

Expression of programmed cell death ligand-1 protein in germinal center B-cell-like and non-germinal center B-cell-like subtypes of diffuse large B-cell lymphoma

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Abstract. Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma worldwide. However, there is still limited information on the expression of programmed cell death ligand-1 (PD-L1), a type 1 transmembrane protein of immunoglobulin B7/CD28 in the DLBCL subtypes. The present study aimed to identify the expression of PD-L1 in germinal center B-cell-like (GCB) subtype and non-germinal center B-cell-like (non-GCB) subtype of DLBCL. A total of 40 patient samples (formalin-fixed paraffin-embedded tissues) consisting of 20 cases of GCB subtype and 20 cases of non-GCB subtype of DLBCL were examined. The PD-L1 protein expressions were evaluated by using immunohistochemical staining in the tumor cells. The results showed a statistically significant difference ($P=0.003$) between the expression of PD-L1 in the GCB subtype and the non-GCB subtype of DLBCL. PD-L1 expression in the tumor cells were observed in 13 cases (65%) of non-GCB subtype and in three cases (15%) of the GCB subtype of DLBCL. In conclusion, it was found that the expression of PD-L1 protein in the tumor cells of the non-GCB subtype of DLBCL was significantly higher as compared with the tumor cells of the GCB subtype of DLBCL.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma, consisting of ~30-40% of all

non-Hodgkin lymphoma cases (1). A multicenter cross-sectional study in Indonesia showed that 68.2% of all patients with non-Hodgkin lymphoma had DLBCL, which more frequently affected males with a median age of 51 years (2).

Based on the gene-expression profiles, DLBCL is divided into three subtypes; the B-cell-like germinal center (GCB) subtype, the activated B-cell-like (ABC) subtype and the third subtype that does not express genes from either the GCB or ABC subtypes. The ABC and the third subtypes of DLBCL are collectively known as the non-germinal center B-cell-like (non-GCB) subtype (3,4). The germinal-center B-cell-like subtype is associated with good clinical outcome and expressed genes characteristic of normal germinal-center B-cells. Meanwhile, the activated B-cell-like subtype was associated with poorer clinical outcome and expressed genes characteristic of activated blood B cells (3).

Tumor cells are known to survive by evading the immune system of the body. The abilities of cancer cells to escape the immune system have been extensively studied, including its immunosuppression capacity (5). Recently, the PD-1/PD-L1 signaling mechanism has emerged in the study of tumor cells immunosuppression as enhancing immune tolerance by inhibition of T cell activation (5).

Programmed cell death-1 (PD-1) or CD279 is a type 1 transmembrane protein of the B7/CD28 family, expressed in several kinds of immune cells such as peripheral activated B and T cells, natural killer cells and distinct dendritic cells (DC) (6). PD-1 is also weakly expressed on the surface of immature T cells and B cells in the thymus and bone marrow during the developmental process (7). PD-1 has two ligands; a programmed cell death ligand-1 (PD-L1)/B7H1 and a programmed cell death ligand-2 (PD-L2)/B7-DC (8). The CD274 gene encodes PD-L1 and PD-L2 is encoded by PDCD1LG2 gene (6). The two of them are located on chromosome 9p.24.1 (6). PD-L1 is expressed on stromal tumor-associated macrophages and lymphocytes. PD-L2 is primarily expressed in antigen-presenting cells (APCs) (9).

Cancer cells may express numerous immune inhibitory signaling proteins that can cause immune cell dysfunction

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and apoptosis. PD-L1 is one of the inhibitory molecules which binds to the programmed cell death-1 (PD-1) molecules expressed on dendritic cells, T cells, B cells and natural killer T cells to suppress anti-cancer immunity (10).

The development of an antibody targeting on immune checkpoint mechanisms such as the PD-1/PD-L1 pathway has led to a clinically significant antitumor response (11). Previous studies showed that expression of PD-L1 in DLBCL patients was related to poor prognosis (12-15). It was decided to investigate PD-L1 expression in the GCB subtype, as compared with those in the non-GCB subtype, because numerous cases of DLBCL are found in Indonesia (2). The hypothesis of the present study was that non-GCB subtype DLBCL had a higher expression of PD-L1 compared with GCB subtype DLBCL. However, information about PD-L1 expression in DLBCL subtypes in Indonesian patients remains limited and the role of PD-L1 as targeted therapy in DLBCL has not been fully elucidated. Therefore, the present study investigated PD-L1 expression in the GCB subtype as compared with those in the non-GCB subtype of Indonesian DLBCL cases.

