Trends in Immunotherapy (2024) Volume 8 Issue 1
doi: 10.24294/ti.v8.i1.3225

ORIGINAL RESEARCH ARTICLE

The expression of autophagy-associated protein LC3, metastasis, and chemotherapy response in colorectal cancer patients receiving uracil-based chemotherapy: An observational study

Mochammad Riskie Aditya Putra, Reno Rudiman, Kiki Lukman*, Bambang Am Am Setya Sulthana, Andriana Purnama, Tommy Ruchimat, Alma Wijaya, Prapanca Nugraha, Andika Rahmawan

Division of Digestive Surgery, Department of Surgery, Faculty of Medicine, Universitas Padjadjaran, Dr. Hasan Sadikin General Hospital, Bandung 40161, Indonesia

*** Corresponding author:** Kiki Lukman, kiki.lukman@unpad.ac.id

ABSTRACT

Background: LC3 serves as a marker for assessing autophagy activity in cancer cells, which is a potential focus for therapeutic interventions. It holds the potential to serve as a predictive indicator of resistance to chemotherapy in colorectal cancer. Thus, this study is aimed at finding the association between LC3 expression, metastasis, and chemotherapy response in colorectal patients. **Methods:** This study was an observational study evaluating the stage and chemotherapy response of a colorectal cancer patient in a tertiary hospital in West Java, Indonesia. The research participants included in this study were 83 subjects. The examination of LC3 was performed with immunohistochemistry. The response evaluation criteria in solid tumors (RECIST) were used for the evaluation of the chemotherapy response. **Results:** A positive LC3 expression was shown in 58 (69.9%) patients. In positive LC3 expression, 20 subjects (27.4%) showed progressive response, 16 subjects (21.9%) showed stable response, 12 subjects (16.4%) showed partial response, and 1 subject (1.4%) showed complete response. Meanwhile, in negative LC3 expression, 12 subjects (16.4%) showed progressive response, 8 subjects (11%) showed stable response, 3 subjects (4.1%) showed partial response, and 1 subject (1.4%) showed complete response. The LC3 expression showed no significant relationship with age, sex, subtype, grade, tumor location, stage, or chemotherapy response ($p > 0.05$). **Conclusion:** The expression of LC3 showed no significant relationship with metastasis or chemotherapy response.

Keywords: autophagy; chemotherapy; colorectal cancer; LC3; metastases

ARTICLE INFO

Received: 9 November 2023
Accepted: 18 December 2023
Available online: 5 March 2024

1. Introduction

Colorectal cancer (CRC) ranks as the third most common type of cancer globally and the third leading cause of death in the United States, with an estimated annual incidence of around 150,000 new cases[1,2]. Meanwhile, in Indonesia, CRC holds the fourth position, with an annual incidence of approximately 35,000 new cases[2]. Research findings suggest an upward trend in the survival rates of individuals diagnosed with colorectal cancer. The 5-year relative survival rate improved from 37.9% during 1998–2002 to 51.5% in 2008–2012. This improvement in survival is likely attributed to the nationwide implementation of a screening program. Early diagnosis appears to significantly enhance treatment outcomes and the chances of survival[3].

Although the exact cause of colorectal cancer remains uncertain, it is understood that the onset of the disease generally results from the interplay of various risk factors, some of which can be modified while

others cannot[4]. Multiple genetic pathways, including chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype pathway, are accountable for the transformation of adenoma into carcinoma[5]. Macroautophagy or autophagy, is the cellular process of delivering proteins, lipids, and intracellular organelles to lysosomes, where cell degradation can occur. After autophagic degradation, intracellular materials are removed from the lysosomal compartment, and recycling occurs in the cytoplasm[6,7]. The autophagy process is also involved in tumorigenesis and can promote or suppress tumors. The tumor suppressive effect is achieved by the degradation of oncogenic protein substrates, toxic proteins, and damaged organelles. However, autophagy-related recycling of intracellular substrates required for mitochondrial activity exerts tumor-promoting effects in cancer cells. Autophagy may also function as a survival pathway for cancer cells. Several studies have shown that autophagy is upregulated in cancer by rat sarcoma (RAS) and is mainly induced in cancer areas under hypoxic conditions, where it supports tumor cell survival[8].

Microtubule-associated protein 1 light chain 3, or LC3, serves as a marker for assessing autophagy activity in cancer cells. It shares similarities with the yeast ATG8 protein and is a ubiquitin-like protein that becomes lipidated and closely associates with autophagosomal membranes. The process of autophagosome formation is identified by the integration of LC3 II (the isoform II of the light chain) within the layers of the vesicle. Immunohistochemistry is a commonly employed technique to effectively gauge autophagosome formation by measuring LC3 expression[8,9]. Only a limited number of studies have examined the significance of LC3 expression as a prognostic factor in CRC. Park et al.'s 2013 study[10] revealed the prognostic impact of LC3 expression in CRC, while Wu et al.'s 2015 study[11] did not indicate the prognostic relevance of LC3 expression in CRC patients. Lock et al.'s 2011 study demonstrated that the activation of oncogenic RAS, which can stimulate tumor growth, is adequate to heighten basal autophagy, although the correlation between autophagy and KRAS mutations remains ambiguous[12]. Research by Wu et al. in 2015 showed a significant relationship between LC3 expression and lymph node (LN) or lymphatic metastases[13]. CRC patients with high LC3 expression showed a lower likelihood of experiencing LN metastasis ($p < 0.001$)[13]. Research by Zhao et al. in 2017 showed a significant relationship between LC3 expression and metastasis. CRC patients with low expression of LC3 showed a higher likelihood of experiencing metastasis ($p < 0.001$)[14].

