RESEARCH ARTICLE

Editorial Process: Submission:08/08/2023 Acceptance:12/11/2023

Clinicopathological Study of *PD-1/PD-L1* Expression in Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma (CLL/SLL) with Emphasis on Large B-Cell Richter Transformation

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Abstract

Objective: In the era of immunotherapy, inhibition of the *PD-1/PD-L1* immune checkpoint pathway has changed the therapeutic landscape for many tumors. Limited studies were performed on the expression of *PD-1* in chronic lymphocytic lymphoma/small lymphocytic lymphoma (CLL/SLL) and its Richter transformation into diffuse large B-cell lymphoma (DLBCL-RT). This study aims to evaluate *PD-1/PD-L1* expression and their prognostic role in CLL/SLL, DLBCL-RT, and DLBCL-de novo patients. **Material and methods:** This retrospective study was conducted on 96 cases (38 CLL/SLL, 11 DLBCL-RT and 47 DLBCL-de novo) that were retrieved from the pathologic and clinical databases at the Oncology Center, Mansoura University. Immunohistochemical evaluation of *PD-1* and *PD-L1* was assessed in tumor cells and the microenvironment in those patients. **Results:** This study demonstrated positive expression of *PD-1* in CLL/SLL patients, mainly in proliferation centers. Moreover, it showed a higher prevalence of *PD-1* expression in DLBCL-RT (9/11 patients) than in DLBCL-de novo (5/47 patients) ($P < 0.001$). Tumor cells revealed positive *PD-L1* expression in 5/47 DLBCL-de novo patients and negative *PD-L1* expression in all CLL/SLL and DLBCL-RT patients. *PD-1* was positive in reactive T-cells, and *PD-L1* was positive in background histiocytes and dendritic cells in all studied cases. *PD-1* positive expression in tumor cells was considered an independent poor prognostic factor for overall survival (OS) in DLBCL patients ($P = 0.04$). In addition, DLBCL-RT had a significantly shorter OS than DLBCL-de novo ($P = 0.005$). **Conclusion:** The high prevalence of *PD-1* expression in DLBCL-RT patients supports the promising and potential role of anti-*PD-1* immunotherapy in the treatment of DLBCL-RT patients.

Keywords: Immunotherapy- immunohistochemical evaluation- DLBCL-RT- survival.

Asian Pac J Cancer Prev, **24 (12)**, 4243-4252

Introduction

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a low-grade B-cell neoplasm that involves bone marrow, peripheral blood, lymph nodes, and spleen (Campo et al., 2017). CLL/SLL is the most common leukemia in adults in Western countries. It accounts for 25 to 30% of all leukemias in the United States, with a male predominance (Siegel et al., 2020). About 2% to 9% of patients develop an aggressive lymphoma, most commonly diffuse large B-cell lymphoma (Richter transformation, DLBCL-RT) (He et al., 2018). Patients with DLBCL-RT have a dismal prognosis and a poor response to standard chemoimmunotherapy (Eyre et al., 2017).

Programmed cell death 1 (*PD-1*)/programmed cell death ligand 1 (*PD-L1*) pathway is an immune checkpoint pathway that has a key role in the maintenance of self-tolerance and the control of exaggerated immune responses. However, in tumor cells, this pathway is utilized to inhibit anti-tumor immune responses and to evade immune surveillance. Blockade of this pathway has emerged as an effective anti-cancer immunotherapy (Xie et al., 2019; Annibali et al., 2018).

Immunotherapy targeting *PD-L1* has led to an alternative therapeutic approach for various tumors, such as non-small cell lung carcinoma (NSCLC) (Rangachari and Costa, 2019). *PD-L1* is expressed in many tumors, such as non-small cell lung carcinoma, head and neck squamous cell carcinoma, and gastric carcinoma (Pant and Harlalka, 2023; Attia et al., 2022). Immunohistochemical expression of *PD-L1* is used as a predictive biomarker to

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predict immunotherapeutic response (Tsimafeyeu et al., 2020). Anti-*PD-1* clinical trials are ongoing in CLL/SLL and DLBCL-RT. Ding et al., (2018), in a phase 2 clinical trial, have reported a promising 44% response of blocking antibody for *PD-1* (Pembrolizumab) in DLBCL-RT (4/9 patients). However, studies regarding *PD-1* and *PD-L1* expression in CLL/SLL and DLBCL-RT concerning clonal cells and tumor microenvironment are still limited in the literature.

The aim of this study is to demonstrate the frequency of immunohistochemical expression of *PD-1* and *PD-L1* in tumor cells and the tumor microenvironment in CLL/SLL, DLBCL-RT, and DLBCL-de novo patients and compare the results with those reported in the literature. Furthermore, this study investigates the possible prognostic and predictive value of *PD-1* and *PD-L1* expression in those patients.

Materials and Methods

Patients

This study is a retrospective analysis of collected data and tumor samples (formalin-fixed paraffin-embedded tissue) from patients diagnosed with typical CLL/SLL (29 patients), aggressive CLL/SLL (9 patients), DLBCL-RT (11 patients), and DLBCL-de novo (47 patients) referred to the surgical pathology laboratory at the Oncology Center, Mansoura University (OCMU), Egypt, through the period from January 2016 to December 2019. Only patients with complete clinical information, accessible paraffin blocks, and excisional surgical lymph node biopsy were included in this study. Meanwhile, tru-cut biopsy, trephine biopsy, and fine needle aspiration (FNA) were not included in this study.