Materials and methods

A total of 40 patients samples in the form of formalin-fixed paraffin-embedded tissues (FFPE) diagnosed as GCB and non-GCB subtypes of DLBCL in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia/Dr. Cipto Mangunkusumo National Central General Hospital, Jakarta, Indonesia during the period of 2014 to 2017 were consecutively retrieved from the archives and reviewed by authors (MH, AH, EH). The tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature. Cases without sufficient FFPE materials were excluded from the study. There were 20 cases of GCB subtype and 20 cases of non-GCB subtype of DLBCL.

Expressions of PD-L1 in DLBCL FFPE samples were evaluated according to the standard immunohistochemistry protocols; chorionic villi (placenta) taken from the archives of the Department of Anatomical Pathology of Universitas Indonesia/Dr. Cipto Mangunkusumo National Central General Hospital was used as a positive control. Unstained sections of 4 μ m thickness were cut. After deparaffinization and rehydration, the slides were blocked with 3% hydrogen peroxide for 10 min and then washed under running water for 5 min. Antigen retrieval was conducted with pH 9 Tris-EDTA buffer, in a decloaking chamber, at temperature of 95°C for 30 min. After washing in PBS pH 7.4 for 5 min, 10% normal horse serum (Thermo Fisher Scientific, Inc.) blocking solution was applied for 30 min at room temperature to block non-specific protein. Then, each slides was incubated with PD-L1 primary antibody (Rabbit polyclonal PD-L1 antibody; GeneTex, Inc.; cat. no. GTX104763) with a dilution of 1:500 for 1 h at room temperature. After repeated washing, the slides were incubated with a ready-to-use secondary antibody polymer (Histofine Simple Stain MAX PO kit; cat. no. 414151F; Nichirei Biosciences Inc.) for 30 min at room temperature. This secondary antibody conjugated to an amino acid polymer and multiple enzyme molecules. After repeated washing, the slides were incubated with diluted diaminobenzidine chromogen buffer substrate for 3 min at room temperature.

Table I. Clinicopathological characteristics of 40 cases with DLBCL investigated.

Characteristics	GCB subtype	Non-GCB subtype
Total subjects	20	20
Median age (year-old)	48	55
Sex		
Male	10	11
Female	9	10
Sites		
Nodal	11	10
Extranodal	9	10

DLBCL, diffuse large B-cell Lymphoma; GCB, germinal center B-cell-like.

Counterstaining was performed with Mayer's hematoxylin for 30 sec at room temperature.

All slides were evaluated qualitatively in five representative fields at x400 magnification under a light microscope (Leica Microsystems GmbH; $\geq 1,000$ tumor cells). Positive staining of PD-L1 expression was shown by brown color in the tumor cells membrane (12). ImageJ software version number 1.51 (National Institutes of Health) was used for cell counting. Expressions of PD-L1 were evaluated in tumor cells. The sample was considered positive for PD-L1 expression if the frequency of PD-L1 expressing cells was $>30\%$ (12).

Data obtained were then analyzed by using SPSS version 20.0 (IBM Corp.). The distribution normality of the data was determined by using the Shapiro-Wilk test. The statistical significance value conducted by using a Fisher's exact test and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics of DLBCL patients. The present study investigated 40 cases of DLBCL. The subjects were divided into two groups, consisted of 20 cases of GCB subtype and 20 cases of non-GCB subtype, which consisted of 21 males and 19 females patients. In the male group, there were 10 GCB subtype cases and 11 cases of non-GCB subtype. In the female group, there were nine cases of germinal center B-cell-like (GCB) subtype and 10 non-GCB subtype cases. The median age of patients with GCB subtype and non-GCB subtype were 48 and 55 years, respectively. Of the 20 GCB subtype cases, nine cases were presented in the extranodal sites and 11 cases in the lymph nodes. Meanwhile, of the 20 non-GCB subtype cases, 10 cases were presented in the extranodal sites and 10 cases in the lymph nodes (Table I).

Expression of PD-L1 in DLBCL tumor cells. PD-L1 staining was conducted in the GCB subtype and the non-GCB subtype of DLBCL samples. The result was later compared with the positive control, which was trophoblastic cells of chorionic villi

Table II. Distribution of PD-L1 expression in Diffuse large B-cell Lymphoma subtypes.

DLBCL Subtypes	Expression of PD-L1 in tumor cells		P-value ^a
	Positive	Negative	
Nodal and extranodal			
GCB	3/20 (15%)	17/20 (85%)	0.003
Non-GCB	13/20 (65%)	7/20 (35%)	
Nodal			
GCB	2/11 (18.2%)	9/11 (81.8%)	0.361
Non-GCB	4/10 (40%)	6/10 (60%)	
Extranodal			
GCB	1/9 (11.1%)	8/9 (88.9%)	0.001
Non-GCB	9/10 (90%)	1/10 (10%)	

^aFisher's exact test. DLBCL, diffuse large B-cell Lymphoma; GCB, germinal center B-cell-like.

