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Article

Association between tumour-infiltrating lymphocyte-T CD8+ and programmed death-ligand 1 protein with the occurrence of metastasis in colorectal cancer patients: An observational study

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Abstract: Background: Tumour-infiltrating lymphocytes CD8+ (TILs CD8+) as an anticancer immune response and Programmed Death-Ligand 1 (PD-L1) as an immune checkpoint molecule expression are important in colorectal cancer (CRC) as their expression correlates with the immune status and the progression of cancer. The primary objective of this study is to examine the relationship between TILs CD8+ and PD-L1 expression levels and CRC metastasis to provide insights into the immune microenvironment's role in CRC progression and metastasis, potentially improving patient outcomes and treatment response. **Methods:** This study was an observational study involving colorectal cancer patients who were treated at a tertiary hospital in Bandung, West Java, Indonesia. There were 40 patients included in the study. Tumour tissue samples were collected from patients, and immunohistochemical staining was performed to evaluate TILs CD8+ infiltration and PD-L1 expression levels. Clinical data, including metastasis occurrences, were collected from the cancer registry. **Results:** Analysis of TILs CD8+ and PD-L1 expression levels in CRC patients revealed significant findings regarding metastasis occurrence and cancer staging. There was a notable positive correlation between PD-L1 expression and TILs-CD8+ infiltration in the presence of distant metastasis and advanced cancer stage ($p < 0.05$). However, no significant association was observed between TILs CD8 and PD-L1 expression levels and lymphatic metastasis. **Conclusion:** This study highlights the potential prognostic value of TILs CD8+ and PD-L1 expression levels in predicting CRC progression and metastasis. However, the lack of a significant correlation with lymphatic metastasis suggests the need for further investigation into the underlying mechanisms. Understanding the roles of PD-L1 and TILs-CD8+ expression in CRC metastasis may facilitate the development of targeted therapeutic strategies to improve patient outcomes.

Keywords: cancer progression; immune response; immunomodulation; prognostic markers; therapeutic targets; tumour microenvironment

1. Introduction

Colorectal cancer (CRC) represents a significant global health burden, ranking third in prevalence worldwide and posing a substantial threat to public health [1]. In Indonesia, CRC holds the position of the fourth most prevalent cancer, further underscoring its impact on the local population [1]. The intricate pathogenesis of CRC involves various molecular pathways, including chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP),

highlighting the complexity of its development. At the forefront of the immune response against CRC are tumour-infiltrating lymphocytes CD8+ (TILs CD8+), renowned for their potent anti-tumour activity [2]. The TILs CD8+ play a pivotal role in identifying tumour-specific antigens and directly targeting transformed cells, contributing significantly to tumour rejection. Additionally, these cells orchestrate the production of critical cytokines such as interleukin-2 (IL-2), IL-12, and interferon-gamma (IFN-γ), which enhance the activation and function of other immune cells, ultimately leading to the targeted elimination of tumour cells [2,3].

Conversely, programmed death-ligand 1 (PD-L1) acts as a key regulator of immune evasion within the tumour microenvironment. Physiologically, PD-L1 engages with its inhibitory receptor, programmed cell death protein 1 (PD-1), on T lymphocytes, dampening their activation and impeding their anti-tumour function. However, in the tumour microenvironment, PD-L1 expression escalates through various mechanisms, triggering abnormal PD-L1/PD-1 signaling. This aberrant activation curtails T cell proliferation and differentiation, inducing apoptosis and impeding cytokine secretion, thereby promoting tumour invasiveness and metastasis [3]. The advent of immunotherapy, particularly immune checkpoint inhibitors (ICI), has revolutionised CRC treatment paradigms. Targeting the PD-1/PD-L1 axis has emerged as a promising avenue, bolstered by studies suggesting enhanced immune responses against tumors. Consequently, assessing PD-L1 expression status has become pivotal in determining appropriate ICI therapy regimens, highlighting the clinical significance of this biomarker [4].

Despite advancements, comprehensively understanding the intricate interplay between TILs CD8+ and PD-L1 expression in CRC metastasis remains imperative for optimising therapeutic approaches and improving patient outcomes. Hence, this study attempts to investigate the expression patterns of TILs CD8+ and PD-L1 in CRC patients, with a particular focus on metastasis occurrence. By delving into the nuances of the immune microenvironment in CRC, this research aims to identify potential prognostic markers and therapeutic targets, thereby fostering advancements in CRC management strategies.

