE) (8).

Autophagy can induce proliferation in synovial fibroblast cells in patients with rheumatoid arthritis and also regulate the resistance to apoptosis in these cells. Autophagy plays a role in delivering citrullinated peptides to T cells and activating them (9). In addition, new studies show that increased autophagy is associated explicitly with inflammation leading to rheumatoid arthritis, and many drugs used to treat this disease may reduce the autophagy process (10). For example, chloroquine (CQ) and hydroxychloroquine (HCQ), which are highly effective drugs in treating RA, can regulate autophagy (11). Considering the role of immune cells in the progression of rheumatoid arthritis and the importance of autophagy in regulating the internal homeostasis of cells, in this study, we determined the expression levels of two genes related

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to autophagy (i.e., Beclin-1 and Atg5) in the peripheral blood cells of two groups of Rheumatoid arthritis patients, (i.e., primary patients and patients under treatment) were examined compared to healthy people because by identifying the changes in the expression of these genes in the group of untreated rheumatoid arthritis patients and the treated group, it is possible to understand the effects of the common drugs used to treat RA on the expression of these genes. In the future, along with the medications used to treat rheumatoid arthritis, these genes can be targeted as a new therapeutic approach and used to improve the treatment of rheumatoid arthritis patients.

Materials and Methods

Collection of samples

This study is analytical-cross-sectional. Samples were randomly collected from two groups of rheumatoid arthritis patients and a group of healthy people as a control group. Peripheral blood samples were collected from patients in the six months of summer and fall of 2021. After the diagnosis of patients with rheumatoid arthritis by a rheumatology specialist according to EULAR/ACR criteria and confirmatory tests, 40 patients (average age 52 years), and 40 healthy individuals (average age 49 years) were sampled. The groups under investigation in this study were matched in terms of age and sex. The number of patients studied was selected based on previous studies in this field. Patients with a history of autoimmune disease, joint disease, severe infection, and cancer, as well as people who used tobacco, were excluded from the study. First, the necessary explanations about the project were given to the people, and each participant participated in this study by declaring personal consent and filling out the consent form. Disease severity (DAS-28) was calculated based on the number of swollen joints, the number of painful joints, and ESR based on the following formula:

DAS-28= 0.56(TJ) $^{1/2}$ + 0.28 (SJ) $^{1/2}$ + 0.7 In(ESR) +0.14 (GH)

(TJ: Number of Tender joints from 28 joints; SJ: Number of swollen joints from 28 joints; GH: Global Health).

RNA extraction and cDNA synthesis

RNA extraction from peripheral blood cells was performed immediately after sampling using the RNX PLUS kit protocol. The quality and kit of extracted RNA were evaluated by gel electrophoresis and nanodrop device (Thermo Scientific, Waltham, MA, USA), respectively. Synthesis of cDNA was done using Transcriptor First Strand cDNA (Roche, Basel, Germany).

Investigation of Beclin-1 and Atg5 gene expression

Using real time PCR technique, the relative expression of Beclin-1 and Atg5 was measured in two groups including patients and healthy individuals. In this study, the GAPDH gene was used as a housekeeping gene (Table 1).

The primers of these three genes were designed using the Oligocalc and Outoanalyzer online sites and were blasted using the NCBI online site to check their specificity. Real time PCR reaction was performed using TAKARA's Syber Green master mix and Roche Life Science Lightcycler® 96.

The PCR reaction was performed in a volume of 20 µL according to the protocol of the kit and to control the work in duplicate on each sample. The time schedule of the amplification device includes three stages: 1 preincubation cycle at 95°C for 30 seconds, 45 amplification cycles at 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and finally 1 cycle of melting was done at 95°C for 5 seconds, 60°C for 60 seconds, and 95°C for 1 second. Using the designed primers (Table 1), the expression value of each Beclin-1 and Atg5 gene was calculated as a difference in threshold cycle value (CT) compared to the GAPDH gene based on Levak's formula. Then, using the Quantile Normalization method, these data were normalized. After calculating the ΔCT, the CT difference of the reference gene from the target gene for each individual, the formula $2^{-\Delta CT}$ was used to obtain the expression level of each of the Beclin-1 and Atg5 genes. Finally, the average expression of these genes was obtained for the three studied groups.