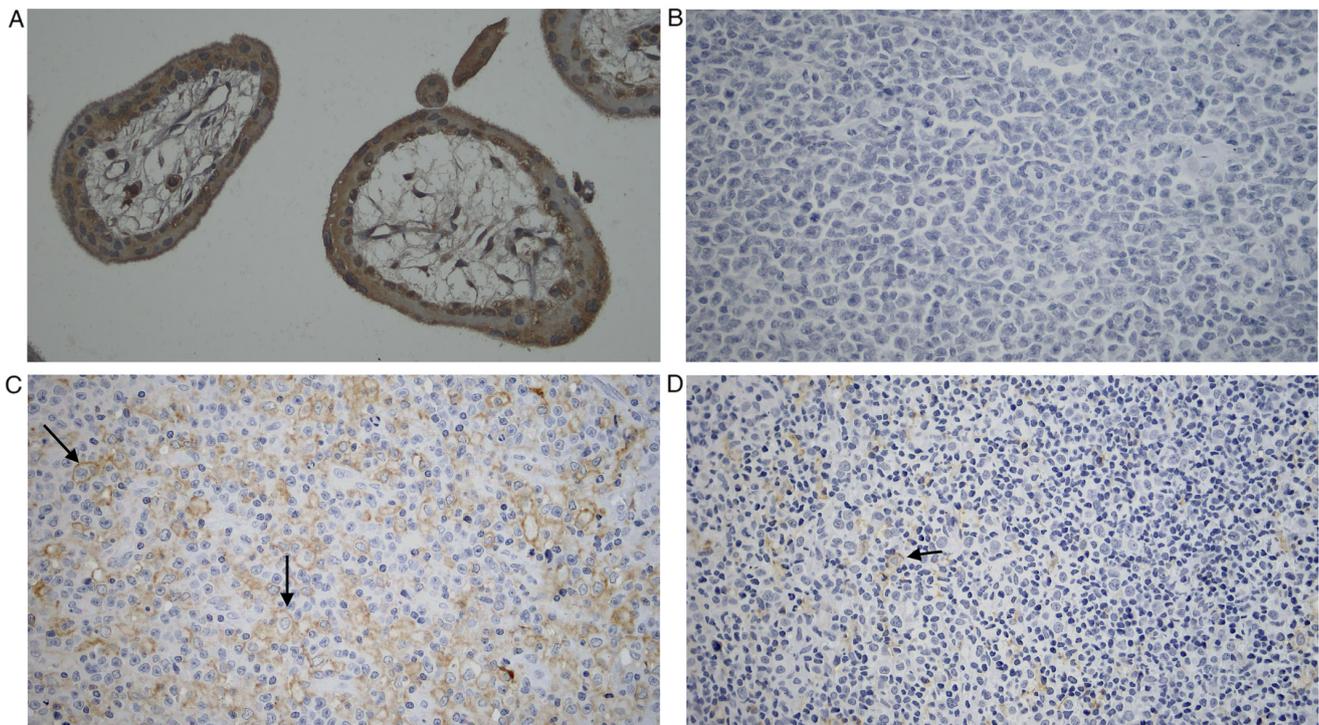


Figure 1. Immunohistochemical staining of PD-L1. (A) PD-L1 expression in positive control (trophoblastic cells of chorionic villi). (B) Negative control of PD-L1 in DLBCL section (without primary antibody). (C) PD-L1 expression in a case of non-GCB subtype of DLBCL was shown strongly in tumor cell membrane (arrow). (D) PD-L1 expression in a case of GCB subtype of DLBCL was shown weakly in tumor cell membrane (arrow). Magnification, x400. PD-L1, programmed cell death ligand-1; DLBCL, diffuse large B-cell Lymphoma; GCB, germinal center B-cell-like.

(Fig. 1). Marked expression of PD-L1 was shown on positive control tissue and no expression in negative control tissue. Meanwhile, there was a more robust PD-L1 expression in the non-GCB subtype of DLBCL compared with the GCB subtype.

The distribution of PD-L1 expression in tumor cells based on the cut-off value of $\geq 30\%$ positively stained cells was described in Table II. The average value for PD-L1 positive expression in tumor cells was 54.725 ± 17.704 , while the average for negative PD-L1 expression in tumor cells was 13.399 ± 9.896 . The Fisher's exact test showed $P=0.003$; there was a significant difference between the expression of PD-L1

in the GCB subtype and the non-GCB subtype of nodal and extranodal DLBCL. Fisher's exact test revealed no significant difference between PD-L1 expression in GCB and non-GCB of nodal DLBCL ($P=0.361$), but revealed significant difference between PD-L1 expression in GCB and non-GCB of extranodal DLBCL ($P=0.001$).

