Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Correlation between PD-1 and sPD-L1 expression levels in peripheral blood of DLBCL patients and their clinicopathological characteristics

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Article Info

Article history:

Received: July 24, 2023
Accepted: January 10, 2024
Published: February 29, 2024

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Abstract

Molecular pathology and clinical characteristics play a crucial role in guiding treatment selection and predicting the prognosis of diffuse large B-cell lymphoma (DLBCL). The programmed cell death protein 1 (PD-1) and its ligand (PD-L1), have emerged as pivotal regulators of immune checkpoints in cancer. The objectives of this study are to investigate the correlation between the expression levels of PD-1 and soluble PD-L1 (sPD-L1) in the peripheral blood of DLBCL patients, analyze their clinicopathological characteristics, and identify the optimal beneficiary group for PD-1/PD-L1 blockade. Peripheral blood samples were collected from 36 DLBCL patients before their initial treatment at Shandong Cancer Hospital between December 2018 and July 2019. The expression levels of PD-1 and sPD-L1 were measured using flow cytometry and enzyme-linked immunosorbent assay (ELISA), respectively. The clinicopathological characteristics, including age, sex, Ann Arbor stage, International Prognostic Index (IPI) score, response to treatment, etc., were recorded for each patient. The surface expression of PD-1 on peripheral blood T cells was significantly higher in DLBCL patients compared to healthy controls. There was a significant association between elevated PD-1 expression levels and the advanced Ann Arbor stage (P=0.0153) as well as the B group (P=0.0184). Higher sPD-L1 levels were associated with the GCB subtype according to Hans's classification (P=0.0435). The expression levels of PD-1 and sPD-L1 in the peripheral blood of DLBCL patients are significantly correlated with advanced disease stage, B group, and GCB subtype according to Hans's classification. This suggests that the PD-1/PD-L1 axis play a critical role in specific subgroups of DLBCL. Targeting this axis could serve as a potential therapeutic strategy to enhance the clinical outcomes of DLBCL patients. Further studies are necessary to explore the prognostic implications of PD-1 and sPD-L1 expression levels in DLBCL patients.

Keywords: Diffuse large B-cell lymphoma, PD-1, sPD-L1, Peripheral blood, Clinicopathological characteristics, Immune checkpoints.

1. Introduction

The PD-1/PD-L1 pathway serves as a dual mechanism, providing protection against immune-mediated damage while facilitating tumor cells' evasion of immune attacks. PD-1/PD-L1 has demonstrated promise in various cancer types, including non-small cell lung cancer [1], melanoma [2], renal cell carcinoma [3], and hematological malignancies, such as relapsed/refractory classic Hodgkin lymphoma (HL) [4]. Over recent years, the expression levels of PD-1 or PD-L1 proteins in tumor-infiltrating lymphocytes and tumor cells have emerged as potential biomarkers indicating cancer patients' prognosis [5-8]. Nonetheless, the impact of PD-1 and PD-L1 expression in peripheral blood lymphocytes and plasma on the effectiveness of immune checkpoint inhibitors remains uncertain. Therefore, this study focused on assessing PD-1 expression on the surface of T cells and B cells in peripheral blood, along with measuring soluble PD-L1 levels in peripheral blood plasma.

The selection of treatment strategies and the prediction of diffuse large B-cell lymphoma (DLBCL) prognosis involve considering important factors such as molecular pathology and clinical features. Numerous markers, such as Bcl-2, Ki67, c-Myc, β2MG, lactate dehydrogenase (LDH), IPI, and GCB [9-15], have been identified as being associated with DLBCL prognosis. Therefore, in this study, we analyzed the expressions of PD-1 and sPD-L1 and investigated their correlation with clinicopathological features. This analysis aimed to provide valuable data for evaluating the therapeutic efficacy and prognosis of DLBCL based on PD-1/PD-L1 expression and to identify the subgroup that would benefit the most from PD/PD-L1 blockade.

