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The Correlation of Light Chain 3B Immunohistochemical Expression with Histopathological Grading in Colorectal Adenocarcinoma

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ABSTRACT

Background
Colorectal cancer ranks third highest of all malignancies worldwide and ranks fourth out of the 10 most common cancers encountered in Indonesia. More than 90% of colorectal cancers are adenocarcinoma type. The mechanism of autophagy, has a dual role in tumorigenesis, namely as a tumor suppressor and as a tumor promoter. Microtubule-associated protein 1 light chain 3B (LC3B) is one of the widely used autophagy markers. The role of autophagy requires further research so that LC3B can be considered as one of the predictors of prognosis, especially in colorectal adenocarcinoma. The aim of this research is to determine the correlation of Light Chain 3B immunohistochemical expression with histopathological grading of colorectal adenocarcinoma.

Methods
Cross-sectional analytic study of 41 samples diagnosed with colorectal carcinoma at H. Adam Malik General Hospital Medan. Staining was performed using a polyclonal antibody rabbit Light Chain 3B immunohistochemistry (GeneTex) which would appear positive in the cytoplasm of tumor cells at a dilution of 1: 500. LC3B expression was assessed semiquantitatively with low and high expression categories. Data analysis was tested with the Spearman correlation test.

Results
LC3B immunohistochemical expression has a very strong correlation with the histopathological grading of colorectal adenocarcinoma and in a positive direction where the higher the colorectal adenocarcinoma grade, the higher the LC3B immunohistochemical expression, and vice versa (p=0,0001; r=0,763).

Conclusion
There was a significant relationship between LC3B immunohistochemical expression and histopathological grading of colorectal adenocarcinoma.

Keywords: Colorectal adenocarcinoma, colorectal cancer, histopathology grading, Light Chain 3B

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INTRODUCTION

The mortality rate of colorectal cancer ranks third highest of all malignancies worldwide.[1] By 2030 the number of newly diagnosed cases and deaths related to colorectal cancer is expected to reach more than 2.2 million and 1.1 million cases.[2] Based on GLOBOCAN data in 2020, the incidence of colorectal cancer ranks third in the world, with 1.9 million new cases (10%) and the second leading cause of death after lung cancer at 935 thousand cases (9.4%). In Indonesia, colorectal cancer is the fourth most common cancer with 34,189 new cases (8.6%) and is the second most common cancer in men after lung cancer. The incidence of colorectal cancer reaches 12.4 per 100,000 adult population with a mortality of 6.7 per 100,000 population.[3]

More than 90% of colorectal cancers are adenocarcinoma subtypes with typical features of invasion through the muscularis mucosa to the submucosa. Several factors such as grading, tumor stage (depth of tumor invasion), lymphatic invasion, vascular invasion, and perineural invasion may affect the prognosis of colorectal adenocarcinoma.[4] Despite advances in the diagnosis and treatment of colorectal adenocarcinoma, such as surgery and neoadjuvant therapy, and palliative care, the reported 5-year overall survival rate is still low (23.2%) and the 5-year cumulative mortality rate is still quite high at 71.3%. Therefore, studies are needed to find biomarkers that are more effective in predicting the prognosis of colorectal adenocarcinoma and to find anti-tumor therapy that targets the biological and molecular features of cancer that are expected to be more effective in reducing mortality and improving the quality of life.[5,6]

Several studies aimed at the mechanism of autophagy have been investigated in several types of cancer to assess the role of autophagy in predicting cancer prognosis. Autophagy plays a dual role in tumorigenesis, where in early stages, it plays a role in cell survival and suppresses carcinogenesis, while in advanced stages, autophagy induces survival, dormancy, growth, and metastasis of cancer cells.[6,7] Microtubule-associated protein 1 light chain 3B (LC3B) is one of the most widely used autophagy markers. The LC-3 family has 3 isoforms, namely LC-3A, LC-3B, and LC-3C. LC3 protein is a basic component of the inner and outer membrane of autophagy, so it may be used as an autophagy marker.[5,6] LC3 is a marker of autophagy and plays a role in autophagy in colorectal cancer. However, the relationship between autophagy and colorectal cancer is still controversial. Elevated LC3 expression is positively correlated with long-term survival in patients with colorectal cancer, so it can be used as a marker of colorectal cancer prognosis. According to a study by Schmitz et al although increased LC3 expression was slightly associated with poor prognosis, in the group with KRAS mutation, overexpression of LC3 was significantly associated with decreased survival. Increased autophagy levels are also thought to be associated with inadequate chemotherapy response and decreased survival.[7-9]

