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The Role of High Expression of BECN1 and CD68 in Prognostic Evaluation for Colorectal Cancer Patients

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Abstract: Objective: To explore the changes in the expression of autophagy-related protein BECN1 and tumor-associated macrophage marker CD68 in colorectal cancer and investigate their association with clinicopathological features and prognosis in colorectal cancer patients. Methods: Sixty colorectal cancer patients were selected as study subjects. Immunohistochemistry was used to detect the expression of BECN1 and CD68 in both colorectal cancer tissues and adjacent non-cancerous tissues. Based on immunohistochemistry results, patients were divided into a BECN1 high-expression group (33 cases) and low-expression group (27 cases) and a CD68 high-expression group (33 cases) and low-expression group (27 cases). Survival rates and survival times of the two groups were compared using the Kaplan-Meier survival curve and Log-Rank test. The correlation between BECN1 and CD68 expression in colorectal cancer tissues was analyzed. RT-qPCR was employed to examine changes in macrophage-associated markers after BECN1 interference. Results: Expression levels of BECN1 and CD68 in colorectal cancer tissues were significantly higher than those in adjacent tissues and were positively correlated with the TNM stage. Survival analysis showed that patients in the BECN1 and CD68 high-expression groups had shorter overall survival compared to those in the low-expression group ($P < 0.05$). BECN1 and CD68 levels in colorectal cancer patients were positively correlated ($P < 0.001$). BECN1 interference markedly reduced the expression of macrophage markers and decreased M2 polarization. Conclusion: Abnormal expression of BECN1 and CD68 in colorectal cancer patients is associated with TNM stage and poor prognosis, suggesting that BECN1 and CD68 can serve as important indicators for postoperative prognostic evaluation.

Keywords: Colorectal cancer; Autophagy; BECN1; Tumor-associated macrophages; CD68

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1. Introduction

The incidence of colorectal cancer (CRC) has risen to third among all malignancies, while mortality ranks second [1],

with the incidence rate showing a yearly upward trend [2]. Currently, surgical resection combined with radiotherapy and chemotherapy remains the primary treatment approach for colorectal cancer; however, prognosis remains poor for many patients with advanced disease. Therefore, further exploration of biomarkers and therapeutic targets that impact colorectal cancer prognosis is urgently needed, with the aim of achieving precise prognostic evaluation and personalized treatment.

Beclin-1 (BECN1) is a key regulatory factor in cellular autophagy that controls autophagy induction and other cellular transport processes [3]. Recent studies have shown that BECN1 can influence tumor drug resistance and the maintenance of cancer stem cell properties, thus playing a role in the occurrence and development of various tumors [4]. Increasing evidence suggests that autophagy also regulates the plasticity and functional polarization of tumor-associated macrophages (TAMs) in the tumor microenvironment, further affecting tumor progression [5]. CD68 is a transmembrane glycoprotein primarily expressed on macrophages and is a key biomarker for quantifying total TAMs. In colorectal cancer tissues, the count of CD68-positive TAMs is positively correlated with tumor invasion depth, lymph node metastasis, and tumor stage [6]. However, there are no reports in the literature regarding the expression patterns of CD68 and BECN1 in colorectal cancer tissues and their impact on CRC prognosis.

Given this, the present study evaluated the role of BECN1 and CD68 in the progression of colorectal cancer. Analysis of BECN1 and CD68 expression in colorectal cancer revealed abnormal expression in cancer tissues, with negative correlations to clinical prognosis. Additionally, BECN1 interference significantly reduced macrophage marker expression and decreased M2 macrophage polarization, indicating that BECN1 and CD68 could serve as potential molecular therapeutic targets for treatment and prognosis in colorectal cancer.

