RESEARCH ARTICLE

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Different Immunohistochemical Expression of CTLA-4 in Diffuse Large B-Cell Lymphoma and Its Associated Prognostic Factors

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Abstract

Background: Diffuse large B-cell lymphoma (DLBCL) is classified into germinal center B-cell-like (GCB) and non-GCB subtypes. This study aimed to evaluate the immunohistochemical expression of CTLA-4 in these subtypes and its association with prognostic factors. **Methods:** This retrospective study analyzed 50 cases of DLBCL. Clinical and histopathological data were collected, and CTLA-4 expression was assessed using immunohistochemistry. Statistical significance was determined using an unpaired t-test. **Result:** The average CTLA-4 expression in DLBCL was 50.46 cells per high-power field. Higher CTLA-4 expression was observed in the non-GCB subtype, patients younger than 60 years, females, those with stage III-IV disease, involvement of more than one extranodal site, and a low International Prognostic Index (IPI) score (0–2). A significant association was found between CTLA-4 expression and age (p = 0.045, 95% CI: 44.67–75.86). **Conclusion:** CTLA-4 expression was present in the tumor microenvironment of DLBCL. Higher expression was significantly associated with patients younger than 60 years.

Keywords: CTLA-4- DLBCL- IPI

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Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive lymphoid neoplasm characterized by the presence of B cells that are three times larger than normal lymphocytes. It is the most common lymphoma in adults, accounting for approximately 30–40% of all non-Hodgkin lymphoma cases [1–4]. DLBCL is a highly heterogeneous disease with complex pathophysiological alterations and diverse clinical outcomes [5, 6]. Various genetic alterations and pathway activations contribute to its development, including *BCL2, BCL6*, and *MYC* rearrangements; *EZH2, GNA13, KMT2D,* and *TP53* mutations; and activation of pathways such as NF-κB, PI3K/AKT, and JAK/STAT signaling [2]. Additionally, the activation of negative regulatory circuits plays a crucial role in immune evasion, diminishing immune surveillance and promoting tumor progression.

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is an immune inhibitory molecule upregulated within the tumor microenvironment (TME) of DLBCL. It inhibits T-cell activation by competing with CD28 for the B7 ligand on antigen-presenting cells [7]. This upregulation impairs immune system recognition and elimination of tumor cells, thereby facilitating tumor growth. Studies have reported significant CTLA-4 expression in various lymphoma subtypes, including DLBCL [7, 8], with increased expression correlating with poor prognosis [9–13]. These findings have also supported the development of immune checkpoint blockade therapies, which have shown promise in refractory solid tumors [7, 8].

Based on the cell of origin, DLBCL is classified into two molecular subtypes: germinal center B-cell-like (GCB) and non-GCB [2, 4]. In settings with limited access to gene expression profiling, immunohistochemical staining for CD20, Bcl-6, MUM1, and CD10 using Hans's algorithm can aid in classification [2, 14]. This classification enables more precise treatment strategies based on the biological characteristics of the disease. Patients with the non-GCB subtype often experience a more aggressive disease course and have poorer outcomes with standard chemoimmunotherapy. The 5-year survival rate for non-GCB DLBCL is 39%, compared to 59% for the GCB subtype [15].

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In addition to molecular subtyping, the International Prognostic Index (IPI) is essential for risk stratification in DLBCL. The IPI includes five clinical factors: age, serum lactate dehydrogenase (LDH) level, Eastern Cooperative Oncology Group (ECOG) performance status, Ann Arbor disease stage, and extranodal involvement. However, few studies have investigated variations in CTLA-4 expression in relation to these prognostic factors.

CTLA-4 plays a crucial role in the TME of DLBCL [9]. Understanding its expression in tumor-infiltrating lymphocytes could help predict patient response to immune checkpoint inhibitors and improve treatment outcomes. However, limited information is available regarding the differences in CTLA-4 expression between GCB and non-GCB subtypes and its association with DLBCL prognostic factors.

This study aimed to evaluate CTLA-4 expression in DLBCL subtypes and its relationship with prognostic factors, particularly those included in the IPI.

