Saleh *et al.*
Egyptian Journal of Medical Human Genetics 2022, **23**(1):155
https://doi.org/10.1186/s43042-022-00367-w

Egyptian Journal of Medical Human Genetics

RESEARCH

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Autophagy-related genes in Egyptian patients with Behçet's disease

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Abstract

Background: Behçet's disease (BD) is a chronic, multi-systemic, recurrent condition that affects the vascular, ocular, mucocutaneous, and central nervous systems. The diagnosis of this disease depends on its clinical features, which are similar to those observed in several diseases, such as Parkinson's disease, pemphigus vulgaris, systemic lupus erythematosus, Crohn's disease, and Sjögren's syndrome. Lysosome-mediated autophagy is a catabolic, cytoprotective mechanism that maintains cell homeostasis by degrading undesired long-lived proteins and recycling nutrients. The aim of this study was to evaluate the correlations between some autophagy-related genes (*ATG5*, *ATG7*, *ATG12*, *LC3b*, *mTOR*) and the pathogenesis and immunopathology of BD. The expression levels of the genes were evaluated by quantitative polymerase chain reaction (qPCR) in 101 individuals that are classified into two groups. Group 1: ($n=71$) BD patients, Group 2: ($n=30$) healthy controls.

Results: Patients with BD had lower mRNA expression levels of *ATG5* and *mTOR* and higher levels of *LC3b* mRNA than the controls. No significant differences in the levels of both *ATG7* and *ATG12* were observed between the two groups. According to the area under the curve analysis, *LC3b* was considered the best candidate biomarker among the selected markers for the diagnosis of BD. The mRNA expression of *ATG5* was significantly correlated with patient age and the presence of oral ulcers. The mRNA expression of *ATG7* was significantly associated with age and the presence of erythema nodosum and vascular lesions, whereas that of *LC3b* was significantly correlated with the presence of pustules.

Conclusion: These findings indicated that elevated levels of *LC3b* were strongly associated with BD. Likewise, the levels of *ATG5* and *ATG7* were associated with the complications and outcomes of this disease. Additional assessments of the mRNA expression levels of these autophagy-related genes might prove beneficial in diagnosing this autoimmune disorder.

Keywords: Autoimmune disease, Autophagy, Behçet's disease, *ATG*, *LC3b*, *mTOR*

Introduction

Behçet's disease (BD, #OMIM 109650) is an uncommon systemic disease of unknown etiology, primarily defined by recurring oral ulcer attacks (60%–90%) that occur at least three times in 12 months. This symptom is considered as the earliest manifestation of the disease. Additionally, genital ulcers (48%–85%), ocular lesions (45%–90%), neurological manifestations (2.3%–38.5%), and other systemic manifestations may develop over several years [1]. The 1994 International Chapel Hill Consensus Conference, which was updated in 2012, classified BD as variable vessel vasculitis (VVV) because the vasculitis associated with this disease does not involve any predominant vessel; this disease can affect any size or type (arteries, veins, and capillaries) of the blood vessel [2]. BD often leads to serious systemic problems, such as

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blindness, and the main causes of mortality are associated with neurological and cardiovascular complications [3]. The prevalence of BD differs worldwide, with an estimated ratio of 3.6 per 100,000 in the Egyptian population [4]. Moreover, organs affected BD is made more difficult by occurring of vessels thrombosis of any type. In male patients with active disease thrombosis is prevalent and is a major root of death and morbidity. Hyperactivation of neutrophils has been demonstrated in patients with BD, possibly due to the presence of the chromosome 6 human leukocyte antigen (HLA)-B51 allele [5].

Mucocutaneous involvement is prevalent in BD. In the current study, four patients had lost sight. The prevalence of oral ulcers in BD is estimated to range between 97 and 100%. Oral ulcers make it difficult to chew, speak, and drink, thereby decreasing the patient's quality of life [6]. Approximately 90% of the patients in the present study developed oral ulcers. As reported by an earlier study, the oral ulcers are considered as the majority of BD symptoms (98.2%), followed by genital ulcers (62.4%), ocular involvements (53.2%), erythema nodosum (53.2%), acniform lesions (51.8%), arthritis (38.6%), gastrointestinal symptoms (25.1%), neurogenic disorders (9.0%) and vascular involvements (8.1%). In addition, it has been previously observed how *HLA* and disease symptoms correlate (41.5% with HLA-B*51 and 24.1% with HLA-A*26) [7]. Acneiform lesions are the most common type of skin lesions that occur primarily on the trunk and extremities of patients with BD. Lesions found on other parts of the body other than the face were considered to be more specific for BD [8]. Arthritis and genital ulcers are more common in females, whereas ocular manifestations, acneiform lesions, and HLA-B*51 are more common in males [7].

Various genetic and environmental factors are involved in the initiation and development of this disease, but the most robustly associated risk factor is the HLA-B51 allele [9]. Four main supporting criteria for evidence genetic influence on BD susceptibility include instances of familial aggregation, unusual geographical distribution, association with HLA class I antigens (HLA-B51) and gene polymorphisms that affect immunological response [9]. Several genetic studies have shown that HLA-B is significantly correlated with cytokine production [10].

Several clinical criteria are used to diagnose BD owing to the lack of a specific diagnostic test [11]. However, it is difficult to reach a diagnosis in some cases because of the time it takes for the typical symptoms to manifest. In addition to the signs and symptoms, the diagnosis of this disease is made based on the positive clinical criteria commonly known as the "International Clinical Criteria for Behçet's Disease." Recurrent mouth sores are generally necessary for a diagnosis of BD and are the ideal benchmark for clinical diagnosis. Moreover, recurring at least two of additional signs as eye inflammation, skin and genital sores confirm the diagnosis of BD [12]. The pathergy test is considered as one of the major criteria, according to the "International Study Group for Behçet's Disease" [13]. Blobner first identified the pathergy reaction, which appears as an erythematous papule or pustule at the injection site 24–48 h after an intradermal puncture, in 1937 [14]. The pathergy test reveals skin hyperreactivity and is used as an additional test in BD diagnosis. The pathergy test is currently not standardized, and the most popular procedure is the intradermal insertion of a 20–22 gauge needle at a 45-degree angle into at least two separate places on the forearm's avascular area [15]. A positive result of this test suggests that a minor injury is causing the immune system to overreact. Hence, the pathergy reaction test is not specific because it can give positive results with other conditions. Moreover, a positive pathergy phenomenon is only seen in small percentage of patients with BD. Therefore, there is an urgent need to search for efficient and specific diagnostic tests for this disease [16, 17].