2. Material and methods

2.1. Study design and setting

The present study had an analytical observational study and retrospective cross-sectional design to examine the relationship between TILs CD8+ and PD-L1 expression in anatomical pathology preparation of colorectal cancer patients at a tertiary hospital in Bandung, Indonesia. The inclusion criteria included intact paraffin blocks that can still be analysed as well as patient medical record data that can be accessed. Exclusion criteria were paraffin blocks that were damaged or incomplete so that they were difficult to analyze. This study was conducted between June 2023 and February 2024. The minimum sample size was calculated using the formula for the difference test of two proportions. Based on Sitompul et al.'s, it is known that the proportion of high stromal TILs expression in patients with PD-L1 expression in total tumour cells and high TILs is 0.375 [5]. The desired level of significance is 0.05, and the desired power is 80%. Based on calculations using the formula mentioned before,

it is known that the minimum number of samples required is 36 samples.

2.2. TILs CD8+ and PD-L1 expression using immunohistochemical staining

The formalin-fixed paraffin-embedded tissue blocks were cut to the thickness of a 3-μm-thick section and then incubated in a 38 ℃ incubator overnight. The specimen was deparaffinized with xylol and rehydrated with a series of ethanol (alcohol) solutions of decreasing concentration. To block nonspecific endogenous peroxidase activity, treat the treat the specimen with a peroxidase solution in methanol, incubate the specimen for 10–15 min, and then rinse with running water. Epitope retrieval was performed on the sample through an antigen retrieval process using ethylenediamine tetra acetic acid (EDTA) solution in a decloaking chamber at a temperature of 1000 ℃ for as long as 20 min. Immunohistochemistry staining was performed using a labelled streptavidin-biotin immunoperoxide complex. The primary antibodies used in this study were anti-PDL-1 rabbit monoclonal (SP142, Abcam, Inc., Cambridge, USA) with a dilution of 1:300 and anti-CD8 rabbit monoclonal (clone SP16, Abcam, Inc., Cambridge, USA) with a dilution of 1:200. After applying the primary antibody, incubate, then rinse in PBS twice for 5 min each. The secondary antibody used was Star Trek Universal HRP Detection STUHRP700L10-KIT (Biocare). After secondary antibody, apply counterstain (Hematoxylin Mayer's), incubate for 10 min, then rinse with distilled water and apply bluing agent with 0.25% lithium carbonate for 10–20 s.

The assessment of TILs CD8+ T immunohistochemical expression is done semi-quantitatively by observing the distribution of immunohistochemical staining. Lymphocytes showing positive CD8 immunohistochemical expression are stained brown on the cell membrane and partially in the cytoplasm. Each slide is evaluated in the entire tumour area using microscopic examination (×400). Five areas without necrosis is selected for evaluation. Adjacent normal tissue, or fibrosis, is not included in the evaluation. The expression of CD8+ T lymphocytes in the stromal area adjacent to tumour cells is examined and assessed based on the percentage of positive cells. Density scores are used for the analysis of CD8+ T lymphocyte cell expression, and the cutoff value for the percentage of positive cells is determined as follows: <20% (score 0), 20%–50% (score 1), >50% (score 2). Score 0 for negative, score 1 for low, and score 2 for high.

The assessment of PD-L1 immunohistochemical expression is performed semi-quantitatively by observing the proportion of TC showing membrane staining of any intensity. Tumour cells showing PD-L1 immunohistochemical expression are stained brown on the cell membrane and/or cytoplasm. Each slide is evaluated in the entire tumour area using microscopic examination (×400). Five areas without extensive inflammation or necrosis are selected for evaluation. Adjacent normal tissue or fibrosis are also not included in the evaluation. Positive PD-L1 expression is evaluated in invasive tumour mass groupings. The expression of PD-L1 in tumour cells is studied and assessed based on the percentage of positive cells. The tumour cell score (percentage of positive cells x all tumour cells) is used for PD-L1 expression analysis, and the cutoff value for the percentage of positive cells is determined as low or negative if stained <5%, weak positive if stained ≥5%, and strong positive if stained

≥50%.

2.3. Metastasis data collection

Data collection from patients was carried out through medical records, the colorectal cancer registry, and the anatomical pathology laboratory. Patient data taken included age, gender, and stage of CRC. The data on lymphatic metastases was taken from the pathology examination, and the data on distant metastases was taken from the X-rays or abdominal CT scan.

