

Programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), and EBV-encoded RNA (EBER) expression in Hodgkin lymphoma

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Abstract Programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) are new targets in cancer immunotherapy. PD-1 protein is an immune checkpoint expressed in many tumors. Epstein–Barr virus (EBV) is present in malignant Hodgkin/Reed–Sternberg (HRS) cells in approximately 40–50 % of Hodgkin lymphoma (HL). The aim of this study is to evaluate the clinical and prognostic importance of PD-1 and/or PD-L1 in HL and also to determine the association between EBV-encoded RNA (EBER) and PD-1/PD-L1. Formalin-fixed, paraffin-embedded tissue samples from 87 cases with HL were analyzed in this study. Immunohistochemical staining was performed to detect the PD-1 and PD-L1 expressions. Chromogenic in situ hybridization for EBER was performed using fluorescein-labeled oligonucleotide probes. PD-1 and PD-L1 expressions were found in 20 % of the cases. The EBER positivity was found in 40 cases (45 %). It has been found that co-expression of PD-1 and PD-L1 was associated with shorter survival although PD-1 or PD-L1 expressions were not found to be related with survival. Overall survival (OS) and disease-free survival (DFS) in cases without PD-1 and PD-L1 expressions were 135 and 107 months, respectively. OS and DFS in cases with co-expression for PD-1 and PD-L1 were 24 and 20 months, respectively, and these differences were found to be statistically significant for both OS and DFS

($p=0.002$ and $p=0.003$, respectively). Cox regression analysis showed that co-expression of PD-1 and PD-L1 was found to be an independent risk factor for prognosis (OR 6.9, 95 % CI 1.9–24.3). Targeting PD-1 and/or PD-L1 is meaningful due to the 20 % expression of each in HL, and we did not find an important association between PD-1 and PD-L1 and EBER expression in HL. Very poor outcome in cases with co-expression of PD-1/PD-L1 suggests new avenues to detect the new prognostic markers and also therapeutic approaches in HL.

Keywords Hodgkin lymphoma · PD-1 · PD-L1 · EBV · Prognosis · Targeted treatment

Introduction

Programmed death-1 (PD-1) protein is one of the most important immune checkpoints and its receptor is expressed by activated T cells. PD-1 mediates immunosuppression and is expressed on T cells after activation [1]. PD-1 is present on exhausted T cells, B cells, NK cells, dendritic cells, and activated monocytes [2]. PD-1 has two ligands known as programmed death-ligand 1 (PD-L1) (B7-H1) and PD-L2 (B7-DC) [3–5]. The physiological role of PD-1 is to guarantee T cell homeostasis and to provide balance between T cell activation and proliferation. Binding of the ligand PD-L1 to PD-1 expressed on the surface of activated T cells delivers an inhibitory signal and reduces cytokine production and proliferation. So, PD-L1/PD-1 interactions confer tumor escape from immune control [6]. This signaling is important for both tumor biology and treatment including the interactions between tumor, tumor microenvironment, and immune system and to target associated pathways in malignant tumors. PD-1/PD-L1 axis has been implicated in prognosis in several solid

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tumors, and results of many studies suggest that PD-1 pathway is a rationale target for therapeutic inhibition [7]. Tumor PD-L1 expression is a hot topic as a possible predictive biomarker for patient benefit from PD-1 pathway. It has been shown many times that blockade of PD-1 or PD-L1 by monoclonal antibodies may lead to significant antitumor effects in various types of malignant tumors [8, 9]. On the other hand, it has been shown an association between Epstein–Barr virus (EBV)-associated neoplasias and PD-1 and/or PD-L1 expression in some lymphomas [10, 11]. Here, we presented the study about the expression patterns of PD-1 and PD-L1 in Hodgkin lymphoma (HL) and their association with EBV status and also prognostic value of PD-1/PD-L1 pathway in this disease.

