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Research Article

High Expression of CXCR4, Instead of PD-L1, Is Correlated with Poor Clinical Response to R-CHOP Therapy in Diffuse Large B-cell Lymphoma Patients

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Abstract:

Background: Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin Lymphoma worldwide. Rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone (R-CHOP) are the current standard therapy. About 10% cases of DLBCL were refractory to these immunochemotherapy and may have poor prognosis. Latest studies showed that C-X-C chemokine receptor type 4 (CXCR4) and programmed death ligand 1 (PD-L1) were involved in DLBCL biology and correlated with patients' poor survival. This study aimed to investigate correlation of CXCR4 and PD-L1 expression with clinical response to R-CHOP in DLBCL and their potential benefit as future predictive biomarkers.

Methods: Thirty four cases of newly diagnosed DLBCL, not otherwise specified (NOS), were selected from Hasan Sadikin General Hospital, Bandung, West Java, Indonesia, during 2015-2018. Treatment outcomes were retrieved from patients' medical records. The expression of CXCR4 and PD-L1 in patients' tumor tissues were detected using immunohistochemistry.

Results: Five of 34 cases (14.70%) showed poor clinical response to R-CHOP. High CXCR4 expression was detected in 11 of 34 cases (32.35%) and was correlated significantly with poor clinical response [p = 0.029, PR (95% CI) = 8.364 (1.055-66.291)]. High PD-L1 expression was detected in 10 of 34 cases (29.41%) and was not correlated with poor clinical response (p = 0.138).

Conclusion: High expression of CXCR4 increases probability of poor clinical response to R-CHOP in patients with DLBCL, but not of PD-L1. This finding provides the ground for further research which relates to CXCR4 as a future predictive biomarker for this challenging malignancy.

Keywords: diffuse large B-cell lymphoma, CXCR4, PD-L1, R-CHOP.

Introduction

Non-Hodgkin Lymphoma (NHL) is the 11th most common malignancy all over the world with 509,590 cases and 248,724 death cases. There are 14,164 cases NHL with 7,565 death cases in Indonesia.[1] Diffuse large B-cell lymphoma (DLBCL) is the most common NHL subtype accounts for 25-35% cases in developed countries with a higher percentage in developing countries.[2] It is a highly aggressive malignancy with heterogenity in clinical manifestations, molecular genetics, response to therapy and prognosis. Median survival is less than one year in untreated patients.[3]

The current standard therapy used for DLBCL patients is R-CHOP which is given in 6-8 biweekly cycles, it consists of rituximab, a monoclonal antibody anti-CD20 considered as immunotherapy owing to its mechanism using patients immune system to eliminate tumor cells, and CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine and prednisone), cytotoxic agents inducing apoptosis in tumor cells. Overall response rate (ORR) to R-CHOP therapy is 89.3% with 6 years survival rate 74.3%.[4] However, there are about 10% cases of DLBCL refractory to these regimen.[5] In other studies, reported refractory cases in DLBCL are 13%.[6] Refractory cases of DLBCL have poor prognosis and those patients will be given more aggresive treatment. Predictive biomarkers are highly required in order to introduce more effective treatment in DLBCL cases.[7]

C-X-C chemokine receptor type 4 (CXCR4) is a chemokine receptor specific to its ligand C-X-C motif chemokine ligand type 12 (CXCL12) which has an important role in lymphoid system development. CXCR4 is normally expressed in B cell, T cell, as well as epithelial and endothelial cells. Interaction of CXCR4 and CXCL12 (CXCR4/CXCL12 axis) involves in cell proliferation, migration and survival.[8]

Programmed death ligand 1 (PD-L1) is a ligand to programmed death protein 1 (PD-1). PD-1 is expressed in cytotoxic T cell, B cell, dendritic cell, macrophage and NK/T cell. Interaction between PD-1/PD-L1 (PD-1/PD-L1 axis) induces inhibitory signals which inhibit proliferation and stimulate dysfunction of PD-1 expressing immune cells. PD-1/PD-L1 axis creates local immunosupressive condition in tumor environment by increasing T regulatory activity, decreasing cytotoxic immune cells (CD8+ T cell and NK cell) as well as phagocytic immune cells (macrophage and neutrophil) activities.[9-11]

Previous studies reported that CXCR4 and PD-L1 may express in tumor cells of DLBCL and have a great role in biological process of this malignancy. High CXCR4 and PD-L1 expression were also correlated with DLBCL patients' poor survival.[9, 12] This present study hypothesized that higher

CXCR4 and PD-L1 expresion has negative correlation with clinical response to R-CHOP in DLBCL.

