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The role of the Beclin-1: a gene related to autophagy in rheumatoid arthritis

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Abstract

Background Rheumatoid arthritis (RA) is a chronic autoimmune disorder. Autophagy, a regulator of cell homeostasis, can impact innate and adaptive immune cells activation and contribute to the pathogenesis of RA. The purpose of this study was to assess the significance of autophagy in RA, by investigating the autophagy signaling Beclin-1 in RA patients.

Results In RA patients, the Beclin-1 gene expression level was higher than the healthy controls with a statistically highly significant difference ($P < 0.001$) where the gene expression mean was 3.33 ± 0.45 in patients and 0.98 ± 0.070 in controls. There was a significant positive correlation between Beclin-1 gene expression and disease duration ($p = 0.013$*), erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF) titer ($P = 0.018$*, 0.027*, and 0.023* respectively). Beclin-1 gene overexpression is significantly correlated with disease activity parameters (DAS 28, patient and physician global health assessment). Furthermore, the Beclin-1 gene overexpression is highly correlated with the disability index, Modified Health Assessment Questionnaire (MHAQ) ($P < 0.001$).

Conclusion The elevated autophagy-related gene Beclin-1 expression in RA patients can contribute to RA probability, high disease activity, and severity. Therefore, suppressing autophagy may be a therapeutic target for RA.

Keywords Rheumatoid arthritis, Autophagy, Beclin-1, Autophagy related genes

Background

Rheumatoid arthritis (RA) is the most prevalent significant autoimmune disease triggered by genetic and environmental factors characterized by articular and extra-articular manifestations. Synovitis, which is its clinical hallmark and is responsible for the inflammatory and destructive features of RA, is caused by an increase in the activity of lymphocytes and macrophages as well as local mesenchymal cells that are named fibroblast-like synovial cells(FLS) [1]. Cells degrade the cytoplasmic components through a natural mechanism called autophagy to produce energy. Autophagy regulates homeostasis and controls the immune responses to self and foreign antigens by affecting lymphocyte growth, survival, and proliferation [2].

Dysregulated autophagy contributes to the pathogenesis of various autoimmune diseases. However, the influence on disease course is unique in each type of disease. While inhibition of autophagy ameliorates diseases including systemic lupus erythematosus, multiple sclerosis, and RA. On the other hand, it might exacerbate others such as psoriasis, psoriatic arthritis, and inflammatory bowel diseases [3]. Even in RA, cell type-specific dysfunction of autophagy pathways may have either a therapeutic or exacerbating role in RA pathology [4].

The dual effect of autophagy in RA is seen in RA synovial fibroblasts by both mediating cell death induced by endoplasmic reticulum stress, and inhibiting apoptosis triggered by proteasome inhibition. This is particularly important in joint inflammation leading to synovial

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hyperplasia in response to proinflammatory microenvironment [5]. Also, defective autophagy in macrophages facilitates the generation of pathological post-translational modifications of peptides rendering them immunogenic [6]. Moreover, autophagy-defective T cells along with lymphopenia allow autoaggressive T cells to thrive, eventually leading to the clinical presentation of RA [7].

Furthermore, the survival of inflammatory cells, including synoviocytes and lymphocytes that release the inflammatory cytokines, was discovered to depend on the activation of autophagy in RA. Also, autophagy plays a crucial part in osteoclasteogenesis and citrullination via Tumor necrosis factor–alpha(TNF-α) dependent mechanism [8, 9]. Along with Major Histocompatibility complex class II moieties, autophagy also has been recognized as having a role in the presentation of cytoplasmic antigens, which are crucial for maintaining self-tolerance [10].

Chloroquine and hydroxychloroquine (HCQ), two medications that can block the cellular process of autophagy via their ability to accumulate in lysosomes are used in the treatment of RA [11]. Moreover, in vitro and in vivo experiments have shown that they can prevent the antigen from being presented to T cells and the development of osteoclast precursors into mature osteoclasts. In both of these processes, autophagy is actively engaged [12].