Materials and Methods

Study design

This retrospective study included all identified cases of DLBCL diagnosed at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia/Dr. Cipto Mangunkusumo National Hospital (FKUI/RSCM), Jakarta, Indonesia, from 2014 to 2019. Ethical approval was obtained from the Faculty of Medicine, Universitas Indonesia (KET-172/UN2.F1/ETIK/PPM.00.02/2022), and the study was conducted in accordance with the Helsinki ethical guidelines. The requirement for informed consent was waived by the ethical board (ND.883/UN2.F1/ETIK/PPM.00.02).

Study Population and Data Collection

The inclusion criteria comprised DLBCL cases with sufficient immunohistopathological assessment, including hematoxylin and eosin (H&E) staining and comprehensive immunohistochemical profiling for non-Hodgkin lymphoma markers (Bcl-6, MUM1, CD10, and Ki67). Cases were excluded if they lacked essential clinical data, such as age, sex, or prognostic variables from IPI, including ECOG performance status, LDH level, Ann Arbor disease stage, and the number of extranodal sites involved. Additionally, cases with non-representative H&E slides or inadequate paraffin-embedded tissue blocks were excluded. The sample size was determined using a standardized formula for categorical-numerical unpaired analytical sampling. A minimum of 20 cases per group was required, with an assumed 20% dropout rate.

Histopathological and Immunohistochemistry Evaluation

Two certified pathologists independently reviewed each case to confirm the DLBCL diagnosis and classify tumors into GCB and non-GCB subtypes using the Hans algorithm. Selected paraffin-embedded tissue blocks were stained for CTLA-4 using immunohistochemistry. The staining protocol involved incubation with a primary CTLA-4 antibody (Biocare Medical, LLC. UMAB249 clone) at a 1:150 dilution for one hour, followed by application of a secondary antibody (Novolink detection kit ® RE7140-CE) for 30 minutes. CTLA-4 expression was evaluated using ImageJ® software. Two pathologists analyzed CTLA-4 immunohistochemical staining by identifying TME areas specifically lymphocytes and histiocytes with clear staining in hotspot regions. TME areas were assessed at five different sites using a 40× objective lens. The number of CTLA-4-positive lymphocyte and histiocyte was counted in each high-power field (cells/HPF), and positive staining was defined as CTLA-4 expression on the membrane or cytoplasm of lymphocytes and histiocytes.

Statistical Analysis

Data analysis was performed using SPSS 25.0. Demographic characteristics were summarized as categorical variables (frequencies and percentages), while CTLA-4 expression was reported as a mean value with a 95% confidence interval (minimum–maximum). A normality test indicated that CTLA-4 expression followed a non-normal distribution; therefore, logarithmic base 10 transformation was applied to achieve normality. Differences in mean CTLA-4 expression between groups were analyzed using an unpaired t-test. A p-value <0.05 was considered statistically significant.

Table 1. Demographic Profile and Prognostic Factors of DLBCL patients

Variable	DLBCL		
	GCB n (%)	non-GCB n (%)	Total N (%)
Age (years, mean±SD)	50.38±12.57	49.31±10.76	50
Sex			
Male	14 (58.3)	9 (34.6)	23 (46)
Female	10 (41.7)	17 (65.4)	27 (54)
IPI factors			
Age			
<60	20 (83.3)	21 (80.8)	41 (82)
>60	4 (16.7)	5 (19.2)	9 (18)
ECOG PS			
<2	24 (100)	23 (88.5)	47 (94)
>2	0	3 (11.5)	3 (6)
Ann Arbor stage			
I, II	18 (75)	20 (76.9)	38 (76)
III, IV	6 (25)	6 (23.1)	12 (24)
LDH			
Normal	1 (4.2)	1 (3.8)	2 (4)
Elevated	23 (95.8)	25 (96.2)	48 (96)
Extranodal site involvement			
<1	22 (91.7)	24 (92.3)	46 (92)
>1	2 (8.3)	2 (7.7)	4 (8)
IPI score			
0−2 (low)	22 (91.7)	22 (84.6)	44 (88)
3−5 (high)	2 (8.3)	4 (15.4)	6 (12)