2. Materials and methods

2.1. General information

Sixty cases of colorectal cancer and adjacent non-cancerous tissues were selected from patients treated at Yantai Shan Hospital, affiliated with Binzhou Medical University, between January 2015 and September 2020. Among these, 18 patients were male and 42 were female, aged between 33 and 73 years, with an average age of 56.19 ± 10.55 years. TNM staging revealed 30 cases in stages I–II and 30 cases in stages III–IV. Inclusion criteria: confirmed colorectal cancer by pathological biopsy; complete clinical and follow-up data. Exclusion criteria: preoperative radiotherapy or chemotherapy; loss of contact during follow-up; presence of other malignant tumors. This study was approved by the Ethics Committee of Binzhou Medical University (Approval No. 2022-121).

2.2. Cell lines, antibodies, and reagents

Table 1 shows the cell lines, antibodies, and reagents used in this experiment.

Table 1. Cell lines, antibodies, and reagents

Cell line/antibody/reagent	Company	Cell line/antibody/reagent	Company
THP-1 cells	Servicebio	IL-4, IL-13	Univ-bio
PMA	MCE	RT-qPCR reagents	Vazyme
Fetal bovine serum	Cellmax	Mouse anti-human CD68 monoclonal antibody	Santa Cruz Biotechnology
BECN1 interference RNA	Ribobio	Mouse anti-human BECN1 monoclonal antibody	Proteintech
Lipo2000	Vazyme	Hematoxylin, neutral resin	Solarbio

2.3. Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining was performed according to the HE staining kit (Beijing Solarbio Bioscience & Technology Co., Ltd.) instructions:

(1) Deparaffinization to water: xylene I for 5 minutes, xylene II for 5 minutes, absolute ethanol for 2 minutes, 95% ethanol for 1 minute, 80% ethanol for 1 minute, 75% ethanol for 1 minute, and rinsed with running water.
(2) Nuclear staining: stained with hematoxylin for 5 minutes, and rinsed with tap water for 20 minutes.
(3) Eosin staining: stained with 80% eosin for 1 minute, and eosin for 16 seconds.
(4) Hydration: 80% ethanol for 1 minute, 95% ethanol for 1 minute, absolute ethanol for 1 minute, and xylene for 1 minute.
(5) Mounting: sealed with neutral resin.

2.4. Immunohistochemical staining

DAB staining was used to detect the expression of BECN1 and CD68, following the polymer method kit (Shanghai Gene Technology Co., Ltd.) instructions:

(1) Deparaffinization to water: Paraffin sections were deparaffinized in xylene and gradient ethanol to water, then rinsed three times with PBST containing 0.25‰ Triton X-100 for 5 minutes each.
(2) Blocking endogenous peroxidase: Incubated with a non-specific staining blocker for 10 minutes to remove endogenous peroxidase, then rinsed three times with PBST.
(3) Antigen retrieval: Added citric acid antigen retrieval solution and used microwave heating to expose antigen sites; cooled to room temperature and rinsed three times with PBST.
(4) Blocking: Blocking with 10% goat serum (PBST-diluted) at room temperature for 30 minutes.
(5) Primary antibody overnight: added primary antibody (BECN1 1:1000, CD68 1:500) and stored at 4°C overnight.
(6) Secondary antibody and color development: Rinsed three times with PBST, added enzyme-labeled goat anti-mouse/rabbit IgG polymer, incubated at 37°C for 30 minutes, and developed with DAB.
(7) Counterstaining with hematoxylin, differentiation with hydrochloric acid, dehydration, clearing, and sealing with neutral resin.

2.5. Immunohistochemical staining result evaluation

Two experienced pathologists independently scored cancer and adjacent tissues in a blinded manner. The scoring criteria for BECN1 and CD68 immunostaining was based on clinical data, utilizing the semiquantitative immunoreactivity score (IRS) system [7]. Scores were assigned based on cellular staining intensity and the percentage of positive cells, as outlined in **Table 2**. The final score was calculated by multiplying the staining intensity and positive cell percentage scores from the same section, ranging from 0 to 12.

Table 2. Semiquantitative immune reaction scoring (IRS) system

Staining intensity	Percentage of positive cells
0: Negative	1: 0%–25%
1: Weak	2: 26%–50%
2: Moderate	3: 51%–75%
3: Strong positive	4: 76%–100%

2.6. Cell transfection and culture

THP-1 cells were induced by PMA (100 ng/mL) to form M0 cells, which were then transfected with BECN1 interference RNA (si-BECN1). After 24 hours of culture, cytokines IL-4 and IL-13 (10 ng/mL) were added to induce M2 polarization. After 24 hours, cells were collected for RT-qPCR analysis.