Beclin-1 is a core component of the class III phosphatidylinositol 3-kinase (PI3K-III) complex, which plays an important role in membrane trafficking and restructuring involved in autophagy, endocytosis, cytokinesis, and phagocytosis, it modulates the lipid kinase activity of PI3K-III catalytic unit Vacuolar protein sorting (VPS34), which generates phosphatidylinositol 3-phosphate (PI(3)P) [13]. Through PI(3)P production, the Beclin 1-VPS34 complex via its central coiled-coil domain (CCD), and evolutionarily conserved domain (ECD) enables the recruitment of several autophagy proteins involved in the nucleation of the autophagosome and provides a platform for important autophagy proteins involved in autophagosomes biogenesis and proper maturation [13, 14].

A potential new therapeutic target for treating active RA is lowering the expression level of genes related to autophagy activation [15, 16]. Therefore, this study was designed to evaluate the expression level of Beclin-1 in RA patients and to investigate its association with disease activity and severity parameters.

Subjects and methods

This study is a case-control one in which four hundred and eight subjects were involved; they were classified into two hundred four RA patients and two hundred four normal healthy persons matched for sex and age. The local Ethics Committee accepted the protocol of this study. Inclusion criteria included RA patients who were diagnosed according to the American College of Rheumatology/European League Against Rheumatism new RA criteria [17], both sexes, 18–60 years old range and a disease duration ≥ 1 year with the ability to give consent, However, exclusion criteria were patients with other chronic illness or combined with other autoimmune diseases as systemic lupus erythematosus, secondary Sjogren syndrome.

Clinical assessment

Both groups of subjects were subjected to history taking, general examination, musculoskeletal examination including inspection (for joint swelling, deformity as flexion contractures or muscle wasting), palpation (for hotness, tenderness), and range of movement (active and passive). Disease activity assessment included morning stiffness, patient and physician global health assessment by Disease Activity Scale (DAS28), VAS (0–10) comprises general health (GH), an ESR, and a count of tender and swollen joints (range 0–28) [18]. Physical disability assessment by a self-applied questionnaire, the modified Health Assessment Questionnaire HAQ (MHAQ), was used to inquire about daily activities and perceived levels of difficulty [19].

Laboratory investigations

Including complete blood count (CBC) was done on Sysmex xs 500i (System, Japan), Rheumatoid factor and CRP were done on Cobas 6000-c502 auto analyzer (Roche Diagnostics, Germany), Anti-CCP on Cobas e411 (Roche diagnostics, Japan) and ESR on vision ESR Analyzer (YHLO, China).

Total RNA isolation and RT-PCR analysis

Peripheral blood samples were subjected to RNA isolation using an RNA purification kit (GENEzol triRNA pure kit), as stated by the manufacturer's instruction then RNA concentrations were measured by Fluorometer (Qubit 3.0, Invitrogen, life technology, Malaysia). RNA (1 µg) was utilized for the complementary DNA (cDNA) synthesis via ABT H-minus cDNA synthesis kit (Applied Biotechnology Catalogue number: ABT009) as stated by the manufacturer's procedure. after that, Real-time polymerase chain reaction RT-PCR (QuantoStudio 5, Thermofisher, Singapore) was performed in 20 µl final volume which included 10 µl TOPreal qPCR 2X preMIX (SYBR Green with low ROX) (Jena Bioscience), 1 µl of each forward primer, reverse primer and cDNA and 7ul nuclease-free water. This PCR mixture was done for both the target gene (Beclin1) and the housekeeping or reference gene

(GADPH) which is used for target gene normalization. The primer sequences used were as follows: Beclin-1, forward: 5′-CCAGGAACTCACAGCTCCATT-3′, reverse: 5′-ATGAATCTGCGAGAGACACCA-3′ and GAPDH (housekeeping or reference gene) forward: 5′-TGGGTG GAATCATATTGGAAC3′, reverse: 5′-TCAACGGAT TTGGTCGTATTG-3′ (Jena Bioscience).

Thermal amplification cycles were initial denaturation at 95 °C for 15 min then 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 20 s then at 95 °C for 15 s for the product characterization by melting curve analysis. Expression levels of each sample as the threshold cycle (CT) were adjusted to GADPH reference gene expression. The ΔCT for each sample was calculated by subtraction the CT value of the reference gene from the CT value of the target gene, ΔΔCT values were defined as the difference between ΔCT of the case and ΔCT of the control. Then, by using the $2-\Delta\Delta CT$ method, the relative gene expression was calculated.