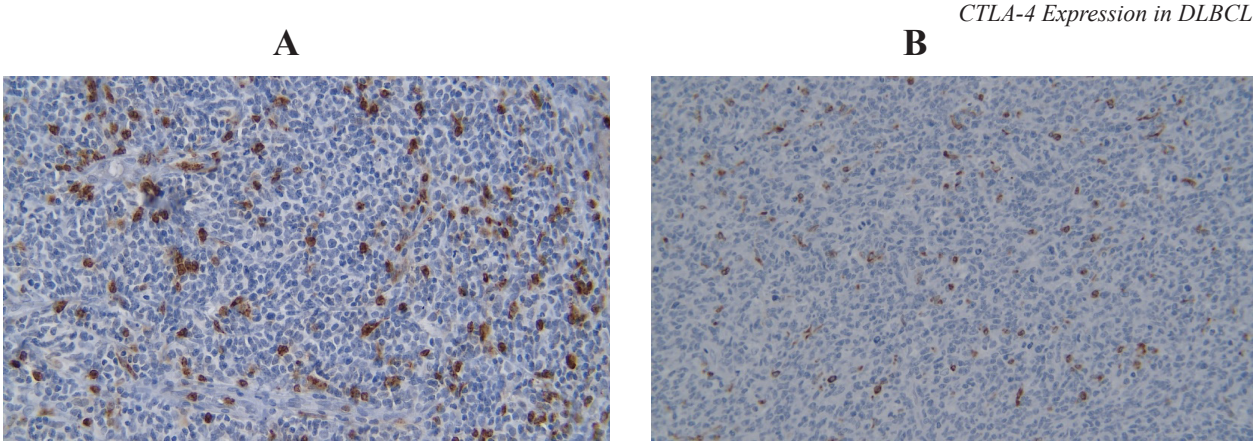

Figure 1. The Positive Immunohistochemical Staining of CTLA-4 in GCB subtype, 400x (A) and non-GCB subtype, 100x (B) of DLBCL. The positive distribution of CTLA-non-GCB is more diffuse in comparison to the GCB subtype.

Table 2. Mean CTLA-4 Immunohistochemical Expression of DLBCL Subtypes

	Mean CTLA-4 expression (95% CI for Mean)*	P value**
DLBCL (n=50)	50.46 (39.72−64.22)	
GCB (n=24)	40.5 (28.18−58.88)	0.076
Non-GCB (n=26)	61.66 (44.67−85.11)	

*Data shown are geometric average in mean; **Non−paired t test of the transformed data using log10

Results

This study included 50 cases of DLBCL, comprising 24 cases classified as the GCB subtype and 26 cases as the non-GCB subtype. Table 1 presents the basic demographic characteristics and factors contributing to the IPI prognostic score for both subtypes. Patients with GCB and non-GCB subtypes had mean ages of 50 and 49 years, respectively. In both subtypes, the majority of patients were under 60 years old. The GCB subtype had a higher proportion of males, whereas the non-GCB subtype had a higher proportion of females. Most patients in both subtypes were classified as disease stage I–II, with elevated LDH levels, involvement of fewer than one extranodal site, and a low (0–2) IPI score.

The immunohistochemical expression of CTLA-4 in this study was observed in lympho-histiocytic cells, with a mean value of 50.46 cells/HPF. As shown in Table 2, CTLA-4 expression was higher in the non-GCB subtype than in the GCB subtype. Figure 1A–B illustrates the distinct distribution of CTLA-4 immunohistochemical expression in both subtypes.

Table 3 presents CTLA-4 expression across different demographic and clinical parameters associated with the IPI prognostic score. Higher CTLA-4 expression was observed in females (p = 0.149), patients with ECOG performance status <2 (p = 0.409), Ann Arbor stage III/IV (p = 0.218), and those with more than one extranodal site involved (p = 0.393). Notably, CTLA-4 expression was significantly higher in patients younger than 60 years compared to those older than 60 years (p = 0.045). Although not statistically significant, patients with a low IPI score exhibited higher CTLA-4 expression than those with a high IPI score (p = 0.417).