Statistical analysis

Under the assumption of quantification and normality, ANOVA and t-test were used. All statistical analyzes were done using Statistic Package for Social Science (SPSS) version 16 software (SPSS Inc., Chicago, IL, USA). Data were reported as Mean±SD. The level of statistical significance was $P<0.05$.

Results

The clinical and laboratory characteristics of the studied subjects are specified in Table 2.

The expression of the Beclin-1 gene in patients shows a significant increase compared to control subjects. The expression level of this gene in patients is 2.21 compared to the control condition ($P<0.05$) (Figure 1).

Atg5 gene expression increased in untreated patients compared to control subjects, which was statistically signficant. The expression level of this gene has increased in patients by 1.53 times compared to the control condition

Table 1. The sequence of primers for gene expression study.

| Gene		Sequence	Product Length	Annealing Temp.
GADPH	Forward	CTTTTAACAGGGGCTGTCTGGT	205bp	63°C
	Reverse	ACTTGATTTTGGAGCCCCGGA		
Beclin-1	Forward	CGTTAGCTGCATACTCTGTGT	152bp	61°C
	Reverse	TGACTGCCTCCTGTCTTTCTTCAA		
Atg5	Forward	CTGGATATCCCCTTTATTGTTATG	104bp	59°C
	Reverse	AGTGTCCTAGTGGCAACTGTCCA		

Table 2. Clinical and laboratory characteristics of the studied groups.

Variable	Patients (n=40)	Control (n=40)
Age (year)	52.17 ± 2.48	49.11 ± 6.43
ESR (mm/h)	31.42 ± 6.22	-
DAS28	4.17 ± 0.194	-

The data is in Mean ± SD format. ESR: erythrocyte sedimentation rate, DAS-28: Disease Activity Score28.
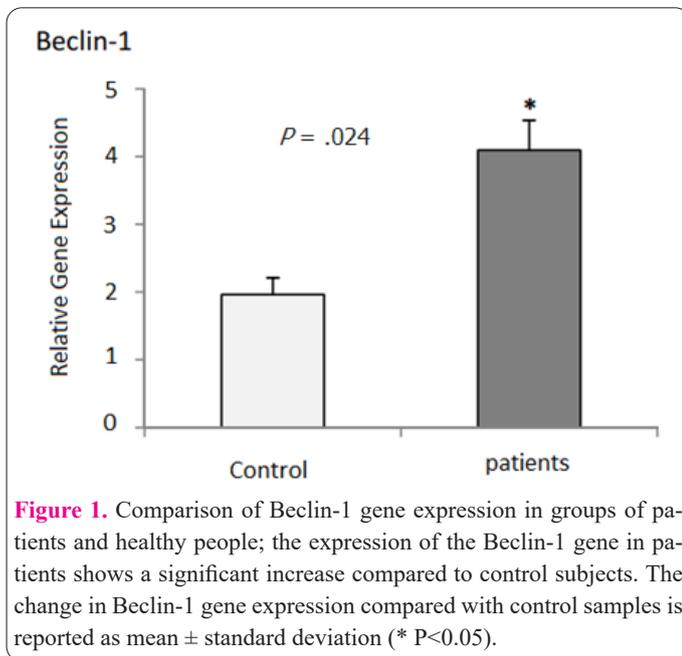
Figure 1. Comparison of Beclin-1 gene expression in groups of patients and healthy people; the expression of the Beclin-1 gene in patients shows a significant increase compared to control subjects. The change in Beclin-1 gene expression compared with control samples is reported as mean ± standard deviation (* P<0.05).
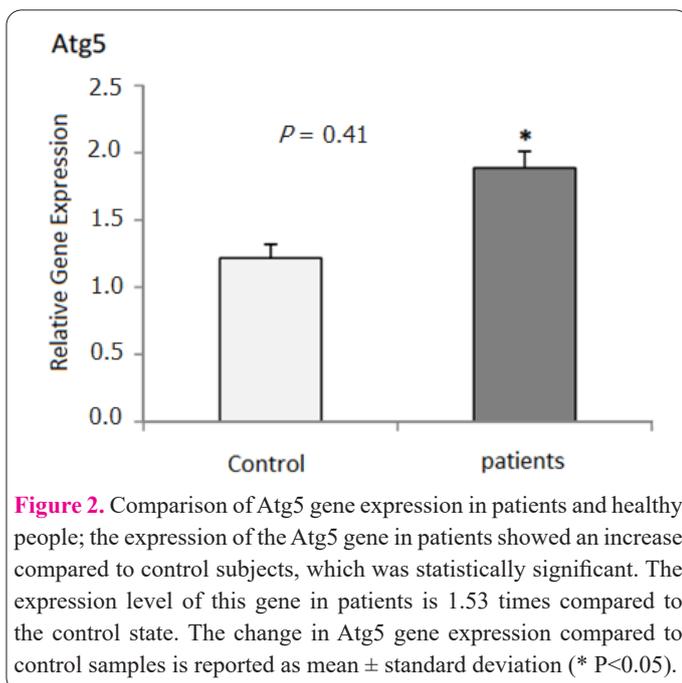
Figure 2. Comparison of Atg5 gene expression in patients and healthy people; the expression of the Atg5 gene in patients showed an increase compared to control subjects, which was statistically significant. The expression level of this gene in patients is 1.53 times compared to the control state. The change in Atg5 gene expression compared to control samples is reported as mean ± standard deviation (* P<0.05).