Materials And Methods

Sample Selection

This cross-sectional study was started after having been approved by Research Ethical Comittee Universitas Padjadjaran (No. 40/UN6.KEP/EC/2019). Cases of DLBCL, NOS, which were diagnosed in Hasan Sadikin General Hospital Bandung, the referral hospital of West Java Province, Indonesia, during period of 2015 until 2018, were retrieved from the anatomic pathology reports. All cases were diagnosed based on the criteria of 2016 World Health Organization (WHO). Patient demographics, clinical data and treatment outcome information were obtained from the medical records. Clinical response were classified as response (complete or partial) and nonresponse (stable or progressive disease) based on revised response evaluation criteria in solid tumors (RECIST 1.1). Among 322 cases of DLBCL during that period, only 34 cases could be included. The rest of the cases were excluded because the data were incomplete, the patients had not received R-CHOP therapy for minimum 4 cycles or formalin-fixed paraffin embeded (FFPE) tissues were unavailable.

Immunohistochemistry Assay and Evaluation

Immunohistochemistry (IHC) was done by applying the method of labeled streptavidin-biotin immunoperoxide complex using One Step Neopoly Detection Kit (Biogear Scientific, BioVentures, Inc., Iowa, USA). Primary antibodies used were rabbit polyclonal anti-CXCR4 antibody (1:100 dilution; cat No. GTX13854, GeneTex. Inc., California, USA) and rabbit monoclonal anti-PD-L1 antibody (1:300 dilution; clone 28-8, cat No. ab205921, Abcam, Inc., Cambridge, UK). Placenta and tonsil tissue were used as external positive controls for CXCR4 and PD-L1, respectively. IHC staining was performed on 4 μm-thick, FFPE tissue sections manually with antigen retrieval methods (0.01M ethylenediamintetra-acetic acid buffer at pH 6.0) as described previously. Counterstaining was done using Mayer's hematoxylin.

The expression of CXCR4 and PD-L1 were assessed using a semiquantitative scoring system (histoscore) in all of the immunohistochemical-stained sections according to the method used by Middle et al.[13] and Laurent et al.[14] CXCR4 positivity was defined by membrane and/or cytoplasmic staining in tumor cells whereas PD-L1 positivity was defined by complete or partial membrane staining with or without cytoplasmic staining in tumor cells. Diffuse blush was considered negative. The staining intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). The percentage of positively stained cells were scored as 0 (none), 1 (1-24%), 2 (25-49%), 3 (50-74%), or 4 (75-100%) for CXCR4 and were scored as 0 (≤10%) and 1 (>10%) for PD-L1. The final score was calculated by multipliying the staining intensity and precentage score, ranging from 0 to 12 for CXCR4 and 0 to 3 for PD-L1. The patients were divided to high or low expression using the median score of each marker as cutoff points (median score of 6 for CXCR4 and 1 for PD-L1). The IHC slides were examined blindly by two pathologist (BMD and BSH) independently without knowledge of clinicopathologic information. Any discrepancies in result were solved and a consensus score was reached.

Statistical Analysis

Numeric data of patient characteristics were recorded as mean, median, range and standard deviation. Comparisons of clinical data between groups were carried out using the Chi-Square test or Fisher's exact test if the criterias for Chi-Square test were not fulfilled. p-values were calculated and $p < 0.05$ was considered statistically significant. The analysis was performed using Statistical Package for Social Science (SPSS version 24.0, IBM Corp., Armonk, NY, USA).

Results

Patient Characteristics

Clinical data of the patients is shown in Table 1. Thirty four DLBCL cases were included, consisted of 21 male and 13 female patients, with a median age of 49 years (range 34-86 years). Cases with early clinical stage (stage I and II) were more than those with advanced stage (stage III and IV). Only 5 of 34 cases did not respond to R-CHOP. Correlation of clinical characteristics with response to R-CHOP were analyzed using Fisher's exact test (Table 1) showed nonsignificant result ($p > 0.05$).

Table 1. Clinical characteristics and their correlation with R-CHOP response

Variable	R CHOP Response		n (%)	p-value*
	Nonresponse n = 5 (14.7%)	Response n = 29 (85.3%)		
Age				0.383
=60 years	2	17	19 (55.9%)	
>60 years	3	12	15 (44.1%)	
Gender				1.000
Male	3	18	21 (61.8%)	
Female	2	11	13 (38.2%)	
B symptoms				1.000
Yes	2	13	15 (44.1%)	
No	3	16	19 (55.9%)	
Primary tumor				0.648
Nodal	2	16	18 (44.1%)	
Extranodal	3	13	16 (47.1%)	
Extranodal involvement >1				1.000
Yes	2	9	11 (32.4%)	
No	3	20	23 (67.6%)	
Clinical stage				1.000
Early (stage I-II)	3	19	22 (64.7%)	
Advance (stage III-IV)	2	10	12 (35.3%)	

Note: *p-value of bivariate analysis using Fisher's exact test, two-tailed.