In the early stages of colon cancer, the primary surgical approach involves excision, either local or wide, with or without anastomosis. For the advanced stages of the disease, resection is considered the most effective option. In cases of rectal cancer, adjuvant therapy may involve radiation, particularly to preserve organ function. This type of treatment is applicable for both resectable and non-resectable cases. Research from a meta-analysis of major phase III clinical trials has indicated that the addition of 5FU adjuvant chemotherapy to surgical treatment can increase survival rates by 2.3% to 5.7%. This chemotherapy has evolved to incorporate a combination of leucovorin, which can regulate 5FU activity and reduce its toxicity. Fluorouracil, chemically known as 5-fluoro-2,4(1H,3H)-pyrimidinedione, is a pyrimidine antimetabolite chemotherapeutic drug. Its mechanism of action involves inhibiting the methylation of deoxyuridylic acid to thymidylic acid by blocking the enzyme thymidylate synthase. Consequently, this leads to a deficiency of thymine, ultimately inhibiting the synthesis of deoxyribonucleic acid (DNA), and to a lesser extent, it hinders the formation of ribonucleic acid (RNA), which is crucial in cell division and growth. Adjuvant chemotherapy is typically recommended for high-risk stage II cases. Current guidelines suggest the use of 5FU-Leucovorin chemotherapy and an oxaliplatin-based regimen as the standard adjuvant therapy. In stage IV, tumor resection can be performed[15]. Multiple research studies have indicated that blocking the process of autophagy in cancerous cells could intensify cell death caused by chemotherapy, thereby identifying autophagy as a potential focus for therapeutic interventions. Additionally, an investigation revealed that reducing the expression of LC3 in colon cancer through the use of lentiviral shRNA could amplify apoptosis induction by 5-FU[10]. This finding implies that autophagy-related proteins, such as LC3, might serve as predictive indicators of resistance to chemotherapy in colorectal cancer. Therefore, there is a requirement for data that establishes the link between

the expression of LC3 and the response to chemotherapy, which can help in the evaluation of treatment choices and the formulation of personalised therapy plans. The primary objective of this study is to examine how the expression of LC3 is associated with both metastasis and the response to chemotherapy in individuals diagnosed with colorectal cancer.

2. Methods

2.1. Study design and setting

This research is a prospective observational study. This study was held in a tertiary general hospital in West Java, Indonesia, and follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines[16]. This study used data taken from the results of anatomic pathology and medical records of all colorectal cancer cases that came to the digestive surgery polyclinic and the emergency room of a tertiary general hospital in West Java, Indonesia. The research subjects in this study were colorectal cancer patients in the Digestive Surgery Division of a tertiary general hospital in West Java, Indonesia, from October 2022 to September 2023. There were 83 patients who were randomly selected using the manual simple random method from the colorectal cancer patients who underwent chemotherapy. The patient selection is shown in **Figure 1**. The inclusion criteria for this study are colorectal cancer patients with complete registry data, including pathology examinations; patients who are over 18 years old; and patients who had complete chemotherapy in the same hospital and who have agreed to be the subject of this study. We excluded colorectal patients who had a history of discontinuation of chemotherapy and stage I colorectal cancer. The examination of LC3 expression was carried out by the immunohistochemistry (IHC) method of paraffin blocks using a biopsy or surgical sample from a colorectal cancer patient's tissue. The chemotherapy responses were measured using the response evaluation criteria for solid tumors (RECIST) version 1.1[17]. The hospital's ethical committee approved this study with No. Ethical Approval LB.02.01/X.6.5/167/2023. Every research participant signed an informed consent form before participating in the study. Univariate statistical tests were used to find the relationship between the expression of LC3 and the characteristics of colorectal cancer patients, including their chemotherapy response, using Chi Square, and a *p*-value less than 0.05 was considered significant.

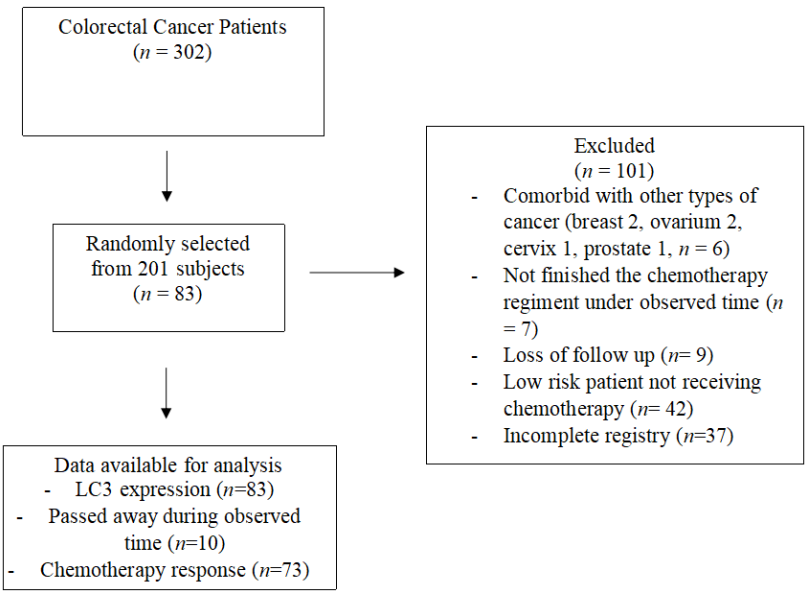
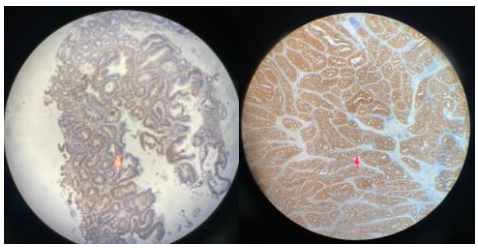


Figure 1. The flowchart of the study selection process.