2.4. Data analysis

The data was processed using SPSS version 26.0. Statistical analysis for categorical data was performed using the Chi-Square test, with a *p* value less than 0.05 considered significant.

3. Results

Of the 40 research subjects, the mean age was 57.33 years with a standard deviation of 12.95 years, and the median age was 59 years. The age ranged from 22 to 88 years, with a range of 66 years. The majority of the subjects were over 50 years old (77.5%). Regarding gender, 45.0% were male and 55.0% were female. Adenocarcinoma was the most common histological type (92.5%), followed by mucinous adenocarcinoma (2.5%) and signet ring cell carcinoma (5.0%). Most tumours were well-differentiated (80.0%). The most common location of the tumour was the rectum (67.5%), followed by the colon (32.5%). In terms of cancer staging, 57.7% of the participants were at stage III, followed by stage IV (30.0%) and stage II (12.5%). Lymphatic metastasis was present in 87.5% of participants, while distant metastasis was present in 30.0%, primarily in the liver. Regarding immune markers, 57.5% of participants showed normal expression of TILs CD8+, while 42.5% showed excessive expression. For PD-L1 expression, 37.5% showed normal expression, 42.5% showed weak positive expression, and 20.0% showed strong positive expression (**Table 1**).

Table 1. Characteristics of study subjects.

Variable		**Proportion (%)**
Age	Mean	57.33 ± 12.95 years
	Median	59 years
	Minimum	22 years
	Maximum	88 years
	Range	66 years
Age	<50	9 (22.5%)
	≥50	31 (77.5%)
Gender	Male	18 (45.0%)
	Female	22 (55.0%)

Table 1. (*Continued*).

Variable		Proportion (%)
Pathological anatomy	Adenocarcinoma	37 (92.5%)
	Mucinous adenocarcinoma	1 (2.5%)
	Signet ring cell	2 (5.0%)
Grade	Well differentiated	32 (80.0%)
	Moderately differentiated	3 (7.5%)
	Poorly differentiated	2 (5.0%)
	Specific	3 (7.5%)
Location	Caecum	2 (5%)
	Ascending colon	4 (10%)
	Transverse colon	3 (7.5%)
	Descending colon	1 (2.5%)
	Sigmoid colon	3 (7.5%)
	Rectum	27 (67.5%)
Location	Colon	13 (32.5%)
	Rectum	27 (67.5%)
Stage	II	5 (12.5%)
	III	23 (57.7%)
	IV	12 (30%)
Lymphatic metastasis	No lymphatic metastasis	5 (12.5%)
	Lymphatic metastasis	35 (87.5%)
Distant metastasis	No distant metastasis	28 (70%)
	Liver	12 (30%)
TILs CD8+	Negative expression	23 (57.5%)
	High expression	17 (42.5%)
PD-L1	Negative expression	15 (37.5%)
	Weak positive expression	17 (42.5%)
	Strong positive expression	8 (20%)

The association between various factors and the expression levels of TILs-CD8+ in colorectal cancer patients is presented in **Table 2**. For age, a statistically significant association was found ($p = 0.030$), with a higher proportion of patients over 50 years old exhibiting excessive TILs CD8+ expression compared to those under 50 years old. However, no significant association was observed between gender and TILs CD8+ expression ($p = 0.289$). Similarly, there was no significant association between TILs CD8+ and histological type, tumour grade, location, and staging ($p > 0.05$). However, a significant association was observed between TILs CD8+ expression and distant metastasis ($p = 0.030$), indicating a higher proportion of patients with distant metastasis showing excessive TILs-CD8+ expression.

Table 2. Relationship between TILs CD8+ expression and characteristics of research subjects.