Statistical analysis

Statistical Package for Social Science (SPSS) (Version 20.0.) Armonk, NY: IBM Corp.) was used for data analysis. Quantitative data were represented as mean ± standard deviation (SD) and median with range. Qualitative variables were given in the form of numbers and percentages. The Mann–Whitney *U* test was used for non-normally distributed variables, the Student *t* test was employed to compare two groups of regularly distributed variables. Spearman's correlations were calculated to assess the correlation between the Beclin-1 levels with various study variables. If the significant probability (*P* value) was ≤ 0.05, the results were considered statistically significant, and highly significant if $p \leq 0.001$.

Results

The current study involved two hundred and four RA patients, their mean age was 44.56 ± 11.3 years with a mean disease duration of 7.9 ± 3.53 years, most of them were females 89.22%, one-third of them had hypertension (34.4%). Regarding the laboratory findings, the ESR and the CRP means were high (28.07 and 8.32) indicating active disease, and the means of positive (RF and AntiCCPs) were (26.45 and 27.73) respectively. The majority of patients were on antimalarial (HCQ) (88.2%) and methotrexate (52%), (Table 1).

Concerning RA disease activity and severity measures, DAS 28 mean was 4.37 indicating moderate disease activities, the morning stiffness mean was 19.90 min, the patient and the physician global health means were 5.34 ± 2.12 and 4.75 ± 1.41 respectively, all the previous assessment measures coincide with moderated disease

Table 1 Clinical characteristics of the RA patients' group (*N* = 204)

Variable	Value
Age (years)	
Mean ± SD	44.56 ± 11.3
Median, range	45 (22–70)
Disease duration	
Mean ± SD	7.9 ± 3.53
Median, range	6 (1–30)
Sex	
Male, no %	22 (10.87%)
Female, no%	184 (89.22%)
Comorbidities: No %	
• HTN	70 (34.3%)
• Diabetes	30 (14.7%)
• HCV	8 (3.9%)
• ILD	8 (3.9%)
• Ischemic heart	6 (2.9%)
• None	82 (40.2%)
Laboratory findings:	
ESR	
Mean ± SD	28.07 ± 0
Median (range)	30 (0–67)
CRP	
Mean ± SD	8.32 ± 5.65
Median (range)	8 (3–12)
RF	
Mean ± SD	26.45 ± 1.41
Median (range)	22 (5–52)
Anti-CCP	
Mean ± SD	27.73 ± 18.38
Median (range)	25 (10–60)
Disease activity (DAS)	
Mean ± SD	4.37 ± 0. 66
Median, range	4.33 (2.4–7.63)
Grades:	
• Remission:	8 (3.9%)
• Mild:	29 (14.2%)
• Moderate:	89 (43.6%)
• Severe:	78 (38.2%)
Patient global health	
Mean ± SD	5.34 ± 2.12
Median, range	5 (1–10)
Physician global health	
Mean ± SD	4.75 ± 1.41
Median, range	5 (1–8)
Morning stiffness (min)	
Mean ± SD	19.90 ± 3.53
Median, range	15 (0–60)
MHAQ	
Mean ± SD	0.98 ± 0.17
Median, range	1 (0–2.5)

Table 1 (continued)

Variable	Value
MHAQ grade	
• Normal	15.7 (32%)
• Mild	54.9 (112%)
• Moderate	41 (20.1%)
• Severe	19 (9.3%)
Treatments No %	
HCQ	180 (88.2%)
MTX	106 (52%)
LEF	88 (43.13%)
SSZ	77 (37.7%)
AZA	14 (6.86%)
Steroids	56 (27.5%)
NSAIDS	34 (16.6%)

Anti-CCP Anti-cyclic citrullinated peptide, *AZA* azathioprine, *HCQ* hydroxychloroquine, *HCV* hepatitis C virus, *HTN* hypertension, *ILD* interstitial lung disease, *LEF* leflunomide, *MHAQ* modified health assessment questionnaire**,***MTX* methotrexate, *NSAIDS* non-steroidal anti-inflammatory drugs, *SSZ* salazopyrine