(P<0.05) (Figure 2).

Discussion

In the present study, the expression level of two genes related to autophagy, i.e., Beclin-1 and Atg5, in the peripheral blood cells in the group of untreated patients with rheumatoid arthritis was higher than in the control group, and these results are statistically significant for both of these genes. In the early stages of rheumatoid arthritis, immune cells such as activated T cells and monocytes accumulate in joint areas. These cells produce pro-inflammatory cytokines that affect synovial fibroblasts and are involved in the pathogenesis of rheumatoid arthritis (12). The factors that play a role in activating these cells are influential in the progression of rheumatoid arthritis. As mentioned, one of the essential mechanisms in maintaining the internal homeostasis of cells is the autophagy process (13). This study selected two critical genes in the autophagy process for investigation. Beclin-1 is one of the important genes in the induction of the autophagy process and is involved in the nucleation of autophagosomes in the membrane and the activation of other autophagy-initiating factors. On the other hand, Atg5 forms a complex with Atg7 in the lipidation of the microtubule-associated light chain (LC3). Also, the evolution of the auto-phagosome membrane plays a role. The increase in the expression of these genes can indicate an increase in the autophagy process in patients with rheumatoid arthritis. Increasing autophagy in these conditions can provide the subunits of nutrients needed for the cells and maintain the immune cells' activity in rheumatoid arthritis (14).

There is not much information about the role of autophagy in rheumatoid arthritis. But a study conducted in 2016 shows that excessive activation of T cells in rheumatoid arthritis and their resistance to apoptosis may result from increased autophagy flow (15). The results obtained in our study regarding Beclin-1 and Atg5 genes were consistent with this study. Also, in addition to making nutrients available to cells, autophagy also plays a role in delivering citrullinated peptides to T cells through the MHCII supply route, thus stimulating the activation of T cells (7). Since many peripheral blood cells are made up of T cells, the increase in these genes can be related mainly to T cells (16, 17). A study conducted on rats shows that the administration of methotrexate can activate apoptosis and autophagy pathways by affecting the factors related to these pathways. For example, methotrexate can increase AMPK in rheumatoid arthritis patients (19). AMPK is an intracellular energy sensor regulating autophagy pathways under different conditions by affecting factors such as mTORC1 and FOXO3 (20). Also, in a study, researchers showed that using combined treatments consisting of methotrexate and an autophagy inhibitor has more effective results on the healing process of rheumatoid arthritis patients (12). However, the exact mechanism of the effect of methotrexate on the autophagy process in patients with rheumatoid arthritis needs more studies. Prednisolone is a glucocorticoid drug commonly used to treat rheumatoid arthritis, reducing the progression of this disease (21).