	Negative expression	**High expression**	**Chi-square (*p* value)**
Age			
Early onset (<50)	8 (20%)	1 (2.5%)	**0.030**
Late onset (≥50)	15 (37.5%)	16 (40%)	
Gender			
Male	12 (30%)	6 (15%)	0.289
Female	11 (27.5%)	11 (27.5%)	
Pathological anatomy			
Adenocarcinoma	21 (52.5%)	16 (40%)	0.160
Mucinous adenocarcinoma	1 (2.5%)	0	
Signet ring cell	1 (2.5%)	1 (2.5%)	
Grade			
Well differentiated	17 (42.5%)	15 (37.5%)	
Moderately differentiated	3 (7.5%)	0	0.454
Poorly differentiated	1 (2.5%)	1 (2.5%)	
Specific	2 (5%)	1 (2.5%)	
Location			
Caecum	2 (5%)	0	
Ascending colon	3 (7.5%)	1 (2.5%)	
Transverse colon	2 (5%)	1 (2.5%)	0.522
Descending colon	0	1 (2.5%)	
Sigmoid colon	1 (2.5%)	2 (5%)	
Rectum	15 (37.5%)	12 (30%)	
Location			
Colon	8 (20%)	5 (12.5%)	0.720
Rectum	15 (37.5%)	12 (30%)	
Stage			
II	3 (7.5%)	2 (5%)	0.077
III	10 (25%)	13 (32.5%)	
IV	10 (25%)	2 (5%)	
Lymphatic metastasis (*N*)			
No lymphatic metastasis	3 (7.5%)	2 (5%)	0.904
Lymphatic metastasis	20 (50%)	15 (37.5%)	
Distant metastasis			
No distant metastasis	13 (32.5%)	15 (37.5%)	**0.030**
Liver	10 (25%)	2 (5%)	
PD-L1 expression			
Negative expression	8 (20%)	7 (17.5%)	0.722
Weak positive expression	11 (27%)	6 (15%)	
Strong positive expression	4 (10%)	4 (10%)	

Table 3 illustrates the association between PD-L1 expression and subject characteristics. The distribution of PD-L1 expression levels across different age groups, gender, and histological type showed no significant difference. However, a statistically significant association was observed between PD-L1 expression and cancer staging ($p = 0.009$). For instance, among subjects at stage II, 7.5% had normal expression, none showed weak positive expression, and 5% exhibited strong positive expression. In contrast, among those at stage IV, no subjects had normal expression, 22.5% displayed weak positive expression, and 7.5% showed strong positive expression. Furthermore, a significant association was noted between PD-L1 expression and the presence of distant metastasis ($p = 0.004$). Notably, 37.5% of subjects without distant metastasis had normal PD-L1 expression, whereas 22.5% of those with distant metastasis exhibited weak positive expression and 7.5% showed strong positive expression. However, no significant association was observed between TILs CD8+ and PD-L1 expression ($p = 0.722$).

Table 3. Relationship between PD-L1 expression and characteristics of research subjects.

	Negative expression	**Weak positive expression**	**Strong positive expression**	**Chi-square (*p* value)**
Age				
Early onset (<50)	3 (7.5%)	4 (10%)	2 (5%)	0.955
Late onset (≥50)	12 (30%)	13 (32.5%)	6 (15%)	
Gender				
Male	7 (17.5%)	8 (20%)	3 (7.5%)	0.892
Female	8 (20%)	9 (22.5%)	5 (12.5%)	
Pathological anatomy				
Adenocarcinoma	14 (35%)	15 (37.5%)	8 (20%)	0.348
Mucinous adenocarcinoma	1 (2.5%)	0	0	
Signet ring cell	0	2 (5%)	0	
Grade				
Well differentiated	13 (32.5%)	11 (27.5%)	8 (20%)	
Moderately differentiated	1 (2.5%)	2 (5)	0	0.453
Poorly differentiated	0	2 (5%)	0	
Specific	1 (2.5%)	2 (5%)	0	
Location				
Caecum	0	1 (2.5%)	1 (2.5%)	
Ascending colon	2 (5%)	2 (5%)	0	
Transverse colon	2 (5%)	0	1 (2.5%)	0.586
Descending colon	1 (2.5%)	0	0	
Sigmoid colon	0	2 (5%)	1 (2.5%)	
Rectum	10 (25%)	12 (30%)	5 (12.5%)	
Location				
Colon	5 (12.5%)	5 (12.5%)	3 (7.5%)	0.919
Rectum	10 (25%)	12 (30%)	5 (12.5%)	

Table 3. (*Continued*).