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Doi: http://dx.doi.org/10.14715/cmb/2024.70.2.7

2. Material and methods

2.1. Patients and healthy volunteers

This study aimed to investigate prognostic factors in patients diagnosed with diffuse large B-cell lymphoma (DLBCL). Peripheral blood (PB) samples were collected from 36 DLBCL patients between December 2018 and May 2019, before chemoradiotherapy. To establish a control group, blood samples were collected from 15 volunteers. Clinicopathological data, such as age, gender, tumor location, staging, grouping, lactate dehydrogenase (LDH) levels, and other relevant information, were recorded from the time of diagnosis to select potential prognostic factors. The study was approved by the hospital's ethics committee, and informed consent was obtained from all patients. Sample collections were performed by the principles outlined in the Declaration of Helsinki.

2.2. Sample treatment

The specimen underwent centrifugation for 5 minutes at a speed of 1500 rotations. Following this, 1 ml of the supernatant was carefully extracted and stored in a refrigerator at a temperature of -80°C using the gradient cooling method. Furthermore, additional specimens were collected, with 100 μl being obtained. These specimens were then treated with erythrocyte lysate to separate and obtain the mononuclear cells.

2.3. Flow Cytometry

To experiment, the following steps should be followed: First, collect 100 ml of whole blood. Next, add 1 μL of the antibody to the whole blood and gently mix to ensure proper distribution. Allow the mixture to incubate for 20 minutes to facilitate antibody binding. Subsequently, incorporate 2 mL of red blood cell lysate into the mixture and gently mix again. Incubate the combined solution for an additional 10 minutes. After the incubation, perform two washes on the cells by adding 2 mL of PBS and centrifuging at 300 g for 5 minutes. Take care to remove the supernatant cautiously after each wash cycle. The cells should then be fixed by adding 4% formalin to the cell pellet, ensuring thorough mixing. The cells should then be treated with the following antibodies in different combinations: phycoerythrin (PE)-conjugated CD279 (PD-1; Bio Legend, San Diego, CA, USA), peridinin chlorophyll (PerCP) / Cy5.5-conjugated CD8 (Bio legend, San Diego, CA, USA), allophycocyanin (APC)-conjugated CD19 (Bio legend, San Diego, CA, USA), BV510-conjugated CD4 (BD Bioscience, Franklin Lakes, NJ, USA). Adhere to the recommended concentrations provided in the manufacturers' instructions. Subsequently, analyze the samples using a flow cytometer such as the BD FACS Canto II. Finally, employ FlowJo software (version 10; Tree Star, Ashland, OR, USA) to analyze the data gathered from the flow cytometer, specifically to determine the percentage of PD-1 expression on CD4+T, CD8+T, and CD19+B cells.

2.4. ELISA analysis

The defrosted supernatant was allowed to thaw completely at room temperature in advance. Commercial ELISA kits from CUSABIO, Wuhan, China, were utilized to measure sPD-L1 levels. All procedures were carried out strictly following the provided instructions. In summary, 96-well plates were used to prepare the samples, including the addition of standard continuous diluent and serum samples. Anti-PD-L1 monoclonal antibodies were also included. Subsequently, a horseradish peroxidase coupling agent was added, and the color development was achieved using the tetramethylbenzidine system. The optical density (OD) at 450 nm was determined using a micro titer plate reader after a 15-minute incubation period. In the control group, diluent was substituted for serum and the remaining steps were identical to those in the experimental group. Standard curves were generated by plotting the mean OD values of the 6 standard concentrations on the vertical (y) axis, and their corresponding concentrations on the horizontal (x) axis (450 nm OD, respectively). Finally, the concentration of each sample was estimated based on its OD value.