Table 2 Beclin-1 gene expression in RA patients and control

Group	Mean ± SD	*T*	*P*
Patient ($n=204$)	3.33 ± .45	68.44	0.0001**
Control ($n=204$)	0.98 ± .070		

T independent *t* test

* Significant $P \leq 0.05$

** Highly significant $P \leq 0.001$

activity. Nearly half of the patients (54.9%) had mild MHAQ grades. Beclin-1 gene expression was higher in RA patients than in healthy controls, with a statistically significant difference $P<0.001$, with the gene expression mean being 3.33 ± 0.45 in patients and 0.98 ± 0.070 in controls (Table 2, Fig. 1). Furthermore, Beclin-1 gene expression was higher in patients with RF and AntiCCP positivity, with a median and range of 3.31(2.38–4.43), 3.11(2.39–4.43) respectively giving a highly significant difference $P<0.001$, (Table 3).

Regarding the correlation between Beclin-1 gene expression level and disease activity and severity parameters in RA patients, there was a significant positive correlation between Beclin-1 gene expression and disease duration ($p=0.013$*) (Fig. 2a), indicating that long disease duration is associated with gene overexpression. There was a significant positive correlation between Beclin-1 gene overexpression and high ESR, CRP, and RF titer ($P=0.018$*, 0.027*, and 0.023* respectively). Beclin-1 gene overexpression is significantly correlated with disease activity. The higher the RA disease activity scores (DAS, patient, and physician global health) were the more the Beclin-1 gene overexpression level, ($P<0.001$) (Fig. 2b). Furthermore, the Beclin-1 gene overexpression is highly correlated with the disability index (MHAQ), ($P<0.001$) (Fig. 2c, Table 4).

Discussion

Autophagy is a dynamic cytoprotective process that eliminates degraded products such as misfolded proteins, damaged organelles, and invading pathogens while recycling the essential components for cellular homeostasis and survival under both normal and stressful circumstances [20]. So, autophagy frequently acts as an intermediary between cell death and survival [21]. This process is tightly controlled by complex autophagy proteins which are encoded by several genes associated with autophagy. Beclin-1 protein which is encoded by the Beclin-1 gene (located on the 17q21.31 chromosome) has an important role in the initial stages of autophagy. It serves as a platform for a multiprotein assembly to mediate autophagosome formation [22].

Earlier studies have shown that autophagy contributes to the degenerative progression of joint injury; however, the majority of these studies mostly relied on in vitro animals with few clinical trials [23]. The current study was designed to investigate the Beclin-1 gene expression level as an autophagy inducer in RA and to clarify its association with various disease activity and severity parameters.

The present study showed that the Beclin-1 gene expression level was higher than the healthy controls indicating abnormal autophagy induction in RA. Moreover, the Beclin-1 gene expression level was more in seropositive patients, indicating abnormal autophagy induction in RA disease and demonstrating the significance of the Beclin-1 upregulation in vulnerability to RA. In agreement with a recent study conducted by Kardideh et al. [24] who found that Beclin-1 expression was 3.41 times higher in early RA patients compared to healthy controls, and 1.5 times higher in patients who were not receiving medication.

The role of autophagy in RA was documented by Zhu et al. study [25] who investigated the autophagy-related proteins (beclin1, Atg5, and light chain 3(LC3) expressed in the synovial tissue of RA and osteoarthritis patients and reported that patients with active RA had considerably higher levels of the autophagy-related proteins expressed in their synovial tissue than did patients with osteoarthritis.