The mechanism of autophagy in terms of cancer cells is not yet clearly understood. The role of autophagy as a cytoprotective in cancer cells and as a tumor inhibitor also requires further research so that LC3B as one of the autophagy markers can be considered as a predictor of prognosis, particularly in colorectal adenocarcinoma. The things mentioned above and some research results that are still controversial became the basis for the author to conduct this research. The aim of this study was to determine the frequency distribution of colorectal adenocarcinoma patients based on age, gender, subtype, histopathology grading, and the frequency of Light Chain 3B immunohistochemical expression in colorectal adenocarcinoma, as well as the role of autophagy in colorectal adenocarcinoma through analysis of Light Chain 3B immunohistochemical expression assessment and its correlation with histopathology grading which may represent tumor aggressiveness.

METHODS

The design of this research is an analytic study with a cross-sectional approach. The study was conducted at the Anatomic Pathology Laboratory of H. Adam Malik Hospital Medan using paraffin blocks and slides from surgical resection that had been histologically diagnosed as colorectal adenocarcinoma. Sample calculation was carried out, obtained a total sample of 41 samples, including inclusion criteria for paraffin blocks and slides that were diagnosed as colorectal adenocarcinoma histopathologically using hematoxylin-eosin (HE) staining, where exclusion criteria were incomplete clinical data and missing or unrepresentative paraffin blocks/slides which could not be processed, cut and re-staining. Histopathology grading was assessed microscopically on the components of glandular differentiation, which was divided into a low-grade tumor if ≥50% of glandular formations were found, and categorized as a high-grade

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tumor if 0-49% of glandular formations were found.

Immunohistochemical staining of LC3B was performed using primary rabbit polyclonal antibody (GTX127375; GeneTex) that was diluted at 1:500. Light Chain 3B expression was identified by the presence of brownish dots staining in the cytoplasm of tumor cells. Immunoreactivity was semi-quantitatively evaluated by integrating the percentage of tumor cells stained and the intensity of staining. The intensity of staining was scored as follows: score 0 (not stained/negative), score 1 (weak), score 2 (strong).[10] The percentage of stained tumor cells was categorized into 5 grades: score 0 (0-5%), score +1 (6-25%), score +2 (26-50%), score +3 (51-75%), and score +4 (76-100%). The sum of the intensity and the percentage of stained tumor cells (nominal scale) was considered as follows: 1 = Low expression (score 0-3), 2 = High expression (score 4-6). Immunohistochemical staining was evaluated and scored by two independent pathologists and the researcher. The correlation of LC3B expression level and histopathology grading was determined by Spearman correlation test analysis. All p-value <0,05 (p<0,05) were considered statistically significant.

RESULTS

Forty-one samples of colorectal adenocarcinoma were obtained in this research. Table 1 shows the distribution of sample characteristics based on age, sex, subtype, and histological grade of colorectal adenocarcinoma.

According to Table 1, the median age of the patients was 50 ± 12,65 years old (range 22-82 years old), mostly in the group of age 50-59 years old (36.6%), and they were predominantly men (61%). The histologic grade was categorized as low grade (well and moderately differentiated) in 20 samples (48.8%) and high grade (poorly differentiated) in 21 (51.2%) samples. Of 41 samples of colorectal adenocarcinoma, 33 samples (80.5%) were adenocarcinoma NOS, followed by 5 samples (12.2%) were mucinous adenocarcinoma, 2 samples (4.9%) were medullary adenocarcinoma, and 1 sample (2.4%) was serrated adenocarcinoma. Low expression of Light Chain 3B immunohistochemical expression was noted in 17 samples (41.5%), meanwhile, 24 samples (58.5%) displayed high expression.

Table 1. Characteristics of 41 patients with colorectal adenocarcinoma.