2.5. Statistical analysis

Statistical analysis was performed using IBM Statistic Package for Social Science (SPSS) Statistics 22 (IBM SPSS, Armonk, NY, USA). The Chi-square test was used to compare qualitative variables across different groups, while the T-test was used for quantitative variables between different groups. Spearman's rank correlation tests were conducted to assess the correlation between PD1 and SPD-L1 expression levels with efficacy. A P-value of < 0.05 was considered statistically significant. Graphical representations of the data were generated using GraphPad Prism software version 8.0 (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Patient characteristics

The clinicopathological characteristics of DLBCL patients in the study cohort are shown in Table 1.

The upper limit of LDH (Lactate dehydrogenase) is 245 U/L and the upper limit of β2MG (Beta-2 microglobulin) is 2.7 mg/L in normal individuals. The staging of DLBCL (Diffuse Large B-cell Lymphoma) is determined using the Ann Arbor staging system. Based on the National Comprehensive Cancer Network International Prognostic Index (NCCN-IPI) score, a score ≤ 3 is categorized as the low or low-moderate-risk group, while a score >3 is classified as the medium or medium-high-risk group. The classification of DLBCL according to the HANS model involves differentiating it into GCB (Germinal Center B-cell-like) subtypes and non-GCB subtypes. Among the 15 healthy controls, the median age was 35 years (ranging from 30 to 62 years) with a male-to-female ratio of 7:8.

3.2. The level of PD-1 in peripheral blood lymphocytes of DLBCL patients is higher compared to that of healthy individuals.

The expression level of PD-1 in CD4+T and CD8+T cells in the peripheral blood of DLBCL patients was significantly higher compared to that of the healthy control group (P=0.0013, Figure 1a; P=0.0252, Figure 1b). However, there was minimal significant difference in PD-1 expression between DLBCL patients and healthy controls on CD19+B cells (P=0.0438, Figure 1c).

3.3. The relationship between the expression levels of PD-1/sPD-L1 and clinicopathological features.

The flow cytometry diagram of representative PD-1 expression on the cell surface of CD4+, CD8+, and CD19+ is shown in Figure 2. The correlation between the expression level of PD-1/ sPD-L1 and clinicopathological features is

Table 1. Clinicopathological features of DLBCL patients.

Clinical parameters	n (%) [1]	n (%) [2]	n (%) [3]
Age (years)			
<65	10(62.5)	7(54)	14(70.0)
≥65	6(37.5)	6(46)	6(30.0)
Gender			
Female	7(43.8)	5(38.5)	11(55.0)
Male	9(56.2)	8(61.5)	9(45.0)
LDH (grouping)			
0(normal)	5(31.2)	4(30.8)	10(50.0)
1(abnormal)	11(68.8)	9(69.2)	10(50.0)
β2MG (grouping)			
0 (normal)	11(68.8)	8(61.5)	7(35.0)
1(abnormal)	5(31.2)	5(38.5)	13(65.0)
Extracapsular extension			
No	3(18.8)	2(15.4)	4(21.1)
Yes	13(81.2)	11(84.6)	15(78.9)
B symptoms			
No	11(68.8)	11(68.8)	15(83.3)
Yes	5(31.2)	5(31.2)	3(16.7)
Ann Arbor staging			
I-II	3(18.8)	3(23.1)	10(50)
III-IV	13(81.2)	10(76.9)	10(50)
Hans classification			
GCB	7(58.3)	5(50.0)	16(80)
Non-GCB	5(41.7)	5(50.0)	4(20)
KI67			
<80%	5(35.7)	4(30.8)	6(30)
≥80%	9(64.3)	9(69.2)	14(70)
BCL-6			
+	8(66.7)	6(60.0)	12(68)
-	4(33.3)	4(40.0)	6(32)
C-Myc			
+	6(54.5)	4(44.5)	8(50.0)
-	5(45.5)	5(55.5)	8(50.0)
MUM1			
+	6(50.0)	4(40.0)	11(57.9)
-	6(50.0)	6(60.0)	8(42.1)
CD10			
+	5(41.7)	3(30.0)	12(66.7)
-	7(58.3)	7(70.0)	6(33.3)
NCCN-IPI			
≤3	6(37.5)	5(38.5)	9(50.0)
>3	10(62.5)	8(61.5)	9(50.0)

1: Represents the number and percentage of patients receiving detection of PD-1 expression level on $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes. 2: Represents the number and percentage of patients receiving detection of PD-1 expression level on $CD19^+$ B lymphocyte surface. 3: Represents the proportion and percentage of patients receiving peripheral blood sPD-L1 test.