Table 3. Different CTLA-4 Expression in Various Demographic and Prognostic Factors of *DLBCL*

Variable	Mean CTLA-4 expression (95% CI for Mean)*	P value**
Sex		
Male	41.69 (28.84−60.26)	0.149
Female	58.88 (42.66−81.28)	
IPI factors		
Age		
<60	57.54 (44.67−75.86)	0.045
>60	26.91 (15.85−45.71)	
ECOG PS		
<2	51.29 (39.81−66.07)	0.409
>2	35.48 (7.08−173.78)	
Ann Arbor stage		
I, II	46.77 (35.48−61.66)	0.218
III, IV	64.57 (40.74−104.71)	
Extranodal site involvement		
<1	50.12 (38.02−64.57)	0.393
>1	61.66 (33.88−112.20)	
IPI score		
0−2 (low)	51.29 (38.90−66.07)	0.417
3−5 (high)	45.71 (23.99−87.1)	

*Data shown are geometric average in mean; **Non-paired t test of the transformed data using log10

Discussion

The expression of *CTLA-4* in the DLBCL tumor microenvironment (TME) has not been extensively studied. However, previous research on solid tumors has reported that high CTLA-4 expression in the tumor microenvironment (TME) is associated with worse patient prognosis [10–13]. In this study, we observed CTLA-4 expression in lympho-histiocytes within DLBCL tumors, with a mean value of 50.46 cells/HPF. This finding may

provide valuable insights for future strategies involving anti-CTLA-4 therapies for DLBCL treatment. The upregulation of immunological inhibitory molecules, such as CTLA-4, suppresses T-cell activation in the tumor microenvironment, thereby impairing the immune system's ability to recognize and eliminate tumor cells, ultimately promoting tumor progression.

The non-GCB subtype of DLBCL is associated with a poorer prognosis than the GCB subtype, as evidenced by a lower five-year survival rate in the non-GCB group compared to the GCB group in the era of rituximab therapy [15]. Based on this, we hypothesized that CTLA-4 expression would be higher in the non-GCB subtype. Although not statistically significant, our findings showed a trend toward higher CTLA-4 expression in the non-GCB subtype compared to the GCB subtype. However, it should also be noted that both DLBCL subtypes exhibit considerable heterogeneity in their biological characteristics and prognostic outcomes. Notably, cases classified as the GCB subtype may still have poor prognoses when they harbor double-hit mutations [16].

Previous studies have reported significantly higher CTLA-4 expression in patients older than 60 years, correlating with poorer prognosis in thymoma patients [13, 17]. This may be attributed to the well-documented decline in immune system function with aging. Age-related cancer development has been linked to increased levels of inflammatory mediators such as IL-6, IL-8, and IL-10, as well as a reduction in fibroblast activity and immunosenescence, leading to decreased functionality of effector immune cells [18]. However, in contrast to these findings, our study demonstrated a significant increase in CTLA-4 expression in patients younger than 60 years. This discrepancy is likely due to differences in sample sizes and the distinct immunophenotyping methods used to assess CTLA-4 expression. While our study measured CTLA-4 expression via immunohistochemical staining, prior studies employed flow cytometry, which provides a more objective and quantitative assessment compared to immunohistochemistry.

The age range of DLBCL patients in this study was 24 to 79 years, with the majority being under 60 years old. Female patients exhibited higher CTLA-4 expression compared to male patients, though this difference did not reach statistical significance. The impact of sex on DLBCL outcomes remains inconsistent across studies. A study by Hedstrom et al. found no statistically significant difference in survival rates between genders [19]. However, prognosis may vary depending on sex due to differences in hormone levels. The higher prevalence of DLBCL in males has been attributed to the absence of estrogen's protective effects [19, 20]. Estrogen plays a protective role by reducing IL-6 levels, while elevated IL-6 levels are associated with poorer survival outcomes in DLBCL patients [21].

ECOG performance status is a crucial tool for assessing a patient's functional capacity for systemic anticancer therapy and predicting cancer prognosis [22]. It is associated with survival duration, treatment response, quality of life, and comorbidities. Additionally, the ECOG score correlates with patient age and cancer stage [23]. In this study, no significant differences were observed between CTLA-4 expression and ECOG performance status.