Patients and methods Formalin-fixed, paraffin-embedded tissue samples from 87 cases with newly diagnosed HL were analyzed in this study. NCCN risk scoring system was used to determine the risk group of the patients. All the cases had been treated with ABVD regimen (doxorubicin, bleomycin, vinblastine, DTIC) as a first-line treatment. Salvage regimen was DHAP (dexamethasone, Ara-C, cisplatin) regimen in the majority of cases in the first relapse. Immunohistochemical staining was performed on 5- μ m sections from tissue using antibody to PD-1 (MRQ-22, Ventana) and PD-L1 (Monoclonal antibody CD274/PDL1 AM26531AF-N.Acris Germany). The visualization system used was the BenchMark XT (Ventana) with heat-induced epitope retrieval (CC1 solution, Ventana) and the iView DAB detection kit (Ventana). The visualization system used was the BenchMark XT with enzymatic digestion (ISH Protease 2, Ventana) and the iView_{Blue} detection kit (Ventana). Cases stained with anti-PD-1 were scored according to intensity of cytoplasmic and/or membranous positivity as follows: 0 (no staining), 1+ (weak or equivocal staining), 2+ (moderate staining), or 3+ (strong staining). Tumor cells, Hodgkin/Reed–Sternberg (HRS) cells, and peritumoral microenvironment were evaluated separately. Tumor positivity was considered when more than 5 % tumor population was stained. Microenvironment was considered positive when more than 20 % of all cell population was stained. HRS cells were evaluated as positive or negative, regardless of intensity and rate. PD-L1 was evaluated cytoplasmic and/or membranous positive and negative for HRS cells and microenvironment by immunohistochemistry. PD-L1 positivity in malignant cells and microenvironment was made according to the scoring system used in a previous article [9, 10]. Chromogenic in situ hybridization for EBV-encoded RNA (EBER) was performed using fluorescein-labeled oligonucleotide probes (INFORM EBER Probe, Ventana). EBER was considered as positive in case of dark-blue nuclear staining.

Statistical analyses The variables between the groups were analyzed by using the chi-square test and McNemar's test. Cohen's kappa was used for agreement statistics. Strength of agreement was evaluated by kappa statistic (<0.00 is poor. 0.00–0.20 is slight, 0.21–0.40 is fair, 0.41–0.60 is moderate, 0.61–0.80 is substantial, 0.81–1.00 is almost perfect). The predictors of survival were analyzed by the Kaplan–Meier method and compared by the Mantel log-rank test. Cox proportional hazard regression model was applied to identify multivariate predictors (forward procedure, Wald method). The results are reported as mean \pm SD, median, number (*n*), and percent (%). A *p* value <0.05 was considered as significant. Statistical analyses were performed using the statistical package SPSS v 22.0.

Findings

Male to female ratio was 46:41. Mean age was 35.3 \pm 14.5 (range between 15 and 71). Sixty percent of the cases had early-stage disease while 40 % had advanced stage disease. According to the risk scores, 15 cases had early stage-favorable disease, 34 cases had early stage-unfavorable disease, and 38 cases had advanced stage disease. Table 1 shows the demographic features of the patients.

PD-1 expression was detected in 18 cases (20 %), and PD-L1 expression was detected in 18 cases (20 %). EBER-ISH was detected in 40 cases (45 %). Figures 1, 2, and 3 show the

Table 1 Demographic features of the cases

		Number	Percent
Sex	Male	46	52.9
	Female	41	47.1
Stage	1	6	6.9
	2	46	52.9
	3	20	23.0
	4	15	17.2
Risk score	Early stage-favorable	15	17.2
	Early stage-unfavorable	34	39.1
	Advanced stage	38	43.7
Histology	Unknown	14	16.1
	Lymphocyte predominant	3	3.4
	Nodular sclerosing	32	36.8
	Mixed cellular	36	41.4
	Lymphocyte depleted	2	2.3
Extranodal involvement	No	56	64.4
	Yes	15	17.2
	Unknown	16	18.4
Mediastinal mass	Yes	37	42.5
	No	32	36.8
	Unknown	18	20.7