Discussion

In the present study, the incidence of DLBCL in male patients was not significantly higher compared with in female patients.

It was found that the location of nodal and extranodal DLBCL in the present study were only slightly different as compared with other study. Shi *et al* (16) showed that DLBCL mostly developed in nodal areas, while only ~37.4% of DLBCL cases were located extranodal. The present study found that PD-L1 expression in the DLBCL cases was significantly higher in the non-GCB subtype compared with GCB subtype. This result was consistent with other previous Asian population studies, which show a higher expression of PD-L1 in the non-GCB subtype of DLBCL compared with in GCB subtypes (12-14,17). These studies also demonstrated that high PD-L1 expression in DLBCL is associated with poor clinical outcomes. Higher expression of PD-L1 in the non-GCB subtype of DLBCL is also associated with poor clinical outcomes. Kwon *et al* (14) showed that strong PD-L1 expression is associated significantly with the presence of B symptoms and Epstein-Barr virus (EBV) infection. Kiyasu *et al* (12) note that PD-L1 expression level is positively correlated with the number of PD-1-positive T cells in activated B-cell-like (ABC)-subtype DLBCL specimens, but is negatively correlated with the number of fork-head box P3 (FOXP3)-positive regulatory T cells in GCB-subtype DLBCL specimens. The poor clinical outcomes of patients with ABC-subtype DLBCL can be associated with PD-L1 expression in tumor cells. By contrast, the lack of PD-L1 expression in GCB-subtype DLBCL specimens can be a possible explanation for the favorable prognosis associated with this disease subtype (17). However in a study performed by Kwon *et al* (14), PD-L1 expression level is not significantly different between non-GCB subtype of DLBCL and GCB subtype ($P=0.271$).

Microenvironment PD-L1 (mPD-L1) can be defined as non-malignant cells abundantly found in the tumor micro-environment (12). Some studies have been conducted to find the association between the PD-1/PD-L1 and its prognosis in DLBCL (12,17). Patients with a low number of PD-1⁺ and PD-L1⁺ have worse outcomes compared with PD-L1⁻ or mPD-L1⁻ in DLBCL cases (12). The finding recommends that PD-L1 expression in DLBCL might reflect clinical features. The study also stated that PD-L1⁺ was significantly associated with lower overall survival compared with those with PD-L1⁻. By contrast, mPD-L1 positivity did not have any correlation with survival (12).

A previous study performed double staining with PD-L1/PAX5 as an alternative way to improve the interpretation of PD-L1 positivity in DLBCL (12). PAX5 is a broader B-cell marker compared with CD 20. The present study did not performed PAX5 analysis however, focusing on the PD-L1 expression. Future study in genetic analysis (chromosome 9 translocation) may consider the involvement of PAX5.

Increased expression of PD-L1 in DLBCL tumor cells can be considered as an independent prognosis marker. The results of present study suggested that the PD-L1 expressions in DLBCL may become a potential immunotherapy marker. DLBCL patients, particularly those with non-GCB subtype might benefit from PD-L1/PD-1 axis inhibition. Further investigation by using PD-L1 as a biomarker of response to such treatment would be recommended.

The present study can be viewed as a preliminary study to describe PD-L1 expression in DLBCL in Indonesia because it is taken from the top referral hospital in Indonesia, which although central, receives lymphoma specimens from remote

areas as well. A future study could be performed using a larger sample sizes and multicenter study.

A limitation to the present study is that it did not correlate the immunohistochemical data with the therapeutic outcome or survival of the patient (due to the position of our department/laboratory as a central referral center for diagnosis), while the diagnosed patients were then treated in various hospitals across the country, some without well-established records of the outcome. It is hoped that improvements in Indonesian infrastructures will enable analysis of the treatment outcome as well.

In conclusion, there was a significant difference in the expression of PD-L1 protein in the non-GCB subtype of DLBCL as compared with GCB subtypes of DLBCL. The expression of PD-L1 protein in the non-GCB subtype was higher compared with that in the GCB subtype.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RA designed the study, performed the experiments, drafted the manuscript, collected various clinical data and performed the statistical analysis. MFH, AA, ASH and ESRH contributed to the review of the manuscript, interpreted the data, assisted with the experiments, revised the manuscript critically and made substantial contributions regarding manuscript concept and judgment. RA and ASH confirm the authenticity of all the raw data. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

All experiments were approved by the Faculty of Medicine, Universitas Indonesia Research Ethics Committees, with protocol number 0171/UN2.F1/ETIK/2018.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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