Table 2. Relationship between the expression level of PD-1 on lymphocyte surface and sPD-L1 in peripheral blood and clinicopathological characteristics

Clinical parameter	*P* ($CD4^+PD1^+$)	*P* ($CD8^+PD1^+$)	*P* ($CD19^+PD1^+$)	*P* (sPD-L1)
Age (years)	0.1536	0.4556	0.2916	0.2008
Gender	0.2806	0.8973	0.1159	0.6628
LDH (grouping)	0.555	0.769	0.326	0.1179
β2MG (grouping)	0.3127	0.4723	0.1386	0.0535
Extracapsular extension	0.1779	0.2212	0.4837	0.6190
B symptoms	0.0184	0.8930	0.4023	0.5130
Ann Arbor staging	0.0153	0.3222	0.5309	0.1797
Hans classification	0.3116	0.9949	0.8601	0.0435
KI67	0.3315	0.9113	0.5247	0.9993
BCL-6	0.0626	0.8873	0.5011	0.8537
C-Myc	0.4381	0.8247	0.8511	0.3108
MUM-1	0.0906	0.8650	0.9639	0.4595
CD10	0.3690	0.7167	0.6030	0.6234
NCCN-IPI	0.8413	0.5621	0.1492	0.1060

P<0.05 was statistically significant.

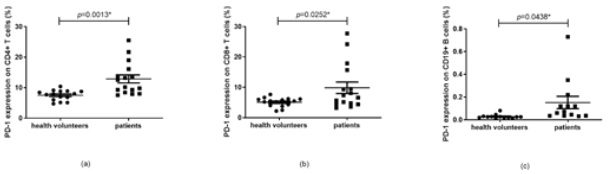

Fig. 1. Comparison of PD-1 expression in peripheral blood lymphocytes between DLBCL patients and healthy volunteers. The methylation ratio of HLA-G in patients with ITP was analyzed utilizing the Sequenom EpiTYPER system to calculate the average values for both females and males. Each data point on the graph represents an individual sample. The mean values, along with the standard error of the mean, are provided. A comparison between the groups was carried out using the unpaired t Student's test (*P<0.05).

shown in Table 2. PD-L1 expression was significantly correlated with Symptom B (See Figure 3a) and Ann Arbor stage (See Figure 3b). However, there was no significant correlation between PD-1 expression and other clinicopathological factors. sPD-L1 is associated with the HANS model GCB subclassification (refer to Figure 3c).

3.4. The relationship between PD1/sPD-L1 and short-term efficacy

Out of the 16 patients with PD-1 expression levels, 14 of them received R-CHOP treatment. Similarly, among the 20 patients with sPD-L1 expression, 18 of them were treated with R-CHOP and one patient was lost to follow-up. The evaluation of the efficacy after 4 cycles of chemotherapy was conducted based on the 2014 Lugano evaluation criteria. The association between the expression of PD-1 and sPD-L1 in peripheral blood and the efficacy after 4 cycles is presented in Table 3.

Table 3. The relationship between PD1/sPD-L1 and short-term efficacy.

	Short-term efficacy (case)				P-value
	CR	PR	SD	PD	
CD4+PD-1+	7	5	0	2	0.967
CD8+PD-1+	7	5	0	2	0.392
CD19+PD-1+	7	5	0	2	0.179
sPD-L1	13	3	1	0	0.743

P<0.05 was statistically significant.

