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Identification of tumor infiltrating lymphocyte CD8 in Indonesian colorectal cancer population: a cross-sectional study

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ABSTRACT

Background: Colorectal cancer is now the third most common cancer both globally and in Indonesia. An important component of the adaptive immune system, tumor-infiltrating lymphocytes (TILs) cluster of differentiation 8 (CD8+) are key in the immune response to malignancies, which were previously believed to be the primary initiators of anti-tumor immunity. A higher TIL CD8+ levels in the tumor microenvironment and a better prognosis have been associated with anticancer benefits in a number of cancers. This study aimed to describe the characteristics of colorectal cancer patients with increased tumor-infiltrating lymphocyte CD8+ expression and their relationship with clinicopathological features.

Methods: A cross-sectional investigation was conducted. Patients in the digestive surgery division with colorectal cancer served as the study's research subjects. There were 36 study subjects that were randomly selected from the available specimens. Examination of tumor-infiltrating lymphocytes CD8+ expression was carried out by the immunohistochemistry method.

Result: Expression of tumor-infiltrating lymphocytes CD8+, was positive in nine (25%) patients, and normal expression was shown in 27 (75%) patients. Most of the patients had adenocarcinomas histologically (91.7%), and 66.7% of patients were older than 50, with 77.8% of the tumors located at the rectum. Univariate analysis of the clinicopathological characteristics related to the tumor-infiltrating lymphocytes CD8+, showed a non-significant relationship statistically ($p > 0.05$).

Conclusion: There was no relationship between clinicopathological characteristics and tumor-infiltrating lymphocyte CD8+ expression in colorectal cancers.

Keywords: Colorectal Cancer, immunohistochemistry, infiltrating lymphocytes, tumor-infiltrating lymphocytes.
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INTRODUCTION

The last report for estimated global cancer statistics in 2020 showed that colorectal cancer (CRC) new cases reached 1,880,000, and the incidence of colorectal cancer is the third highest after breast and lung cancer.[1] In Southeast Asia, the crude mean incidence of CRC was 6.95 per 100,000. In Indonesia, the incidence rate reached 17.2/100,000 people.[2,3]

The survival rate of CRC has significantly increased thanks to improved screening practices and the use of various treatments, which have been made possible by a deeper understanding of the pathophysiology of colorectal cancer.[4] However, with recurrent or advanced metastases, over 40% of CRC patient's relapse. As a result, substantial research is currently being done to find ways to overcome resistance and relapse as well as more efficient targets, including targeted therapy and immunotherapy.[5]

The host immune system's intrinsic processes have been successfully used in immunotherapy during the past ten years to eradicate malignant cells, transforming the therapeutic environment for a variety of solid and non-solid tumors. A rising number of studies, including those involving lymphocytes (including T cells, B cells, and natural killer (NK) cells), macrophages, dendritic cells, and neutrophils, which show a wide range of patient-to-patient variance, are supporting the significance of tumor immune infiltration.[6] For a very long period, it was believed that colorectal cancer was immunogenic and difficult to treat with immunotherapy. Due to advancements in the molecular identification of tumor-associated antigens recognized by T cells and methods for determining antigen-specific T cell responses, the scientific community's viewpoint on this issue has now changed.[7,8]

Tumor-infiltrating lymphocytes (TILs) CD8+, an essential part of the adaptive immune system, it is crucial for immunological protection against intracellular pathogens such viruses, bacteria, and tumors, which were thought to be the main sources of anti-tumor immunity.[8-11] Immunosuppressive cells do make up a part of tumor-infiltrating lymphocytes, but the tumor selectively recruits and/or directs these cells to preserve the immune-privileged microenvironment. TILs, on the other hand, might sometimes be seen as the

immune system's attempts to combat tumor responses.[11] Several molecules generated by CD8+ T lymphocytes, including perforin, granzymes, granulysin, Fas ligand, and tumor necrosis factor (TNF), participate in the cytotoxicity process. By identifying tumor antigens and obliterating altered cells directly, TILs CD8+ induce tumor rejection. Interleukin-2 (IL-2), IL-12, and interferon (IFN) are produced by effector CD8+ T cells in the tumor microenvironment, enhancing TILs CD8+ and resulting in the targeted destruction of tumor cells.[8-11]