2.2. LC3 expression using IHC analysis

Hematoxylin and eosin (HE) staining was used to examine tissue samples for colorectal cancer diagnosis. Utilizing the immunohistochemistry (IHC) method, the expression of LC3 was examined in the identified primary tumor and subsequently compared with neighboring non-cancerous tissues. After being fixed with formalin, 4 m slices of block paraffin tissue were cut, dewaxed, rehydrated, and blocked using hydrogen peroxide. The slices were microwaved in 10 mm citrate buffer in order to extract the antigen (pH 6.0). On the sections, a rabbit polyclonal antibody against human LC3 (Abcam, ab48394, Cambridge, UK) was then incubated for a further night at 4 °C. The slices were then subjected to a secondary antibody labelled with horseradish peroxidase for 30 min. Once the sections were prepared with diaminobenzidine tetrahydrochloride, hematoxylin was utilised as a counterstain. IHC analysis was done seven days after preparation to prevent antigen degradation. The negative controls were made using phosphate-buffered saline (PBS) instead of the primary antibody, and the positive controls were supplied by Abcam. A staining of 0 percent was considered negative, 5 percent was considered weak or class 1-LC3 score, 6 to 35 percent was considered class 2-LC3 score, 36 to 65 percent was considered class 3-LC3 score, and 66 to 100 percent was considered class 4-LC3 score. Specimens with a class 4-LC3 score were considered positive or showed overexpression of the LC3 (**Figure 2**).



(a) **(b)**

Figure 2. Assessment of LC3 expression with IHC: **(a)** weak staining of LC3 score less than 80%; **(b)** class 4-LC3 score or overexpression with staining 81%–100%.

2.3. Metastasis data and chemotherapy response evaluation using RECIST

All the subjects in this study completed uracil based chemotherapy, repeated every two weeks for 12 cycles. There were three regiment used in the study. First, the fluorouracil, folinic acid, and irinotecan (FOLFIRI) regimen, with one and half hours of 180 mg/m^2 irinotecan, followed with two hours of 400 mg/m^2 folinic acid, and followed by a bolus of 400 mg/m^2 fluorouracil and a 46-hour infusion of 2.4–3 g/m^2 fluorouracil. Second, the fluorouracil, leucovorin, and oxaliplatin (Folfox 6) regimen, with two hours of 400 mg/m^2 leucovorin and oxaliplatin 100 mg/m^2, followed by a bolus of 400 mg/m^2 fluorouracil and a 46-hour infusion of 2.4–3 g/m^2 fluorouracil. Third, two days set of the fluorouracil and leucovorin (de Gramont) regimen, with two hours of 200 mg/m^2 leucovorin and a 400 mg/m^2 bolus injection dosage of fluorouracil, was continued with a 600 mg/m^2 infusion of fluorouracil for 22 h[18,19].

Table 1. The response evaluation criteria in solid tumors (RECIST) version 1.1. based on abdominal CT scan for colorectal cancer.

Categorical response	Description
Complete response	Disappearance of all the tumors and all the lymph nodes affected.
Partial response	More than 30% decrease in the total diameter of the solid tumor.
Stable disease	No changes of the tumor, or the changes neither complete or partial response.
Progressive disease	More than 20% increase in the total diameter of the solid tumor, or new lesions, including metastasis.

Metastasis data were collected from the cancer register. Lymphatic metastasis or distant metastasis were collected. The response evaluation criteria in solid tumors (RECIST) version 1.1 was used as a standard for

grouping the chemotherapy response before and after the completion of the chemotherapy regiment as shown in **Table 1**. The comparison of size was derived from abdominal computed tomography scans obtained before and after chemotherapy.

2.4. Statistical analysis

The data that has been collected is processed and computerized; numerical data is presented as mean and standard deviation. Categorical data is presented as percentages. The comparison of the variables was based on the chi square test performed using the SPSS 26 software (SPSS Inc., Chicago, IL, USA). The p-value < 0.05 indicates a significant relationship.

3. Results

3.1. Subject characteristics

There were 83 research subjects, and the characteristics of the research subjects are shown in **Table 2**. There were 50 female and 33 male patients. The mean age was 58.04 years ± 11.4, and 74.7% of patients were older than 50. Most of the patients showed adenocarcinoma histologically (90.4%), and 68.7% showed well-differentiated tumors. There were 52 (62.7%) rectal cancer patients and 31 (37.3%) colon cancer patients. There were 35 (42.2%) stage III cancer patients and 34 (41%) stage IV cancer patients. Lymphatic metastases were found in 53 (63.9%) patients. Liver metastases were found in 29 (34.9%) patients. The majority of the patients show high expression of LC3 (69.9%).

Table 2. Characteristics of research subjects.

Variable		**Proportion (%)**
Age	Mean	58.04 ± 11.4 years
	Median	58 years
	Minimum	31 years
	Maximum	88 years
	Range	57 years
Age group	<50 years	21 (25.3%)
	≥50 years	62 (74.7%)
Sex	Male	33 (39.8%)
	Female	50 (60.2%)
Histologic type	Adenocarcinoma	75 (90.4%)
	Mucinous adenocarcinoma	6 (7.2%)
	Signet ring cell	1 (1.2%)
	Neuroendocrine	1 (1.2%)
Grade	Well diff	57 (68.7%)
	Moderately diff	10 (12%)
	Poorly diff	8 (9.6%)
	Specific	8 (9.6%)
Tumor Location	Colon	31 (37.3%)
	Rectum	52 (62.7%)
Stage	II	14 (16.9%)
	III	35 (42.2%)
	IV	34 (41%)

Table 2. (*Continued*).

