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Original Article

Expression of Beclin1 in the colonic mucosa tissues of patients with ulcerative colitis

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Abstract: To investigate the expression of Beclin1 in the colonic mucosa tissue of patients with ulcerative colitis (UC), which acts as a regulator of autophagy and might play a part in the disease progression potentially. A total of 112 patients were selected from September 2013 to November 2014, and their colonic mucosal tissues were collected as the subject of study. Among them, 75 cases were diagnosed with ulcerative colitis (UC), 37 cases were diagnosed with irritable bowel syndrome (IRS) during the same time, which was set as the control group. The mucosal tissues were processed with ELISA and IHCA to measure the expression level of Beclin1, and correlation analysis was performed to demonstrate its role in the disease progression. The expression level pf Beclin1 was significantly higher in the UC patients compared with the control group ($P<0.05$). Meanwhile, it's positively correlated with the severity of disease, the endoscopic classification and the pathologic staging results, which has statistical significance ($P<0.05$). Beclin1 was expressed at a higher level in UC patients, and correlated with the severity of the disease, indicating the abnormal regulation of autophagy in the disease progression.

Keywords: Beclin1, autophagy, ulcerative colitis, inflammatory bowel disease, irritable bowel syndrome

Introduction

Ulcerative colitis (UC) is a chronic inflammatory condition which is characterized by relapsing and remitting episodes of inflammation limited to the mucosal and the submucosal layer of the colon. It almost invariably involves the rectum and typically extends in a proximal and continuous fashion, so the other portions of the colon could also be affected [1]. Although the pathogenesis of inflammatory bowel disease (IBD) remains unclear, a number of risk factors have been identified, including environmental factors such as smoking and diet [2], or genetic susceptibility [3]. Patients with UC usually present with diarrhea which is frequently associated with blood. Associated symptoms include colicky abdominal pain, urgency, and tenesmus. Fever, fatigue, and weight loss could also occur [4]. The molecular mechanism of the pathogenesis of UC has been proposed to be connected with the disruption of intestinal mucosal homeostasis between injury and protection.

Autophagy is an evolutionarily conserved catabolic pathway in which cytoplasmic components are sequestered in vacuoles known as autophagosomes, and degraded by lysosomes [5]. It is a cytoprotective mechanism that protects cells exposed to situations of stress, such as toxins and metabolic stress [6]. The formation of autophagosome, including nucleation and expansion of the autophagosomal membrane, is dependent on the activity of Atg proteins, including the Beclin1 complex, the Atg12 and light chain 3 systems [7]. Additionally, the disruption of its regulation has been connected with the pathogenesis of multiple diseases, such as infectious diseases, neurodegenerative disorders, autoimmune diseases, and malignancies [8, 9]. The aim of this study is to investigate the role of Beclin1 in the pathogenesis of UC by measuring its expression level in the patients' colonic mucosa, and further dem-