Most tumor-infiltrating immune cell subtypes were recently studied, and CD8+ T cells were found to have the highest effect on patient survival.[12] Pages et al. and Galon et al. conducted the initial analysis of the function of TILs CD8+ in prognosis in a sizable cohort of CRC patients.[13,14] Several studies have shown that increased TILs levels in the tumor microenvironment have been linked to anticancer effects and a better prognosis in a number of malignancies. Additionally, tumors from patient cohorts that were divided into groups based on the immune infiltrate's density and whether metastases were present or not showed that a sufficient immune infiltrate, along with the successful initiation and differentiation TILs CD8+, is necessary for the successful suppression of metastasis development.[12,15] This study aims to describe the characteristics of CRC patients with TILs CD8+ expression and their relationship with clinicopathological features.

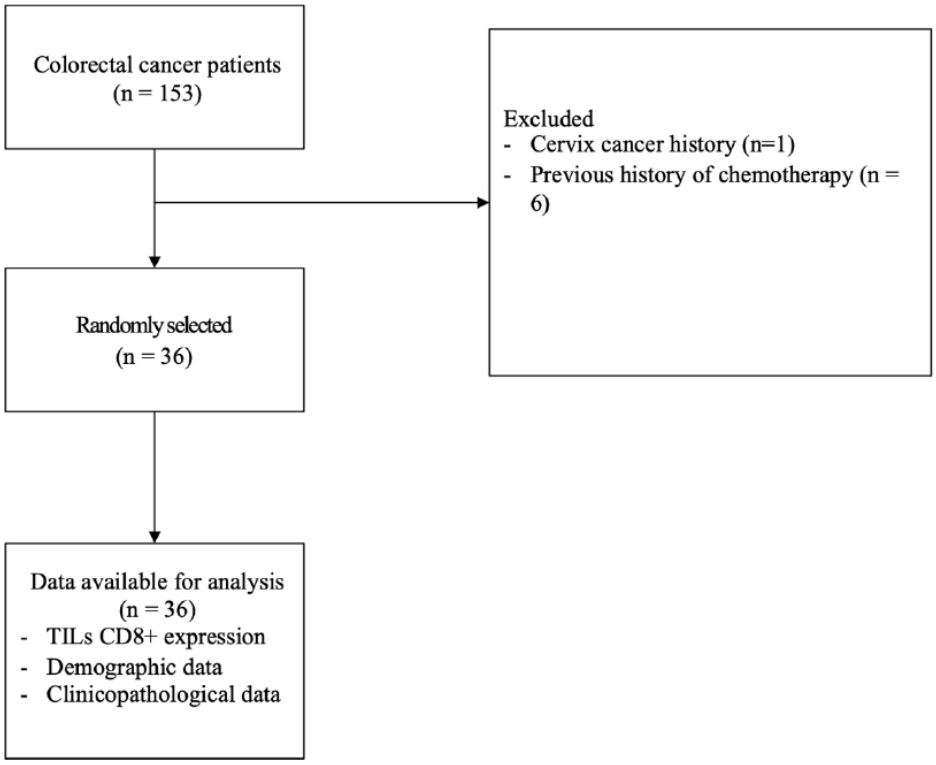

Figure 1. The flowchart of the study selection process.