Contrarily to autophagy, apoptosis is a programmed cell death and the interaction between these two processes affects cellular behavior. Recent research reveals that autophagy and apoptosis have a role in the control of the immune system and the promotion of self-tolerance in rheumatic disorders [26]. According to this

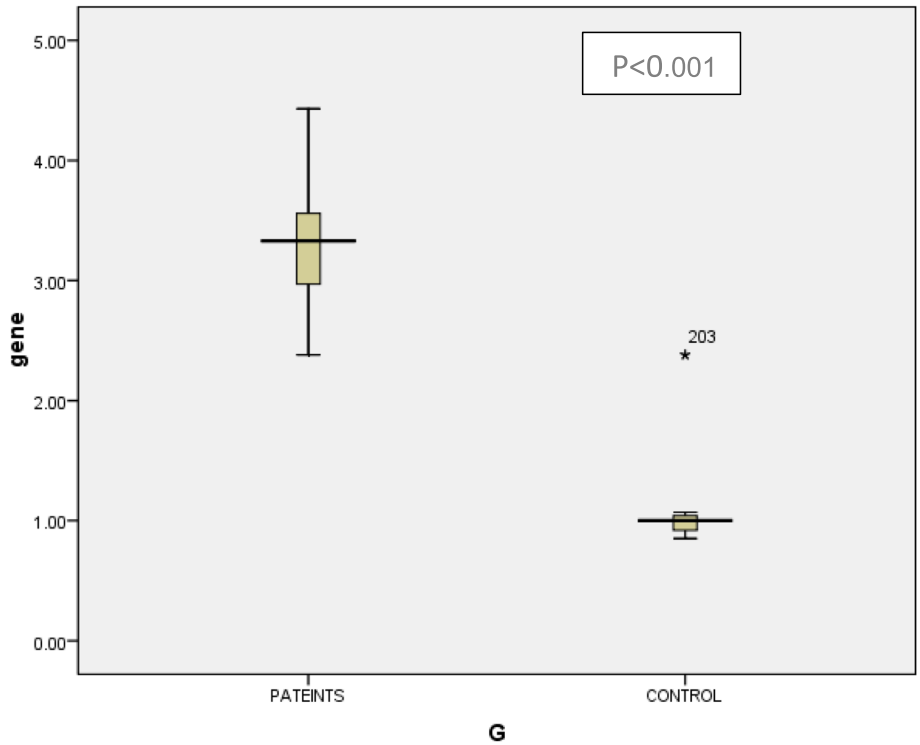

Fig. 1 Beclin-1 gene overexpression and RA patients and healthy control. In RA patients Beclin-1 gene expression was higher than the healthy control with a statistically high significant difference $P \leq 0.001$ where the gene expression mean was 3.33 ± 0.45 in patients and 0.98 ± .070 in controls

Table 3 Relationship between the Beclin-1 gene expression and seropositive (RF& anti-CCP) in patients with RA

Group	Number	Beclin Median(range)	U test	*P*
Anti-CCP	Negative (43)	2.91 (2.38–4.29)	2.23	0.001
	positive (161)	3.11 (2.39–4.43)		
RF	Negative (74)	2.9 (2.38–4.14)	2.88	0.001
	positive (130)	3.31 (2.38–4.43)		

RF rheumatoid factor, *Anti-CCP* anti-cyclic citrullinated peptide, Mann-Whitney *U* test

* Significant $P \leq 0.05$

** Highly Significant $P \leq 0.001$

theory, prior research has shown that FLS hyperplasia in RA is caused by decreased apoptosis, which is primarily proven by elevated amounts of anti-apoptotic proteins and down-regulated pro-apoptotic factors as a result of autophagy activation which in turn contribute to progressive synovial thickening [27, 28].

Recently, Xu and colleagues suggested that RA patients' resistance to treatment may be influenced by the balance between autophagy and apoptosis and discovered a substantial negative association between apoptosis and autophagy. The findings of this study suggest that RA FLS may use the Beclin-1-dependent autophagic pathway as a survival mechanism to avoid the disruption by the anti-metabolite methotrexate to maintain cell viability to treat RA more successfully, It is worthwhile to combine MTX with an autophagy inhibitor [29].

Autophagy disturbs the bone metabolism equilibrium causing osteoclasteogensis via TNF-a dependent mechanism [30]. Osteoclasts from RA synovia have been found to express autophagy-related molecules such as Beclin-1 and Atg7 more frequently [16]. Additionally, it has been shown that the use of autophagy inhibitors significantly reduces bone degradation in experimental arthritic mice models [31, 32].