BD has long been considered as an autoimmune disorder with unknown clear causes, but it shares many clinical symptoms with other autoinflammatory diseases. BD aetiology has traditionally been attributed to immune system disorders. Both adaptive and innate immune cells are implicated in disease pathogenesis [18]. The loss of normal immunological control, along with the activation and migration of neutrophils to the inflammatory lesion site, is thought to play a major role in the pathogenesis of BD. Patients with BD show hallmark depletion in the natural killer (NK) cells in the peripheral blood, a shift that corresponded to the presence of the disease, accompanied by a reduction in the cytokine IL-10 and regulatory T cells (Tregs). Conversely, over production of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-8 and IL-17 level that correlated with BD activity [19].

Autophagy (autophagocytosis) is a natural, intracellular regulated mechanism characterized by the removal of aggregated proteins, infectious organisms, and damaged organelles via lysosomes, and hence plays a multitude of physiological and pathological functions [20]. Macroautophagy, microautophagy, and chaperon-mediated autophagy are the three most prevalent types of autophagy [21]. Autophagy pathways and their mediators have a crucial function in the immune system and inflammatory process; they may help prevent autoimmune disorders and inflammatory conditions. Hence, autophagy dysfunction can contribute to the pathogenesis of several autoimmune diseases [22].

Autophagy is carried out by autophagy-related genes (ATG). Genetic screening performed on the budding yeast *Saccharomyces cerevisiae* led to the identification of the first genes involved in autophagy [23]. Numerous genes, such as the immune-related GTPase family M protein (*IRGM*), *ATG2A*, *ATG5*, *ATG7*, *ATG12*, ATG16 like 1 *(ATG16L1)*, leucine-rich repeat kinase 2), death-associated protein [24], mechanistic target of rapamycin (*mTOR*), regulatory associated protein of mTOR (*RAPTOR*), rapamycin insensitive companion of mTOR (*RICTOR*), and microtubule-associated protein light chain 3b (*LC3b*), have been examined in BD [10]. Nonetheless, there is a lack of research concerning these genes in Egyptian patients with BD.

Atg5 is a crucial protein found in the phagophoric membrane of the autophagic vesicle [25]. It is activated by Atg7 to form a complex with Atg12 and Atg16L1. Atg7 is required for the formation and expansion of autophagosomes [26, 27]. Atg12 is activated by Atg7, following which it conjugates with Atg5; this Atg12-Atg5 conjugate forms a complex with Atg16L [28]. The LC3-1 C-terminus conjugates with the Atg12-Atg5-Atg16L complex and is associated with the phagophore membranes to form LC3-II. The Atg12-Atg5-Atg16L complex separates from the autophagosome following the development of the autophagosome [29]. LC3-B was thought to be involved in the regulation of the assembly and disassembly of microtubules [30]. LC3 is a key protein in the autophagy pathway and is involved in the selection of the autophagy substrate and biogenesis of the autophagosome. LC3 is the most widely used marker of autophagosomes [31]. mTOR, also known as the mechanistic target of rapamycin and FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1), is a kinase encoded by the *mTOR* gene in humans [32]. mTOR forms connections with other proteins and is a key element of the two different protein complexes, mTOR complex 1 (mTORC1), and mTOR complex 2 (mTORC2), which control many biological activities [33]. Active mTORC1 is located on lysosomes, and mTOR inhibition occurs by damaging the lysosomal membrane through various exogenous or endogenous agents [34]. However, mTOR suppression during the damage of lysosomes promotes autophagy's reaction, resulting in lysophagy, which eliminates the destroyed lysosomes [35].

A study by Adeeb et al. [36] identified some probable diagnostic biomarkers, such as the HLA-B*51, chemokines CCR1 and CCR3, cytokines TNF, ILs, and IFNs, T lymphocytes, inflammasomes, autoantibodies, and fecal calprotectin. In addition, hypovitaminosis D has been linked to various inflammatory diseases, including BD. Vitamin D has immunosuppressive effects and is recommended as a therapeutic tool for many autoimmune disorders [37]. According to Do et al. [38], vitamin D reduces the expression of toll-like receptor (TLR) in the monocytes of BD patients; additionally, TLR-2 and TLR-4, which are crucial for the etiology of BD, are responsible for inducing inflammation. Thus, vitamin D may be useful as a therapy option for patients with this type of inflammation. However, despite the appearance of these candidate markers, there is currently insufficient widespread data to justify their adoption and incorporation into the most recent classification standards [38].

The present study was conducted to screen a panel of differentially expressed autophagy-related genes (*ATG5*, *ATG7*, *ATG12*, *LC3b*, *mTOR*) that have been postulated to play a role in the immunopathology and pathogenesis of BD. Additionally, correlations between the clinical heterogeneity and prognosis of the disease and the selected specific autophagy markers were determined.

Subjects and methods

Patients

This prospective study comprised 101 subjects (71 BD patients and 30 healthy controls) recruited from the Internal Medicine inpatient ward and Rheumatology and Clinical Immunology outpatient clinics at the Internal Medicine Department, Kasr Elainy, School of Medicine, Cairo University Hospitals. Written informed consents were signed from all the patients, family members or legal guardians, as appropriate according to the organization of ethical guidelines. The aim, methods and duration of the study were fully explained to all participants. The anonymity and confidentiality of the patient's data were ensured by coding the datasheets. Informed consent for the use of the detailed medical history, demographic, and medication profile information was obtained from the subjects.

The study was approved by the Institutional Bioethics Committee of the National Research Center (Registration number: 19047) and conducted in accordance with the ethical guidelines of the Declaration of Helsinki [39].

BD was diagnosed by an expert rheumatologist based on the "International Clinical Criteria for Behçet's Disease." Exclusion criteria: (1) BD mimics diseases e.g.: vasculitis, (2) patients with other autoimmune diseases, (3) diabetic patients, (4) patients with rheumatic diseases, (5) patients with current infections such as HCV, (6) malignancies and (7) endocrine diseases were also excluded.

Drugs and treatment protocol

Demographic and clinical information were collected from the records. The currently used medications such as disease-modifying anti-rheumatic drugs (DMARDs), Colchicine, Azathioprine, Prednisolone, Rituximab and Etanercept and others such as anticoagulants (Warfarin

or Marivan), Cyclophosphamide (Endoxan) were also recorded.