Case categorization

CLL/SLL patients were divided into two categories: typical/regular CLL/SLL and aggressive CLL/SLL. The latter was defined by the presence of expanded or confluent proliferation centers (PCs) (broader than a 20x field) and a high proliferation index, which in turn is defined by either >2.4 mitoses/PC or Ki-67 >40%/PC (Gine et al., 2010) (Figures 1 A and 1 D).

Regarding DLBCL patients, the only defined parameter for DLBCL-RT is a history of CLL/SLL. On the other side, DLBCL-de novo patients had no previous history of any lymphoproliferative disorders. DLBCL cases were classified into geminal center B-cell origin (GCB) and non-geminal center B-cell origin or activated B-cell type (ABC) using Hans algorism (Rotaru et al., 2019).

Clinical and laboratory data

All clinical and laboratory data of the 38 CLL/SLL, 11 DLBCL-RT, and 47 de novo DLBCL patients were retrospectively retrieved from the pathologic and clinical databases of OCMU. The follow-up data of concern included: the follow-up duration reported in months; progression-free survival (PFS) was defined as the period from the date of diagnosis to the time of progression or relapse; and overall survival (OS) was defined as the period from the date of diagnosis till the time of disease-specific death or the last follow-up visit of the patient. Progression of the disease was detected when CLL/SLL patients developed sudden onset of B symptoms that were accompanied by lymphadenopathy detected by physical examination or imaging studies.

Immunohistochemistry

Tissue sections from excised lymph nodes were cut at a thickness of 4 μm and immunostained by the following primary antibodies with the DAKO autostainer:

- Ki-67 mouse monoclonal antibody (Dako, Santa Clara, United States, ready to use) for unstained cases.
- *PD-L1* rabbit monoclonal antibody (Quartett, Berlin, Germany, 1:100, clone QR1, ready to use).
- *PD-1* rabbit monoclonal antibody (Quartett, Berlin, Germany, 1:100, clone QR2, ready to use).

IHC was conducted with Autostainer Link 48, using its optimized reagents with pharmDx kits EnVisionTM FLEX Visualization Systems (Link code K8000), and EnVision FLEX Hematoxylin (Link code K8008) regarding the user's guide standardized technique pre-programmed into the autostainer software. FFPE sections are pre-treated with heat-induced epitope retrieval (HIER) using the 3-in-1 specimen preparation procedure was done with these parameters: pre-heat temperature equals 65°C; epitope retrieval equals 97°C for 20 minutes; cool down to 65°C. The automated protocol depends on an indirect biotin-avidin system using a universal biotinylated immunoglobulin secondary antibody and diaminobenzidine (DAB) substrate. After completion of the staining procedure, the sections were dehydrated, then cleared and mounted (Dako, 2017).

PD-1 and *PD-L1* staining intensity, and cellular percentage of the neoplastic B-cells, and background total cellularity were documented. Staining for PD1 and *PD-L1* was scored as follows: 0, no staining; 1+, equivocal to weak staining; 2+, moderate staining; 3+, strong staining (He et al., 2018). Follicular T-cells showed strong 3+ *PD-1* staining and served as an internal control. On the other hand, *PD-L1* revealed membranous staining in reactive histiocytes, and dendritic cells served as an internal control for *PD-L1*.

PD-1 positivity in neoplastic B-cells was defined as positive membranous and/or cytoplasmic staining in ≥30% of CLL/SLL clonal cells and large B-cells in DLBCL cases (He et al., 2018).

Statistical analysis and data interpretation

Data analysis was performed by SPSS software, version 18 (SPSS Inc., PASW statistics for Windows version 18). Chicago: SPSS Inc. Qualitative data were described using numbers and percentages. Quantitative data were described using the median (minimum and maximum) for non-normally distributed data and the mean ± Standard deviation for normally distributed data after testing normality using the Kolmogrov-Smirnov test. The significance of the obtained results was judged at the (≤0.05) level. Chi-Square, Fischer exact test, and Monte Carlo tests were used to compare qualitative data between groups as appropriate. Kaplan-Meier test: used to calculate overall survival using log rank χ^2 to detect the

effect of risk factors affecting survival. Cox regression was used to assess predictors of survival with the calculation of the hazard ratio.

Ethical considerations

Approval of the committed Institutional Research Board (IRB) at the Faculty of Medicine, Mansoura University, Egypt (Code Number: MDP.20.06.42) was obtained for this study.

Results

PD-1 IHC evaluation in CLL/SLL patients

PD-1 was expressed in ≥ 30% of tumor cells in 20/38 patients (14 regular CLL/SLL and 6 aggressive CLL/SLL) (Figures 1.B and 1.E) and in < 30% of tumor cells in 16/38 patients (13 regular CLL/SLL and 3 aggressive CLL/SLL). The remaining two patients were completely negative for *PD-1* staining in tumor cells. *PD-1* was expressed in mild (10/36), moderate (19/36) or strong (7/36) cytoplasmic and/or membranous staining. *PD-1* was expressed mainly in proliferation centers. However, neoplastic cells other than PCs were also positive for *PD-1*.

PD-L1 IHC evaluation in CLL/SLL patients

PD-L1 expression was evaluated in background immune cells. *PD-L1* was expressed in 35 cases in background immune cells with variable intensity and percentage (26 regular CLL/SLL and 9 aggressive CLL/SLL) (Figures 1.C and 1.F). Three patients with regular CLL/SLL cases were negative. Tumor cells were negative for *PD-L1* in all patients.