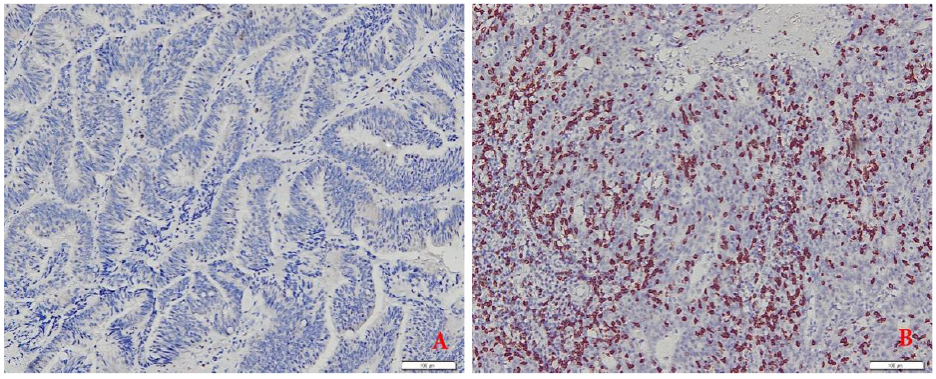

Figure 2. Assessment of TILs CD8+ expression with IHC, A. IHC score negative: no staining or weak staining of tumor cells, B. IHC score positive: intense membrane more than 75% of tumor cells.

PATIENTS AND METHODS

Study design and setting

The study was cross-sectional and adhered to the STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) standards.[16] It took place in the tertiary hospital of West Java, Indonesia. Patients with colorectal cancer who came to digestive surgery at a tertiary general hospital in West Java from February to March 2023 served as the study's research participants. There were 36 study subjects that were randomly selected from available specimens, as shown in Figure 1. Colorectal cancer patients with anatomical pathology confirmation who are older than 18 and who have consented to participate in this study were included. Patients with colorectal cancer who had received systemic therapy—radiotherapy or chemotherapy—and were afflicted with another cancer, such as lung, ovarian, or breast cancer—were excluded. The examination of TILs CD8+ was carried out by the immunohistochemistry (IHC) method of paraffin blocks for the histological diagnosis of colorectal cancer. The hospital ethics committee approved the study prior to sample collection.

TILs CD8+ Expression using IHC Analysis

Tissue samples were examined histologically by hematoxylin and eosin (HE) staining for the diagnosis of CRC, and for TIL CD8+ expression analysis, all samples were immunohistochemically stained with CD8+ antibodies. Each of the paraffin blocks was cut to a thickness of 4 microns and then deparaffinized with xylol and alcohol. Antigen retrieval was performed using a decloaking tool at a temperature of 950°C for as long as 20 minutes for CD8+.

Table 1. Characteristics of research subjects

Variable		Value
Age	Mean	54.89 ± 14.55
	Median	56
Age, n (%)	< 50	12 (33.3)
	≥ 50	24 (66.7)
Sex, n (%)	Male	17 (47.2)
	Female	19 (52.8)
Histologic, n (%)	Adenocarcinoma	33 (91.7)
	Signet Ring Cell	2 (5.6)
	Mucinous Adenocarcinoma	1 (2.8)
Grade, n (%)	Well Diff	28 (77.8)
	Moderately Diff	4 (11.1)
	Poorly Diff	1 (2.8)
	Specific	3 (8.3)
Tumor location, n (%)	Colon	8 (22.2)
	- Caecum	2 (5.6)
	- Ascending colon	2 (5.6)
	- Transverse colon	1 (2.8)
	- Sigmoid	3 (8.3)
	Rectum	28 (77.8)
Stage, n (%)	I	2 (5.6)
	II	13 (36.1)
	III	9 (25)
	IV	12 (33.3)
Metastases, n (%)	Liver	9 (25)
	Lung	1 (2.8)
	Liver and bone	2 (5.6)
TILs CD8+, n (%)	Normal expression	27 (75)
	Over expression	9 (25)

TILs CD8+ = Tumor-infiltrating lymphocytes CD8+

Immunohistochemistry staining was performed using a labelled streptavidin-biotin immunoperoxide complex. The primary antibodies used in this study were anti-CD8 mouse monoclonal (144B, Cellmarque, California, USA) with a dilution of 1:100. The secondary antibody used was Star Trek Universal HRP Detection STUHRP700L10-KIT (Biocare). Positive TILs CD8+ expression was based on the intense staining of more than 75% of tumor cells, as shown in Figure 2.