Clinical investigation and diagnosis

Detailed information about the medical history and medication profile was obtained. Ophthalmological fundus and slit lamp examinations were performed for those presenting with ocular manifestations. All the participants were subjected to laboratory assessments, including hemoglobin (Hb; mmol/l), white blood cell count (WBC; 10^9/l), platelet count (plt; $10.^9$/l), erythrocyte sedimentation rate (ESR; mm/s), and immunological profiles for rheumatoid factor (RF), C-reactive protein (CRP), and antinuclear antibody (ANA). The relative expression levels of the autophagy markers were determined by quantitative polymerase chain reaction (qPCR)

Relative expression of autophagy markers by quantitative polymerase chain reaction (qPCR)

Five milliliters of blood were collected in EDTA tubes by vein puncture, and the total RNA was extracted using a QIAamp RNA blood Mini Kit (Cat. No. 52304; Qiagen, USA) [40]. The total RNA was reverse transcribed into first-strand complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368813, Applied Biosystems, USA) [41]. The relative mRNA expression levels of *ATG5*, *ATG7*, *ATG12*, *LC3b,* and *mTOR* were measured by qPCR using the Roche real-time PCR system (light cycler 480, product no. 05015278001; Roche life science, UK) and the SYBR Green master mix (Cat. No. K0252; Thermo Scientific, USA) [42].

Sequence of primers for genes is illustrated in Table 1. The values were normalized based on the expression level of the endogenous housekeeping gene (*β-actin)*, which was used as a denominator. The relative changes in gene expression between patient and healthy control groups were determined using the light cycler 480 real-time PCR system software 1.5.0 SP3. The thermal cycling was performed using a three-step cycling protocol: Initial step (95 °C, 10 min for 1 cycle), denaturation step (95 °C, 15 s for 40 cycles), annealing step (59 °C, 30 s for 40 cycles), and finally extension step (72 °C, 30 s for 40 cycles).

Table 1 Primer sequences for the selected studied autophagy genes and housekeeping gene

Gene	Primer
ATG5	Forward: 5′-AAAGATGTGCTTCGATGTGT-3′ Reverse: 5′-CACTTTGTCAGTTACCAACGTCA-3′
ATG7	Forward: 5′-ATGATCCCTGTAACTTAGCCCA-3′ Reverse: 5′-CACGGAAGCAAACAAACAACTTCAAC-3′
ATG12	Forward: 5′-TAGAGCGAACACGAACCATCC-3′ Reverse: 5′-CACTGCCAAAACACTCATAGAGA-3′
LC3b	Forward: 5′-GATGTCCGACTTATTCGAGAGC-3′ Reverse: 5′-TTGAGCTGTAAGCGCCTTCTA-3′
mTOR	Forward: 5′-GCAGATTTGCCAACTTCGG-3′ Reverse: 5′-CAGCGGTAAAAGTGTCCCCTG-3′
Actin	Forward: 5′-AGGCCAACCGCGAGAAGATGACC -3′ Reverse: 5′-GAAGTCCAGGGCGACGTAGCAC -3′

Statistical analysis

The results were statistically analyzed using the Minitab 17.1.0.0 software for windows (Minitab Inc., 2013, Pennsylvania, USA). Continuous data were presented as mean and standard deviation, whereas categorical data were described as frequency counts and percentages. The normality of the data was examined using the Shapiro–Wilk test. Comparisons between two continuous groups were performed using the independent t-test or Mann–Whitney test, and those between two or more categorical groups were analyzed using the Chi-square test.

The accuracy of the mRNA expression of *ATG5*, *ATG7*, *ATG12*, *mTOR*, and *LC3b* was assessed using the receiver operating characteristics (ROC) curve analysis, assuming that the null hypothesis of the area under ROC (AUROC) was 0.5 (power, 80%; confidence interval [CI], 95%, and BD prevalence, 1%) [43].

A sample size calculated with minimum total number of 90 using *MedCalc* (MedCalc Software v.13, Ostend, Belgium) software for windows. The quality of calculated AUROC was as follows: (0.90–1 = excellent), (0.80–0.90 = good), (0.70–0.80 = fair), (0.60–0.70 = poor) and (0.50–0.60 = fail). General linear model (GLM) used to estimate the association between clinical features of patients and relative mRNA expression of examined genes. All tests were two sided, p considered significant if < 0.05.

Results

Patient demographics

A total of 101 subjects aged (10–65) years were included in this study. All 71 patients consented for detailed medical history, demographic, and medication profile examination. A summary of the clinical characteristics of all participants are represented in Table 2. The 71 patients presented with a mean Hb of 7.94 ± 1.3, WBC of 7.1 ± 2.99, platelet count of 296.7 ± 237.8, and ESR of $5.9^{*}10^{-4} \pm 5.2^{*}10^{-4}$. Furthermore, 31 (43.66%) patients were positive for CRP. The immunological profiles for RF and ANA showed negative results in all 71 patients.

The mRNA expression levels of the autophagy-related genes

To examine the autophagy-related genes expression in patients with BD, we used qPCR technique to investigate

Table 2 Demographic characteristics of all participants

	Control subjects (*n* = 30)	BD patients (*n* = 71)	*p* value
Age (years) (mean ± SD)[1]	35.46 ± 12.72	35.32 ± 11.77	0.9
Gender[2]	Male 2 (6.67%)	Male 51 (71.83%)	< 0.01
	Female 28 (93.33%)	Female 20 (28.17%)	
Disease duration (years) (mean ± SD)[1]	–	4.67 ± 4.38	–
Family history (Yes/No)[2]	–	8/63	–
Mouth ulcers[2]	–	64 (90.14%)	–
Genital ulcers[2]	–	53 (74.65%)	–
Erythema nodosum[2]	–	6 (8.45%)	–
Pustules[2]	–	18 (25.35%)	–
Arthralgia[2]	–	19 (26.76%)	–
Arthritis[2]	–	10 (14.08%)	–
Major vascular lesions[2]	–	18 (25.35%)	–
CNS complications[2]	–	6 (8.45%)	–
Ocular problems[2]	–	53 (74.65%)	–
GIT illness[2]	–	6 (8.45%)	–
Pulmonary diseases[2]	–	3 (4.23%)	–
Fever[2]	–	3 (4.23%)	–
Headache[2]	–	6 (8.45%)	–
Treatment status[2]			–
Steroid dose (mg)	–	9.72 ± 10.45	
Steroid (Prednison or Solumedrol IV)	–	58 (81.69%)	
Colchicines	–	50 (70.42%)	
Anticoagualtion (Warfarin or Marivan)	–	6 (8.45%)	
Azathioprine	–	35 (49.3%)	
Cyclophosphamide (Endoxan)	–	12 (16.9%)	
Cyclosporin	–	2 (2.82%)	
Biological treatment (Rituximab and Etanercept)	–	2 (2.82%)	

Statistical analysis by: [1]Student t-test and [2]Pearson-chi square

GIT Gastrointestinal tract, *CNS* Central nervous system, *IV* Intravenous

the levels of mRNA expression of *ATG5*, *ATG7*, *ATG12*, *LC3b* and *mTOR* genes as shown in Table 3 and Fig. 1. The expression levels of *ATG5* and *mTOR* were significantly decreased ($p = 0.004$ and 0.01, respectively), whereas that of *LC3b* was significantly upregulated ($p < 0.001$) in the BD patients when compared to those in the healthy controls. No significant differences in the expression levels of *ATG7* and *ATG12* were observed between the two groups ($p = 0.24$ and 0.41, respectively) Table 3.