Also, studies show that prednisolone can increase apoptosis in immune cells, including T cells, by increasing the expression of pro-apoptotic proteins. Regarding hydroxychloroquine, it should be mentioned that this compound is used as an anti-malarial drug (22). Still, it has been used in treating autoimmune diseases such as rheumatoid arthritis

in recent years. Hydroxychloroquine can directly inhibit the integration of autophagosomes with lysosomes (23). Thus it is known as a potent suppressor of the autophagy process. On the other hand, studies show that TNF-dependent autophagy may play a role in resistance to apoptosis in this disease because it is well-known that inhibition of autophagy by anti-TNF drugs may activate apoptosis (24). According to the information related to the mechanisms of action of these drugs, it can be said that the effects of these drugs are likely to decrease the level of autophagy and the resistance to apoptosis (increased apoptosis) in the peripheral blood cells of patients with rheumatoid arthritis (25). Although the available evidence shows that methotrexate can increase autophagy, it should be noted that this drug can also activate apoptosis (26). These two pathways are closely related and act in opposite directions in most cellular situations. Which of these two mechanisms is more dominant in cells largely depends on the environmental signals of these cells (27). On the other hand, combining methotrexate with drugs such as prednisolone (increasing apoptosis) and hydroxychloroquine (inhibiting autophagy) can lead cells to decrease autophagy. Reducing autophagy can reduce the activity of immune cells, reduce resistance to apoptosis in these cells, and generally facilitate the healing process of patients (28).

In general, this study showed that the expression of autophagy genes increases in the peripheral blood cells of rheumatoid arthritis patients. This way, we raised the importance of autophagy in the progression of rheumatoid arthritis. To clarify the exact mechanism of autophagy in rheumatoid arthritis, it is suggested to evaluate other genes and factors effective in this path.

Funding

Conflict of Interests

The authors declared no conflict of interest.

References

1. Weyand CM, Goronzy JJ. The immunology of rheumatoid arthritis. Nat Immunol 2021; 22(1): 10-18.

2. Favalli EG, Ingegnoli F, De Lucia O, Cincinelli G, Cimaz R, Caporali R. COVID-19 infection and rheumatoid arthritis: Faraway, so close!. Autoimmun Rev 2020; 19(5): 102523.

3. Aletaha D, Smolen JS. Diagnosis and Management of Rheumatoid Arthritis: A Review. Jama-J Am Med Assoc 2018; 320(13): 1360-1372.

4. Zhang F, Wei K, Slowikowski K, et al. Defining inflammatory cell states in rheumatoid arthritis joint synovial tissues by integrating single-cell transcriptomics and mass cytometry. Nat Immunol 2019; 20(7): 928-942.

5. Nguyen DX, Cotton A, Attipoe L, Ciurtin C, Dore CJ, Ehrenstein MR. Regulatory T cells as a biomarker for response to adalimumab in rheumatoid arthritis. J Allergy Clin Immun 2018; 142(3): 978-980.

6. Rana AK, Li Y, Dang Q, Yang F. Monocytes in rheumatoid arthritis: Circulating precursors of macrophages and osteoclasts and, their heterogeneity and plasticity role in RA pathogenesis. Int Immunopharmacol 2018; 65: 348-359.

7. Vomero M, Barbati C, Colasanti T, et al. Autophagy and Rheumatoid Arthritis: Current Knowledges and Future Perspectives. Front Immunol 2018; 9: 1577.

8. Chadha S, Behl T, Bungau S, et al. Focus on the Multimodal Role of Autophagy in Rheumatoid Arthritis. Inflammation 2021; 44(1): 1-12.

9. Feng FB, Qiu HY. Effects of Artesunate on chondrocyte proliferation, apoptosis and autophagy through the PI3K/AKT/mTOR signaling pathway in rat models with rheumatoid arthritis. Biomed Pharmacother 2018; 102: 1209-1220.

10. An Q, Yan W, Zhao Y, Yu K. Enhanced neutrophil autophagy and increased concentrations of IL-6, IL-8, IL-10 and MCP-1 in rheumatoid arthritis. Int Immunopharmacol 2018; 65: 119-128.