In the current study, Beclin-1 gene expression was strongly correlated with disease duration, high ESR, CRP, and RF titer; Beclin-1 gene overexpression is significantly correlated with disease activity (DAS 28, patient, and physician global health). Furthermore, the Beclin-1 gene overexpression is highly correlated with the disability index (MHAQ). Despite Zhu had assessed Beclin genes in the synovium [25] but they also, showed that the blood levels of many RA activity-related indicators, including CRP, ESR, CCP, and RF, were highly linked with the autophagy level in the synovium. In Egyptian systemic

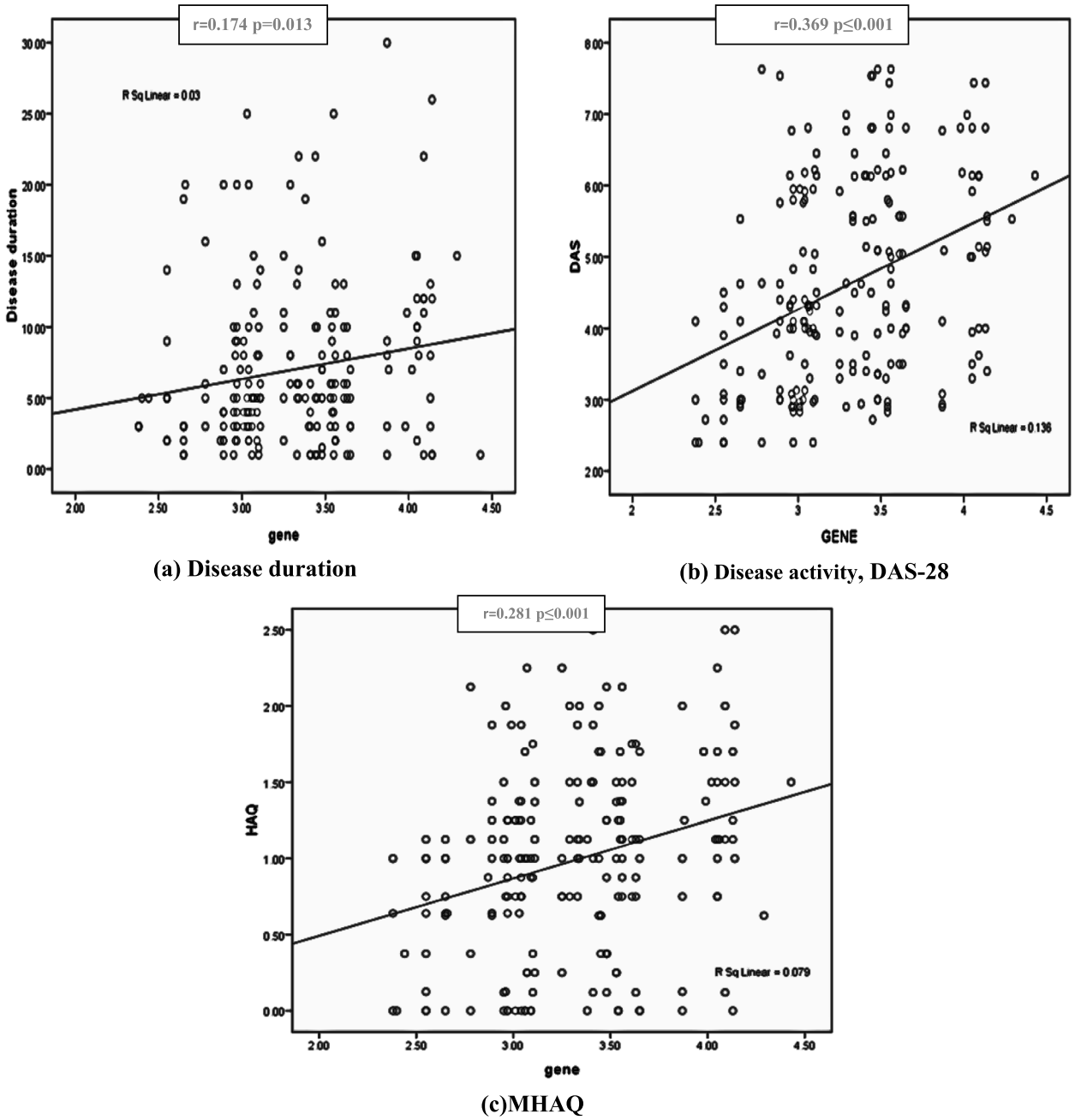

Fig. 2 Correlation between Beclin-1 gene overexpression and different RA patient's parameters. **a** There was a significant positive correlation between Beclin-1 gene expression and disease duration ($p = 0.013$*). **b** Beclin-1 gene overexpression is significantly correlated with disease activity. The higher the RA disease activity scores (DAS, patient and physician global health) the more the Beclin-1 gene overexpression level, ($P < 0.001$). **c** The Beclin-1 gene overexpression is highly correlated with the disability index (MHAQ), ($P < 0.001$)

lupus erythematosus patients, the variant alleles of ATG-5, and Beclin-1 were more common in patients younger than 30, anemic patients, and anti-double-stranded DNA (dsDNA) patients [33].

To our knowledge, no other previous studies assessed the correlation between RA activity and autophagy genes expression. Our research found that the high Beclin-1 gene expression level in RA patients is associated with developing more active and severe disease so its analysis is necessary to predict the disease severity and may also contribute to early aggressive and more effective therapy by using drugs regulating autophagy and gene therapy.

Table 4 Correlations between Beclin-1 gene expression and disease activity and severity parameters in RA patients

Variable	*R*	*P*
Age	0.064	0.361
Disease duration	0.174	**0.013***
RF	0.159	**0.023***
ANTICCP	0.020	0.773
ESR	0.166	**0.018***
CRP	0.156	**0.027***
DAS	0.369	**0.0001****
Patient global health	0.335	**0.0001****
physician global health	0.341	**0.0001****
MHAQ	0.281	**0.001****

* Significant $P \leq 0.05$

** Highly Significant $P \leq 0.001$

Limitations