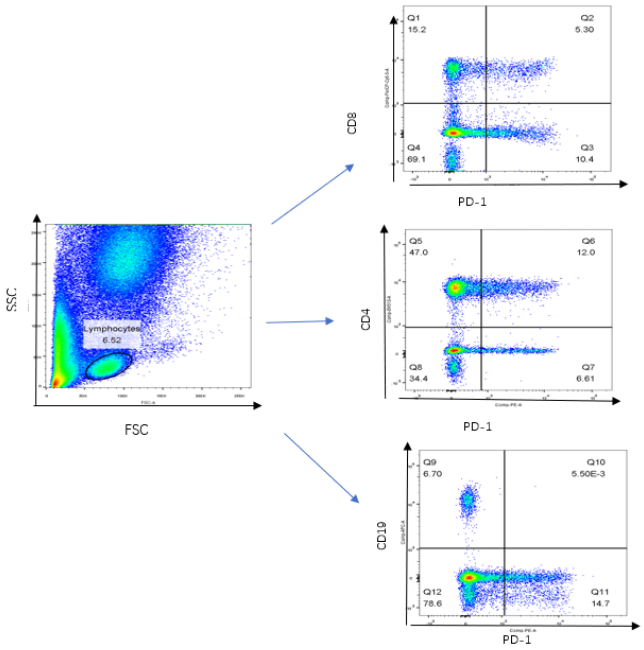

Fig. 2. Flow cytometry for detection of PD-1 expression in CD4+, CD8+ and CD19+ cells.

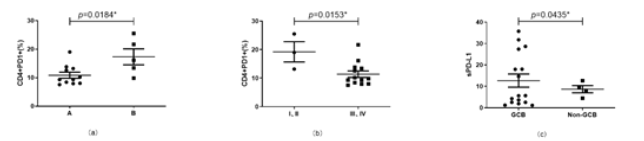

Fig. 3. Relationship between the expression level of PD-1/ SPD-L1 and clinicopathological features. The methylation ratio of HLA-G in patients with ITP was analyzed utilizing the Sequenom EpiTYPER system to calculate the average values for both females and males. Each data point on the graph represents an individual sample. The mean values, along with the standard error of the mean, are provided. A comparison between the groups was carried out using the unpaired t Student's test (*P<0.05).

4. Discussion

The blockade of PD-1/PD-L1 pathways has shown

promising anti-tumor activity in DLBCL treatment (7). However, not all patients respond to this treatment, and it can cause severe side effects. Immunological parameters in the tumor microenvironment, particularly PD-L1 expression in tumor tissue, have been reported as predictive biomarkers for response to PD-1 inhibitors [16]. However, patients with lower values may still benefit from therapy, but biopsies have inherent invasiveness and limitations: inability to sample the same area sequentially and potential lack of representativeness due to tumor heterogeneity [17]. Evidence suggests that immunological biomarkers found in peripheral blood can reflect the immunological milieu of the tumor microenvironment [18]. Blood-based biomarkers are considered ideal due to their dynamic nature, potential to reflect real-time treatment effects and easy accessibility. However, despite the advantages provided by working with PBMC, there is still a lack of reliable cellular biomarkers in this compartment, which necessitates further investigation. This study aimed to identify patients who are most likely to respond to PD-1 inhibitors before treatment. We hypothesized that a higher baseline level of sPD-L1 and/or PD-1 in peripheral blood could predict the response to PD-1 inhibitors.