Table 3 Relative mRNA expression profile of studied autophagy genes

Gene	Control subjects (*n* = 30)		BD patients (*n* = 71)		*p* value
	Median	Interquartile range	Median	Interquartile range	
ATG5	$9.49*10^{-3}$	$(6.52*10^{-3}–1.89*10^{-2})$	$4.62*10^{-3}$	$(2.18*10^{-3}–1.08*10^{-2})$	0.004
ATG7	$5.29*10^{-3}$	$(3.14*10^{-3}–9.74*10^{-3})$	$4.46*10^{-3}$	$(2.16*10^{-3}–9.20*10^{-3})$	0.24
ATG12	$1.70*10^{-2}$	$(1.37*10^{-2}–3.09*10^{-2})$	$1.43*10^{-2}$	$(8.86*10^{-3}–3.35*10^{-2})$	0.41
LC3b	$7.00*10^{-3}$	$(5.39*10^{-3}–1.63*10^{-2})$	$6.69*10^{-2}$	$(2.77*10^{-2}–1.24*10^{-1})$	< 0.001
mTOR	$1.70*10^{-2}$	$(1.37*10^{-2}–3.09*10^{-2})$	$7.44*10^{-3}$	$(5.03*10^{-3}–2.79*10^{-2})$	0.01

Continues data represented as median and interquartile range (IQR), *p* considered significant if < 0.05

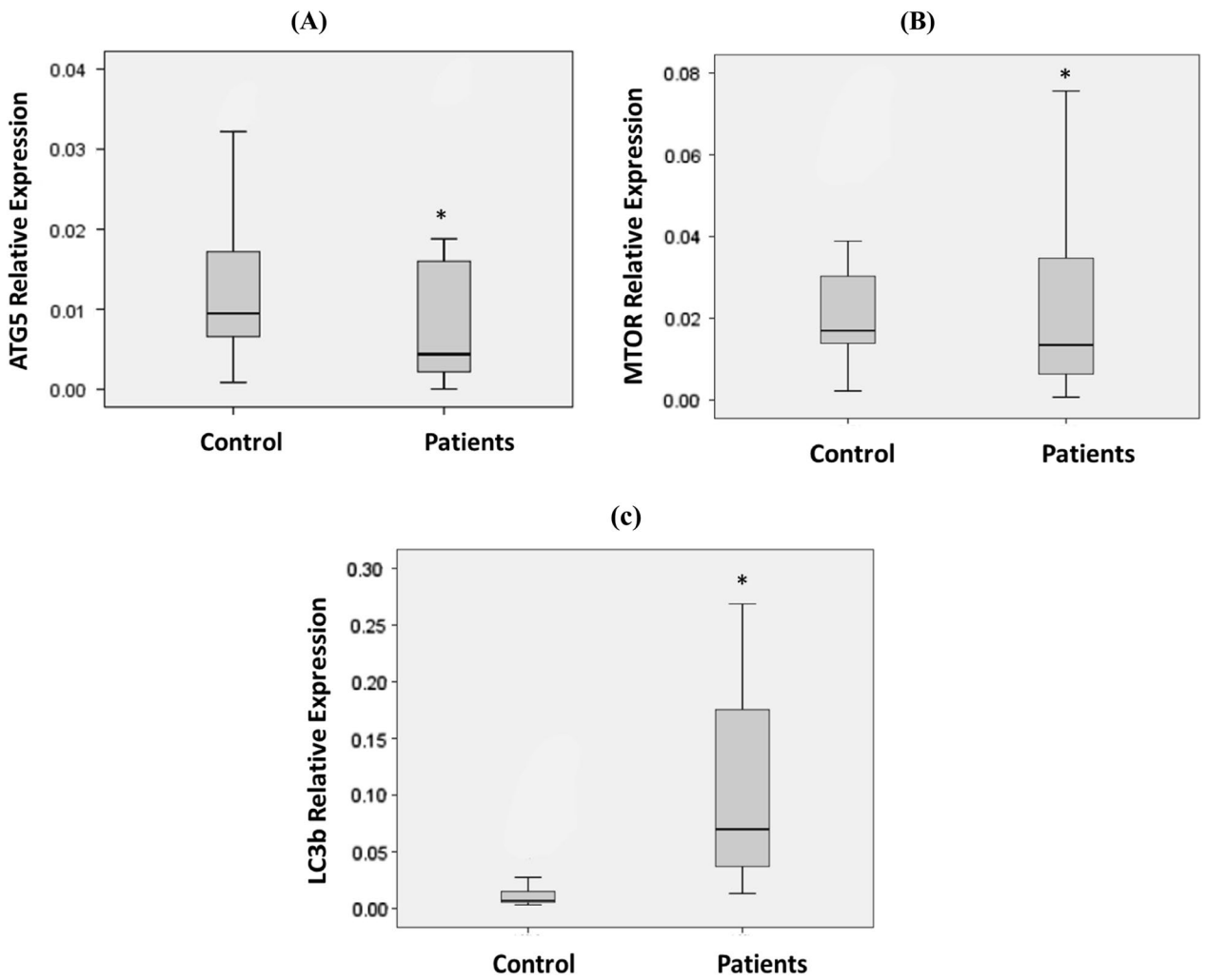

Fig. 1 Relative expression of autophagy-related genes, BD $n=71$, HC $n=30$, **A**: *ATG5*, **B**: *mTOR*, **C**: *LC3b*. Values are expressed as means ± SD.*p* value < 0.05

Association between the expression levels of the autophagy-related genes and the clinical parameters of patients with BD

The clinical features of the 71 patients with BD and the association of each marker with various clinical criteria are examined and shown in Table 4. The mRNA expression level of *ATG5* was significantly associated with patient age (mean = 35.3; $p=0.001$) and the incidence of an oral ulcer (90.1%; $p=0.01$). Likewise, the expression level of *ATG7* was significantly associated with patient age ($p=0.001$) and the presence of erythema nodosum (8.4%; $p=0.02$) and vascular lesions (25.3%; $p=0.04$). LC3b expression was significantly associated with the presence of pustules (25.4%; $p=0.03$).