	Negative expression	Weak positive expression	Strong positive expression	Chi-square (*p* value)
Stage				
II	3 (7.5%)	0	2 (5%)	**0.009**
III	12 (30%)	8 (20%)	3 (7.5%)	
IV	0	9 (22.5%)	3 (7.5%)	
Lymphatic metastasis (*N*)				
No Lymphatic metastasis	3 (7.5%)	0	2 (5%)	0.114
Lymphatic metastasis	12 (30%)	17 (42.5%)	6 (15%)	
Distant metastasis				
No distant metastasis	15 (37.5%)	8 (20%)	5 (12.5%)	**0.004**
Liver	0	9 (22.5%)	3 (7.5%)	

4. Discussion

Our study indicates that the majority of patients with colorectal cancer are over 50 years old, with 31 out of a total of 40 patients (77.5%) being over 50 years old. This study's findings are consistent with the general profile of colorectal cancer patients, the majority of whom are over 50 years old [6,7]. The data shows that the risk of colorectal cancer increases significantly every decade of life, with the risk increasing by one percent (1%) for every 10-year increase in age after reaching 50 years old [6–8]. Referring to colorectal cancer epidemiology data, colorectal cancer cases in men and women show nearly similar prevalence, with prevalence rates of 1:23 (4.3%) in males and 1:25 (4.0%) in females [7,8].

The results of the anatomical pathology examination in this study are consistent with previous studies, which show that adenocarcinoma is the most commonly found type in colorectal cancer, accounting for up to 90% of total cases, with various differentiation levels such as comedo, medullary, micropapillary, mucinous, and signet ring cell types. In clinical practice, moderately differentiated adenocarcinoma is the most common diagnosis (~70%), while poorly differentiated or well-differentiated adenocarcinoma each account for 20% and 10% of colorectal cancer cases, respectively [9]. Existing literature shows an increase in the proportion of colorectal cancer patients with tumours in the rectum, from 27% in 1995 to 31% in 2019. Approximately 4 out of 10 colorectal cancer patients aged 50–64 is diagnosed with tumours in the rectum. The latest epidemiological data for 2019 shows that 60% of new colorectal cancer cases are advanced-stage diseases, with 22% of them having distant metastases [10].

It is known that high TILs-CD8+ expression may indicate resistance to metastasis in colorectal cancer, while positive PD-L1 expression triggers higher metastasis rates. The results of this study align with the theory, showing that the majority of advanced-stage colorectal cancer patients experience metastasis with low TILs-CD8+ expression and high PD-L1 expression, as shown in other studies [3,11,12].

As previously mentioned, colorectal cancer is caused by genetic changes in tumour cells and is influenced by tumour-host interactions. Recent studies have shown a direct correlation between the density of T cell subpopulations, including TILs

CD8+, and favourable clinical outcomes in colorectal cancer, supporting the primary role of T cell immunity in suppressing colorectal tumour progression. Excessive TILs expression is also associated with deficiencies in DNA MMR proteins and MSI. Recent research indicates that MSI and TILs are potential tumour markers for predicting the outcomes of immune checkpoint blockade in colorectal cancer. Upon examining several study characteristics, the relationship between TILs CD8+ in this study was significantly associated with age. This briefly indicates a larger proportion of high TILs CD8+ expression, with high expression occurring more frequently in advanced-onset subjects. Research on the relationship between TILs CD8+ expression and study characteristics has been extensively conducted before. One such study by Liu et al. found that TILs CD8+ had no significant association with age, as well as with gender, grade, anatomical pathology, and MMR protein expression [13]. Similar findings were reported in a study by Huang et al. However, Huang's study showed a significant association between TILs CD8+ expression and tumour location, pT stage, pN stage, TNM stage, tumour differentiation, and MMR status ($p < 0.05$) [14]. In a similar cross-sectional study by Irawan et al., no significant association was found between TILs CD8+ expression and age, as well as with gender, anatomical pathology, grade, tumour location, and colorectal cancer stage ($p > 0.05$) [15]. Another similar study by Yin et al. showed no significant association between TILs-CD8+ expression and age, as well as with gender, tumour location, differentiation, pT stage, vascular invasion, and lymphatic metastasis ($p > 0.05$) [16]. From the several studies outlined before, it can be concluded that this study is not entirely consistent with previous research. The majority of studies show no significant association between TILs CD8+ expression and various characteristics of the research subjects. The physiological process regarding the relationship between TILs CD8+ and age is still unclear. Therefore, further research is needed on the relationship between age and TILs CD8+ expression with a larger and more diverse sample.

Besides age, this study also found a significant association between TILs CD8+ and distant metastasis ($p = 0.03$). Unlike age, this finding aligns with other similar studies. Broadly, metastasis in colorectal cancer is associated with TILs CD8+ biological and mechanistic functions, such as transcriptional control through epigenetic regulation of cell proliferation and apoptosis processes. These mechanisms may still occur in early-stage colorectal cancer, whereas in advanced stages, TILs CD8+ may no longer induce apoptotic signals, leading to metastasis.