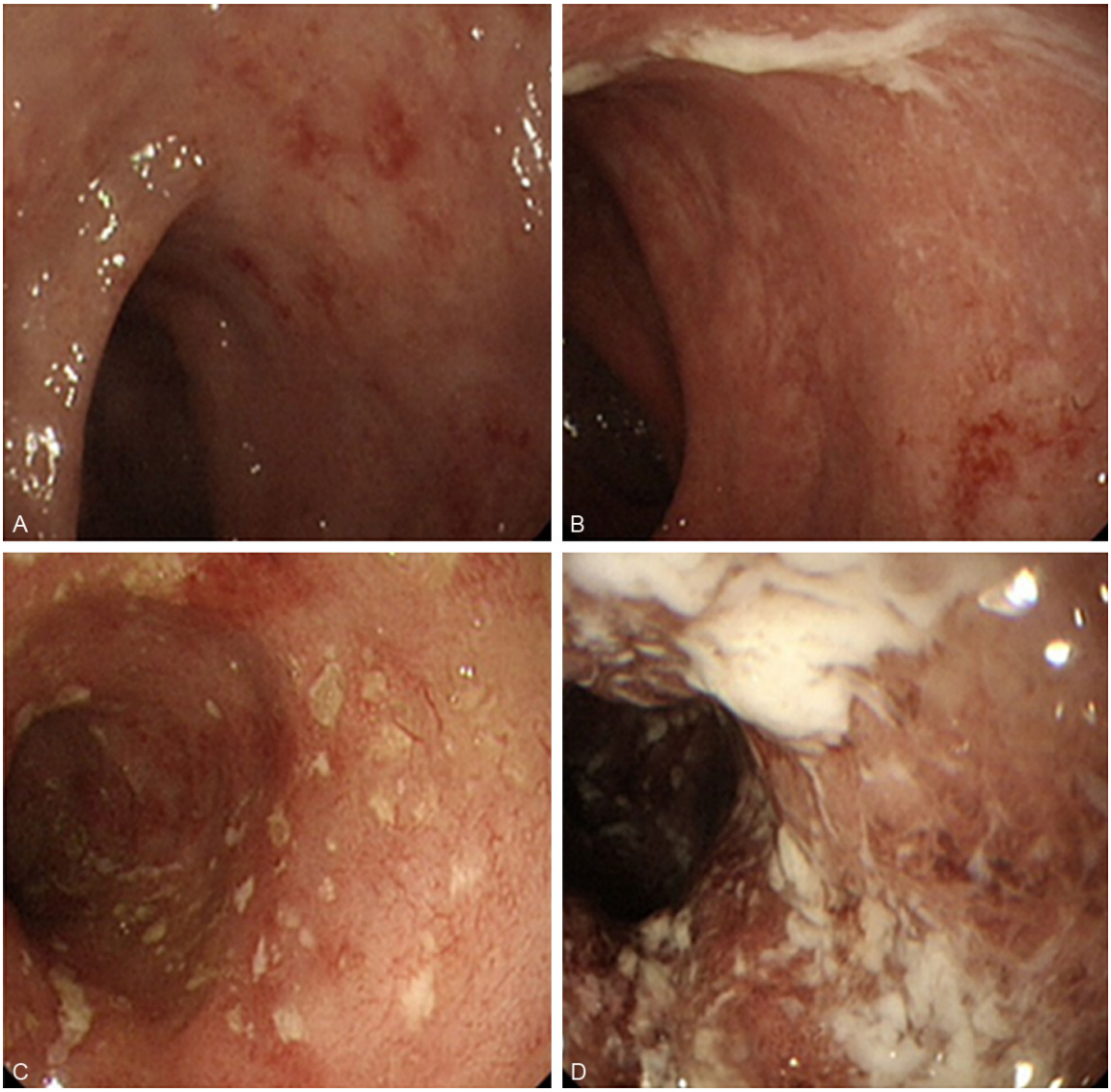

Figure 1. The mucosal findings of the rectum from patients of different endoscopic gradings. A. Grade I; B. Grade II; C. Grade III; D. Grade IV.

onstrate that Beclin1 could potentially be used as a clinical marker.

Materials and methods

Study design and participants

The authors independently coordinated the trial, managed the database, performed the analyses, and wrote the drafts of the manuscript. 75 cases of symptomatic inpatients and outpatients with diagnosed UC under endoscope in the department of gastroenterology were collected from Sep 2013 to Nov 2014. Among them, there included 40 male and 35 female, whose age ranged from 20 to 78 and the mean age was (49.84±14.93). The diagnoses were made with the reference from the Diagnostic Criteria of UC from the Chinese Society of Gastroenterology in Chinese Medical Association (CMA). Meanwhile, another 37 patients diagnosed with irritable bowel syndrome (IRS) were recruited into the study as the external control group (21 male, 16 female, mean age 41.81±13.99). There were no statistical difference in the age and gender distribution between the two groups. The local research ethics committee approved the study protocol and written informed consent was obtained from all patients. The following information was

Table 1. The expression of Beclin1 in UC group and control group

Groups	n	Beclin1 (ng/mL)	t	P (two-tails)
UC	75	2.54±1.20	3.22	0.002
Control	37	1.77±1.19		

Table 2. The expression of Beclin1 in the affected tissues and normal tissues from the UC group

Tissues	n	Beclin1 (ng/mL)	t	P (two-tails)
Affected	48	2.38±1.24	7.71	0.000
Normal	48	1.00±0.42		

collected for each participant: baseline characteristics (gender, age, family history); symptomatic presentation (stool property, frequency of bloody purulent stool, abdominal pain, etc.); laboratory tests (white blood cells, hemoglobin, erythrocyte sedimentation rate, serum albumin, etc.); endoscopic examination results, complications, pathological examination results, malignancy, etc. Specimens were collected simultaneously from the lesion sites (rectum, sigmoid colon) and the normal tissues (ascending colon 5-10 cm from the ileocecal valve), the latter was used as an internal control. The IBS patients examined at the same day were used as the external control, with the specimens collected at the same sites.