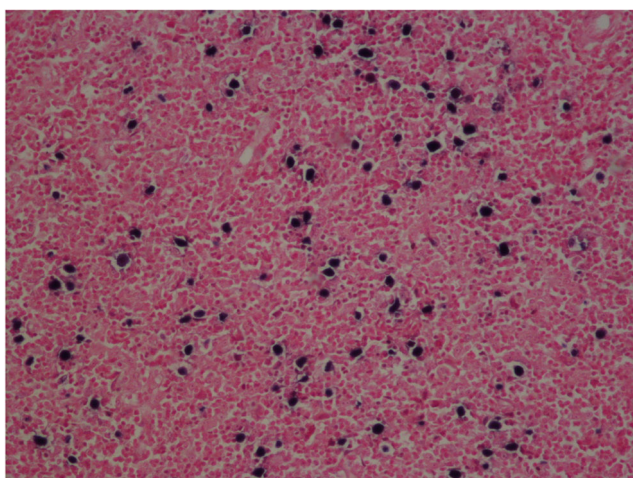


Fig. 1 EBV ISH (x400)

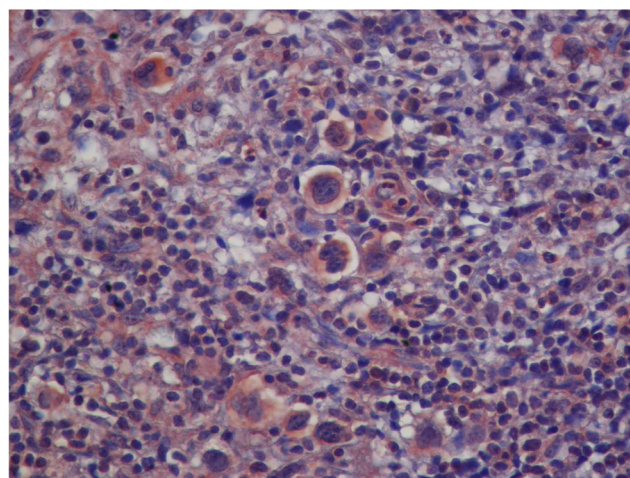


Fig. 3 PD-L1 expression

EBV, PD-1, and PD-L1 expressions, respectively. Table 2 shows the associations between EBV, PD-1, and PD-L1. We did not find an association between EBV and PD-1 (kappa coefficient=0.08) and between EBV and PD-L1 (kappa coefficient=-0.01). There was a fair association between PD-1 and PD-L1 (kappa coefficient=0.23).

Immunohistochemically, three cases had 2+ staining and 15 cases had 1+ staining with PD-1. We did not detect 3+ staining with PD-1. PD-L1 was found to be positive in 18 cases, and staining was detected in HRS cells and microenvironment by immunohistochemistry.

Table 3 shows the results of survival analyses according to clinical and prognostic factors. When we evaluated the clinical parameters, we found that both overall survival (OS) and disease-free survival (DFS) were longer in patients with early stage than in patients with advanced stage disease (*p* values were 0.024 and 0.001, respectively). Additionally, OS and DFS were found to be shorter in cases with extranodal involvement as compared without extranodal disease (*p* values were 0.004 and 0.001, respectively) and in cases with high-

risk disease compared with low-risk disease (*p* values were 0.024 and 0.001, respectively).

Although median OS and DFS were shorter in cases with EBV(+) compared with EBV(-) (100 vs. 142 months for OS and 107 vs. 100 months for DFS), differences were not statistically significant for both (*p*=0.064 and *p*=0.129, respectively). Figure 4 shows OS in cases with or without EBV.