Variable		Proportion (%)
Lymphatic metastasis	Negative	30 (36.1%)
	Positive	53 (63.9%)
Distant Metastasis	Negative	47 (56.6%)
	Liver	29 (34.9%)
	Others (lung, bone, uterus, etc.)	7 (8.4%)
LC3 expression	High expression	58 (69.9%)
	Normal expression	25 (30.1%)

3.2. Univariate analysis of variables and LC3 expression

No variables showed a statistically significant relationship ($p < 0.05$) in univariate analysis with LC3 expression, as shown in **Table 3**.

Table 3. Univariate analysis for subject characteristics with LC3 expression.

	High expression	Normal expression	Chi-Square (*p*-value)
Age			0.858
Early onset (<50)	15 (18.1%)	6 (7.2%)	
Late onset (≥50)	43 (51.8%)	19 (22.9%)	
Sex			0.343
Male	25 (30.1%)	8 (9.6%)	
Female	33 (39.8%)	17 (20.5%)	
Histologic type			0.141
Adenocarcinoma	51 (61.4%)	24 (28.9%)	
Mucinous adenocarcinoma	6 (7.2%)	0	
Signet ring cell	0	1 (1.2%)	
Neuroendocrine	1 (1.2%)	0	
Grade			0.541
Well diff	38 (45.8%)	19 (22.9%)	
Moderately diff	8 (9.6%)	2 (2.4%)	
Poorly diff	5 (6%)	3 (3.6%)	
Specific	7 (8.4%)	1 (1.2%)	
Tumor location			0.411
Colon	20 (24.1%)	11 (13.3%)	
Rectum	38 (45.8%)	14 (16.9%)	
Stage			0.549
II	9 (10.8%)	5 (6%)	
III	23 (27.7%)	12 (14.5%)	
IV	26 (31.3%)	8 (9.6%)	
Lymphatic metastasis			0.631
Negative	20 (24.1%)	10 (12%)	
Positive	38 (45.8%)	15 (18.1%)	

Table 3. (*Continued*).

	High expression	Normal expression	Chi-Square (*p*-value)
Distant metastasis			0.185
Negative	31 (37.3%)	16 (19.3%)	
Liver	20 (24.1%)	9 (10.8%)	
Others (lung, bone, uterus, etc.)	7 (8.4%)	0	

3.3. Univariate analysis of variables and chemotherapy response

Table 4. Relationship between expression of chemotherapy response and characteristics of research subjects.

	Chemotherapy response				
	Complete response	**Partial response**	**Stable disease**	**Progressive disease**	**Chi-Square (*p*-value)**
Age					0.120
Early onset (<50)	0	1 (1.4%)	6 (8.2%)	12 (16.4%)	
Late onset (≥50)	2 (2.7%)	14 (19.2%)	18 (24.7%)	20 (27.4%)	
Sex					0.977
Male	1 (1.4%)	7 (9.6%)	10 (13.7%)	13 (17.8%)	
Female	1 (1.4%)	8 (11%)	14 (19.2%)	19 (26%)	
PA					0.883
Adenocarcinoma	2 (2.7%)	15 (20.5%)	22 (30.1%)	27 (37%)	
Mucinous adenocarcinoma	0	0	2 (2.7%)	3 (4.1%)	
Signet cell ring	0	0	0	1 (1.4%)	
Neuroendocrine	0	0	0	1 (1.4%)	
Grade					0.273
Well diff	2 (2.7%)	15 (20.5%)	15 (20.5%)	18 (24.7%)	
Moderately diff	0	0	4 (5.5%)	5 (6.8%)	
Poorly diff	0	0	3 (4.1%)	4 (5.5%)	
Specific	0	0	2 (2.7%)	5 (6.8%)	
Tumor location					0.335
Colon	2 (2.7%)	5 (6.8%)	9 (12.3%)	12 (16.4%)	
Rectum	0	10 (13.7%)	15 (20.5%)	20 (27.4%)	
Stage					**0.037**
II	0	2 (2.7%)	5 (6.8%)	5 (6.9%)	
III	1 (1.4%)	11 (15.1%)	14 (19.2%)	9 (12.3%)	
IV	1 (1.4%)	2 (2.7%)	5 (6.8%)	18 (24.7%)	
Lymphatic metastasis					0.195
Negative	1 (1.4%)	7 (9.6%)	11 (15.1%)	7 (9.6%)	
Positive	1 (1.4%)	8 (11%)	13 (17.8%)	25 (34.2%)	
Distant metastasis					0.377
Negative	1 (1.4%)	9 (12.3%)	18 (24.7%)	14 (19.2%)	
Liver	1 (1.4%)	5 (6.8%)	4 (5.5%)	15 (20.5%)	
Others metastasis	0	1 (1.4%)	2 (2.7%)	3 (4.1%)	
LC3 expression					0.636
Normal expression	1 (1.4%)	3 (4.1%)	8 (11%)	12 (16.4%)	
High expression	1 (1.4%)	12 (16.4%)	16 (21.9%)	20 (27.4%)	

No variables showed a statistically significant relationship ($p < 0.05$) in univariate analysis with chemotherapy response, except the stage variable, with a *p*-value of 0.037 as shown in **Table 4**.

4. Discussion

4.1. Study population characteristics

Based on **Table 2**, it is known that the average age of the research subjects is 58.04 ± 11.4 years, with a median of 58 years. This data highlights that the majority of patients diagnosed with colorectal cancer at a tertiary general hospital in West Java, Indonesia, are typically aged 50 and above. These findings align with epidemiological research indicating that a significant proportion of individuals with CRC are over the age of 50.[20,21] Several studies have extensively referenced data indicating that the likelihood of developing CRC rises during the fifth decade of life, with the risk figure increasing by up to one percent (1%) for every 10 additional years of age starting from the age of 50[15,20–22].