Characteristics	N	%
Age		
<30 years old	2	4.9
30-39 years old	8	19.5
40-49 years old	10	24.4
50-59 years old	15	36.6
60-69 years old	3	7.3
70-79 years old	2	4.9
≥80 years old	1	2.4
Sex		
Male	25	61.0
Female	16	39.0
Histological grade		
Low grade	20	48.8
High grade	21	51.2
Histological subtype		
Adenocarcinoma NOS	33	80.5
Mucinous adenocarcinoma	5	12.2
Medullary adenocarcinoma	1	2.4
Serrated adenocarcinoma	2	4.9
*LC3B immunohistochemical expression		
Low expression	17	41.5
High expression	24	58.5

*Light Chain 3B (LC3B)

Table 2. The correlation between Light Chain 3B expression and histological grading of colorectal adenocarcinoma

Histological grade	Light Chain 3B Expression				p-value
	Low		High		
	n	%	n	%	
Low grade	16	39.0	4	9.8	0,0001*
High grade	1	2.4	20	48.8	

*Spearman correlation test

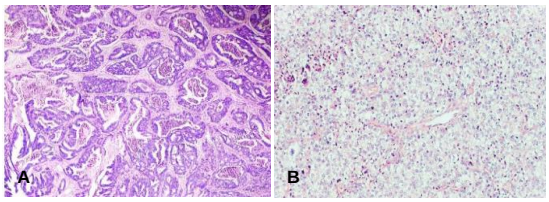

Figure 1. Tumor grade of colorectal adenocarcinoma. A. Low grade (HE, 100 times). B. High grade (HE, 100 times).

Correlation test analyses summarized in Table 2 displayed that LC3B expression levels significantly correlated with the histological grade of colorectal adenocarcinoma (p<0.05). Spearman's correlation coefficient (rho) was 0.763 (r=0.61-0.80) with a positive correlation which means that as histological grade increases, the LC3B expression also tends to increase, and vice versa. Representative histological images of tumor grade, histological

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subtypes, and LC3B expression levels are shown in Figures 1, 2, and 3 respectively.

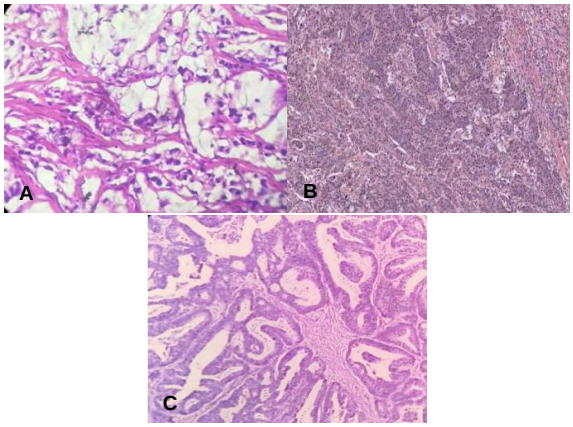

Figure 2. Histological subtype of colorectal adenocarcinoma. A.Mucinous adenocarcinoma colorectal (HE, 100 times). B. Medullary adenocarcinoma colorectal (HE, 100 times). C. Serrated adenocarcinoma colorectal (HE, 100 times).

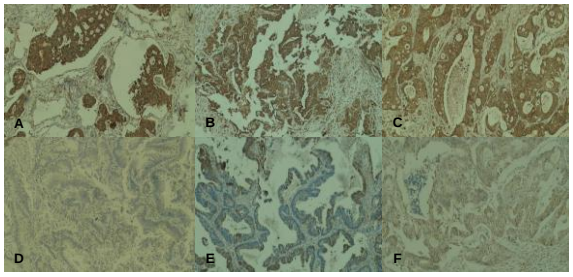

Figure 3. LC3B immunohistochemical expressions. A-C. High expression. D-F. Low expression.