Statistical analysis

Using the statistical analysis program SPSS 26 for the variable (SPSS Inc., Chicago, IL, USA). The mean and standard deviation (SD) were used to describe descriptive data. Categorical data described as percentage. The chi-square test was used to compare the variables. A p value of 0.05 or lower was regarded as significant.

RESULTS

Table 1 showed the research participant's characteristics. Thirty-six patients were a part of the study. Nineteen patients were male and seventeen were female. 66.7% of patients were over the age of 50, and the mean age was 54.89 years plus 14.55. Histologically, adenocarcinomas were seen in the majority of the patients (91.7%), and 77.8% of them had tumors that were well differentiated. There were eight (22.2%) patients with colon cancer and 28 (77.8%) with rectal cancer. Expression of TILs CD8+ was positive in nine (25%) patients, and normal expression of TILs CD8+ was shown in 27 (75%) patients. None of the six variables related to the TILs CD8+ expression were statistically significant ($p < 0.05$) in univariate analysis, as shown in Table 2.

DISCUSSION

The clinicopathological features of the research subjects from this study show a median age of 56 years and an average age of 54.89 ± 14.55 years. The majority of the patients who came for cancer treatment are mostly over 50 years old, as described in late-onset. The results of this study are consistent with the epidemiological studies' findings about the profile of CRC patients, who are mostly over 50. Studies have shown that the risk of CRC increases in the fifth decade of life. According to some research, starting at age 50, the risk rises by up to one percent (1%) for every subsequent 10 years. There were 17 men and 19 women (52.8 percent and 47.2 percent, respectively). There are only slight differences between male and female respondents. In a different study, the prevalence rate of colorectal disease was 1:23 (4.3%) in men and 1:25 (4.0%) in women, with no difference between the sexes.[17,18] As many as 33 subjects (91.7%) had adenocarcinoma, which was the histological diagnostic for colorectal cancer. Signet ring cell carcinoma was found in two subjects (5.6%) and mucus adenocarcinoma was found in one subject (2.8 percent). While 28 subjects (77.8%) shown well differentiation based on grade, four subjects (11.1%) demonstrated moderate differentiation, and one subject (2.8%) demonstrated poor differentiation. According to literature reviews, adenocarcinoma can be found in up to 90% of colorectal cancer. Adenocarcinomas can differentiate into comedo, medullary, micropapillary, mucinous, or signet ring cells. Most literature research on the adenocarcinoma type reveal moderate differentiation, which is as much as 70%, followed by well and poor differentiation, which is about 10-20%.[17–20] This is a contrast comparison for this study where majority of the tumor differentiation was well differentiation.

Based on the tumor's location, there were eight (22.2%) cancers in the colon and 28 (77.8%) cancers in the rectum. Based on the existing literature, about more than half of colorectal cancer occurs in the rectum, followed by the descending and sigmoid colon.[19,20] Most of the study subjects suffered from CRC stages II and

Table 2. Univariate analysis for subject characteristics with TILs CD8+ expression

	Normal expression	Over expression	*p* value
Age, n (%)			
Early onset (<50)	10 (27.8%)	2 (5.6%)	0.414
Late-onset (≥50)	17 (47.2%)	7 (19.4%)	
Sex, n (%)			
Male	14 (38.9%)	3 (8.3%)	0.335
Female	13 (36.1%)	6 (16.7%)	
Histologic, n (%)			
Adenocarcinoma	25 (69.4%)	8 (22.2%)	0.603
Signet Ring Cell	1 (2.8%)	1 (2.8%)	
Mucinous Adenocarcinoma	1 (2.8%)	0	
Grade, n (%)			
Well Diff	20 (55.6%)	8 (22.2%)	
Moderately Diff	4 (11.1%)	0	0.579
Poorly Diff	1 (2.8%)	0	
Specific	2 (5.6%)	1 (2.8%)	
Tumor location, n (%)			
Colon	7 (19.4%)	1 (2.8%)	0.355
Rectum	20 (55.6%)	8 (22.2%)	
Stage, n (%)			
I	1 (2.8%)	1 (2.8%)	
II	9 (25%)	4 (11.1%)	0.390
III	6 (16.7%)	3 (8.3%)	
IV	11 (30.6%)	1 (2.8%)	