Based on sex, the distribution comprised 50 females (60.2%) and 33 males (39.3%). When compared with CRC epidemiological data, the rates of CRC cases show no significant disparity between the sexes, with a prevalence rate of 1:25 (4.0%) in women and 1:23 (4.3%) in men[15,20–22].

The results of the anatomical pathology examination showed that 75 subjects (90.4%) showed adenocarcinoma, followed by 6 mucinous adenocarcinoma subjects (7.2%), one signet ring cell subject (1.2%), and 1 neuroendocrine subject (1.2%). Based on grade, 57 subjects (68.7%) showed good differentiation, 10 subjects showed moderate differentiation (12%), and each had poor and specific differentiation in 8 subjects (9.6%). This aligns with literature studies, indicating that the occurrence of adenocarcinoma in colorectal cancer is found in up to 90% of the total cases. Adenocarcinoma itself can differentiate into various forms, including comedone, medullary, micropapillary, mucinous, and signet ring cell types. Grading of the adenocarcinoma mostly dominated with moderately differentiated (around 70%), followed by 20% of poorly differentiated, and 10% of well differentiated[23].

In this study, 52 subjects (62.7%) exhibited tumors in the rectum, while the remaining 31 subjects (37.3%) had tumors in the colon. The findings of this study are consistent with existing literature, indicating a shift in the distribution of CRC cases concerning tumor location. The percentage of CRC cases situated in the rectum has continued to rise, from 27% in 1995 to 31% in 2019. Approximately four out of 10 diagnoses of CRC in individuals aged 50–64 occur in the rectum[24].

Most of these research subjects, specifically 35 subjects (42.2%), were diagnosed with stage III CRC, followed by 34 subjects (41%), diagnosed with stage IV, with the highest occurrence of metastases found in the liver (in 29 out of 36 patients). Additionally, metastases were observed in other organs such as the lung, bone, and uterus. A smaller number of patients were identified with stage II CRC, amounting to 14 subjects (16.9%). These findings are consistent with the most recent epidemiological data from 2019, which stated that 60% of newly reported cases of CRC were at an advanced stage of the disease, with 22% of these cases showing distant metastases[24].

4.2. Expression of LC3 in colorectal cancer patients

On cancer cells, autophagy has a variety of dynamic effects that can affect the development, progression, and response of the cancer to treatment. Several studies have highlighted the existence of a cross-talk between tumor suppressor genes and/or oncogenes via autophagy-related gene (ATG) pathways[25]. LC3 was the first autophagy marker linked to the development of colorectal cancer[26]. One of the LC3 isoforms, named LC3-II, is expressed excessively in CRC cells, especially at advanced stages, compared with normal colorectal cells[27]. Notably, lower levels of LC3 have been linked to a favorable prognosis in CRC, particularly in its advanced

stages[28]. The autophagy process related to LC3 is involved in one form of the autophagy process, namely macro-autophagy[29,30].

Macro-autophagy delivers cytoplasmic cargo to lysosomes via intermediate double membrane-bound vesicles, referred to as autophagosomes, which fuse with lysosomes to form autolysosomes. Macro-autophagy involves the formation of phagophores, which are precursors of autophagosomes. Both early and late stages of autophagosome production require a number of protein complexes connected to autophagy-related genes (ATGs). The microtubule-associated protein 1 light chain 3 beta (LC3-I) is processed to create LC3-II after being coupled with phosphatidylethanolamine during membrane formation. Lysosomes break LC3-II when they fuse with autophagosomes and possess the necessary enzymes for cargo destruction. Elevations in LC3-II are frequently utilized to gauge autophagic activity[29,31].

5-FU is one of the most effective and most commonly used agents in the treatment of CRC and is the main element in combination chemotherapy regimens. Its antitumor impact primarily stems from the inhibition of thymidylate synthase (TS), leading to tumor cell death[32]. Additionally, 5-FU has the ability to trigger the autophagy process. LC3 serves as a scaffold for the core autophagosome machinery, functioning in autophagosome biogenesis by acting as a selective cargo adapter or regulating signaling proteins like kinase/GTPase/GAP[33]. In cases where the MLH1 gene is robustly linked to CRC, the cancer tends to be more aggressive. MLH1 can elevate LC3 levels, thereby mitigating the anticipated DNA damage effects of chemotherapy agents[34]. Encouraging autophagy in situations where apoptosis is impeded or by directly prompting autophagy signals represents a viable strategy that could be employed in cancer treatment.[35] Consequently, understanding the expression of LC3 in CRC patients is important as it can influence the decision to use 5-FU as a therapeutic protocol.

In this study, the analysis of LC3 expression indicated that 58 out of 83 individuals with CRC (69.9%) exhibited results indicating overexpression. Notably, the proportion of cases demonstrating increased LC3 expression remained consistent with findings from various other studies. Moreover, the univariate analysis of LC3 Chi Square expression did not reveal any significant association between LC3 overexpression and variables such as age, gender, anatomical pathology, grade, tumor location, and stage. The study by Wu et al. in 2015 showed that there was overexpression of LC3 in 175/242 (72.31%) of CRC patients, and it was shown that LC3 overexpression was associated with poor differentiation grade ($p = 0.021$)[10]. In 2016, Schmitz et al. showed that 35 of 127 CRC patients (27.5%) had overexpression of LC3. Significant results were only shown in the relationship between LC3 overexpression and poor differentiation grade ($p = 0.016$)[9]. Analysis between LC3 expression and gender, age, and tumor location showed an insignificant relationship[10,11].