DISCUSSION

In our study, we found that colorectal adenocarcinoma mostly occurred at the age of 50-59 years old (36.6%), with an average age of 50 ± 12.65 years old. This was attributed to dietary and lifestyle patterns that accumulate with age. In contrast to data from 8 countries including the UK and India, where there was an increase in the incidence of colon cancer in the age group below 50 years old. Likewise, countries such as Germany, Australia, the United States, Sweden, Canada, and the United Kingdom had a decreased or stable incidence in the age group of 50 years old or older, but an increased incidence at younger ages. A study in Makassar showed that colorectal cancer was most prevalent in the 51-60 years old group (68/268 patients, 25.4%), followed by the 41-50 years old and 61-70 years old groups (58/268 patients, 21.6% respectively). The phenomenon of increased incidence in young adult patients (under 40 years old) generally has been linked with genetic disorders such as having a history of HNPCC, FAP, intestinal infection Chron disease, and Ulcerative Colitis.[4,11,12-15]

The colorectal adenocarcinoma patients in this study were predominantly men (61,0%). According to the American Cancer Society, men have about 30% higher risk of developing colorectal cancer compared to women. In addition, men diagnosed with colorectal cancer have a worse prognosis and about 40% higher mortality than women. This is attributed to hormonal differences between men and women. Estrogen in women has a protective role against colorectal carcinogenesis, namely by regulating colonic epithelial cell growth and inhibiting colorectal tumor proliferation through estrogen receptors or by reducing secondary bile acids and Insulin-like Growth Factor-I (IGF-1). Moreover, lifestyle factors including smoking and alcoholic beverages also trigger men's susceptibility to this malignancy. Tobacco smoke contains a mixture of thousands of chemicals, more than 60 of which are well-established carcinogens (e.g., N-nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, aldehydes, and metals) that have been known to cause DNA damage. Individuals who habitually drink more than four glasses of alcohol per day increase their chances of developing colorectal cancer by 52%. Acetaldehyde oxidation from ethanol metabolism may cause inflammation of the digestive tract mucosa resulting in abnormal cell growth, in addition, acetaldehyde is also able to bind to other molecules and cause DNA mutations that will trigger carcinogenesis.[4,11,16-19]

Histological grading is one of the parameters to determine the prognosis of colorectal adenocarcinoma. The worse the grading, the worse patient's prognosis. Based on the histological grade, we found that high-grade colorectal adenocarcinoma was more common than low-grade adenocarcinoma. This is not in line with research conducted by Nasution at H. Adam Malik Medan Hospital in 2015-2017, which found that the most common grade of colorectal adenocarcinoma was well-differentiated in 44.4% of cases, followed by moderately differentiated in 35.8% of cases and poorly differentiated in 19.8%. When referring to the use of the 2019 WHO grading classification, the well differentiated and moderately differentiated categories are included in the low-grade category, while poorly differentiated is included in the high-grade category. This difference could be due to the sampling in this study was not limited by time and was taken randomly.[4,20] In our study, there only four

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subtypes were found, dominated by the NOS adenocarcinoma subtype at 80.5%, followed by the mucinous adenocarcinoma subtype at 12.2%, medullary adenocarcinoma at 4.9%, and serrated adenocarcinoma at 2.4%. This is in accordance with WHO 2019 that most cases were diagnosed as adenocarcinoma NOS.[4] Research conducted by Li et al in China also showed that adenocarcinoma NOS was the most common subtype (93.7%).[21]

A total of 41.5% of samples showed LC3B expression with a low expression category, while 58.5% of samples showed high expression. This result was not different from the study by Niklaus et al in which colorectal adenocarcinoma samples with high expression of LC3B were dominated (59.9%) compared to low expression.[6] The high-grade colorectal adenocarcinoma mostly had high expression of LC3B as well (48.8%). Likewise, the low-grade tumor mostly showed low expression of LC3B, which was around 39%. However, there was a high-grade tumor that showed LC3B low expression (2.4%) and a low-grade tumor that showed high expression of LC3B (9.8%). Increased apoptosis mentioned in the literature may worsen the grading of the tumor, caused by tumor cells losing their differentiation, and tumor cells will try to increase the process of autophagia to maintain their survival.[7,22]

The number of solid areas in a tumor can also worsen the histological grading. In high-grade colorectal adenocarcinoma, there are fewer glandular components found, and they are replaced with solid areas and later tend to experience hypoxia. If a tumor cell experiences a state of hypoxia, the tumor cell will try to maintain its survival in order to maintain the energy balance in tumor cells by increasing the process of autophagia. This explains why in high-grade tumors, there is a high autophagia process. Similarly, in this study, high LC3B expression was found in high-grade colorectal adenocarcinoma. In solid tumors, including colorectal cancer, the expression of autophagia markers, such as Beclin-1, LC3B, p62, and Rab-7 tends to be high.[7,22-26] In this study, there was only 1 high-grade serrated adenocarcinoma subtype that showed low LC3B expression. In addition, there were also 4 low-grade colorectal adenocarcinoma samples that showed high LC3B expression, namely adenocarcinoma NOS and mucinous adenocarcinoma subtypes. There is no definitive explanation in terms of this, but MSI status and KRAS mutations seem to have an important role that still needs further study.[27]