The median OS and DFS were found to be shorter in cases with PD-1 expression than in those without PD-1 expression (100 vs. 135 months for OS and 107 vs. 100 months for DFS); differences were not significant (*p*=0.928 and *p*=0.607, respectively). Similarly, the median OS and DFS were found to be shorter in cases with PD-L1 expression (79 months for both OS and DFS) than in those without PD-L1 expression (135 months for OS and 100 months for DFS); again, differences were not significant (*p*=0.232 for OS and *p*=0.305 for DFS). Although PD-1 or PD-L1 expressions were not associated with survival times, we found that co-expression of PD-1 and PD-L1 was associated with shorter survival. Median OS and DFS times in cases not expressing PD-1 and PD-L1 were 135 and 107 months, respectively. However, these times were 24 and 20 months in cases with co-expression, and these

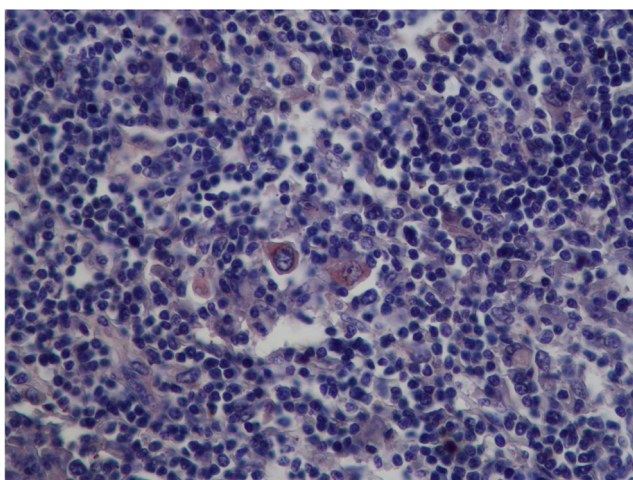


Fig. 2 PD-1 expression

Table 2 Association between EBV and PD-1, PD-L1

		EBV			Kappa coefficient McNemar test
		Negative	Positive	Total	
PD-1	Negative	39	30	69	0.08
	Positive	8	10	18	
PD-L1	Negative	37	32	69	-0.01
	Positive	10	8	18	
Total		47	40	87	
		PD-1			Kappa coefficient McNemar test
		Negative	Positive	Total	
PD-L1	Negative	58	11	69	0.23
	Positive	11	7	18	
Total		69	18	87	

Table 3 Results of survival analyses according to clinical and prognostic factors

	Total/Ex (n)	Censored (%)	Overall survival (OS)			Disease-free survival (DFS)		
			Mean (month)	Median (month)	p	Mean (month)	Median (month)	p
EBV								
Negative	47/12	74.5	117.7	142		99.5	107	
Positive	40/13	67.5	75.3	100	0.064	69.4	100	0.129
PD-1								
Negative	69/20	71.0	106.5	135		93.0	107	
Positive	18/5	72.2	96.7	100	0.928	77.9	100	0.607
PD-L1								
Negative	69/19	72.5	110.5	135		93.7	100	
Positive	18/6	66.7	67.7	79	0.232	62.0	79	0.305
Co-expression of PD-1 and PD-L1								
PD-1 and PD-L1(-)	58/18	69.0	105.4	135		92.3	107	
PD-1 or PD-L1(+)	22/3	86.4	116.5	100		88.5	100	
PD-1 and PD-L1(+)	7/4	42.9	30.9	24	0.002	13.7	20	0.003
Stage								
1+2	52/10	80.8	118.4	135		108.5	107	
3+4	35/15	57.1	86.2	78	0.024	51.6	74	0.001
Extranodal involvement								
Yes	56/10	82.1	123.0	143		111.7	108	
No	15/7	53.3	61.1	70	0.004	43.3	70	0.001
Risk score group								
Early stage-favorable	15/2	86.7	131.6	142		99.8	107	
Early stage-unfavorable	34/6	82.4	122.7	135		113.3	108	
Advanced stage	38/17	55.3	79.4	78	0.005	49.0	70	0.000
Total	87/25	71.3	105.9	135.0		89.8	100	

differences were found to be statistically significant for both OS and DFS ($p=0.002$ and $p=0.003$, respectively). Figure 5 shows OS curves according to co-expression of PD-1 and PD-L1. Cox regression analysis showed (Table 4) that co-

expression of PD-1 and PD-L1 was found to be independent risk factors that related with prognosis. The odds ratio was found to be 6.9 (95 % CI 1.9–24.3, $p=0.002$) for the cases having co-expression of PD-1 and PD-L1 (both PD-1 and PD-L1(+)).