Staging criteria

The severity of disease was based on the ulcerative colitis practice guidelines in adults from American College of Gastroenterology [10]: Mild (S1)-patients have four or fewer stools per day, with or without blood, no signs of systemic toxicity, a normal erythrocyte sedimentation rate (ESR); Moderate (S2)-patients with moderate clinical disease have frequent loose, bloody stools (>4 per day), mild anemia not requiring blood transfusions, and abdominal pain that is not severe; Severe (S3)-patients with a severe clinical presentation typically have frequent loose bloody stools (≥6 per day) with severe cramps and evidence of systemic toxicity as demonstrated by a fever (temperature ≥37.5°C), tachycardia (HR ≥90 beats/minute), anemia (hemoglobin <10.5 g/dL), or an elevated ESR (≥30 mm/hour).

The endoscopic extent and severity of UC [11]: I-mucosal hyperemia, fuzzy vascular network, ecchymosis; II-mucosal hyperemia and edema, increased brittleness, visible superficial ulceration, contact bleeding; III-ulcer fusion, spontaneous bleeding, exudation; IV-massive ulcer fusion, large amounts of mucus and bloody exudate (**Figure 1**).

The pathological staging standard [12]: I-mucosa lamina propria infiltrated by a small number of neutrophils (<10/HPF), a small amount of crypts are involved; II-mucosa lamina propria infiltrated by more neutrophils (10~50/HPF), more than 50% of the crypts are involved; III-mucosa lamina propria infiltrated by a large number of neutrophils (>50/HPF), crypt abscess; IV-apparent acute inflammation and ulceration in lamina propria.

Study reagents and instruments

One part of the specimens collected was fixed with 10% formaldehyde, and the other part was placed in the Corning 2.0 ml tubes and cryopreserved in the -70°C refrigerator. Other reagents and instruments used in the sample processing including: Beclin1 ELISA kit (Cloud-Clone Corp, USA), MS-352 microplate reader (Finland), AC8 microplate washer (Finland), mice anit-human Beclin1 monoclonal antibody (SC48-381, Santa Cruze Biotech, USA), sheep anti-rabbit/mice antibodies labeled by HRP (Zhongshanjingqiao Biotech, China), double-amino benzidine (DAB) chromogenic reagent kit (Zhongshanjingqiao Biotech, China).

ELISA for Beclin1

The specimens were homogenized and the proteins were extracted. 50 μL solutions, either consisting of 10 μL sample was mixed with 40 μL diluent or standard solutions with a range of concentrations, were added into the holes on the microplates. 50 μL antibody solutions labeled with HRP with were added into the holes, which then were sealed by the microplate sealers. The microplate was then incubated in the water bath (37°C) for 60 min. The solutions in each hole were disposed and washing buffer was added into each hole, standing for 1 min and the washing buffer was then disposed. The microplate was washed like this for 5 times. 50 μL reaction substrate A and B were injected into the holes, which were then incubated in darkness (37°C) for 15 min until 50 μL stopping buffer was added. The OD value at the

Table 3. The expression of Beclin1 in the affected tissues from patients of different disease characteristics

Disease characteristics	n	Beclin1 (ng/mL)	t	P (two-tails)
Severity	75		0.477	0.000
Mild	31	2.03±0.88		
Moderate	26	2.36±1.02		
Severe	18	3.69±1.20		
Involvement	75		0.244	0.035
Rectum	15	2.18±1.10		
Sigmoid	20	2.45±1.42		
Left colon	13	2.51±1.15		
Total colon	27	2.81±1.08		
Endoscopic grading	75		0.555	0.000
I	26	1.97±0.87		
II	26	2.09±0.81		
III	10	3.36±1.01		
IV	13	3.94±1.14		
Pathological grading	75		0.722	0.000
I	29	1.76±0.90		
II	18	2.22±0.52		
III	14	2.94±0.68		
IV	14	4.15±1.04		
Clinical pattern	75		0.092	0.435
Primary	18	2.45±1.19		
Chronic recurrent	36	2.48±1.08		
Chronic continuous	17	2.43±1.19		
Explosive	4	3.90±1.88		