PD-1 IHC evaluation in DLBCL patients

In DLBCL-RT (Figures 2.A and 2.D), *PD-1* was expressed in ≥ 30% of tumor cells in 9/11 patients (Figures 2.B and 2.E) and in < 30% of tumor cells in 2/11 patients. In contrast, in DLBCL-de novo (Figures 3.A and 3.D), *PD-1* was expressed in ≥ 30% of tumor cells in 5/47 patients (Figure 3.E) and in < 30% of tumor cells in 1/47 patients. While most patients (41/47) were negative (Figure 3.B). The patients expressed *PD-1* in mild (1/17), moderate (10/17) or strong (6/17) cytoplasmic and/or membranous staining.

Expression of PD-L1 in tumor cells in studied DLBCL patients

PD-L1 revealed negative expression in the tumor cells of all DLBCL-RT patients. In addition, the majority of DLBCL-de novo patients (38/47) were negative for *PD-L1*. *PD-L1* revealed positive expression in ≥30% of tumor cells in 4/47 DLBCL-de novo patients (Figure 3.C) and in <30% of tumor cells in 5/47 patients.

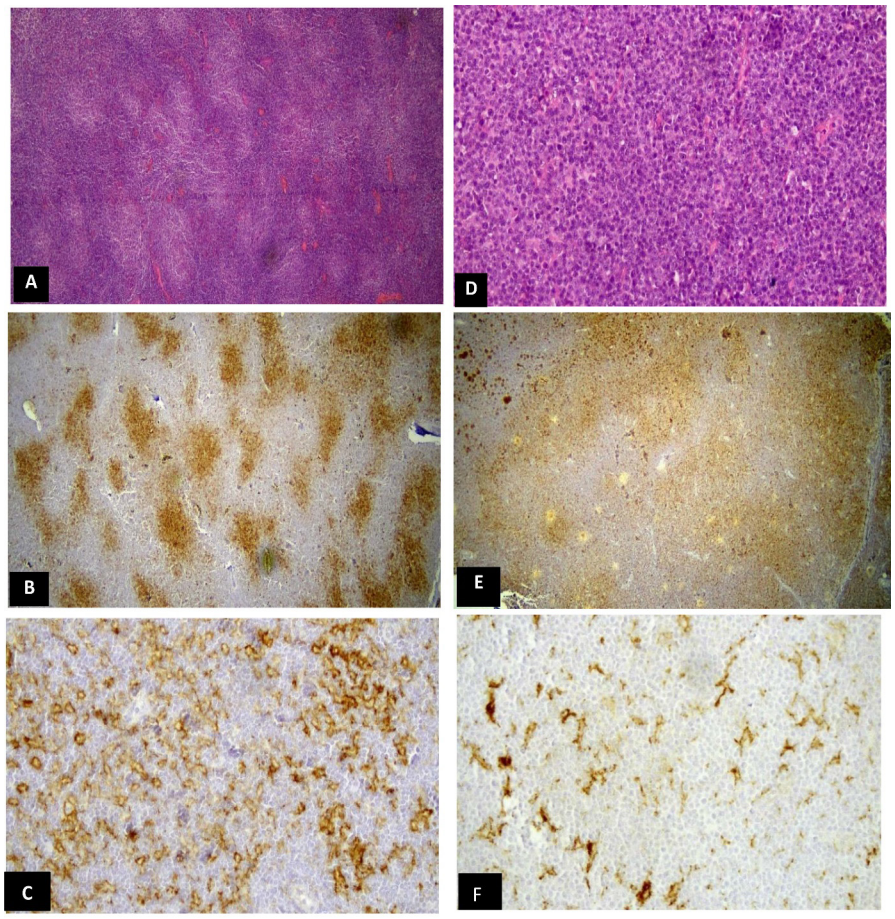

Figure 1. A. Pseudo-follicles in a case of atypical CLL/SLL (H&E; x40). B. Expression of PD-1 mainly in PCs (IHC; DABx40). C. Strong expression of PD-L1 in reactive histiocytes (20%) (IHC; DABx200). D. A case of atypical CLL/SLL with expanded PCs (broader than x20 microscopic field) (H&E; x200). E. Expression of PD-1 in about 70 % of tumor cells, mainly in PCs (IHC; DAB x40). F. Expression of PD-L1 in reactive histiocytes (10%) (IHC; DABx400).

Table 1. Summary of PD-1 and PD-L1 Expression in Studied CLL/SLL, DLBCL-RT and DLBCL- de novo.

	Typical CLL/SLL	Aggressive CLL/SLL	DLBCL-RT	DLBCL- de novo
Total number of cases	29	9	11	47
PD-1 in tumor cells				
Negative	2/29	0/9	0/11	41/47
<30	13/29	3/9	2/11	1/47
≥30	14/29	6/9	9/11	5/47
PD-L1 in tumor cells				
Negative	29/29	9/9	11/11	38/47
<30	0/29	0/9	0/11	4/47
≥30	0/29	0/9	0/11	5/47

CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; RT, Richter transformation; PD-1, programmed death receptor 1; PD-L1, programmed death ligand 1.

Expression of PD-L1 in background immune cells in studied DLBCL patients

PD-L1 revealed membranous staining in background immune cells, mostly histiocytes and dendritic cells, in the studied DLBCL (Figures 2.C, 2.F and 3.F). It showed expression in less than 8% of background cells in 14/58 patients and more than 8% of background cells in 44/58 patients. A summary of *PD-1* and *PD-L1* expression in studied CLL/SLL, DLBCL-RT, and DLBCL- de novo is illustrated in Table 1.