Association between autophagy markers and BD diagnosis

The ROC curve and cutoff values for the selected autophagy markers were calculated to identify the predictive marker for BD Table 5 and Fig. 2. The accuracies of the expression levels of *ATG5* and *mTOR* were demonstrated by the AUC values (68% and 66%, respectively; $p=0.004$ and 0.01, respectively). *LC3b* expression showed accuracy with an AUC of 88% ($p<0.001$). A cutoff point of > 0.024 for the *LC3b* expression proved most useful for diagnosing BD, with a sensitivity and specificity of 80%. Likewise, a cut point of < 0.0034 showed a sensitivity of 39% and specificity of 90% for *ATG5,* while that of < 0.0081 showed a sensitivity of 54% and specificity of 90% for *mTOR* expression. Despite the increase in the specificity for the expression levels of *ATG5* and

Table 4 Association of disease manifestation with relative mRNA expression of different studied autophagy genes

Genes	*ATG5*		*ATG7*		*ATG12*		*mTOR*		*LC3b*	
	F	***P***	**F**	***P***	**F**	***P***	**F**	***P***	**F**	***P***
Age	4.97	0.000	4.38	0.001	1.18	0.351	0.38	1.00	0.33	1.00
Male	1.67	0.212	2.72	0.112	0.05	0.821	1.24	0.281	0.00	0.961
Family history	1.22	0.281	3.02	0.102	1.30	0.273	0.01	0.912	0.11	0.743
Mouth ulcers	8.87	0.011	1.20	0.293	0.84	0.373	0.26	0.622	0.95	0.343
Genital ulcers	0.27	0.613	0.56	0.463	0.93	0.352	0.34	0.573	0.13	0.722
Erythema nodosum	1.27	0.271	6.08	0.021	0.00	0.972	0.06	0.811	1.06	0.321
Pustules	1.93	0.182	1.34	0.261	0.04	0.851	0.49	0.493	5.83	0.033
Arthralgia	0.47	0.501	0.55	0.472	1.04	0.321	0.04	0.843	0.77	0.391
Arthritis	0.39	0.543	0.71	0.412	1.44	0.242	1.00	0.331	0.01	0.942
GIT illness	0.01	0.922	0.23	0.643	0.70	0.412	0.28	0.602	1.04	0.322
Ocular problems	3.81	0.063	2.98	0.101	0.00	0.963	0.00	0.982	0.05	0.823
CNS complications	0.16	0.691	0.34	0.571	0.83	0.371	1.36	0.263	0.01	0.911
Major vascular lesion	1.36	0.263	4.99	0.043	1.59	0.221	1.00	0.333	0.03	0.862
Pulmonary diseases	0.28	0.602	0.94	0.342	0.85	0.373	0.75	0.402	0.03	0.871

F General linear model with stepwise elimination, *P p* value and *p* considered significant if $p < 0.05$

Table 5 Correlation between autophagy markers and BD diagnosis

	ATG5	*mTOR*	*LC3b*
AUC	68%	66%	88%
Cutoff points	< 0.0034	< 0.0081	> 0.024
Sensitivity	39%	54%	80%
Specificity	90%	90%	80%
PPV	46%	54%	47%
NPV	87%	90%	95%
p value	0.004	0.01	< 0.001

AUC area under curve, *PPV* positive predictive value, *NPV* negative predictive value, *p* value is considered significant if < 0.05

mTOR, their sensitivity values were low. Alternatively, the increase in both the sensitivity and specificity for *LC3b* expression indicated its significant association with BD.

Discussion

BD is a persistent inflammatory multi-systemic condition that affects numerous organs and has broad clinical manifestations involving the mucocutaneous, genital, ophthalmic, vascular, and gastrointestinal systems. The symptoms are more pronounced in those with chronic inflammation and endothelial dysfunction [5]. BD is a form of chronic vasculitis that affects vessels of all sizes and is defined by the presence of recurrent oral ulcers, genital ulcers, and ophthalmic issues (particularly uveitis), thus forming the triple-symptom complex [44]. These manifestations are caused by chronic inflammation due to the infiltration of neutrophils and lymphocytes [45].

BD is equally prevalent among males and females in some geographic regions and more prevalent among males in others [46]. BD is classified as a systemic vasculitis associated with significant morbidity and mortality, particularly with an early age onset in males [47]. In addition, a study by Kural-Seyahi et al. [48] demonstrated that the disease has a more severe course and higher mortality among male patients. Moreover, Maldini et al. [49] stated that both male gender and the presence of the HLA-B51 allele are consistently associated with a severe disease course and poor prognosis in BD. Likewise, HLA-B51 was more common among men with BD [49]. Male patients are more likely to be affected at a younger age, have a more severe uveitis, present with worse visual acuity, and suffer vision loss over time [50].

BD is diagnosed based on clinical features, which are commonly encountered in other autoinflammatory diseases such as (Pemphigus vulgaris, Sjögren syndrome, Parkinson disease, Systemic lupus erythematosus, and Crohn's Disease) [51]. This could lead to an inaccurate diagnosis; hence, the identification of reliable blood markers for a more accurate diagnosis is crucial.

Autophagy is a lysosome-mediated cytoprotective catabolic mechanism that upholds cellular homeostasis by the degradation and reuse of the unwanted intracellular constituents of long-lived proteins and recycling nutrients. These biological processes are performed by autophagy proteins via several pathways that affect the functions of the immune system, especially the production of B and T lymphocytes [24]. Several studies have suggested strong correlations between BD and the autophagy-related inflammatory and immune features