Table 4. The immunohistochemical grading of the expression of Beclin1 in the UC group and control group

	UC (%)		Control (%)	
0	16	14.0	18	15.8
+	21	18.4	13	11.4
++	18	17.5	6	5.3
+++	20	65.8	0	0
Total	59	78.67	19	51.35

Pearson test x^2=17.072, P=0.001.

wavelength of 450 nm was measured for each hole within 15 min. According to the standard working curve plotted based on the standard solutions, the concentration of Beclin1 for each specimen was then measured.

Immunohistochemical assay for Beclin1

To quantify the Beclin1 expression in the tissues with the immunohistochemical method. The specimens were fixed with formaldehyde, embedded with paraffin and cut into sections as thick as 5 μm. The sections were processed according to the routine protocols, and the primary antibody (mice anti-human Beclin1 antibody) of the appropriate concentration was then added onto the slides. After incubating in the 4°C refrigerator for one night, the secondary antibodies (sheep anti-rabbit/mice antibodies) labeled with HRP were added and incubated for another 30 min in room temperature. Then the solution was washed and developed according to the routine protocol. Another set of solution with the primary antibody replaced by PBS solution was used as the negative control.

It's already known that Beclin1 is distributed widely in the cell membrane and cytoplasm. Two experienced pathologists were required to review each slice independently by selecting 5 high power fields (×400) on each slice, counting 100 cells in each field, and scoring the expression of Beclin1. The scoring system was determined as follows: 1) based on the signal intensities: no staining 0; faint yellow 1; brown yellow 2; brown 3. 2) based on the percentage of positive cells: <5% 1; 5%-30% 1; 30%-60% 2; 60%-90% 3; >90% 4. The two scores based on signal intensities and percentage of the positive cells were then multiplied to generate the total scores. And the total scores were graded as follows: negative (-) 0; weak positive (+) 1-4; moderate positive (++) 5-8; strong positive (+++) 9-12.

Statistical analysis

Statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, USA). The quantitative data were represented with x±s, and the comparisons between groups were performed with t-test. The expression levels of Beclin1 in the tissue from patients of different disease staging were further compared by ANOVA analysis. The immunohistochemical results were analyzed with Pearson test. The Spearman rank correlation analysis was used