Association between PD-1 expression in tumor cells and clinical and laboratory findings in all studied CLL/SLL patients

There was no statistically significant association between *PD-1* expression and various clinical or laboratory data except for elevated ESR (P = 0.039). Other associations were insignificant: patients' age; gender; B-symptoms; associated HCV and HBV infection; lymphadenopathy status; the size of the excised lymph node; lymphocytosis; anemia; thrombocytopenia;

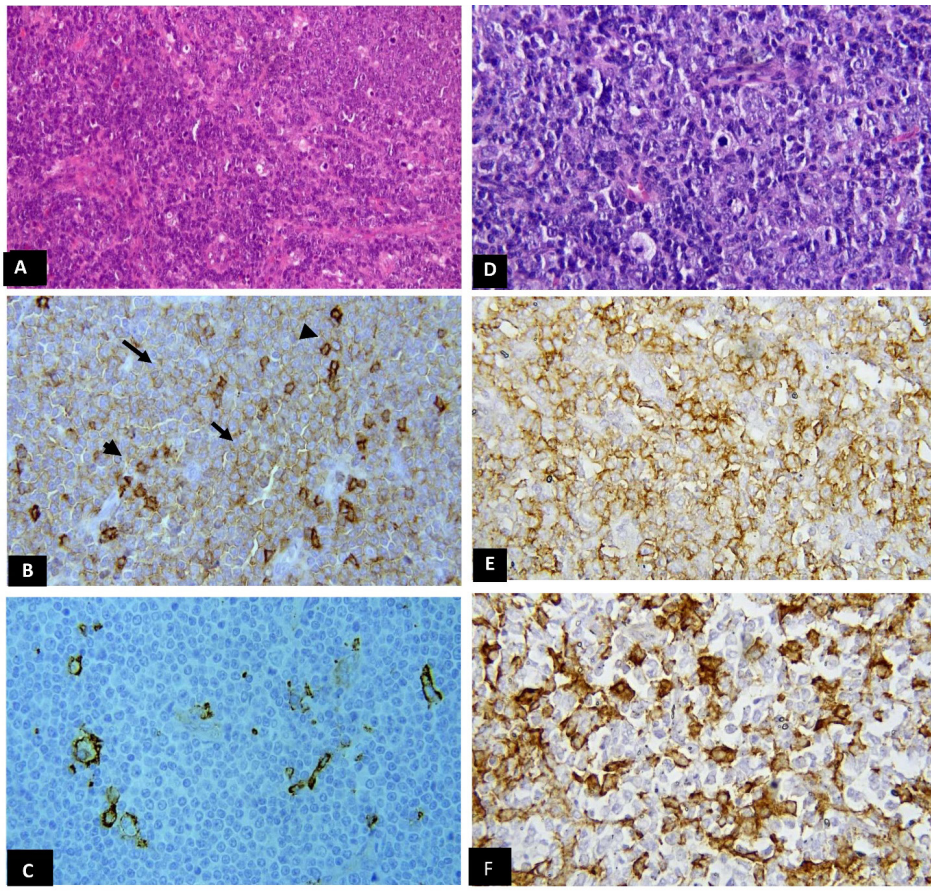

Figure 2. A. A case of DLBCL-RT; Diffuse sheets of large sized lymphocytes showing large cells with frequent mitoses (H&E; x200). B. Black arrows; Moderate membranous reaction for PD-1 tumor cells (IHC; DABx400). Arrows' head; Reactive T cells. C. Strong membranous reaction for PD-L1 in background histiocytes (about 5%) (IHC; DABx400). D. A case of DLBCL-RT (H&E; x400). E. Strong membranous reaction for PD-1 in about 90 % of tumor cells (IHC; DABx400). G. Strong cytoplasmic/membranous reaction for PD-L1in background histiocytes (about 40%) (IHC; DABx400).

Table 2. Association between PD-1 Expression in Tumor Cells and Clinical Laboratory Data in DLBCL Cases.