In this study, the expression of PD-L1 was significantly associated with advanced colorectal cancer stages. Other studies support this finding. Shang et al. found higher PD-L1 expression in stages III and IV compared to stages I and II [17]. Kim et al. also showed a significant association between PD-L1 expression and advanced stages of colorectal cancer [18]. However, Lee et al. found no significant association between PD-L1 expression and colorectal cancer stage [19]. The difference in results may be due to differences in sampling techniques.

In this study, PD-L1 expression showed no significant links with age, gender, anatomical pathology differentiation, tumour location, or TILs CD8+ expression. Some studies also found no significant connection between PD-L1 expression and age [20,21]. However, Lee et al. reported that older patients tended to have higher PD-L1 expression [19]. Wang et al. found no significant link between PD-L1 expression and

gender [21]. On the other hand, some studies did find a significant association between PD-L1 expression and TILs CD8+ expression. For instance, Liu et al. [13] and Xin et al. [22] reported significant relationships between PD-L1 expression and TILs-CD8+ expression. Differences in results could be due to the study's population not fully representing the broader scenario.

Until now, two different mechanisms of increased PD-L1 expression in tumour cells have been reported: intrinsic immune resistance and adaptive immune resistance. The former is an increase in PD-L1 expression as a result of constitutive oncogenic signals in tumour cells. In contrast, adaptive immune resistance refers to the induction of PD-L1 expression on tumour cells in response to local inflammatory signals (such as interferon) generated by active anti-tumour immune responses (activation of cytotoxic T cell pathways and/or Th1 pathways). This then leads to the emergence of PD-1 expression on T cells. When binding to PD-L1 or other ligands, PD-1 inhibits kinases involved in T cell activation via the SHP2 phosphatase, leading to T cell apoptosis, although additional signalling pathways may also be induced [23].

This study indicates that PD-L1 expression is significantly associated with distant metastasis. Shan et al. showed similar results [20]. However, Masugi et al. found no significant association between CD274 expression, the gene encoding the PD-L1 protein, and distant metastasis in patients with colorectal cancer [24]. The difference in results with this study may be due to differences in the analysis methods used, although referring to the same aspect, PD-L1. This study also did not show a significant association between PD-L1 expression and lymphatic metastasis (p = 0.114). Several other studies have also reported similar findings [19,21].

The PD-L1 is a key immunoregulatory molecule that, upon interaction with its receptor, PD-1, can suppress CD8+ cytotoxic immune responses both in physiological and pathological pathways [25]. Physiologically, PD-L1 can bind to inhibitory receptors found on the surface of T lymphocytes, namely PD-1, thus suppressing T cell activation [20,26]. The normal function of PD-L1 in the immune system is to maintain a balance between protective immunity and immune tolerance in the body. However, in the tumour microenvironment, PD-L1 expression increases through various mechanisms, leading to abnormal PD-L1/PD-1 signalling transduction activation. This can inhibit T cell proliferation and differentiation, inducing the apoptosis of T cells [20]. CD8+ T cells play a crucial role in tumour rejection by identifying tumour-specific antigens and directly destroying transformed cells [3]. The binding between PD-1 and PD-L1 can inhibit T cells from eradicating tumour cells and inhibit the effector function of T cells already activated by tumour-antigen-bearing APCs [12]. Abnormal activation of PD-L1/PD-1 signalling transduction also leads to inhibited signal transduction and secretion of various cytokines, leading to invasion and metastasis of cancer cells [20].

The study found a significant link between TILs-CD8+ expression and distant metastasis in colorectal cancer, which aligns with previous research. For example, Liu et al. discovered that TILs-CD8+ expression was tied to lymphatic metastasis and advanced TNM stages [13]. Huang et al. also showed that TIL-s-CD8+ expression was closely associated with lymph node metastasis and advanced TNM stages [14]. Similarly, Saleh et al. demonstrated the relationship between TILs-CD8+ expression and CRC metastasis, highlighting the importance of biological mechanisms like

transcriptional control and regulation of cell processes in advanced disease stages. They also observed negative regulation of apoptotic processes, particularly in stage IV, suggesting limitations in TILs-CD8+'s ability to induce apoptosis in advanced stages [27].