The current monocentric investigation focuses exclusively on one autophagy gene regulator. It would be helpful to conduct additional multicentric research involving more autophagy signaling pathways and regulators to pinpoint a particular regulatory factor as a novel therapeutic target for avoiding and managing the clinical manifestations of RA.

Conclusion

Beclin-1 gene expression as a measure of autophagy activation is upregulated in RA, autophagy is crucial for the survival of the inflammatory cells as well as a key factor in citrullination and osteoclasteogenesis in RA. Lowering the expression of genes involved in autophagy activation may represent a novel therapeutic target for the treatment of active RA.

Abbreviations

Anti-CCP	Anti-cyclic citrullinated peptide
Atg	Autophagy-related genes
CCD	Central coiled-coil domain
cDNA	Complementary deoxyribonucleic acid
CT	Cycle threshold
DAS	Disease activity score
dsDNA	Double-stranded deoxyribonucleic acid
FLS	Fibroblast-like synovial cells
GADPH	Glyceraldehyde-3-phosphate dehydrogenase
HCQ	Hydroxychloroquine
LC3	Light chain 3
MHAQ	Modified Health Assessment Questionnaire
MHC	Major histocompatibility complex
PCR	Polymerase chain reaction
PI3K-III	Phosphatidylinositol 3-kinase class III complex
(PI (3) P	Phosphatidylinositol 3-phosphate
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
TNF	Tumor necrosis factor
VAS	Visual Analogue Scale
VPS34	Vacuolar protein sorting

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Authors' contributions

All authors contributed. Shymaa and Doaa collected the clinical data of the patients. Asmaa and Nora did the investigations. All shared in the writing process. All authors read and approved the final manuscript.

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Availability of data and materials

We approve the availability of our data upon request.

Declarations

Ethics approval and consent to participate

An official permission was obtained from Institutional Review Board NO. (ZU-IRB # 10099-7-11-2022). at Faculty of Medicine, Zagazig University Hospitals and from the Rheumatology& Rehabilitation and Clinical pathology Departments at the same University. The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki 1964) for studies involving humans. A written informed consent was obtained from the participants.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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