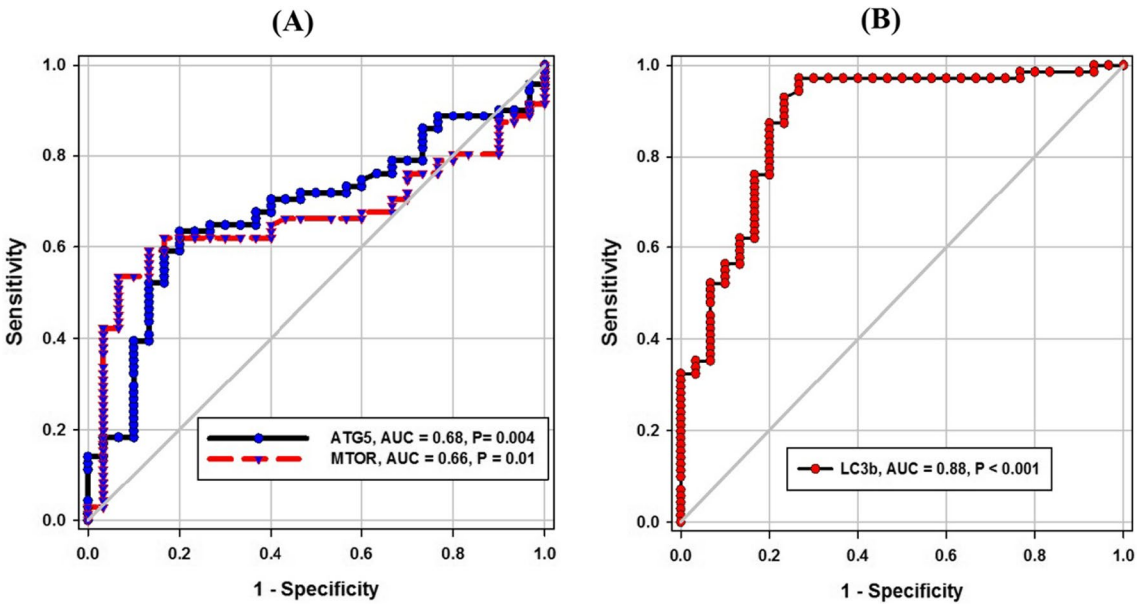

Fig. 2 ROC curve of **A** *ATG5*, *mTOR*, and **B** *LC3b* expression

[52–54]. The dysregulation of some autophagy-related genes has been examined in BD [10, 24].

The present study was conducted to screen a panel of differentially expressed genes related to autophagy (ATG5, ATG7, ATG12, LC3b, mTOR) to ascertain their use as markers for the diagnosis of BD in the Egyptian population. The expression of *LC3b* was upregulated and significantly correlated with the diagnosis of BD. Although the expression levels of *ATG5* and *mTOR* were significantly decreased in the BD patients, they demonstrated low sensitivity. No significant differences in the expression levels of *ATG7* and *ATG12* were observed in the current study. These data were partially in agreement with the quantitative data of a previous study [10], wherein the mRNA expression levels of all ATGs, including ATG5, ATG7, ATG12, LC3b, mTOR, RAPTOR, and RICTOR, were downregulated in M1 macrophages from BD patients when compared to those in healthy individuals. On the other hand, higher levels of *ATG5* mRNA in BD patients compared to healthy controls [24]. The mRNA expression levels in the present study confirm the correlation between the inflammatory complications of BD and the disruption of the autophagy process. A similar finding was reported in another study done by Liang et al. [55] which demonstrated that macroautophagy played a role in Parkinson's disease, a neurodegenerative disorder that shared its autoimmune features with BD; the authors observed abnormal expression levels for *ATG5*, *ATG7*, *ATG12*, and *LC3b*.

Several triggers, including food deprivation [56], hypoxia [57], oxidative stress [58], pathogen infection [59], and endoplasmic reticulum stress [60], can trigger the evolutionarily conserved process of autophagy through different signaling pathways.

Apoptosis can be prevented by the mammalian target of rapamycin (mTOR), which can also encourage cell proliferation in the presence of nutrients and cytokines [61]; while stress and food deprivation prevent mTOR from activating several molecular complexes, comprising the transmembrane protein complex, the PI3K complex, the unc-51-like kinase (ULK) complex, and two ubiquitin-like protein conjugation systems (Atg12 and LC3), to initiate autophagy [62].

Assembly of the ULK complex triggers the start of autophagy by phosphorylating AMBRA1 to activate the PI3K complex. Membrane nucleation is regulated by class III PI3K and Beclin-1. In order to fundamentally prevent the premature fusion of the vesicles and lysosomes, the Atg5- Atg12-Atg16 complex is drawn to the pre-autophagosomal structure (PAS). Here, it makes contact with the phagophore's outer membrane [63].

The second ubiquitin-like system enhances the interaction between phosphatidylethanolamine (PE) and Atg8 / microtubule-associated protein 1 light chain 3 (LC3). A pathogen ingested by LC3 is destroyed and degraded at a higher rate because of its high affinity for the lysosome, while coupled to the phagosome [64]. LC3 is processed into LC3II via Atg4, Atg7, and Atg3 [65] and is necessary for the expansion and completion of the

autophagic membrane and is present on its outer and inner surfaces of it. The Atg5-Atg12-Atg16 complex gets out of autophagosome. Atg9 is necessary for the formation of the intraluminal vesicles and the localized acidification within the autolysosome [66]. It is translocated to the autophagosome formation site, where it provides for the phagophore membrane [67], following which the autophagosome merges with the lysosome to form the autophagosome [68], Fig. 3.

The inflammatory complications of BD may be interpreted by the downregulation of the *ATG5* gene, which inhibits the formation of autophagosomes in mammalian cells or mice, and enhances the inflammatory reaction [57]. These inflammatory reactions may occur due to the hypersensitivity of T cells against multiple antigens. Following T-cell activation, the monocytes are activated through CD40-CD154 interactions and various T-cell-derived cytokines (IFN-γ and TNF-α) to produce IL-12, which enhances the shift to Th1 responses. Consequently, this abnormal T-cell activation leads to neutrophil activation, which can be triggered by cytokines such as IL-8, IL-17, IFN-γ, and TNF-α. Also, circulating proteins (C3, C4, C5, IgA, and Haptoglobin) were increased [69]. Atg5 and Atg16L1-deficient macrophages produce significant amounts of proinflammatory cytokines after lipopolysaccharide stimulation [70]. One study reported a direct association between low *ATG5* levels and autoimmunity; the genome-wide association study showed that many autoimmune diseases, such as multiple myeloma, SLE, rheumatoid arthritis, and systemic sclerosis, were correlated with low *ATG5* levels [71]. Alternatively, another study reported that the upregulation of *ATG5* was associated with blood pressure, insulin sensitivity, and glucose homeostasis [72].