However, this study is also inconsistent with another study by Alsalman et al., which grouped patients based on pathological stage. Early-stage colon cancer represented patients with completely resected tumours and no further evidence of involvement of surrounding organs, lymph nodes, or distant sites. In contrast, advanced colorectal cancer was defined as locally advanced colorectal cancer that could not be operated on, or metastatic colorectal cancer. A sub-analysis was performed for these groups by comparing the expression of different immune markers. Alsalman's study showed no significant variation in the levels of expression of FoxP3, CD25, CTLA-4, and LAG-3 in TILs-CD8+ among patients with early and advanced stages [28].

The CD8 has previously been known as an important biomarker of T lymphocytes. Many studies have reported the clinical significance and prognostic value of its expression in immune cells infiltrating tumours in CRC. Studies have also shown that tumour growth and poor prognosis in CRC patients are associated with decreased activation of CD8+ T cells and disruption of cell proliferation. The association of TILs CD8+ expression with metastatic events in CRC may occur due to various factors [3,11].

Lipid metabolism is one of the key biological mechanisms that can control the activation and phenotype of immune cells or alter or programme their functions. Here, genes related to cholesterol biosynthesis were down-regulated in TILs CD8+ from patients with advanced CRC stage; this may reduce the activation of CD8+ T cells and disrupt their ability to release cytolytic molecules such as granzyme. Saleh et al. found that genes related to T cell activation and migration, cytolysis, adaptive immune response, cytokine secretion, and IFN-γ signalling were down-regulated in TILs CD8+ patients with advanced disease tages. These data indicate that TILs CD8+ from advanced disease stages may have limited T cell migration and recruitment to tumour sites, impaired activation, and a limited antitumour response, thereby potentially triggering higher metastasis [27].

In addition to lipid metabolism, it was also found that hypoxia is a hallmark of the tumour microenvironment, which can negatively affect the cytotoxic activity of CD8+ T cells and lead to tumour aggressiveness and therapy resistance. Studies have found that genes related to cellular response to hypoxia are up-regulated in TILs CD8+ from advanced stages, potentially leading to T cell apoptosis, increased secretion of suppressive cytokine IL-10, and limited capacity for T cell proliferation, thus also triggering metastasis [28]. In conclusion, transcriptomic profiling of sorted TILs CD8+ from CRC patients and comparative analysis between advanced and early stages reveal biological mechanisms and molecular signalling pathways that undergo changes during disease progression.

This research includes a comprehensive portrayal of colorectal cancer patient demographics, showcasing a diverse sample characterised by variations in age, gender, and tumour features. The inclusion of variables related to the immune response, such as TILs-CD8+ and PD-L1 expression, enriches the analysis by shedding light on the

immune dynamics within the tumour microenvironment. However, this study's strength may be tempered by its small sample size, which could limit statistical power and the generalizability of findings to broader populations. Moreover, the retrospective design introduces inherent biases and limitations, including potential discrepancies in data collection and the inability to establish causal relationships between variables.

5. Conclusion

This study highlights a significant positive association between TILs CD8+ expression and PD-L1 expression and the occurrence of distant metastasis and stage in colorectal cancer. However, no significant association was found with lymphatic metastasis. In light of these findings, the researchers provide several recommendations for future investigations. Firstly, there is a need for further research into other immune factors that may contribute to immune system inhibition or activation with metastasis, such as regulatory T cells, natural killer (NK) cells, or other cytokine factors. Exploring these factors could provide deeper insights into the mechanisms underlying metastasis and the immune response in colorectal cancer. Secondly, subsequent studies could delve into analysing the relationship between treatment and response to immunotherapy, particularly focusing on the effects of PD-L1 blockade and the role of TILs CD8+ as predictors of response to such therapy. Understanding how these factors interact could help tailor more effective treatment strategies for colorectal cancer patients. Lastly, conducting further research with a larger sample size would be beneficial to validate the findings of this study. A larger sample size would enhance the reliability and generalizability of the results, providing more robust evidence for clinical decision-making and potential therapeutic interventions in colorectal cancer management.

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Ethical approval: This study was carried out according to the Declaration of Helsinki ethical research. Approval for this research was obtained from the Research Ethics Committee of the Faculty of Medicine, Padjadjaran University, Bandung, Indonesia, with the number LB.02.01/X.6.5/490/2023 dated 9 February. To maintain the privacy of research subjects, data will be stored securely, and all information regarding identifiable research subjects will be kept confidential. Data will be presented in group form anonymously.

Conflict of interest: The authors declare no conflict of interest.

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