The presence of PD-1+ tumor-infiltrating lymphocytes (TILs) has been established as a prognostic indicator for various tumor types [19-21]. Ahearne et al. identified PD-1+ TILs as a reliable prognostic factor for DLBCL [22]. However, the expression of PD-1 on peripheral blood T cells did not consistently match the PD-1 expression observed in the tumor microenvironment through immunohistochemistry (IHC) [23]. MacFarlane et al. propose that PD-1 expression on peripheral blood mononuclear cells can serve as a biomarker for disease severity and prognosis [24]. Taking this into account, we examined the expression of PD-1 on the surface of peripheral blood T cells. Our study revealed higher levels of PD-1 expression on the surface of peripheral blood T cells in DLBCL patients compared to normal individuals, which aligns with previous findings [23]. Although PD-1 was expressed in CD19+ B cells, the expression level was relatively low, and the level of CD19+PD-1+ cells did not significantly differ from that of normal individuals. This discrepancy may be attributed to the limited sample size. Regarding the relationship between PD-1 expression and clinicopathology, our analysis demonstrated a significant correlation between CD4+PD-1+ expression and staging and B grouping.

There are two forms of PD-L1 expression: membrane-bound and soluble. Overexpression of membrane-bound PD-L1 in tumor tissue has been associated with poor prognosis in various malignant tumors [25, 26]. In recent years, the soluble form of PD-L1 (sPD-L1) has emerged as a potential player in immunosuppression and is associated with the poor prognosis of malignant tumors [27-29]. Elevated levels of sPD-L1 in plasma have been found to correlate with decreased complete response rates and poorer survival in DLBCL patients, even with high-dose chemotherapy [30, 31], although conflicting results have been reported by Keane et al. [32]. Previous studies have indicated that high PD-L1 expression may be caused by genetic abnormalities or chromosomal changes [33], particularly structural alterations of the 9p24.1 region. Georgiou et al. demonstrated the genetic basis of PD-L1 overexpression in DLBCL, with cytogenetic alterations affecting the PD-L1/PD-L2 locus being more frequently observed in the non-germinal center B cell-like (non-GCB) subtype of DLBCL. This suggests that treatments targeting the PD-1-PD-L1/PD-L2 axis could potentially benefit DLBCL patients, especially those belonging to the more aggressive non-GCB subtype [34]. However, in our study, when analyzing the relationship between sPD-L1 and clinical features, we found that sPD-L1 expression was associated with the Hans classification. Specifically, sPD-L1 expression was higher in DLBCL patients classified as GCB compared to those classified as non-GCB. It is worth noting that the small sample size may have influenced these findings, and further investigation in a larger cohort is necessary to validate these observations. Notably, Rossville et al. did not observe a correlation between PD-L1 expression and sPD-L1 levels in DLBCL patients [31]. This discrepancy emphasizes the importance of further exploration into the origin and clinical significance of sPD-L1.

5. Conclusion

Our analysis revealed strong evidence supporting a connection between the expression of PD-1 in T cells found in the peripheral blood of DLBCL patients and the presence of symptom B and stage I/II. Furthermore, we observed a significant correlation between the expression of sPD-L1 in the peripheral blood plasma of DLBCL patients of the germinal center B-cell (GCB) subtypes. These findings indicate that measuring the levels of PD-1 and sPD-L1 expression could be valuable in assessing the prognosis of individuals diagnosed with DLBCL.

Conflicts of interest

The authors declare no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Shandong Cancer Hospital and Institute, Shandong First Medical University, and Shandong Academy of Medical Science.

Informed consent statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data availability

For original data, please contact phd_jima@163.com.

Author contributions.

J.M. analyzed data, and wrote the paper; J.Q., J.N.Z., and Q.H. performed research; L.W., C.C.C. arranged for obtaining patient informed consents and data collection; L.H.S., L.N.X. designed and reviewed the work. The manuscript was critically revised by all authors.

Funding

This research was funded by the National Natural Science Foundation of China (No. 81500096; No. 82170123 and No. 8150010892), the Natural Science Foundation of

Shandong Province (ZR2021MH115), CSCO Clinical Oncology Research Foundation -Hengrui Tumor Research Fund Project (Y-HR2020QN-0175 and Y-SY201901-0011) and Wu Jieping Medical Foundation Clinical Research Grant Program (320.6750.2021-02-40); Start-up fund of Shandong Cancer Hospital (RCYJ201902).

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