In Zhao et al.'s 2017 study[14], across three distinct cohorts of individuals with CRC, the results showed an overexpression of LC3 in 100 out of 205 cases (48.7%) in the first cohort, 84 out of 160 cases (52.5%) in the second cohort, and 77 out of 161 cases (47.8%) in the third cohort. Analysis between LC3 expression and gender, age, and tumor location showed a non-significant relationship. Meanwhile, LC3 analysis by stage showed significant results ($p = 0.01$) between early stages (stages I and II) and advanced stages (stages III and IV)[14]. Guo et al.'s 2019 study[35,36] revealed a significant upregulation of LC3 in tumor samples compared to normal specimens (64.87% vs. 2.00%, $p < 0.001$), with a sensitivity of 71.4% and specificity of 91.3%. Analysis of the relationship between LC3 expression and variables such as gender, age, family history of cancer, tumor location, and grade showed an insignificant relationship[36].

4.3. Relationship between expression of LC3 and metastasis in colorectal cancer patients

Metastases in CRC can be identified by stage according to the TNM classification from The American Joint Committee on Cancer (AJCC). Metastases that occur in lymph nodes start with the N1 classification, which is from stage III to stage IV. Meanwhile, distant metastases are classified as M, starting from stage IV[37].

In this study, it was found that 53/83 (63.9%) had lymphatic metastasis and 36/83 (43.3%) had distant metastasis. The highest distant metastasis occurs in the liver (29/36). Although there is a comparison of over-expressing LC3 with normal LC3 expression in stage III (23:12) and stage IV (26:8) CRC patients, which shows that metastases can occur more in CRC patients who have over-expressing LC3, univariate chi-square analysis of LC3 overexpression on the stage of CRC patients showed a *p*-value of 0.549, which shows that there is no significant relationship between LC3 overexpression and metastases in CRC patients. This study does not agree with the study by Wu et al.[13], which showed a significant relationship between LC3 expression and lymph nodes or lymphatic metastases. CRC patients with high expression of LC3 showed a lower likelihood of experiencing lymph node metastasis ($p < 0.001$). They also said there is a relationship between excess LC3 and less cell differentiation ($p < 0.05$). The significant correlation between LC3 expression and cell differentiation suggests that cell malignancy may occur due to increased autophagy[11].

Meanwhile, a study from Schmitz et al. in 2016 demonstrated that CRC patients who had low levels of LC3 had a higher chance of experiencing distant metastases ($p = 0.027$)[9]. Just like Schmitz, Wu et al., also demonstrated that LC3 overexpression was connected to less cell differentiation ($p = 0.016$)[13]. This research is also not in accordance with research by Zhao et al. in 2017 [14], which showed LC3 expression was connected with histological differentiation, T stage, N stage, CEA level, and CA19-9 level. The expression of LC3 did not significantly correlate with age, gender, illness subtype, or family history of cancer. Subjects with low LC3 expression had a higher risk of acquiring metastatic colorectal cancer (CRC) compared to individuals with high LC3 expression ($p < 0.001$). It also showed that LC3 expression in 84% of metastatic CRC subjects was low, while 56% of non-metastatic CRC patients were highly expressing LC3 ($p < 0.001$)[14]. In the study of Guo et al. in 2019, the results of the chi-square analysis of the relationship between LC3 expression and synchronous/metachronous metastases showed that the results were not significant with $p = 0.058$[36].

Studies suggest there is an uncertain relationship between LC3 expression and metastasis, as seen in this study. Several research have examined the association between LC3 expression and clinicopathological variables, including age, gender, and tumor site, including this study, did not show a significant relationship. However, from previous studies, low LC3 values have been associated with poorer levels of differentiation and a lower risk of metastases[9,11,14]. It has also been found that low LC3 values are connected, particularly in patients with advanced stages of colorectal cancer, with a favorable response to treatment and a favorable prognosis for survival[28,37,38]. Therefore, LC3 examination is important as a consideration for continued anti-cancer therapy related to autophagy in patients with CRC.

4.4. Relationship between expression of LC3 and chemotherapy responses in colorectal cancer patients

Autophagy is a potential target for therapy since it has been demonstrated in numerous studies that inhibiting it in tumor cells can increase the cell death caused by chemotherapy. It was also found that in colon cancer, decreasing LC3 expression by lentiviral shRNA could increase the induction of apoptosis by 5-FU[10]. This may indicate that autophagy proteins, including LC3, can predict chemoresistance in colorectal cancer. An investigation was done in this study between the chemotherapeutic response and the characteristics of the research subjects, including LC3 expression levels, to show the clinical results obtained after chemotherapy.

The chemotherapy response in this study was classified according to RECIST criteria consisting of progressive disease, stable disease, partial response, and complete response[39]. In this study, positive LC3 expression was found in 20 subjects (27,4%) showing progressive response, 16 subjects (21,9%) showing stable response, 12 subjects (16.4%) showing partial response, and 1 subject (1,4%) showing complete response. Meanwhile, in negative LC3 expression, 12 subjects (16.4%) showed progressive response, 8 subjects (11%) showed stable response, 3 subjects (4.1%) showed partial response, and 1 subject (1.4%) showed complete response. The univariate analysis test of chemotherapy response did not show a significant

relationship between chemotherapy response and age, gender, anatomical pathology, grade, tumor location, metastasis, and LC3 expression.

The prognostic relevance of LC3 expression to the chemotherapy response in CRC has so far only been addressed in a few studies. The study from Park et al.[10] investigated the correlation between autophagy marker expression levels, clinicopathological factors, and patient survival rates in patients receiving adjuvant 5-FU therapy for stage II and stage III colon cancer. Tumor stage, histological grade, primary site, patient age or sex, and other clinicopathological factors were not substantially correlated with LC3 expression levels. The response to the chemotherapy given is assessed by the patient's survival rate. Park et al.[10] showed that there was no significant relationship between LC3 expression levels and patient survival rates (p = 0.387), which means that the effect of LC3 was almost the same for stage II and stage III patients. Shim et al.[40] showed that pathological complete response (ypCR) was inversely related to LC3β expression (p = 0.003) and changes in autophagy-related protein expression (p = 0.046) in colorectal cancer patients who had undergone neoadjuvant chemoradiotherapy and laparoscopic total mesorectal excision. This study showed that pathological complete responses were more frequently found in rectal cancers with lower LC3β expression[40]. In the research of Guo et al. in 2019[36], an analysis was also carried out in the form of the predictive value of LC3 expression for chemotherapy containing cetuximab. In 25 patients with the wild-type KRAS gene who received chemotherapy as first-line treatment, no significant relationship was found between LC3 expression (p = 0.362) and disease control rate or overall response rate[36].