CXCR4 and PD-L1 Immunohistochemical Expression

The immunohistochemical expression of CXCR4 and PD-L1 were evaluated (shown in Figure 1 and 2, respectively). CXCR4 was highly expressed in 11 of 34 cases (32.35%). There were 10 of 34 cases (29.41%) showed high PD-L1

expression.

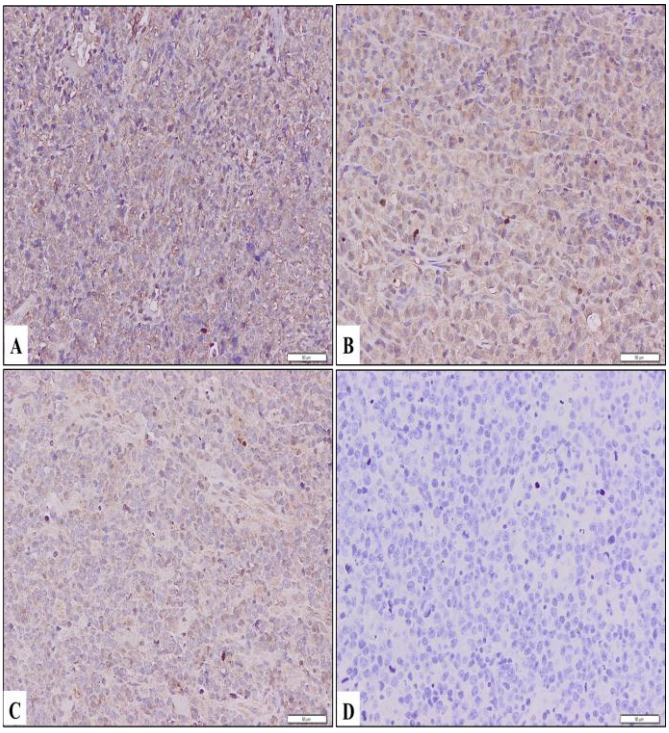

Fig. 1. Representative picture of CXCR4 immunohistochemistry. A. Strong (+3) , B. Moderate (+2), C. Weak (+1), D. Negative (0). (200x magnification).

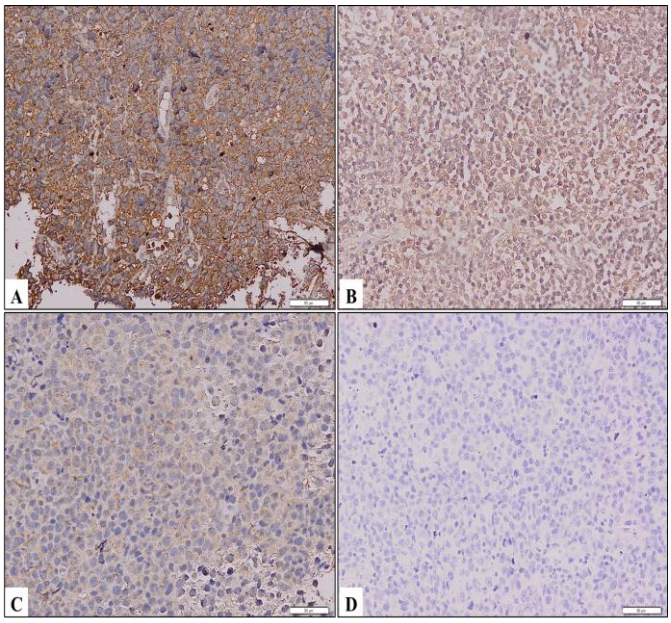

Fig. 2. Representative picture of PD-L1 immunohistochemistry. A. Strong (+3) , B. Moderate (+2), C. Weak (+1), D. Negative (0). (200x magnification).

Correlation of CXCR4 and PD-L1 Expression with Clinical Characteristics and Response to R-CHOP Therapy

CXCR4 expression was significantly correlated with clinical stage (p = 0.0003) but was not correlated with the other clinical characteristics. PD-L1 expression was correlated significantly with patients' gender (p = 0.022) but was not correlated with the other clinical characteristics (Table 2).