2.7. RT-qPCR assay

Total RNA was extracted, and 1 μg RNA was reverse-transcribed using the HiScript II One-Step RT-PCR Kit, followed by real-time PCR analysis using the ChamQ SYBR qPCR Master Mix on the ABI 7500 real-time PCR system in triplicate. PCR conditions were as follows: 95°C for 30 seconds, 95°C for 3 seconds, and 60°C for 30 seconds, with 40 cycles. The expression of each gene was normalized to β-actin mRNA. Primers used for amplification are listed in **Table 3**.

Table 3. Primers for RT-qPCR assay of genes

Primer name	Primer sequence
BECN1 F	CCATGCAGGTGAGCTTCGT
BECN1 R	GAATCTGCGAGAGACACCATC
β-actin F	CATGTACGTTGCTATCCAGGC
β-actin R	CTCCTTAATGTCACGCACGAT
CD68 F	GGAAATGCCACGGTTCATCCA
CD68 R	TGGGGTTCAGTACAGAGATGC
F4/80 F	CAGCGTTCTGGACAAAGTGTG
F4/80 R	CGGAGTGATATTTGCTGAGGGT
CD11b F	GCCTTGACCTTATGTCATGGG
CD11b R	CCTGTGCTGTAGTCGCACT
CCL22 F	ATCGCCTACAGACTGCACTC
CCL22 R	GACGGTAACGGACGTAATCAC
CCL17 F	GGGAGTGCTGCCTGGAGTAC
CCL17 R	CCTGGAGCAGTCCTCAGATGTC
CCL18 F	GCATTCTCACTGTGACGACTCTG
CCL18 R	CTTGGTCCCTATCTTGCTGTTTCTG
IL10 F	GACTTTAAGGGTTACCTGGGTTG
IL10 R	TCACATGCGCCTTGATGTCTG

2.8. Statistical analysis

Data analysis was performed using GraphPad 9.0 software. For continuous data, *t*-tests were used for comparison. Kaplan-Meier survival curves and the Log-Rank test were used to compare survival rates and survival times between patients with high and low BECN1 and CD68 expression. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Expression of BECN1 and CD68 in colorectal cancer tissues

Immunohistochemistry results show that BECN1 is positively located in the cytoplasm, and CD68 is localized on the cell membrane, with staining from light yellow to brown (**Figure 1**). As shown in **Figure 2**, BECN1 and CD68 exhibited weak expression in some adjacent non-cancerous tissues but had significantly higher expression levels in CRC tissues, with the differences being statistically significant ($P < 0.05$). Based on the mean BECN1 expression (7.6), 60 CRC patients were divided into high- and low-expression groups, with the high-expression group above the mean and the low-expression group below it. The high-expression BECN1 group consisted of 33 cases, while the low-expression group consisted of 27 cases. Similarly, the mean CD68 expression (7.25) was used as the threshold, with 33 cases in the high-expression CD68 group and 27 in the low-expression group (**Table 4**).