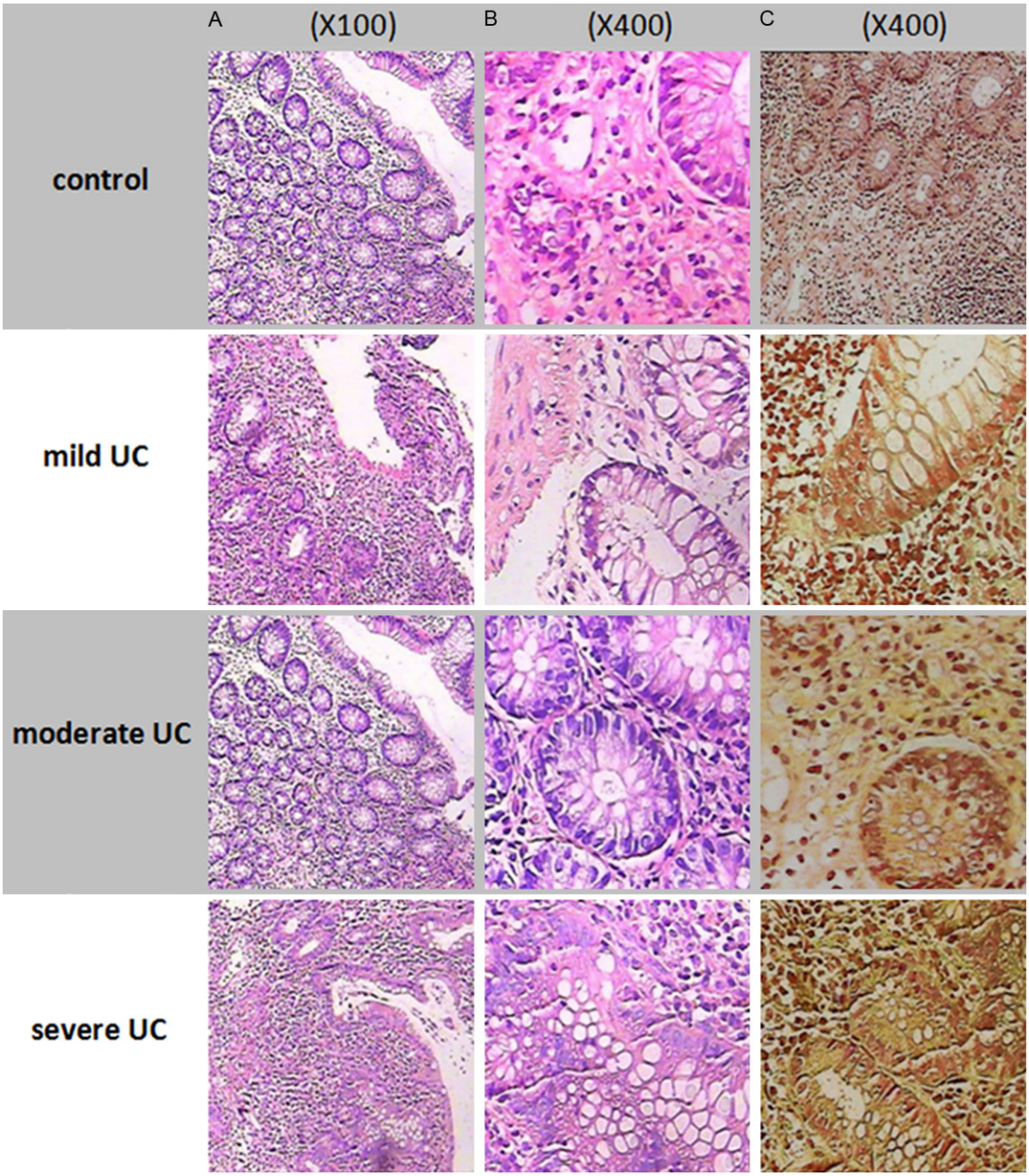

Figure 2. The HE staining results of multiple lesion tissues (A: ×100; B: ×400) and the immunohistochemical staining results of Beclin1 expression in the affected tissues (C: ×400).

to analyze the correlation among groups. *P* value <0.05 was considered statistically significant.

Results

The overall expression level of Beclin1, which was quantified by ELISA, was much higher in the tissues from the UC patients, compared to the corresponding tissues from the IRS patients (**Table 1**). And the difference was significant (P=0.002). The different types of tissues within the UC patients were reviewed by comparing the Beclin1 expression in the lesion tissues and unaffected tissues (**Table 2**). Similar results were observed that the Beclin1 was more expressed in the lesion tissues (P<0.05). Further analysis was performed to investigate

Table 5. The immunohistochemical grading of the expression of Beclin1 in the affected tissues from patients of different disease characteristics

Disease characteristics	n	Beclin1 positive (%)	x^2	P
Severity	75		6.92	0.03
Mild	31	20 (33.90)		
Moderate	26	22 (37.3)		
Severe	18	17 (28.8)		
Involvement	75		7.24	0.07
Rectum	15	11 (18.6)		
Sigmoid	20	18 (30.5)		
Left colon	13	7 (11.9)		
Total colon	27	23 (39.0)		
Endoscopic grading	75		8.77	0.03
I	26	16 (27.1)		
II	26	21 (35.6)		
III	10	10 (16.9)		
IV	13	12 (20.3)		
Pathological grading	75		8.91	0.03
I	29	19 (32.2)		
II	18	13 (22.0)		
III	14	13 (22.0)		
IV	14	14 (23.7)		
Clinical pattern	75		2.00	0.57
Primary	18	15 (25.4)		
Chronic recurrent	36	28 (47.5)		
Chronic continuous	17	12 (20.3)		
Explosive	4	4 (6.8)		

the correlation of Beclin1 expression with the disease characteristics, including the disease severity, disease extent, endoscopic grading results, pathological grading results and the clinical classification (**Table 3**). It has been shown that except for the clinical classifications, the Beclin1 expression levels had been correlated with the other disease characteristics.