Table 2. Correlation of CXCR4 and PD-L1 expression with clinical characteristics

Variable	CXCR4			PD-L1		
	High n =11 (32.4%)	Low n = 23 (67.6%)	p-value[a]	High n = 10 (29.4%)	Low n = 24 (70.6%)	p-value[a]
Age			1.000			0.276
=60 years	6	13		4	15	
>60 years	5	10		6	9	
Gender			1.000			0.022[b]
Male	7	14		3	18	
Female	4	9		7	6	
B symptoms			0.064			0.462
Yes	2	13		5	10	
No	9	10		4	15	
Primary tumor			1.000			1.000
Nodal	6	12		5	13	
Extranodal	5	11		5	11	
Extranodal involvement >1			0.060			0.691
Yes	1	10		4	7	
No	10	13		6	17	
Clinical Stage			0.0003[b]			1.000
Early (I-II)	10	7		3	19	
Advance (III-IV)	1	21		2	10	

Note: [a]p-value of bivariate analysis using Fisher's exact test, two-tailed

[b]p < 0.05 means statistically significant.

Correlation of CXCR4 and PD-L1 expression with clinical response to R-CHOP was summarized in Table 3. High CXCR4 expression was correlated significantly with poor clinical response (p = 0.029). The probability of poor clinical response to R-CHOP in cases with high CXCR4 expression was 8.364-fold higher than those with low CXCR4 expression. However, there was no significant correlation between high PD-L1 expression with clinical response to R-CHOP (p > 0.05).

Table 3. Correlation of CXCR4 and PD-L1 expression with clinical response to R-CHOP

Variable	Clinical Response		Total	p-value[a]	Prevalence ratio (95% CI)
	Nonresponse (n=5)	Response (n=29)			
CXCR4				0.029[b]	8.364
High	4	7	11		(1.055-66.291)
Low	1	22	23		
PD-L1				0.138	.[c]
High	3	7	10		
Low	2	22	24		

Note: [a]p-value of bivariate analysis using Fisher's exact test, one-tailed.

[b]p < 0.05 means statistically significant.

[c]Prevalence ratio calculation could not be done due to nonsignificant result (p > 0.05).

Discussion

Diffuse large B-cell Lymphoma (DLBCL) is an agressive hemathological malignancy. DLBCL can occur in all ages but it is more common in elder individuals with slightly male predominance.[2] DLBCL patients may present with nodal or extranodal mass with rapid tumor growth. Primary extranodal DLBCL accounts for 30-40% of DLBCL.[12] Almost half of DLBCL patients came with early clinical stage.[7] In this present study, most of the patients aged less than 60 years old, male to female ratio is 1.61 showing male predominance, 47.1% of tumors was located primarily extranodal and 64.7% patients were in early clinical stage. The current standard treatment for DLBCL is R-CHOP immunochemotherapy. There were refractory (nonresponse) cases occur in this treatment and in this study, the refractory cases was 14.7%.

Rituximab is a monoclonal antibody anti-CD20, an immunotherapy regimen which eliminates tumor cells by antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CMC), apoptosis and direct growth arrest mechanism. In ADCC mechanism, Fc region of rituximab interacts with Fc receptors in macrophages, granulocytes, and NK cells. CD16 is one of Fc receptors expressed in NK cells. When CD16 interacts with Fc region of rituximab, they will stimulate NK cells to produce IFNɣ which has anti-tumor effect. NK cells induce cytolysis mediated by granzyme and perforin. In CMC mechanism, Fc region of rituximab binds to C1q to activate complement system by classic pathway. This binding then initiates membrane attack complex (MAC) formation to induce cytolysis. In apoptotic mechanism, binding of rituximab and CD20 induces structural changes (cross-linking) of CD20. Crosslinked CD20 inhibits p38, MAPK, NF-Kb, ERK1/ERK2 and AKT survival pathway. Through this apoptotic mechanism, rituximab affects sinergistically with cytotoxic agents. In direct growth arrest mechanism, rituximab induces tumor cells acumulation in G1 phase. This process is mediated by the increase of acid-sphingomyelinase activity and ceramide formation in raft microdomain. Ceramide induces signaling pathway controlling CDK inhibitor such as p27Kip1 through MAPK-dependent mechanism.[15, 16]

Chemotherapy CHOP are cytotoxic agents which eliminate tumor cells by apoptotic mechanism. Cyclophosphamide and hydroxydaunorubicin induce DNA damage which may induce cytochrome C release controled by mitochondrial P53. Cytochrome C then binds to apoptosis-activating factor-1 (Apaf-1) which activates caspase-9 followed by caspase-3.[17, 18] Oncovin (vincristine) disrupts microtubuli needed for mitosis,[19] while prednisone induces apoptosis by its binding to glucocorticoid receptor.[20]