Studies suggest there is an uncertain relationship between LC3 expression and chemotherapy response, as seen in this study. Data on the impact of LC3 expression on chemotherapy response is basically still lacking, so further research is still needed. However, it is known that autophagy can predict chemoresistance in colorectal cancer, so examining LC3 as a biomarker remains important when considering further anti-cancer therapy related to autophagy in patients with CRC.

5. Conclusion

The conclusion of this study is that there is no association between LC3 expression, metastases, and chemotherapy response in colorectal cancer patients. However, the expression of LC3 assessment is an important examination for CRC patients that can help in deciding on personal targeted therapy.

Author contributions

Conceptualization, KL and RR; methodology, MRAP; software, PN; validation, BAASS, AP and TR; formal analysis, KL; investigation, AW; resources, AW and MRAP; data curation, MRAP; writing—original draft preparation, PN; writing—review and editing, PN; visualization, PN; supervision, KL; project administration, AR; funding acquisition, KL. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors extend our gratitude to all of the patients who consented to participate in this research as well as the trainees and surgical residents who made this study possible.

Ethical declaration

The study was given ethical approval by the hospital ethics committee under the number LB.02.01/X.6.5/167/2023. An informed consent form was signed by each research subject before to their involvement in the investigation.

Conflict of interest

The authors declare no conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: A Cancer Journal for Clinicians. 2018, 68(6): 394-424. doi: 10.3322/caac.21492
2. Siegel RL, Wagle NS, Cercek A, et al. Colorectal cancer statistics, 2023. CA: A Cancer Journal for Clinicians. 2023, 73(3): 233-254. doi: 10.3322/caac.21772
3. Dulskas A, Gaizauskas V, Kildusiene I, et al. Improvement of Survival over Time for Colorectal Cancer Patients: A Population-Based Study. Journal of Clinical Medicine. 2020, 9(12): 4038. doi: 10.3390/jcm9124038
4. Kemenkes RI. National guidelines for the management of colorectal cancer (Indonesian). Jakarta: Menteri Kesehatan RI. 2018; pp. 9–27.
5. Yamagishi H, Kuroda H, Imai Y, et al. Molecular pathogenesis of sporadic colorectal cancers. Chinese Journal of Cancer. 2016, 35(1). doi: 10.1186/s40880-015-0066-y
6. Rabinowitz JD, White E. Autophagy and Metabolism. Science. 2010, 330(6009): 1344-1348. doi: 10.1126/science.1193497
7. Levine B, Kroemer G. Autophagy in the Pathogenesis of Disease. Cell. 2008, 132(1): 27-42. doi: 10.1016/j.cell.2007.12.018
8. Wang Y, Zhao Z, Zhuang J, et al. Prognostic Value of Autophagy, Microsatellite Instability, and KRAS Mutations in Colorectal Cancer. Journal of Cancer. 2021, 12(12): 3515-3528. doi: 10.7150/jca.51430
9. Schmitz KJ, Ademi C, Bertram S, et al. Prognostic relevance of autophagy-related markers LC3, p62/sequestosome 1, Beclin-1 and ULK1 in colorectal cancer patients with respect to KRAS mutational status. World Journal of Surgical Oncology. 2016, 14(1). doi: 10.1186/s12957-016-0946-x
10. Park JM, Huang S, Wu TT, Foster NR, Sinicrope FA. Prognostic impact of Beclin 1, p62/sequestosome 1 and LC3 protein expression in colon carcinomas from patients receiving 5-fluorouracil as adjuvant chemotherapy. Cancer Biology & Therapy. 2013, 14(2): 100-107. doi: 10.4161/cbt.22954
11. Wu S, Chenbo S, Yangyang L, et al. Autophagy-related genes Raptor, Rictor, and Beclin1 expression and relationship with multidrug resistance in colorectal carcinoma. Human Pathology. 2015, 46(11): 1752-1759. doi: 10.1016/j.humpath.2015.07.016
12. Lock R, Roy S, Kenific CM, et al. Autophagy facilitates glycolysis during Ras-mediated oncogenic transformation. Yoshimori T, ed. Molecular Biology of the Cell. 2011, 22(2): 165-178. doi: 10.1091/mbc.e10-06-0500
13. Wu S, Sun C, Tian D, et al. Expression and clinical significances of Beclin1, LC3 and mTOR in colorectal cancer. International Journal of Clinical and Experimental Pathology. 2015; 8(4): 3882.
14. Zhao H, Yang M, Zhao B. Beclin 1 and LC3 as predictive biomarkers for metastatic colorectal carcinoma. Oncotarget. 2017, 8(35): 59058-59067. doi: 10.18632/oncotarget.19939
15. Recio-Boiles A, Cagir B. Colon cancer. Available online: https://www.ncbi.nlm.nih.gov/ books/NBK470380/ (accessed on 6 July 2023).
16. Cuschieri S. The STROBE guidelines. Saudi Journal of Anaesthesia. 2019, 13(5): 31. doi: 10.4103/sja.sja_543_18
17. Beer L, Hochmair M, Prosch H. Pitfalls in the radiological response assessment of immunotherapy. memo - Magazine of European Medical Oncology. 2018, 11(2): 138-143. doi: 10.1007/s12254-018-0389-x
18. Cheeseman SL, Joel SP, Chester JD, et al. A 'modified de Gramont' regimen of fluorouracil, alone and with oxaliplatin, for advanced colorectal cancer. British Journal of Cancer. 2002, 87(4): 393-399. doi: 10.1038/sj.bjc.6600467
19. Leicher LW, de Graaf JC, Coers W, et al. Tolerability of capecitabine monotherapy in metastatic colorectal cancer: a real-world study. Drugs in R&D. 2017; 17: 117-24. doi: 10.1007/s40268-016-0154-8
20. Mármol I, Sánchez-de-Diego C, Dieste AP, et al. Colorectal Carcinoma: A General Overview and Future Perspectives in Colorectal Cancer. Int J Mol Sci. 2017, 18(1): 197. doi: 10.3390/ijms18010197
21. Recio-Boiles A, Kashyap S, Tsoris A, et al. Rectal cancer. Available online: https://www.ncbi. nlm.nih.gov/books/NBK493202/ (accessed on 6 July 2023).
22. Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. J Gastrointest Oncol. 2012; 3(3): 153–73. doi: 10.3978/j.issn.2078-6891.2012.030
23. Mattiuzzi C, Sanchis-Gomar F, Lippi G. Concise update on colorectal cancer epidemiology. Annals of Translational Medicine. 2019, 7(21): 609-609. doi: 10.21037/atm.2019.07.91
24. Morgan E, Arnold M, Gini A, et al. Global burden of colorectal cancer in 2020 and 2040: Incidence and mortality estimates from GLOBOCAN. Gut. 2023; 72(2): 338-344. doi: 10.1136/gutjnl-2022-327736
25. Mahgoub E, Taneera J, Sulaiman N, et al. The role of autophagy in colorectal cancer: Impact on pathogenesis and implications in therapy. Frontiers in Medicine. 2022, 9. doi: 10.3389/fmed.2022.959348