Meanwhile, the immunohistochemical measurement showed similar results. There was almost no Beclin1 expressed in the tissues from the control group, while various levels of the protein expression were observed in the UC patients (**Table 4**; **Figure 2**), and correlated with the disease characteristics (**Table 5**; **Figure 2**).

Discussion

Autophagy plays an essential role in maintaining the balance of protein metabolism, energy supply in normal conditions and under cell stress [13], through intracellular lysosomal digestion of aging, damaged the subcellular organelles or incorrectly folded protein. Thus, this process is important in clearance of waste, reconstruction and development [14]. In normal cells, autophagy is kept at a lower level to maintain cell homeostasis [15]. However, autophagy is induced immediately to clear intracellular excess or damaged subcellular organelles and intracellular components, in response to any growth signals or cellular stress, such as ionizing radiation, hypoxia ischemia, and convulsion [16]. In that way, cells are struggled to accustomed to certain environments and survive.

Beclin1 gene, also known as BECN1 gene and homology of yeast ATG6, is a specific gene related to autophagy in mammals. Liang group [17] discovered a protein with 60 ku in the rat model of lethal Sinbis viral encephalitis in 1998 and the gene was named Beclin1. One year later, Aita and others [18] demonstrated that Beclin1 is located in human chromosome 17q21, encoding a 2098 bp-long cDNA sequence, which is highly homologous with yeast autophagy genes apg6/vps30.

Beclin1 was then identified as a critical regulator in the process of autophagy by targeting the autophagy genes (ATG) [9]. Therefore, the expression level of Beclin1 can be an important index for the level of autophagy.

Ulcerative colitis (UC) is an autoimmune disease [19], with features of difficulty in clinical diagnosis, recurrence, poor prognosis, and lack of predictive biomarkers. Recent studies have illustrated that autophagy facilitates the processes of immune response, via clearance of damaged organelles and intracellular pathogens, and participation in the antigen presentation by endogenous MHC-II [20, 21]. UC is predominantly a non-specific intestinal inflammatory disorder, however, it remains unclear whether evaluation of ATG expression can provide deep insight in better understanding of UC.

In this study, we demonstrated that the autophagy protein Beclin1 was mildly expressed in normal colon mucosa and extensively up-regulated in patients with UC. Additionally, the expression significantly increased, as with the progression of the disease. Consistently, the

correlation between the severity of IBD and autophagy level has been demonstrated, assessed by mRNA levels of IRGM (immune-related guanosine triphosphate: an autophagy-related protein). The expression of IRGM in patients with IBD (including UC and CD) was significantly higher than that in control group, and the expression of IRGM increased dramatically with the aggravation of the disease [22]. All these suggest that excess autophagy might be associated with the progression of IBD, and IRGM and BECN could be used potential biomarkers for the diagnosis and assessment of IBD.

In our study, the differential expression of BECN1 in colon mucosa of patients with UC (UC group) and normal control (control group) was assessed by ELISA and immunochemistry. Consistent with previous studies, excessive autophagy was determined in UC group, possibly suggesting its association with colonic inflammation, pathogen infection, auto-immune response and hypoxic ischemic stress. Studies have elucidated the autophagy-mediated negative regulation of inflammatory response, triggered upon PAMP-TLR signaling [23, 24]. Furthermore, autophagy, as a cellular protective strategy, can be induced by ischemia and hypoxia of colon mucosa [25], providing alternative energy sources and removing abnormal organelles and protein. In addition, autophagy occupies an important role in immune mediation, through facilitating the processes of antigen presentation, thymus selection and lymphocyte development, homeostasis maintenance and so forth [26-30]. Autophagy also has impact on vascular functions, including angiogenesis, vascular inflammation and vascular calcification [29-33], which may correlate with the microcirculation disturbance and microthrombus formation of colonic mucosa in the development of UC. Of course, further investigations focused on these questions are needed to clarify the precise mechanism of autophagy in the modulation of UC progression.

In the current investigation, we confirmed the abnormal autophagy status in ulcerative colitis. It is well acknowledged that autophagy is a double-edged sword, the detailed mechanism in the progression of UC remains to be further confirmed. Further clarifications as to whether autophagic inflammatory cells or autophagic colonic cells are causative and whether interference with autophagy with autophagy inhibitor is effective are required.

Disclosure of conflict of interest

None.

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