In this study, we investigated the correlation of immunohistochemical expression of CXCR4 and PD-L1 with clinical response to R-CHOP in patients with DLBCL. The results indicated that the CXCR4 and PD-L1 was highly expressed in some of DLBCL cases. CXCR4 expression was significantly higher in early clinical stage groups. PD-L1 expression was significantly higher in female patients. Previous study showed that neither CXCR4 nor PD-L1 expression was correlated with age, gender, presence of B symptoms, primary tumor location, more than one extranodal involvement or clinical stage.[9, 11, 21]

The chemokine receptor CXCR4 is thought to have biological role several hematologic and non-hematologic malignancies. CXCR4 expression in DLBCL tumor cells may be induced by genetic mutation as reported by Fernandez et al.,[22] there was CXCR4 *WHIM-like* mutation in DLBC patient having MYD88 L265P mutation. CXCR4 may also upregulated by PI3K/AKT, CREB3, PAX3-FKHR, Wnt and Notch pathway through B-cell receptor (BCR) activation.[8]

Interaction of CXCR4 and CXCL12 activates intracellular signals, such as JAK2/STAT3, MEK/ERK and PI3K/AKT pathway to promote cell proliferation, migration, and survival. Jiang et al.[23] reported that CXCR4 downregulation induces osteosarcoma cells apoptosis through inhibition of PI3K/AKT/NF-kB pathway. PI3K/AKT pathway is an intracellular signaling pathway which involves in DLBCL lymphomagenesis. Kim et al.[24] reported that dysregulated PI3K/AKT pathway disrupts apoptotic mechanism in DLBCL tumor cells by inducing phosphorylation-inhibition of pro-apoptotic gene. Therefore, this pathway may interfere with apoptotic mechanisms by R-CHOP as a basis of this study hypothesis. As the result of this study, patients with high expression of CXCR4 were correlated with poor clinical response to R-CHOP. However, it needs to be explored in further studies whether PI3K/AKT pathway is involved or not as an underlying mechanism of poor clinical response to R-CHOP.

Programmed death ligand 1 (PD-L1) is immune check-point creating local immunosupressive condition in the tumor microenvironment through its interaction to PD-1. Li et al.[7] reported that 20-25% of DLBCL cases have PD-L1 expression in tumor cells. PD-L1 expression in DLBC is known to be upregulated by genetic alteration. Gain, amplification, or fusion in PD-L1/PD-L2 locus on chromosome 9p24.1 directly activate PD-L1 promoter.[25] Proto-oncogen *immunoglobulin heavy-chain locus* (IGH), PIM1, and TP53 are translocation partners for PD-L1/PD-L2.[26] PD-L1 expression may be induced by acquiring condition through activation of intracellular pathway regulating PD-L1 gene transcription, such as AP-1/cJUN/JUN-B pathway by latent membrane protein 1 (LMP-1), an Epstein-Barr virus product, JAK3/STAT5 pathway by interferon, and MEK/ERK pathway by HIF-1α and HIF-2α resulting from hypoxic condition due to fast-growing tumor.[9-11]

Immunosupressive condition created by PD-1/PD-L1 axis may interefere with mechanism of action of rituximab as immunotherapy regimen. Liu et al.[27] reported that overactivated PD-1/PD-L1 axis expression facilitates chemoresistance of DLBCL cells to CHOP regimen through PI3K/AKT pathway activation. This study suggests interference of PI3K/AKT pathway activation, which upregulates PD-L1 expression, with mechanism of action of CHOP. However, as the result of this present study, cases with

high expression of PD-L1 were not correlated with poor clinical response to R-CHOP. Mechanism of action of Rituximab other than immune-mediated (direct growth arrest and apoptosis) and mechanism of action of CHOP as cytotoxic agents (apoptosis) are not interfered directly by PD-1/PD-L1 axis. Intracellular pathway other than PI3K/AKT may involve in high PD-L1 expression in these patients. However, these possible mechanisms need to be explored in other studies.

Conclusion

This study revealed that CXCR4 and PD-L1 were highly expressed in some DLBCL cases. CXCR4 instead of PD-L1 immunohistochemical expression is correlated negatively with clinical response to R-CHOP therapy in DLBCL patients. This study provided preliminary data for further investigation of CXCR4 and its predictive value in DLBCL cases.

Conflict of Interest

The authors declare that there are no conflict of interests in this study.

Acknowledgement

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