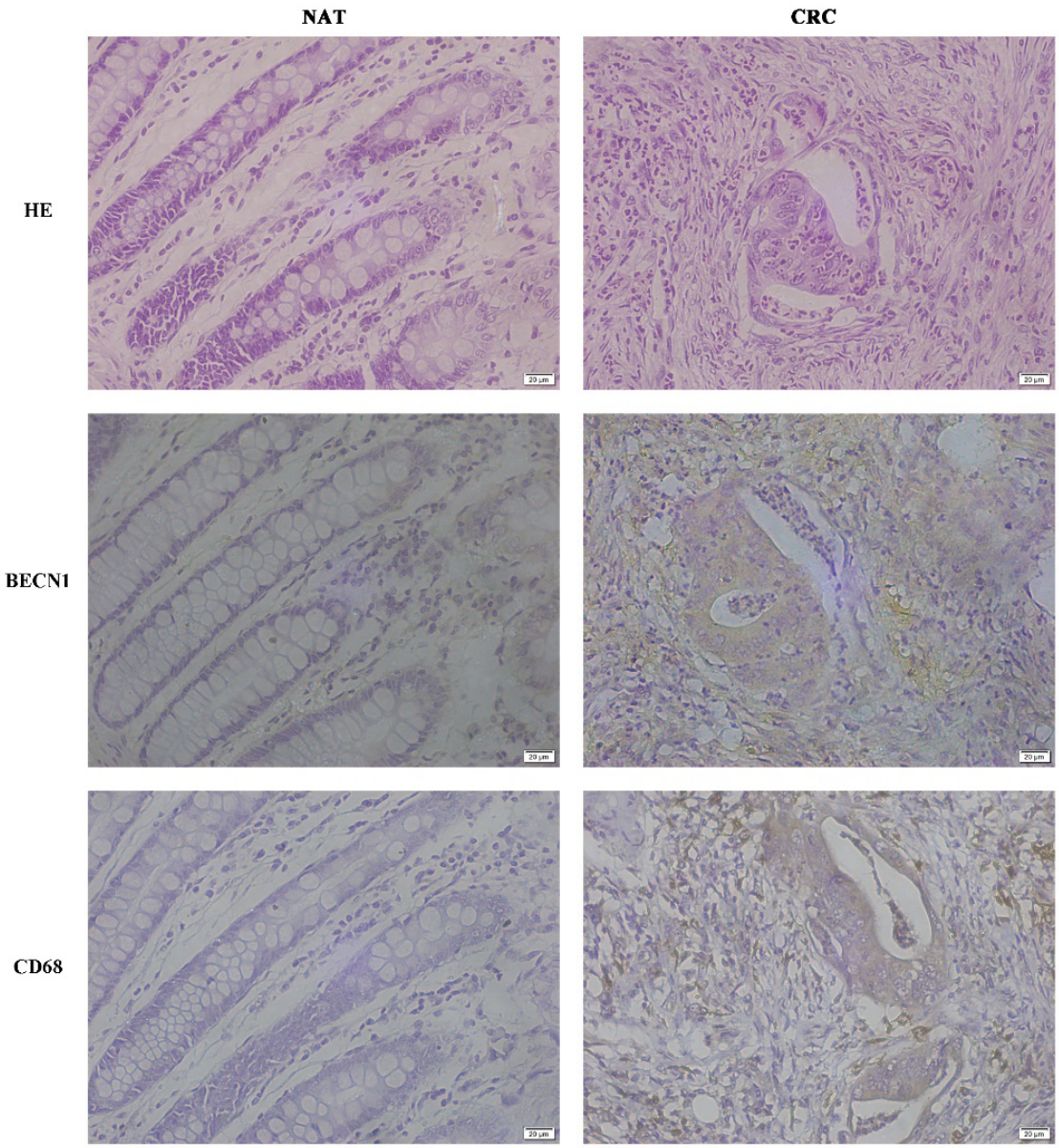

Figure 1. The expression of BECN1 and CD68 in adjacent normal tissues **(left)** and CRC **(right)** (HE and IHC staining, scale bar: 20 μm)