	DLBCL-RT N=11			DLBCL-de novo N=47		
	PD-1 negative/<30 N=2 cases N/%	PD-1≥30 N=9 cases N/%	Test of Significance	PD-1 negative/ <30 N=2 cases N/%	PD-1 ≥30 N=5 cases N/%	Test of Significance
Age						
≤60	1 (50)	3 (33.3)	χ^{2FET}=0.196	23 (54.8)	3 (60)	χ^{2FET}=0.05
>60	1 (50)	6 (66.7)	P=1.0	19 (45.2)	2 (40)	P=1.0
Gender						
Male	1 (50)	7 (77.8)	χ^{2FET}=0.637	26 (61.9)	1 (20)	χ^{2FET}=3.21
Female	1 (50)	2 (22.2)	P=0.491	16 (38.1)	4 (80)	P=0.148
Lymphocytosis (>5 x 109/L)						
Absent	1 (50)	4 (44.4)	χ^{2FET}=0.02	38 (90.5)	5 (100)	χ^{2FET}=0.520
Present	1 (50)	5 (55.6)	P=1.0	4 (9.5)	0	P=1.0
Anemia (HB <10 g/L)						
Absent	2 (100)	6 (66.7)	χ^{2FET}=0.917	32 (76.2)	1 (20)	χ^{2FET}=6.75
Present	0	3 (33.3)	P=1.0	10 (23.8)	4 (80)	P=0.023*
Thrombocytopenia (platelets<100 x 109/L)						
Absent	2 (100)	5 (55.6)	χ^{2FET}=1.39	34 (81)	5 (100)	χ^{2}=1.14
Present	0	4 (44.4)	P=0.491	8 (19)	0	P=0.571
LDH (>190 U/I)						
Not-Elevated	0	0	……	7 (16.7)	0	χ^{2}=0.979
Elevated	2 (100)	9 (100)		35 (83.3)	5 (100)	P=0.322
First hour ESR						
Not-Elevated	0	2 (22.2)	χ^{2FET}=0.543	5 (11.9)	1 (20)	χ^{2FET}=0.393
Elevated	2 (100)	7 (77.8)	P=0.461	37 (88.1)	4 (80)	P=0.821
BM involvement						
BMA						
Negative	1 (50)	4 (44.4)	χ^{2FET}=0.02	37 (88.1)	5 (100)	χ^{2}=0.666
Positive	1 (50)	5 (55.6)	P=1.0	5 (11.9)	0	P=1.0
BMB						
Negative	0	4 (44.4)	χ^{2FET}=1.39	32 (76.2)	4 (80)	χ^{2FET}=0.036
Positive	2 (100)	5 (55.6)	P=0.491	10 (23.8)	1 (20)	P=1.0
Associated malignancy						
Absent	2 (100)	9 (100)	……	40 (95.2)	5 (100)	χ^{2FET}=0.249
Present	0	0		2 (4.8)	0	P=1.0
B-Symptoms						
Absent	0	8 (88.9)	χ^{2FET}=6.52	29 (69.0)	3 (60)	χ^{2FET}=6.52
Present	2 (100)	1 (11.1)	P=0.06	13 (31.0)	2 (40)	P=0.06
Viral infection						
Absent	1 (50)	5 (55.6)	χ^{2MC}=0.356	23 (54.8)	1 (20)	χ^{2FET}=2.16
HCV	1 (50)	3 (33.3)	P=0.837	19 (45.2)	4 (80)	P=0.188
HBV	0	1 (11.1)		0	0	
Lymphadenopathy						
Localized	0	0	……	2 (4.8)	0	χ^{2FET}=0.249
Generalized	2 (100)	9 (100)		40 (95.2)	5 (100)	P=1.0
Size of excised lymph node (centimeters)	3.75±0.35	3.83±1.78	t=0.063 p=0.951	3.39±1.47	2.80±1.15	t=0.871 p=0.388

N, number; χ^2, Chi-Square test; P, Probability value; *statistically significant (P<0.05)

Table 2. Continued

	DLBCL-RT N=11			DLBCL-de novo N=47		
	PD-1 negative/<30 N=2 cases N/%	PD-1≥30 N=9 cases N/%	Test of Significance	PD-1 negative/ <30 N=2 cases N/%	PD-1 ≥30 N=5 cases N/%	Test of Significance
Extra nodal site number						
Absent	2 (100)	5 (55.6)	χ^{2MC}=1.39	31 (73.8)	0	χ^{2MC}=10.89
One site	0	1 (11.1)	P=0.497	7 (16.7)	3 (60)	P=0.004*
>one site	0	3 (33.3)		4 (9.5)	2 (40)	
Ann Arbor Stage						
I/II	0	1 (11.1)	χ^{2FET}=0.244	8 (19.0)	0	χ^2=1.15
III/IV	2 (100)	8 (88.9)	P=1.0	34 (81)	5 (100)	P=0.571
IPI						
0-2	1 (50)	2 (22.2)	χ^{2FET}=0.637	26 (61.9)	2 (40)	χ^{2FET}=0.890
3-5	1 (50)	7 (77.8)	P=0.491	16 (38.1)	3 (60)	P=0.381

N, number; χ^2, Chi-Square test; P, Probability value; *statistically significant (P<0.05)

bone marrow involvement; elevated LDH; extra-nodal involvement; staging.

Association between PD-1 expression in tumor cells and clinical and laboratory findings in all studied DLBCL patients

A significant relationship was observed between *PD-1* expression in DLBCL-de novo patients and anemia (P = 0.023). In addition, there was a significant relationship with the number of extra-nodal involved sites (P = 0.004). Other parameters showed no significant relationship with *PD-1* expression, as shown in Table 2.