Similar to the findings of the current study, environmental cues, including starvation, were reported to suppress mTOR and repress autophagy in another study [73]. Furthermore, a shortage of nutrients following mTOR inhibition resulted in reduced biosynthesis and increased autophagy in BD patients [74]. *mTOR*

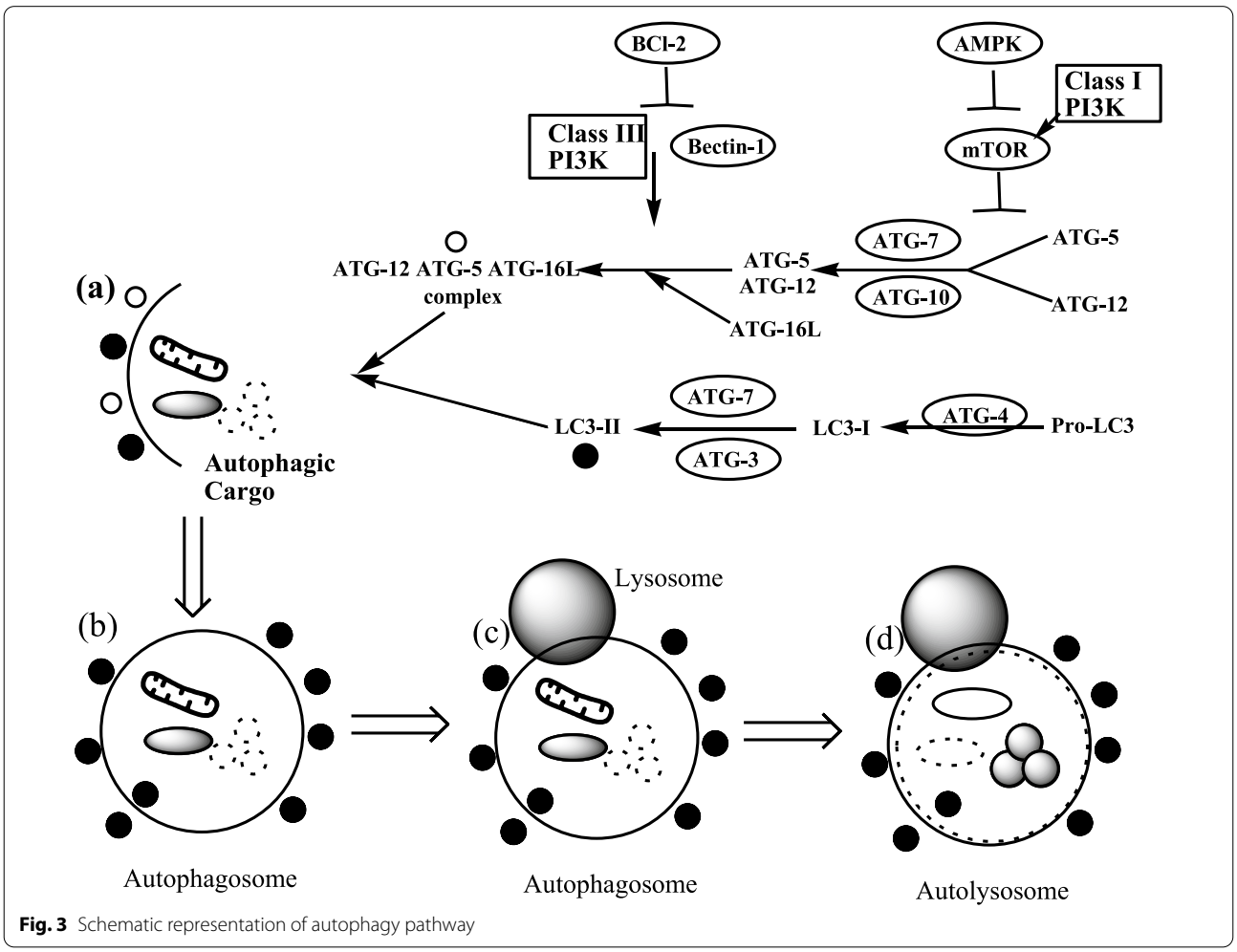

Fig. 3 Schematic representation of autophagy pathway

stimulates anabolic cellular processes that result in growth. Even though autophagy is a fundamental catabolic function in the cell [75, 76]. The conserved serine/threonine kinase *mTOR* belongs to the family of protein kinases called phosphatidylinositol 3-kinase of and is a downstream effector of PI3K/AKT pathway [77].

Inflammation and immunity are intricately correlated with the autophagy pathway and its proteins [78]; autophagy-related proteins play a part in the activation and inhibition of inflammatory and immunological responses. Moreover, inflammatory and immune signals are involved in the activation and inhibition of autophagy, Fig. 4. Therefore, the pathophysiology of many infectious diseases and inflammatory disorders may be caused by deficiencies in autophagy, which might be caused by mutations in the autophagy gene.

The energy depletion could activate adenosine monophosphate (AMP) protein kinase and further stimulate the mTOR substrate complex to amplify the formation of autophagosomes [79]. mTOR has a core function in controlling metabolic programs and is essential for linking metabolism with immune functions [80]. The metabolic apparatus involved in food uptake and glycolysis must be upregulated for T cells to function properly. Thus, mTOR inhibition contributes to the inhibition of T-cell function [81]. Studies have indicated that the activity of mTOR may play a crucial role in incorporating immunological microenvironment cues to control the differentiation of helper cells [82].

LC3 is considered as an autophagy factor that exists in a soluble (LC3-1) or lipidated (LC3-II) form [83]. LC3-1 is transformed into LC3-II and degraded after the fusion of the lysosomal autophagosomes. Moreover, LC3 is a crucial protein in the autophagy pathway, where it plays a role in the selection of the autophagy substrate and biogenesis of the autophagosome [84]. LC3 has been extensively utilized to track the number of autophagosomes and the autophagic activity [85]. Therefore, the quantity