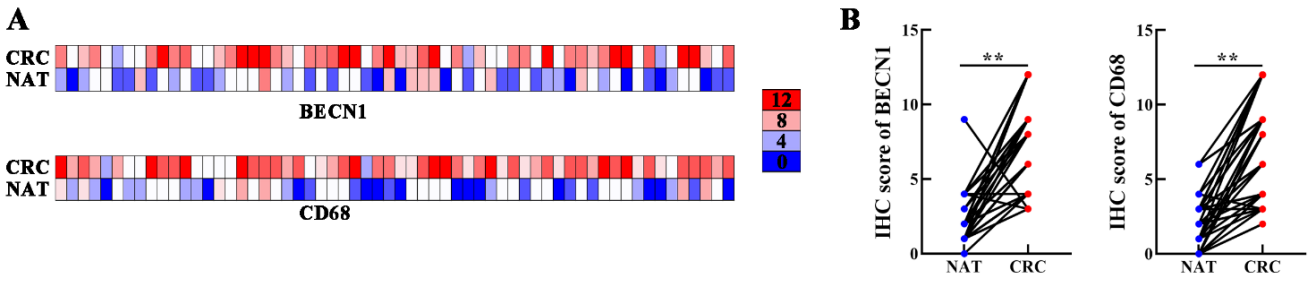

Figure 2. The expression level of BECN1 and CD68 in CRC and adjacent normal tissues. **(A)** Score of BECN1 and CD68 protein expression based on IHC staining in adjacent nonmalignant and CRC specimens (n = 60). **(B)** BECN1 and CD68 protein expression based on the staining index in adjacent nonmalignant and CRC specimens. Values are expressed as the mean ± SEM across different clinical stages (n = 60)

Table 4. Relationship between BECN1 and CD68 expression and clinicopathological features in CRC tissues [n (%)]

Features	BECN1		*P*	CD68		*P*
	High expression	Low expression		High expression	Low expression	
Gender			0.8131			0.6929
Female (n = 42)	24 (57.14)	18 (42.86)		23 (54.76)	19 (45.23)	
Male (n = 18)	9 (50.00)	9 (50.00)		10 (55.55)	8 (44.44)	
Age (years) (Mean 56.19; Range 33–73)			0.3330			0.2522
< 60 years (n = 36)	22 (61.11)			21 (58.33)	15 (41.67)	
≥ 60 years (n = 24)	11 (45.83)			12 (50.00)	12 (50.00)	
Adenocarcinoma (n = 55); Mucinous adenocarcinoma (n = 4); Tubular adenocarcinoma (n = 1)						
TNM stage			< 0.0001			< 0.0001
I–II (n = 30)	7 (23.33)	23 (76.67)		5 (16.67)	25 (83.33)	
III–IV (n = 30)	26 (86.67)	4 (13.33)		28 (93.33)	2 (6.67)	

3.2. Relationship between BECN1 and CD68 expression and clinicopathological characteristics of CRC

Based on the immunohistochemical scoring, further analysis of BECN1 and CD68 expression in relation to the clinicopathological features of CRC showed that both expressions were correlated with the TNM stage. BECN1 and CD68 expression levels were significantly higher in patients with advanced-stage disease (TNM stages III and IV) than in those with early-stage disease (TNM stages I and II) (**Figure 3**). However, no statistically significant differences in BECN1 and CD68 expression were observed based on age or gender (P > 0.05, **Table 4**).

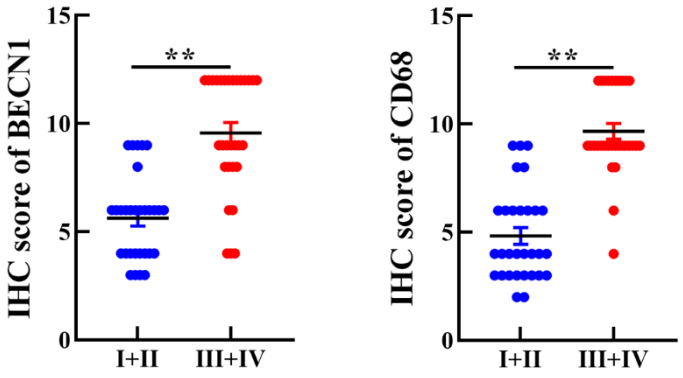

Figure 3. Relationship between BECN1 and CD68 protein levels and TNM stage of CRC

3.3. Relationship between BECN1 and CD68 expression and patient prognosis

The Log-Rank method was used to assess the impact of BECN1 and CD68 expression on the survival time of CRC patients. Using the mean BECN1 expression (7.6) as the threshold, patients in the high-expression group had significantly shorter overall survival than those in the low-expression group (HR = 2.683, 95% CI 1.065–6.763, *P* = 0.0364), as shown in **Figure 4A**. Similarly, using the mean CD68 expression (7.25) as the threshold, patients in the high-expression group had significantly shorter overall survival than those in the low-expression group (HR = 2.775, 95% CI 1.109–6.944, P = 0.0291), as shown in **Figure 4B**. These results indicate that aberrant expression of BECN1 and CD68 in CRC tissues is negatively correlated with patient prognosis.