11. Zhou W, Shen Q, Wang H, et al. Knockdown of YAP/TAZ Inhibits the Migration and Invasion of Fibroblast Synovial Cells in Rheumatoid Arthritis by Regulating Autophagy. J Immunol Res 2020; 2020: 9510594.

12. Chen M, Li M, Zhang N, Sun W, Wang H, Wei W. Mechanism of miR-218-5p in autophagy, apoptosis and oxidative stress in rheumatoid arthritis synovial fibroblasts is mediated by KLF9 and JAK/STAT3 pathways. J Invest Med 2021; 69(4): 824-832.

13. He SD, Huang SG, Zhu HJ, et al. Oridonin suppresses autophagy and survival in rheumatoid arthritis fibroblast-like synoviocytes. Pharm Biol 2020; 58(1): 146-151.

14. Wang W, Li C, Zhang Z, Zhang Y. Arsenic Trioxide in Synergy with Vitamin D Rescues the Defective VDR-PPAR-gamma Functional Module of Autophagy in Rheumatoid Arthritis. Ppar Res 2019; 2019: 6403504.

15. van Loosdregt J, Rossetti M, Spreafico R, et al. Increased autophagy in CD4(+) T cells of rheumatoid arthritis patients results in T-cell hyperactivation and apoptosis resistance. Eur J Immunol 2016; 46(12): 2862-2870.

16. Almutairi K, Nossent J, Preen D, Keen H, Inderjeeth C. The global prevalence of rheumatoid arthritis: a meta-analysis based on a systematic review. Rheumatol Int 2021; 41(5): 863-877.

17. Nahhas AF, Nahhas AF, Alshaikh AA, Webster TJ. Inhibiting Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Variants: Targeting the Spike and Envelope Proteins Using Nanomaterial Like Peptides. J Biomed Nanotechnol 2022; 18(4): 1121-1130.

18. Friedman B, Cronstein B. Methotrexate mechanism in treatment of rheumatoid arthritis. Joint Bone Spine 2019; 86(3): 301-307.

19. Wang W, Zhou H, Liu L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. Eur J Med Chem 2018; 158: 502-516.

20. Sadrjavadi K, Shahbazi B, Fattahi A. De-esterified tragacanth-chitosan nano-hydrogel for methotrexate delivery; optimization of the formulation by Taguchi design. Artif Cell Nanomed B 2018; 46(sup2): 883-893.

21. Hua C, Buttgereit F, Combe B. Glucocorticoids in rheumatoid arthritis: current status and future studies. Rmd Open 2020; 6(1): e000536.

22. Rempenault C, Combe B, Barnetche T, et al. Clinical and Structural Efficacy of Hydroxychloroquine in Rheumatoid Arthritis: A Systematic Review. Arthrit Care Res 2020; 72(1): 36-40.

23. Sharma AN, Mesinkovska NA, Paravar T. Characterizing the adverse dermatologic effects of hydroxychloroquine: A systematic review. J Am Acad Dermatol 2020; 83(2): 563-578.

24. Shi N, Zhang S, Silverman G, Li M, Cai J, Niu H. Protective effect of hydroxychloroquine on rheumatoid arthritis-associated atherosclerosis. Anim Model Exp Med 2019; 2(2): 98-106.

25. Roberts J, Smylie M, Walker J, et al. Hydroxychloroquine is a safe and effective steroid-sparing agent for immune checkpoint inhibitor-induced inflammatory arthritis. Clin Rheumatol 2019;

38(5): 1513-1519.

26. Fang H, Sha Y, Yang L, et al. Macrophage-Targeted Hydroxychloroquine Nanotherapeutics for Rheumatoid Arthritis Therapy. Acs Appl Mater Inter 2022; 14(7): 8824-8837.

27. Jorge A, Lu N, Choi H, Esdaile JM, Lacaille D, Avina-Zubieta JA. Hydroxychloroquine Use and Cardiovascular Events Among Patients With Systemic Lupus Erythematosus and Rheumatoid Arthritis. Arthrit Care Res 2023; 75(4): 743-748.

28. Magne TM, Helal-Neto E, Correa LB, et al. Rheumatoid arthritis treatment using hydroxychloroquine and methotrexate co-loaded nanomicelles: In vivo results. Colloid Surface B 2021; 206: 111952.