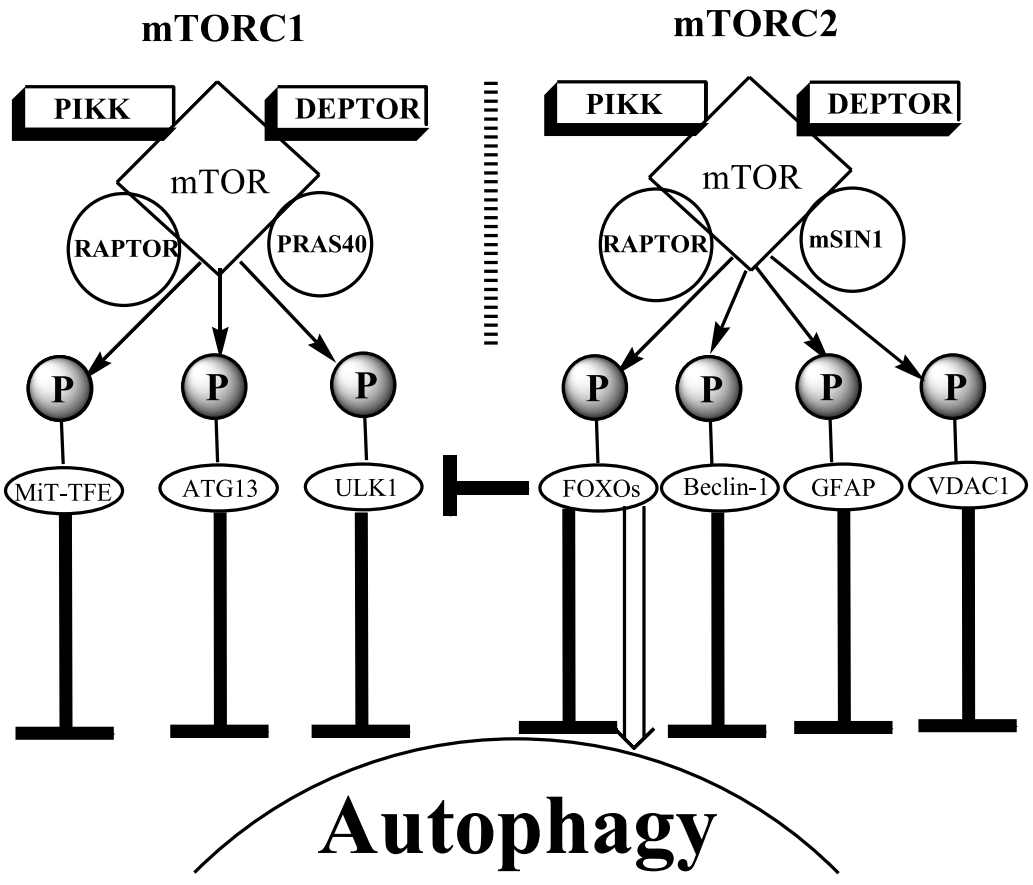

Fig. 4 The two different mTORC1/2 complexes and how autophagy is regulated by them. DEPTOR and PIKK are components shared by *mTOR* complexes, whereas PRAS40 and RAPTOR are special to mTORC1 and RICTOR and mSIN1 are limited to mTORC2. When mTORC1 phosphorylates the MiT-TFE factors, *ATG13* and ULK1, autophagy cannot be positively regulated. Subsequently, Beclin-1, GFAP, and VDAC1 are phosphorylated by mTORC2 to prevent them from also inducing autophagy. The subcellular location of FOXO proteins is altered by mTORC2-mediated phosphorylation, which has a variety of effects on the control of autophagy

of LC3 is associated with the number of autophagosomes and is considered as a predictor of autophagic behavior [30]. However, increased LC3 expression cannot reliably represent increased autophagic activity. It may also suggest a decrease in autophagic function due to fusion blocking following an increase in the number of autophagosomes [34], which may explain the high expression level of LC3b in the current study. A previous study stated that LC3 is a major way of autophagy in phagocytes such as dendritic cells (DCs) and macrophages to remove intracellular pathogens; thus, autophagy involves LC3 and pathogens in single-membrane phagosomes [86].

Moreover, it is clear that in BD there is an ongoing autoimmune cascade resulting from signals from infected cells that the host releases. This immune reaction would override any outside influences. On a favorable genetic landscape, T cells and other antigen-presenting cells would direct the cycle to continue. Uncontrolled adaptive responses would be activated and allowed to continue as a persistent pathogenic presence via autoantigens that activate the dendritic T cells and B cells [87]**.** Both adaptive and innate immune systems are stimulated with a proinflammatory and Th1-type cytokine profile in BD. BD might be accompanied by a genetic mutation that affects an adhesion molecule, a proinflammatory cytokine/chemokine, a transcription factor, or a regulatory component and predispose to early or more intense neutrophil and T-cell responses. This concept, which also explains the "pathergy" or "skin urate" tests, is represented by the increased neutrophil response to urate crystals [88].

In the current study, associations between the expression levels of the autophagy genes and the clinical characteristics of the BD patients were examined at baseline. The mRNA expression level of *ATG5* was significantly associated with patient age and the incidence of oral ulcers, whereas that of *ATG7* was significantly associated with patient age and the presence of erythema nodosum and vascular lesions. However, *LC3b* showed a significant association with the presence of pustules. One study reported that *ATG5*-deficient mice, particularly in the neural cells, may develop gradual impairment of motor function with increased cytoplasmic inclusion bodies in the neurons [89]. In another study, mice lacking the *ATG5* gene died within one day after giving birth [57]. Furthermore, elevated expression levels of several *ATG* genes were found to be significantly linked to a high patient survival rate, thereby indicating the potential of Atg proteins as useful prognostic markers [90].

Conclusion

The present study showed that *LC3b* expression might prove as an accurate and reliable diagnostic marker for patients with BD. The expression of this gene was correlated with the presence of pustules, whereas low levels of *ATG5* were associated with the presence of oral ulcers in patients with BD. Nonetheless, additional national and international collaborative efforts using larger sample sizes are warranted to conduct genetic and/or molecular studies to confirm the findings of this study and identify the most reliable marker for BD.

Abbreviations

BD: Behçet's disease; ATG: Autophagy-related gene; Atg: Autophagy-related protein; LC3: Light chain 3; mTOR: Mammalian target of rapamycin; VVV: Variable vessel vasculitis; HLA-B51: Human leukocyte antigen; RAPTOR: Regulatory associated protein of mTOR; RICTOR: Rapamycin-insensitive companion of mTOR; mTORC1: MTOR complex 1; mTORC2: MTOR complex 2; TLR: Toll-like receptor; ROC: Receiver operating characteristic; AUC: Area under a curve.

Acknowledgements

I thank all of my research team; Dr. Eman, dr. Rania, dr. Abeer and dr. Shimaa. Also I thank my father and my mother for caring my baby girl, Mariam to help me continue my study.

Author contributions

All authors have read and approved the final version of the manuscript. Dr. E.M.S.: is the head of the supervision. Dr. A.R.L.A. and Mrs. D.N.S.: contributed to samples collection from patients in Kasr Alainy school of Medicine; Cairo university. Dr. A.R.L. in addition contributed to full clinical diagnosis of all patients. Dr. A.R. and Mrs. D.N.S.: contributed to conducting practical tests of molecular biology of our study. Dr. R.H.M.: contributed to conducting statistical tests of our study, and with Mrs. D.N.S.: writing and revise the manuscript.

Funding

The study was done on my personal account.

Availability for data and materials

The datasets used and / or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Since the study was done on humans, the approval of Ethics Committee was taken from the National Research Center, Medical Research Ethics Committee, and final ethical approval number: 19047, on May 2, 2019. This was according to the relevant Egyptian laws and with Helsinki Declaration, good medical and laboratory practice (GCP and GLP) guidelines as well as World Health Organization rules regarding the Ethics of scientific research.

Consent for publication

All authors permitted publication and written license was received.

Compting interests

The authors declare that they have no competing interests.

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Received: 5 April 2022 Accepted: 10 November 2022
Published: 23 November 2022

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