TILs CD8+ = Tumor-infiltrating lymphocytes CD8+

IV: 13 subjects (36.1%) and 12 subjects (33.3%), respectively. Subjects with stage IV CRC showed the highest metastasis to the liver (75% of the total metastasis cases).

This demonstrates that the majority of Indonesian CRC patients seek treatment when they have severe clinical symptoms such as constipation, blood in the stool, and abdominal pain as signs of an advanced stage of colorectal cancer.[21]

The expression of TILs CD8+ in this study showed nine subjects (25%) with positive mutation results. In this study, the proportion of KRAS mutations was similar to that in several other studies. In the study by Sideras et al. with 47 patients with stage IV CRC with liver metastases, 47 subjects underwent a TILs CD8+ examination. The majority of TILs expression is shown in the peri-tumoral region when compared to the intra-tumoral region. The median TILs CD8+ density in the intra-tumoral region was 58.6/mm2, and the peri-tumoral median was 883/mm2. Calculation of the ratio of TILs CD8+ to regulatory T-cells in the intra-tumoral region showed this ratio as a predictor of survival (hazard ratio 0.45, 95% confidence interval 0.20-0.99, p = 0.044).[22]

The study by Trabelsi et al. with 106 CRC patients underwent a TILs CD8+ examination in the central tumor (CT) and invasive margin (IM) areas. Positive results expressed as high TILs CD8+ expression in both areas; heterogeneous results expressed as high results in one area; and a negative result expressed as a low result in both areas. Their study showed that from 95 subjects, there were 20/95 (21.05%) with high TILs CD8+ expression, heterogeneous at 49/95 (51.58%), and low at 26/95 (27.37%). The results of the analysis showed that tumors with high TILs CD8+ expression had better overall survival (OS) compared to tumors with low TIL CD8+ expression (P < 0.005), similar to glutamine.[23,24]

Based on the results of this study, TILs CD8+ expression and clinicopathological factors in CRC patients did not show an interrelated relationship. Based on existing research, TILs CD8+ expression was positively connected with improved overall survival in CRC patients, demonstrating a correlation between the two. In a study of several tumors, it was discovered that the number of CD8+ T lymphocytes present in a tumor is the strongest indicator of how well a patient will respond to anti-programmed cell death receptor 1 (PD-1/PD-L1) therapy.[25,26] One of the limitations of this study is the relatively small sample size. For future research, the examination of TILs CD8+ expression can be related to the expression of PDL-1, which can be the basis for implementing immunotherapy in patients with CRC, especially metastatic CRC.

CONCLUSION

No clinicopathological relationship observed between TILs and CD8+ expression in colorectal cancers. However, examination of the expression of TILs CD8+ expression in CRC patients can help unlock the potential of its development in targeted immunotherapy, either for metastatic CRC or at an earlier stage.

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CONFLICT OF INTEREST

There are no disclosed conflicts of interest for the authors.

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ETHICAL STATEMENT

The Dr. Hasan Sadikin Hospital ethics committee approved the project with permission code LB.02.01/X.6.5/08/2023.

AUTHOR'S CONTRIBUTION

All authors contributed to the study's conception and design as well as its drafting and finalization. Elit Irawan, Kiki Lukman, Deny Budiman and Prapanca

Nugraha participated in data collecting. Etis Primastari participated in the examination of the TIL CD8+ expression using iimunohistochemistry methods. Nurhayat Usman, Bambang Am Am Setya Sulthana and Reno Rudiman analyzed the data and interpretation.

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