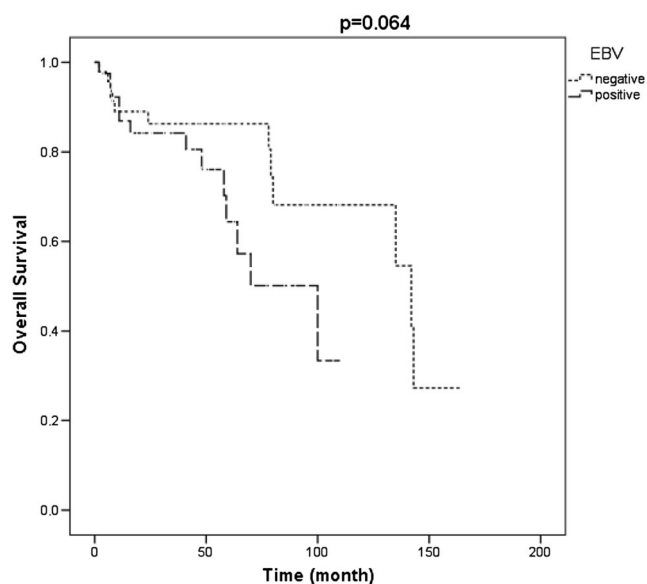
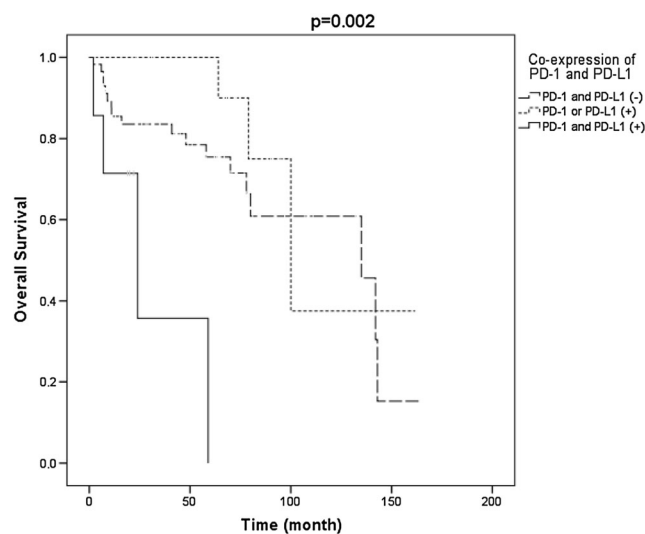
**Fig. 4** Overall survival curves according to EBV groups**Fig. 5** Overall survival curves according to co-expression of PD-1 and PD-L1

Table 4 Results of Cox regression analyses

	OR (95 % CI)	<i>p</i>
Age	1.0 (1.0–1.0)	0.008
Stage (3 or 4)	2.2 (0.9–4.8)	0.064
EBV(+)	2.0 (0.8–4.9)	0.130
Co-expression of PD-1 and PD-L1		0.002
PD-1 or PD-L1(+)	0.5 (0.1–1.5)	0.208
PD-1 and PD-L1(+)	6.9 (1.9–24.3)	0.002