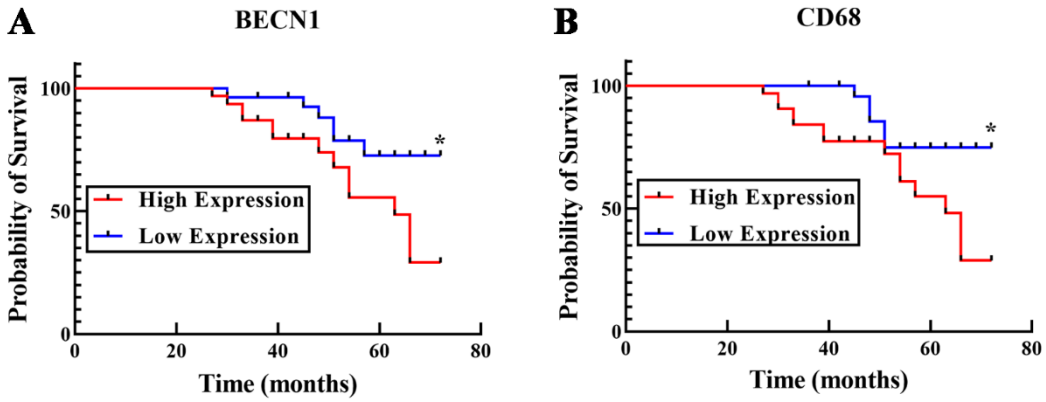

Figure 4. BECN1 and CD68 protein levels and their prognostic significance in CRC. **(A)** Kaplan–Meier curves of overall survival based on BECN1 expression in CRC patients. **(B)** Kaplan–Meier curves of overall survival based on CD68 expression in CRC patients

3.4. Correlation between BECN1 and CD68 expression in CRC tissues

Further analysis of the correlation between BECN1 and CD68 based on immunohistochemistry scores revealed that 81.82% of CRC cases had high expression of both BECN1 and CD68, while 77.78% of cases exhibited low expression of both markers (**Table 5**). As shown in **Figure 5**, a positive correlation was found between BECN1 and CD68 expression (R = 0.3458, $P < 0.001$), suggesting that abnormal expression of these markers plays an important role in the progression of CRC.

Table 5. Correlation of BECN1 and CD68 expression in CRC tissues [*n* (%)]

		BECN1		
		High expression	Low expression	Total
CD68	High expression	27 (81.82)	6 (18.18)	33 (55.00)
	Low expression	6 (22.22)	21 (77.78)	27 (45.00)
	Total	33 (55.00)	27 (45.00)	60 (100.00)

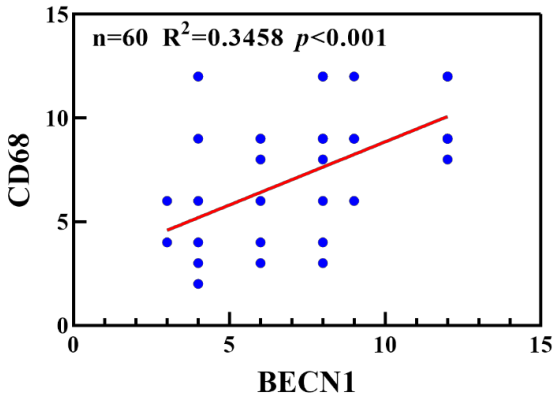

Figure 5. Correlation analysis of BECN1 and CD68 expression levels in human CRC specimens (n = 60)

3.5. BECN1 knockdown reduces macrophage marker expression

To further examine the role of BECN1 in macrophages, human monocytes (THP-1) were induced with PMA to form M0 macrophages. Upon transfection with BECN1 interference RNA (si-BECN1), RT-qPCR analysis of macrophage markers CD68, F4/80, and CD11b revealed that BECN1 knockdown significantly reduced the mRNA levels of these markers (**Figure 6**). These findings suggest that BECN1 may influence macrophage infiltration in CRC, thereby impacting cancer progression.