The Ann Arbor staging system is widely used to determine the disease stage of DLBCL [2, 24]. In this study, 76% of cases were classified as stage I–II. DLBCL is characterized by a rapid increase in tumor mass, and patients with advanced-stage disease tend to have poorer survival outcomes [25]. Although not statistically significant, our findings indicated higher CTLA-4 expression in patients with advanced-stage disease (III–IV).

Tumor cells undergo metabolic reprogramming characterized by increased glucose uptake and heightened lactate production. LDH, a readily measurable biomarker in most clinical laboratories, plays a crucial role in this process. Elevated LDH levels, which serve as a prognostic biomarker in DLBCL, enable tumor cells to evade the immune system by modifying the tumor microenvironment (TME) [23]. In this study, the majority of cases exhibited elevated LDH levels, with only two samples showing normal levels. Elevated LDH at the time of lymphoma diagnosis was associated with increased tumor size and poorer prognosis. A previous study also reported that a 1.5-fold increase in LDH serum levels over three months correlates with a higher risk of relapse in DLBCL patients [23].

According to the literature, DLBCL can occur in both nodal and extranodal sites, with extranodal involvement seen in up to 40% of cases. The most frequently affected extranodal sites include the digestive tract, bone, testicle, spleen, Waldeyer's ring, salivary glands, thyroid, liver, kidney, and adrenal gland [2]. In this study, the most common extranodal sites were in the head and neck. However, no significant difference in CTLA-4 expression was observed between patients with fewer than one extranodal involvement and those with multiple extranodal sites.

The IPI scoring system, introduced over 25 years ago, remains a widely used prognostic tool. It assigns one point for each adverse prognostic factor, including age over 60, elevated LDH levels, Ann Arbor stage III/IV disease, ECOG performance status of 2 or higher, and more than one site of extranodal involvement. In the era of R-CHOP therapy, patients with no IPI risk factors have an overall survival (OS) rate of 94%, while those with 2–3 risk factors have a 79% OS rate, and those with 3–5 factors have a 55% OS rate [24]. A study by Chen et al. found that CTLA-4 expression was higher in the high-risk DLBCL group (IPI score >2) compared to the low-risk group (IPI score <2) [7]. However, in contrast to these findings, this study did not identify a significant association between IPI score and CTLA-4 expression. This discrepancy is likely due to the small sample size, as only six patients in this study had high IPI scores.

A limitation of this study is the subjectivity in evaluating CTLA-4 expression within the stained tumor microenvironment (TME). Future research should incorporate a standardized scoring system to quantify CTLA-4 expression in DLBCL, reducing variability in interpretation. Additionally, further analysis of patient

therapy history and disease outcomes is needed to validate the predictive significance of CTLA-4 expression.

In conclusion, CTLA-4 expression was observed on the lympho-histiocytic cell membrane and/or cytoplasm of DLBCL tumors. While no statistically significant difference in CTLA-4 expression was found between the GCB and non-GCB subtypes, a trend toward higher expression in the non-GCB group was noted. Mean CTLA-4 expression appeared to be higher in patients under 60 years of age, females, those with stage III–IV disease, and those with more than one extranodal site involvement. Among these factors, only age showed a statistically significant association with CTLA-4 expression (p=0.045). No significant correlation was observed between total IPI score and CTLA-4 expression.

Author Contribution Statement

L.R. contributed to conceptualization, data curation, statistical analysis, writing original draft preparation, M.F.H. and A.G.N. are responsible for funding acquisition, supervision, data collection and analysis, and manuscript review and editing. D.K. contributed to statistical analysis, and manuscript writing and editing.

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Approval by Scientific Body

The study is a part of student thesis approved by Department of Anatomical Pathology, Faculty of Medicine Universitas Indonesia.

Ethical Declaration

The study was approved by the ethical committee of the Faculty of Medicine Universitas Indonesia (KET-172/UN2.F1/ETIK/PPM.00.02/2022) and was directed according to Helsinki ethical guideline. Informed consent was waived by ethical board (ND.883/UN2.F1/ETIK/PPM.00.02).

Data Availability

Research data is accessible upon reasonable request to the correspondence author.

Conflict of Interest

The authors declared there is no conflict of interest.

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