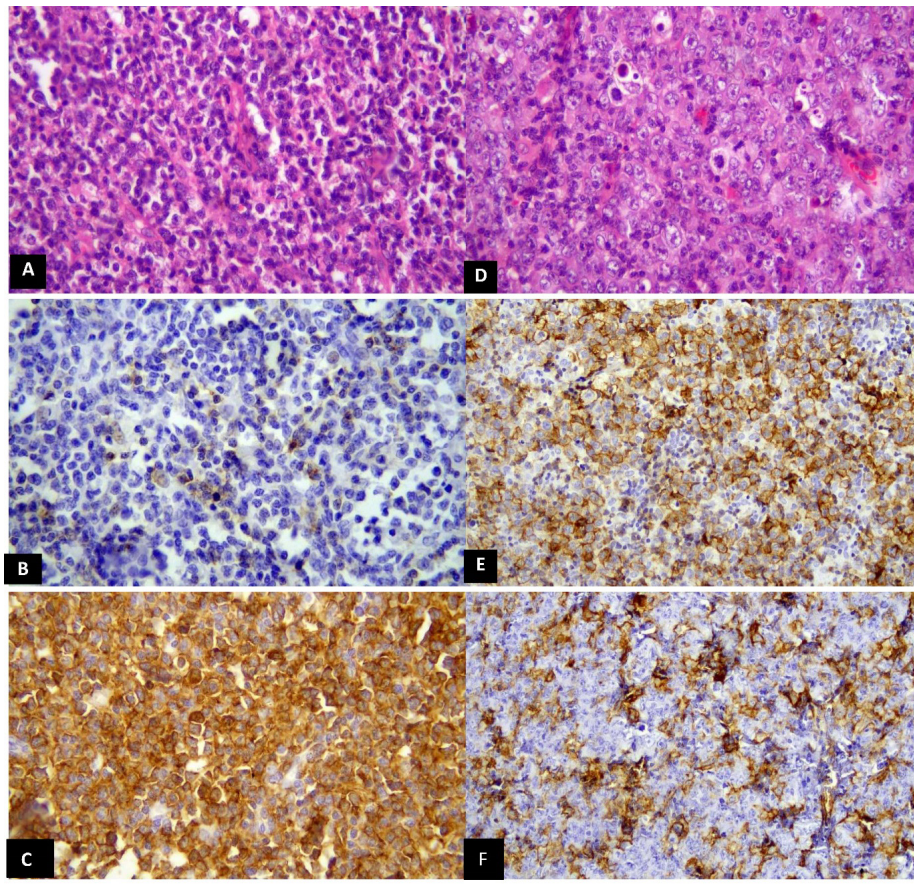

Figure 3. A. DLBCL-de novo of ABC type (H&E; x400). B. Positive reaction for PD-1 in scattered reactive T-cells and negative in tumor cells (IHC; DABx400). C. Strong cytoplasmic/membranous reaction of PD-L1 in tumor cells (90%) (IHC; DABx400). D. DLBCL- de novo of ABC type; (H&E; x400). E. Strong membranous PD-1 expression in 80% of tumor cells (IHC; DABx200). F. Strong membranous reaction of PD-L1 in background histiocytes (about 40%) with negative reaction in tumor cells (IHC; DABx200).

Table 3. Risk Factors for Overall-Survival among Studied CLL/SLL and DLBCL Cases

	Univariate analysis			Multivariate analysis		
	HR (95%CI)	Log rank χ2	P value	HR (95% CI)	B	P value
CLL/SLL cases						
Age						
≤60 (r)	63 (40.1-85.9)	10.25	0.001*	3.56 (1.51-8.38)	1.27	0.004*
>60	16 (15.16-16.84)					
Anemia						
Absent (r)	45 (30.56-59.4)	5.07	0.024*	2.79 (1.13-6.89)	1.03	0.026*
Present	26 (0.001-55.2)					
Ki-67 proliferation index						
≤40	42 (23.17-60.83)	6.55	0.01*	1.59 (0.34-7.44)	0.464	0.556
>40 (r)	16 (11.71-20.29)					
PD-1				----------	--------	----------
Negative	42 (31.09-52.91)	0.051	0.822			
Positive	32 (10.19-53.80)					
PD-L1				----------	-------	----------
Negative	63 (42.25-83.74)	0.227	0.633			
Positive	32 (11.9-52.11)					
CLL/SLL (Typical) (r)	45.0 (30.16-59.84)					
CLL/SLL(Aggressive)	16.0 (10.16-21.84)	10.15	0.001*	4.77 (1.21-18.79)	1.56	0.025*
DLBCL cases						
Age						
≤60 (r)	45 (35-50)	6.94	0.008*	3.26 (1.57-6.78)	1.18	0.002*
>60	16 (10.82-40.81)					
Anemia						
Absent	44 (32.18-55.82)	11.44	0.001*	2.36 (1.12-4.95)	0.857	0.024*
Present	14 (10.98-17.03)					
BM involvement						
BMA						
Negative	40 (29.56-50.44)	7.82	0.005*	1.76 (0.576-5.36)	0.564	0.322
Positive	12 (5.53-18.47)					
BMB						
Negative	40 (30.79-49.21)					
Positive	14 (8.46-19.54)	6.26	0.012*	0.960 (0.336-2.74)	-0.04	0.94
Associated malignancy						
Absent	34 (25.02-42.97)	4.2	0.04*	4.45 (0.889-22.34)	1.49	0.069
Present	13 (12.52-14.48)					
Viral infection				----------	--------	----------
Absent	31 (16.31-45.69)	17.74	<0.001*			
HCV	34 (24.49-43.5)					
HBV	6 (6-6)					
Number of extra-nodal sites						
Absent (r)	39 (24.19-53.8)	12.53	0.002*	1	1	
One site	40 (27.02-52.98)			1.46 (0.6-3.55)	0.378	0.405
> One site	14 (11.08-16.92)			8.19 (3.20-20.92)	2.1	<0.001*
PS				----------	---------	- ---------
1	39 (28.49-49.51)	59.4	<0.001*			
2	14 (0.001-28.78)					
3	14 (14-14)					
4	5 (5-5)					

B, Beta; CI, confidence interval; r,reference; P, Probability; P ≤ 0.05 is significant; HR, Hazard ratio