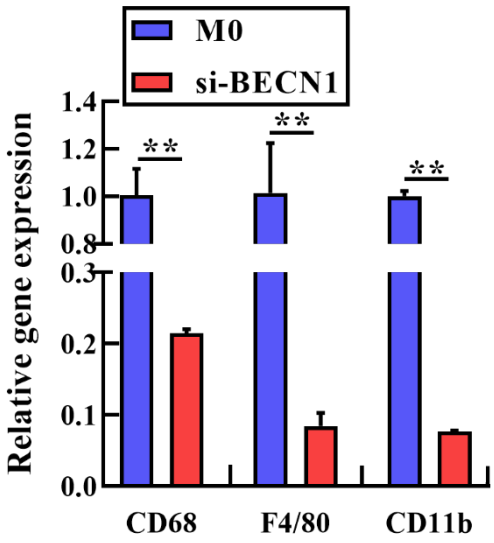

Figure 6. The effect of BECN1 knockdown on macrophage markers

3.6. BECN1 knockdown reduces M2 macrophage polarization

Tumor-associated macrophages in tumor tissues are predominantly M2-type macrophages, which can inhibit T-cell response, thereby promoting tumor growth. To further investigate the effect of BECN1 knockdown on M2 macrophage polarization, M0 macrophages were induced with IL-4 and IL-13 to form M2 macrophages, followed by si-BECN1 transfection. RT-qPCR analysis of M2 macrophage markers CCL17, CCL18, CCL22, and IL-10 revealed that BECN1 knockdown significantly decreased the mRNA levels of these markers (**Figure 7**). These results suggest that BECN1 promotes macrophage polarization towards the M2 type, thereby facilitating CRC progression.

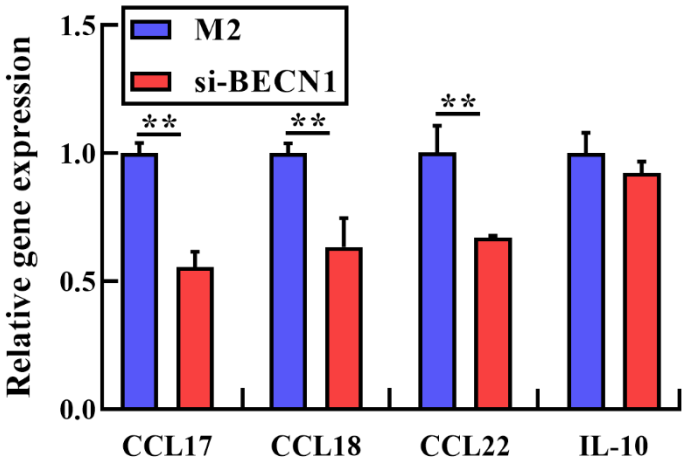

Figure 7. Effect of BECN1 knockdown on M2 macrophage polarization

4. Discussion

With the continuous changes in diet and lifestyle, the incidence and mortality of CRC are rising annually in China. Therefore, early diagnosis of CRC is essential to extend patient survival and improve outcomes. *BECN1* is a key autophagy-related gene that bridges the autophagy and apoptosis pathways by interacting with Bcl-2 [8]. The *BECN1* gene, located at chromosome 17q21, is close to the breast cancer susceptibility gene *BRCA1* [9]. Studies by Cervantes-Diaz *et al.* have shown high BECN1 expression in triple-negative breast cancer, which correlates with poor prognosis [10]. Conversely, research by Li *et al.* indicates that ubiquitin-mediated degradation of BECN1 can inhibit autophagy and thus promote breast cancer progression [11]. In human tumors, BECN1 loss often correlates with BRCA1 deficiency, suggesting that BECN1 may not act as a tumor suppressor in most cancers. Although Hu *et al.* reported that low BECN1 expression contributes to CRC invasion and metastasis [12], recent studies have found that autophagy is upregulated in CRC, contributing to its progression, chemoresistance, and more. Qureshi-Baig *et al.* demonstrated that hypoxia induces autophagy and subsequently triggers ezrin phosphorylation in tumor-initiating cells, ultimately promoting CRC development [13]. Hu *et al.* showed that cytokine IL-6 in the tumor microenvironment activates autophagy through the IL-6/JAK2/BECN1 pathway, which enhances CRC chemoresistance [14]. In a previous study, we also observed that high-mobility group protein B1 (HMGB1) binds to BECN1, activating autophagy and affecting CRC radiosensitivity, with the autophagy activator Tat-Beclin1 significantly promoting CRC progression. This study used immunohistochemistry to analyze BECN1 expression