The assessment of mucinous adenocarcinoma's histological grade is still polemic. In the conventional system, colorectal cancer is graded based on the degree of glandular differentiation where the presence of glandular formation ≥50% is considered as low grade, and other cases showing <50% of glandular structures are considered high-grade tumors. However, historically it is still unclear whether this scoring scheme must be applied in the mucinous adenocarcinoma subtype. In fact, in WHO classification 2000, all mucinous adenocarcinoma subtypes were considered high-grade tumors. Meanwhile, in the newest WHO Classification of Digestive Tumors (2019), it was mentioned that the determination of mucinous adenocarcinoma's grade is based on glandular formations.[4,28] Previous studies reported higher expression levels of LC3 found in colon and colorectal cancer tissues when compared to normal mucosa, along with other autophagia markers such as p62.[6,27,29-32]

In our study, correlation analysis displayed that there was a significant positive correlation (p=0.0001) between LC3B expression and colorectal adenocarcinoma's grade. This indicates that the higher the grading of colorectal adenocarcinoma, the higher the LC3B expression, and vice versa. This finding is supported by a previous study, where high LC3B expression was significantly associated with high colorectal cancer pT status (p=0.02), lymphatic invasion (p=0.002), and nerve plexus invasion (p = 0.06).[32] Another study reported that LC3B expression was significantly associated with tumor differentiation, tumor margins, vascular and nerve plexus invasion, lymph node metastasis, and tumor pStage.[29] These results suggest that autophagia may contribute to tumor progression in colorectal cancer. On the other hand, a study by Shim et al showed that LC3B expression levels were not associated with age, gender, primary disease stage (tumor size and node status), histological grade, or serum tumor markers in patients with rectal cancer. However, low LC3B protein expression in that study was strongly associated with achieving pathologic complete response (ypCR) and pathologic T0 stage after neoadjuvant chemoradiotherapy.[33]The former study showed a statistical trend of shorter overall survival in colorectal cancer patients of KRAS mutation subgroup with positive LC3 expression was found. LC3 positivity was significantly associated with poor tumor differentiation grading (p=0.016). Histological grading, lymph node metastasis, tumor stage, and distant

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metastasis in the study were also significantly correlated with the patient's outcome.[34]

It has been observed that the autophagia process is inhibited in the advanced stage of the tumor, which is indicated by the accumulation of p62 and LC3B, although autophagia is triggered in the early stage as indicated by increased Beclin-1 protein levels.[20] Niklaus et al reported that the combination of dot-like LC3B/dot-like-cytoplasmic p62 staining showed the best prognostic discrimination. Cytoplasmic staining such as high dot-like LC3B/high dot-like p62 was associated with the best prognosis; whereas high dot-like LC3B/low dot-like p62 showed the worst prognosis, indicating the activation of autophagia.[6] It is known that p62 could act as a tumor suppressor because it is able to induce autophagic degradation of regulators of the Wnt signaling pathway. It is speculated that p62 deficiency may upregulate this oncogenic pathway, resulting in increased biological aggressiveness.[34] Therefore, the results of this study need to be supported by the results of other studies evaluating the correlation between colorectal adenocarcinoma grading and other autophagy markers.

CONCLUSION

The most common age of colorectal adenocarcinoma patients in this study was 50-59 years old (15 samples), which were predominantly men as many as 25 samples. The most common colorectal adenocarcinoma subtype was adenocarcinoma NOS with 33 samples and the most common grading was high grade. Immunohistochemical expression of LC3B in colorectal adenocarcinoma predominantly was high expression (58.5%). In addition, the results of statistical analysis summarized a strong correlation between LC3B immunohistochemical expression and histopathological grading of colorectal adenocarcinoma with a positive direction, where the higher the grading of colorectal adenocarcinoma, the higher the LC3B immunohistochemical expression, and vice versa (p=0.0001; r=0.763 (r=0.61-0.8).

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