26. Tanida I, Ueno T, Kominami E. LC3 conjugation system in mammalian autophagy. The International Journal of Biochemistry & Cell Biology. 2004, 36(12): 2503-2518. doi: 10.1016/j.biocel.2004.05.009
27. Chen Z, Li Y, Zhang C, et al. Downregulation of Beclin1 and Impairment of Autophagy in a Small Population of Colorectal Cancer. Digestive Diseases and Sciences. 2013, 58(10): 2887-2894. doi: 10.1007/s10620-013-2732-8
28. Yang M, Zhao H, Guo L, et al. Autophagy-based survival prognosis in human colorectal carcinoma. Oncotarget. 2015, 6(9): 7084-7103. doi: 10.18632/oncotarget.3054
29. Burada F. Autophagy in colorectal cancer: An important switch from physiology to pathology. World Journal of Gastrointestinal Oncology. 2015, 7(11): 271. doi: 10.4251/wjgo.v7.i11.271
30. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. The Journal of Pathology. 2010, 221(1): 3-12. doi: 10.1002/path.2697
31. Devenport SN, Shah YM. Functions and Implications of Autophagy in Colon Cancer. Cells. 2019, 8(11): 1349. doi: 10.3390/cells8111349
32. Vodenkova S, Buchler T, Cervena K, et al. 5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future. Pharmacology & Therapeutics. 2020, 206: 107447. doi: 10.1016/j.pharmthera.2019.107447
33. Lee YK, Lee JA. Role of the mammalian ATG8/LC3 family in autophagy: differential and compensatory roles in the spatiotemporal regulation of autophagy. BMB Reports. 2016, 49(8): 424-430. doi: 10.5483/bmbrep.2016.49.8.081
34. Manzoor S, Saber-Ayad M, Maghazachi AA, et al. MLH1 mediates cytoprotective nucleophagy to resist 5-Fluorouracil-induced cell death in colorectal carcinoma. Neoplasia. 2022, 24(2): 76-85. doi: 10.1016/j.neo.2021.12.003
35. Tang JC, Feng YL, Liang X, et al. Autophagy in 5-Fluorouracil Therapy in Gastrointestinal Cancer. Chinese Medical Journal. 2016, 129(4): 456-463. doi: 10.4103/0366-6999.176069
36. Guo GF, Wang YX, Zhang YJ, et al. Predictive and prognostic implications of 4E-BP1, Beclin-1, and LC3 for cetuximab treatment combined with chemotherapy in advanced colorectal cancer with wild-type KRAS: Analysis from real-world data. World Journal of Gastroenterology. 2019, 25(15): 1840-1853. doi: 10.3748/wjg.v25.i15.1840
37. Amin MB, Greene FL, Edge SB, et al. The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population‐based to a more "personalized" approach to cancer staging. CA: A Cancer Journal for Clinicians. 2017, 67(2): 93-99. doi: 10.3322/caac.21388
38. Guo GF. Autophagy-related proteins Beclin-1 and LC3 predict cetuximab efficacy in advanced colorectal cancer. World Journal of Gastroenterology. 2011, 17(43): 4779. doi: 10.3748/wjg.v17.i43.4779
39. Khokher S, Qureshi MU, Chaudhry NA. Comparison of WHO and RECIST Criteria for Evaluation of Clinical Response to Chemotherapy in Patients with Advanced Breast Cancer. Asian Pacific Journal of Cancer Prevention. 2012, 13(7): 3213-3218. doi: 10.7314/apjcp.2012.13.7.3213
40. Shim BY, Sun DS, Won HS, et al. Role of autophagy-related protein expression in patients with rectal cancer treated with neoadjuvant chemoradiotherapy. BMC Cancer. 2016, 16(1). doi: 10.1186/s12885-016-2250-0