Table 3. Continued

	Univariate analysis			Multivariate analysis		
	HR (95%CI)	Log rank χ2	P value	HR (95% CI)	B	P value
IPI						
0-2	44 (39.0-52.0)	15.9	<0.001*	2.73 (1.3-5.73)	1.004	0.008*
3-5	14 (12.98-15.01)					
Ki-67						
≤55	44 (36.69-51.30)	5.16	0.023*	2.23 (1.11-4.47)	0.802	0.024*
>55	29 (13.73-44.27)					
PD-1 in tumor cells				1.14 (1.05-1.81)	1.102	0.04*
Negative/<30%	34 (21.2-46.8)	6.1	0.014*			
Positive ≥30%	14 (6.67-21.33)					
PD-L1 in immune cells.				----------	---------	----------
<8%	31 (20.27-41.7)	0.09	0.764			
≥8%	30 (14.59-66.26)					
DLBCL-RT	9 (3.61-14.39)	7.89	0.005*	1.325 (1.002-1.96)	1.03	0.047*
DLBCL-de novo	39 (28.69-49.31)					

B, Beta; CI, confidence interval; r,reference; P, Probability; P ≤ 0.05 is significant; HR, Hazard ratio

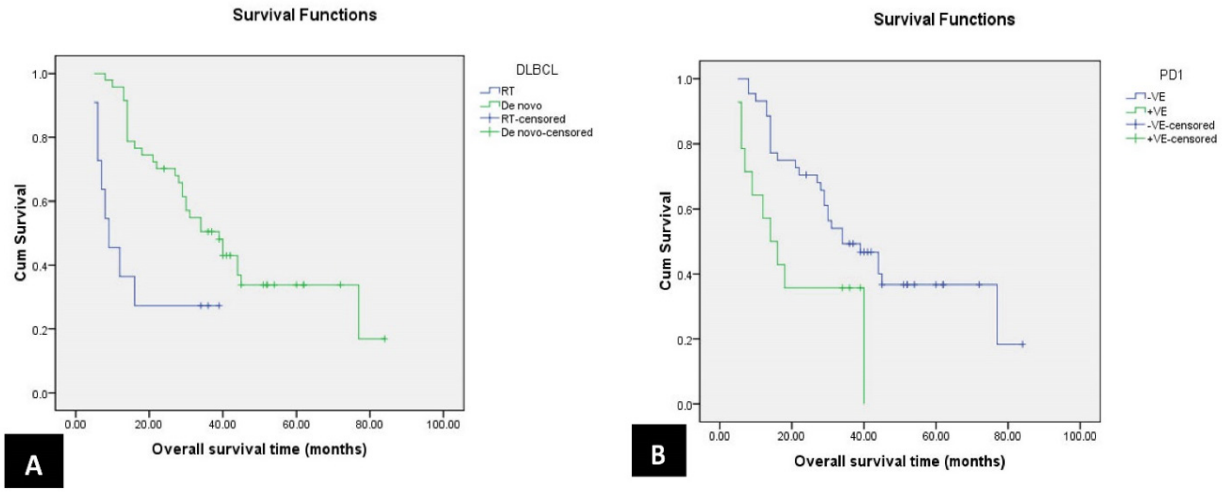

Figure 4. A. Kaplan Meir survival curve describe for patients with DLBCL stratified by DLBCL-RT and DLBCL-de novo. OS was significantly lower in DLBCL-RT cases (log-rank; P=0.05). B. Kaplan Meir survival curve describe for patients with DLBCL stratified by PD-1 positive or negative. OS was significantly lower in PD-1 positive cases (log-rank; P=0.014).

Overall survival (OS) of CLL/SLL studied patients

The median OS for the included CLL/SLL patients was 38 (23.98-52.02) months, and the 2-year survival was (63.2%), which dropped to (49.5%) for 3-year survival.

Analyses of prognostic factors are illustrated in Table 3. Univariate analysis identified four prognostic factors in OS in CLL/SLL patients: patients' age > 60 years (P = 0.001), anemia (P = 0.024), Ki-67 proliferation index > 40% (P = 0.01), and aggressive CLL/SLL cases (P = 0.001). Multivariate analysis revealed that patients' age (P = 0.004), anemia (P = 0.026), and aggressive CLL/SLL (P = 0.025) were identified as significant prognostic factors for OS.

Overall survival (OS) of studied DLBCL patients

The median OS for the included DLBCL patients was 31 (21.19-40.8) months, and the 2-year survival was (62.1%), which dropped to (44.0%) for 3-year survival. Patients with DLBCL-RT had a 9-month median OS that ranged between 3.61 and 14.39 months. However, DLBCL-de novo had longer survival with a median OS of 39 months (28.69-49.31). The median period of transformation from CLL/SLL to Richter transformation was 34 (9-62) months.

As shown in Table 3, univariate analysis identified 12 prognostic factors for OS in DLBCL patients: patients' age > 60 years (P = 0.008), anemia (P = 0.001), positive BMA for deposits (P = 0.005), positive BMB (P = 0.012), associated malignancy (P = 0.04), associated HBV infection (P < 0.001), involvement of > one extra-nodal site (P = 0.002), performance status > 1 (P < 0.001), IPI (3-5) (P < 0.001), Ki-67 proliferation index > 55% (P = 0.023), *PD-1* expression in ≥30% of tumor cells (P = 0.014) (Figure 4.B) and DLBCL-RT (P = 0.005)

(Figure 4.A). On multivariate analysis, patients' age > 60 years (P = 0.002), anemia (P = 0.024), involvement of > extra-nodal site (P < 0.001) and IPI (3-5) (P = 0.008), Ki-67 > 55% (P = 0.024), *PD-1* expression in ≥ 30% of tumor cells (P = 0.04), and DLBCL-RT (P = 0.047) were proven to have independent poor prognostic impact on OS.