Discussion

Programmed death-1 (PD-1) and its ligands, PD-L1 and PD-L2, deliver inhibitory signals, and this signaling regulates the balance between T cell activation, tolerance, and immunopathology. Specific and balanced responses to eradicate pathogens and tumors are very important in immune responses to foreign and self-antigens. For this reason, PD-1/PD-L pathway is very important in the pathogenesis of chronic infections and tumors [2]. PD-1 protein expressed by activated T cells while PD-L1 (B7-H1) and PD-L2 (B7-DC) both expressed by tumor cells and microenvironment [6]. It is very well known that macrophages in microenvironment of HL are very important prognostic parameters [12]. PD-L1 is not expressed by normal epithelial tissues, normal lymph nodes, and plasma cells, but it is aberrantly expressed on various human cancers, lymphoma cells, and myeloma cells [13–15]. PD-L1 may promote Ca progression by disabling the host antitumor response, and its expression on tumor cells is associated with poor prognosis in various malignant tumors including renal cell cancer, breast cancer, pancreas cancer, ovarian cancer, gastric cancer, esophageal cancer, head and neck cancer, and non-small cell lung cancer [16–22].

PD-L1 has an important role in lymphomagenesis. PD-L1 has been detected in various types of lymphomas and lymphoma cell lines. However, expression levels and frequencies are highly variable between less than 5 % and more than 90 % in different types and subtypes of the lymphomas in different studies. Antibodies used in different studies were clone MIH1; eBioscience, BioLegend, Apero Scan Scope XT Workstation and EH12 for PD-L1 and PD-1 [11, 14, 23–27]. PD-L1 expression has been analyzed by using flow cytometry, immunohistochemistry, and fluorescein in situ hybridization in cell lines and lymphoma specimens. We must consider that differences in the specificity and sensitivity of the commercial antibodies used for immunohistochemical staining of PD-1 and/or PD-L1 may explain the differences between studies and indirectly the prognostic value of the PD-1/PD-L1 system [6]. In our study, we used 5 and 20 % cutoff values for tumor and microenvironment.

PD-L1 expression has been studied in HL in a relatively limited number of the studies. HRS cells variably express PD-

L1/B7-H1, whereas tumor-infiltrating T cells express the co-inhibitory receptor: PD-1 [27, 28]. In a study covering 280 cases with classical HL and 3 cases with nodular lymphocyte-predominant HL on a tissue microarray platform, PD-1(+) cells have been observed in all cases of nodular lymphocyte-predominant HL but only in 1 % of classical HLs. With these findings, the authors proposed that the presence of PD-1(+) rosettes around neoplastic cells is typical but not exclusive for nodular lymphocyte-predominant HL [29]. However, in another study, PD-L1 (9p24.to) amplification has been found to be restricted to the HL subtype classical HL and increased PD-L1 expression has been found on tumor cells [27]. In another study, PD-L1 expression has been studied by using immunohistochemistry (IHC), and PD-L1 has been found in the majority of classical and mixed cellular HL subtypes in malignant cells and in tumor-infiltrating macrophages. However, PD-L1 has not been found in nodular lymphocyte-predominant HL in this study [10]. In our study, we found PD-1 expression in 18 cases (20 %) and PD-L1 in 18 cases (20 %). Except one case, all the cases showed more than 20 % staining for PD-1 or PD-L1. As mentioned before, various methods with variable cutoffs have been used in different studies. For this reason, we could not strictly compare our results with other studies. However, our results suggest that both PD-1 and PD-L1 are meaningful targets for HL.

Prognostic significance of PD-1 or PD-L1 expression in solid tumors has been evaluated in many types of solid tumors and limited number of cases with lymphoma. It has been shown that expression of PD-L1 in cancers including colon, gastric, hepatocellular, melanoma, non-small cell lung, ovarian, and renal cell cancers predicts poor outcome [7, 30]. When we looked at the available lymphoma data, we found that increased level of PD-L1 was associated with high proliferative index or poor prognosis. In these studies, it has been proposed that PD-L1 can promote cyclinD1 and CDK4 expression, and this leads to cell arrest in G1/S phase and maintains high growth rates in lymphoma cells. Additionally, it has been suggested that PD-L1 contributes to resistance to the drugs, and it has been shown that blockade of PD-L1 or PD-1 can reverse drug resistance and improves therapeutic efficacy. In diffuse large B cell lymphoma, increased level of PD-L1 has been found to be associated with high proliferative index and poor response to treatment and an association between extranodal involvement, poor PS, and inferior survival [14, 31]. In a HL study, increased amount of PD-1(+) tumor-infiltrating lymphocytes has been found to be a stage-independent negative prognostic factor for OS [27]. Another study about classical HL suggests a critical role for the PD-1/PD-L1 signaling axis with high numbers of tumor-infiltrating macrophages and an inferior clinical outcome through the suppression of antitumor immunity [10]. In our study, although DFS and OS were found to be shorter in cases showing PD-1 or PD-L1(+), we did not find an important difference for