in 60 CRC and adjacent non-cancerous tissues, revealing that BECN1 is abnormally expressed in CRC and associated with TNM staging; patients with high BECN1 expression had shorter survival and poorer prognosis. These results collectively indicate that BECN1 plays a critical role in CRC progression, and inhibiting BECN1-induced autophagy may represent a promising anticancer therapy.

Macrophages are essential in immune response and exhibit varying roles in different disease microenvironments, such as inflammation and tumors [15]. As one of the most common non-tumor cell types in human cancers, including CRC, macrophages display extensive functional plasticity in response to environmental stimuli [16]. In distinct microenvironments, TAMs can polarize into classically activated (M1) or alternatively activated (M2) phenotypes, exerting either tumor-suppressive or tumor-promoting effects [17]. CD68, a characteristic surface marker of macrophages, is abnormally expressed in multiple tumors and correlates with patient prognosis [6]. In an analysis of 148 CRC patients, Shin *et al.* found that CD68-positive macrophages were associated with distant metastasis [18]. In this study, we observed significantly higher CD68 expression in CRC tissues than in adjacent tissues, with a positive correlation between CD68 expression and TNM stage; patients with high CD68 expression had shortened survival and poor prognosis. Chronic inflammation is a critical factor in the development of colitis-associated colorectal cancer, where the extent and duration of the inflammatory response drive the progression of inflammatory bowel disease to CRC. Autophagy aids in protecting the intestinal mucosal barrier and modulating intestinal inflammation [19]. M1 and M2 macrophages play crucial roles in the onset and progression of inflammatory bowel disease, as well as in maintaining intestinal homeostasis and repairing damage [20]. In the tumor microenvironment, macrophages, fibroblasts, and other cells can secrete cytokines such as IL-6, which recruit additional immune cells to amplify the inflammatory response. These cytokines also affect tumor cells by activating intracellular signaling pathways, thus promoting angiogenesis, invasion, metastasis, and immunosuppression. Additionally, activation of intracellular pathways, such as NF-κB, can trigger autophagy, further promoting the secretion of relevant cytokines by tumor cells. This bidirectional positive feedback regulation can further accelerate tumor progression [14]. Our results also indicate a positive correlation between BECN1 and CD68 expression in CRC. Interference with BECN1 markedly reduces macrophage markers and decreases M2 polarization, suggesting that both BECN1 and CD68 could serve as critical factors for evaluating CRC prognosis.

5. Conclusion

In conclusion, BECN1 and CD68 are highly expressed in CRC tissues and associated with TNM staging, with no correlation observed with age or gender. High expression of BECN1 and CD68 correlates with shortened survival in patients, and interfering with BECN1 significantly reduces macrophage markers and decreases M2 polarization, suggesting that BECN1 and CD68 may be valuable prognostic markers for post-surgical evaluation in CRC patients. Further studies will explore their molecular mechanisms *in vivo* and *in vitro*, aiming to provide a theoretical foundation for CRC clinical treatment.

Author contributions

Conceptualization: Yanchao Ma
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Investigation: Yan Zhang, Yuhui Han, Xiuxin Liu
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Writing – review & editing: Yanchao Ma
Visualization: Yan Zhang, Wenjiong Sheng

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Disclosure statement

The authors declare no conflict of interest.

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