Discussion

Immunotherapy has gained significant importance in the fight against cancer over the past few years. Immune checkpoint receptors have an essential role in maintaining homeostasis and self-tolerance (Hatefi et al., 2022). Anti-programmed cell death protein-1/programmed cell death ligand 1 (*PD-1/PD-L1*) monoclonal antibody is one of the main two immune checkpoint inhibitors (ICIs) that have shaped the therapeutic landscape of some types of cancer (Ballas, 2018).

PD-1 is expressed on activated T cells, with particularly high expression by tumor-infiltrating T lymphocytes (TILs) (Pardoll, 2012). It is also expressed on activated B cells, monocytes, NK cells, and T cells in the germinal centers of normal human lymphoid tissues (Postow et al., 2015; Garcia-Lacarte et al., 2021). When *PD-1* binds to *PD-L1*, T-cell function becomes suppressed. Blockade of *PD-1* leads to enhancement of T-cell killing capacity (Pardoll, 2012). Pembrolizumab was the first humanized monoclonal antibody against *PD-1* that gained its first global approval by the United States Food and Drug Administration (FDA) in 2014 for patients with unresectable or metastatic melanoma (Kwok et al., 2016). Refractory classical Hodgkin lymphoma (CHL) or primary mediastinal large B-cell lymphoma (PMBCL) have robust responses with pembrolizumab (Chen et al., 2017; Tomassetti et al., 2019). Patients with DLBCL-RT have a promising response to pembrolizumab in a phase 2 clinical trial (Ding, 2018).

In this study, *PD-1* expression was evaluated in neoplastic B-cells in 38 CLL/SLL, 11 DLBCL-RT, and 47 DLBCL-de novo patients. In those patients, *PD-1* was not only expressed in PCs of CLL/SLL patients (20/38) but was also expressed in neoplastic B-cells other than PCs. In contrast to other studies (He et al., 2018; Gine et al., 2010; Behdad et al., 2019), *PD-1* expression was restricted to PCs. As documented before (Dorfman et al., 2006; Xerri et al., 2008), *PD-1* revealed an intense positive reaction in background reactive T-cells and residual germinal centers.

Regarding DLBCL-RT patients, neoplastic B-cells expressed *PD-1* in all patients (≥ 30% of tumor cells in 9/11 and < 30% in 2/11 patients). Contrary, in DLBCL-de novo cases, *PD-1* was expressed in only 6/47 cases (≥ 30% of tumor cells in 5 and < 30% in 1 patient). This matches with two previous studies (He et al., 2018; Behdad et al., 2019) that demonstrated increased *PD-1* expression in DLBCL-RT (12/15 and 14/17 cases, respectively) and decreased *PD-1* expression in DLBCL-de novo (1/22 and 2/25 cases, respectively). In close similarity with He et al. (2018), who reported *PD-L1* expression in only 1/15 of DLBCL-RT patients, our study revealed *PD-L1* negative expression in tumor cells in all CLL/SLL and DLBCL-RT patients. On the other side, *PD-L1* revealed positive expression in 9/47 of DLBCL-de novo patients (≥30% of tumor cells in 4/47 and in <30% of tumor cells in 5/47 patients). These results coincide approximately with previous studies (He et al., 2018; Behdad et al., 2019) that reported positive expression of *PD-L1* in 1/26 cases and 2/25 cases, respectively. All studied CLL/SLL and DLBCL cases revealed positive *PD-L1* expression in background immune cells.

Our results showed that there was no statistically significant association between *PD-1* expression in CLL/SLL patients with different clinical and laboratory data except for ESR elevation (P = 0.039). On the other side, *PD-1* expression in DLBCL-de novo was significantly associated with the presence of anemia (P = 0.023) and more than one extra-nodal site involvement (P = 0.004).

In our study, old age at the onset, associated anemia, and aggressive CLL/SLL patients were identified as significant prognostic factors for OS in CLL/SLL patients, similar to previous results (Gine et al., 2010; Wierda et al., 2011). *PD-1* expression had no association with the OS of the studied CLL/SLL cases. Overall, the prognosis of DLBCL-RT was inferior to that of patients with DLBCL-de novo. These findings are slightly lower than those obtained from two recent studies (He et al., 2018; Elnair et al., 2020). Moreover, patients' age, presence of anemia, more than one extra-nodal site involvement, IPI (3-5), Ki-67 > 55%, *PD-1* expression in ≥30% of tumor cells, and DLBCL-RT cases were found to be significant prognostic factors for OS.

To summarize, our study documented that *PD-1* is expressed in CLL/SLL tumor cells mainly in proliferation centers. Furthermore, *PD-1* is expressed in most cases of DLBCL-RT. Although *PD-1* expression shows no significant association with overall survival in CLL/SLL patients, it is considered an independent poor prognostic marker associated with short OS in DLBCL cases. Due to the higher prevalence of *PD-1* expression in DLBCL-RT than DLBCL-de novo patients and its association with short OS, we recommend further larger studies to augment our findings and better define the crucial role of *PD-1* in patients of DLBCL-RT and the potential role of anti-*PD-1* immunotherapy in the treatment of DLBCL-RT patients.

Author Contribution Statement

All authors contribute equally; all authors reviewed the results and approved the final version of the manuscript.

Acknowledgements

General

The authors thank Mr. Ibrahim Attia for his precious technical assistance.

Ethical approval

Institutional Research Board (IRB) at the Faculty of Medicine, Mansoura University, Egypt (code number: MDP.20.06.42).

Availability of data

The datasets are available from the corresponding author upon request.

Conflict of interest

The authors declare that there are no relevant financial affiliations or conflicts of interest to disclose.

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