these cases. We found that the cases with PD-1 and PD-L1 co-expression have significantly shorter DFS and OS times as compared with cases not expressing PD axis. However, PD-1 and PD-L1 co-expression was found to be a significant independent risk factor (OR 6.9), and the confidence interval of the parameter was found to be wide (95 % CI 1.9–24.3) due to insufficient sample size (only seven patients and four were censored). Although the number of these cases is low, this point has been found remarkable. Considering this limitation for obtaining more reliable results, further studies are needed in large number of cases.

This finding suggests the considerable immune escape in cases with PD-1 and PD-L1 expression. Also, Cox regression analysis showed prognostic significance of PD-1/PD-L1 system in addition to the age. On the other hand, PD-1 and PD-L1 expressions were evaluated by IHC in cases with nasopharyngeal cancer recently, and they found poorest prognosis for DFS in cases with co-expression of PD-1 and PD-L1, and they concluded that PD-1/PD-L1 co-expression reflects the selective suppression of cytotoxic lymphocytes in the tumor micro-environment [32].

Epstein–Barr virus (EBV) has been implicated in approximately 40 % of classical HLs [33]. There are some studies evaluating the association between EBV and PD-1, or its ligands and results are complex. EBV infection has been implicated in PD ligand expression, and it has been shown that EBV-encoded LMP1 can promote PD-L1 expression in tumor cells [34]. However, in another study covering 12 cases with EBV(+) HL, 11 cases showed strong membranous PD-L1 staining in malignant RS cells while 20 of 29 cases with EBV(–) HL showed PD-L1 positivity. In this study, it has been suggested that EBV status does not predict the presence or absence of PD-L1 expression in HL [10]. Additionally, EBV-encoded RNA ISH has been studied in primary mediastinal large B cell lymphoma, and no significant correlation has been found between EBV positivity and PD ligand [11]. We did not find an important association between EBV and PD-1 or PD-L1.

Monoclonal antibodies targeting PD-1 and PD-L1 have been synthesized and used successfully with durable responses in many solid tumors [35, 36]. Recently, there are many trials establishing the beneficial effects of the PD-1 blockers in lymphomas [7, 37]. The PD-1 inhibitors seem to reverse tumor escape from immune surveillance across several cancers, including melanoma; lung, bladder, and kidney cancers; and HL. These cancers are being targeted with PD-1 inhibitors used alone or in combination with other inhibitors that target specific pathways associated with tumor proliferation. Downregulation of PD-L1 combined with chemotherapy can suppress lymphoma growth, promote antitumor activity, and prolong the survival rate. Targeted therapy with PD-L1 may provide a new promising approach in the treatment of lymphomas [14]. In vitro studies showed that T cells from

primary HL expressing PD-1 responded to PD-1 blockade [28, 29, 38]. Lastly, very good results with nivolumab and pembrolizumab in relapsed/refractory cases with HL have been published and presented [39, 40].

In conclusion, we detected PD-1 or PD-L1 expression in 20 % of the cases. Although each of PD-1 or PD-L1 expression found to be not affecting the clinical outcome, it showed that the co-expression of PD-1 and PD-L1 expressions associated with poor clinical outcome. These results suggest that the PD-1 or PD-L1 are important targets in HL, and monoclonal antibodies targeting this system will be very useful and effective in cases with HL as observed in many solid tumors.

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Conflict of interest The authors declare that they have no conflict of interest.

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