

# Research Paper: Immune Checkpoint Molecules in Primary Diffuse Large B-Cell Lymphoma of the Central Nervous System



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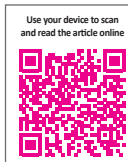
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## ABSTRACT

**Introduction:** Primary Diffuse Large B Cell Lymphoma of CNS (PCNSL) is a rare variant of Diffuse Large B Cell Lymphoma (DLBCL) and presents with an aggressive clinical course and usually resistant to commonly used therapy regimens. Recently, role of immune checkpoint molecules including PD-1 and PD-L1 confirmed in some solid tumors and lymphoma resulting tumor cells escape the immune system and help to survive and to spread. Inhibitors of PD-1 and PD-L1 have shown lasting responses in several solid and some hematological tumors, while limited studies evaluate checkpoint molecules on PCNSL.

**Method:** In this study, we investigated PD-1 and PD-L1 expression by immunostaining on 71 patients with PCNSL and correlation with demographic data, location of the tumor, proliferation rate, cell of origin, and CD8 positive T cell infiltration in tumor microenvironment.

**Results:** 16 from 71 showed PD-1 expression, while PD-L1 expression were 42/71. No association was determined between PD-1/PD-L1 expression and gender, cell of origin, and proliferation rate, but a highly significant difference was determined between the infiltration of CD8 positive T cells in two groups of PD-1/PD-L1 positive and negative.

**Conclusion:** This study revealed expression of check point molecules in remarkable number of PCNSL which may open new therapeutic recommendations in this aggressive lymphoma type.

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## Highlights

- Primary diffuse large B-cell lymphoma of the central nervous system (PCNSL) is a rare variant of Diffuse large B-cell lymphoma limited to the central nervous system.
- PCNSL has a poor prognosis without response to usual therapy for other types of lymphoma.
- Checkpoint molecules have a critical role in the pathogenicity of some malignancies.
- Few research studies have been conducted about the Programmed Death-1 (PD-1) and its ligand PD-L1 (checkpoint molecules) role in the pathogenicity of PCNSL.
- PD-1 and PD-L1 have been detected in some PCNSLs and show association with T cell infiltration.

## Plain Language Summary

Primary diffuse large B-cell lymphoma of the central nervous system (PCNSL) is a rare variant of high-grade lymphoma of the brain which is limited to immune-privileged organs such as the central nervous system, testis, and eyes. This type of high-grade lymphoma is resistant to usual therapy of other similar lymphomas and shows poor prognosis. Immunotherapy and immune checkpoint (programmed death-1 [PD-1] and its ligand PD-L1) inhibitors are new treatment protocols and their use was approved in some solid tumors and few types of lymphoma, including classical Hodgkin lymphoma. However, few research studies have been done on the evaluation of immune checkpoint molecules in the pathogenesis of PCNSL. We investigated these molecules on 71 patients with PCNSL by immunostaining method and noticed the expression of PD-1 and PD-L1 in some of them. This study and similar ones open new therapeutic routes in this aggressive lymphoma type.

### 1. Introduction

**D**iffuse Large B-Cell Lymphoma (DLBCL) is a neoplasm of medium to large B cells and often involves multiple nodal and extranodal regions, whereas some DLBCL variants, including primary diffuse large B-cell lymphoma of the central nervous system (PCNSL), Primary Testicular Lymphoma (PTL), and primary mediastinal large B-cell lymphoma (PMBL), present as localized masses in extranodal specific organs. PCNSL is a rare neoplasm and originates from the brain, spinal cord, leptomeninges, or eye, with no evidence of systemic disease (Nayak et al., 2017). DLBCLs are classified based on molecular characteristics to germinal center B-cell subtype (GCB) and activated B-cell subtype (ABC). Most PCNSLs show an ABC-subtype phenotype and many studies claim that they originate from a late germinal center or early-post germinal center cell (Yang & Liu, 2017). This specific type of DLBCL accompanies with PTL, has restricted homing to the main immune sanctuaries, has poor survival, and is usually refractory to commonly-used therapy regimens (Chapuy et al., 2016) such as high-dose chemotherapy with methotrexate, rituximab, or whole-brain radiotherapy, and bone marrow transplantation (Berghoff et al., 2014; Korfel & Schlegel,

2013). Most patients represent with relapse, and there is limited knowledge of effective therapy (Berghoff et al., 2014). New treatment protocols such as immunotherapy, including monoclonal receptor T-cell therapies, and immune checkpoint inhibitors improve the efficacy of chemoradiotherapy in the treatment of malignancies.

One of the most frequently-studied pathways in immunotherapy is the programmed death-1 receptor (PD-1, CD279) and its ligands PD-L1 (CD274), which has a major role in a variety of neoplastic diseases. PD-1 is expressed by some T cells, B cells, and Natural Killer (NK) cells, and has been diagnosed as an exhaustion marker in some cancer cells (Turris, Andrews, & Vignali, 2015). PD-L1 (CD274) is also expressed at the surface of some B cells, T cells, macrophages, and antigen-presenting cells and on some malignant cells, including a variety of lymphomas. PD-1 and its ligand suppress the immune system and promote the escape of tumor cells from the immune response (Berghoff et al., 2014; Panjwani, Charu, DeLisser, Molina-Kirsch, Natkunam, & Zhao, 2018). In many solid tumors, PD-1 is upregulated by a large proportion of Tumor-Infiltrating Lymphocytes (TILs), whereas its ligands PD-L1 and PD-L2 are expressed by tumor cells (Ahmadzadeh et al., 2009). Binding of PD-L1 to PD-1 inhibits the proliferation of activated T lymphocytes and so creates

the anti-tumor immune response. Several lymphoma types express PD-1 and PD-L1, including mycosis fungoides, Sezary syndrome, nodular sclerosis, mixed cellularity classical Hodgkin's lymphoma, primary mediastinal large B-cell lymphoma, T-cell/histiocyte-rich B-cell lymphoma, EBV-positive and -negative post-transplant lymphoproliferative disorder, EBV-associated DLBCL, plasmablastic lymphoma, extranodal NK/T-cell lymphoma, and primary effusion lymphoma (Chen et al., 2013; Kantekure et al., 2012). Inhibitors of PD-1 (nivolumab) and PD-L1 (pembrolizumab) disrupt this signaling and have shown lasting responses in several solid and some hematological tumors (Brahmer et al., 2012; Callahan & Wolchok, 2013; Gatalica et al., 2014; Wolchok et al., 2013). These medications also improve the outcomes of patients with relapsed/refractory Classical Hodgkin Lymphoma (CHL) and Chronic Lymphocytic Leukemia (CLL), so they provide treatment opportunities in other hematopoietic malignancies (Ansell et al., 2015; Ding et al., 2017).

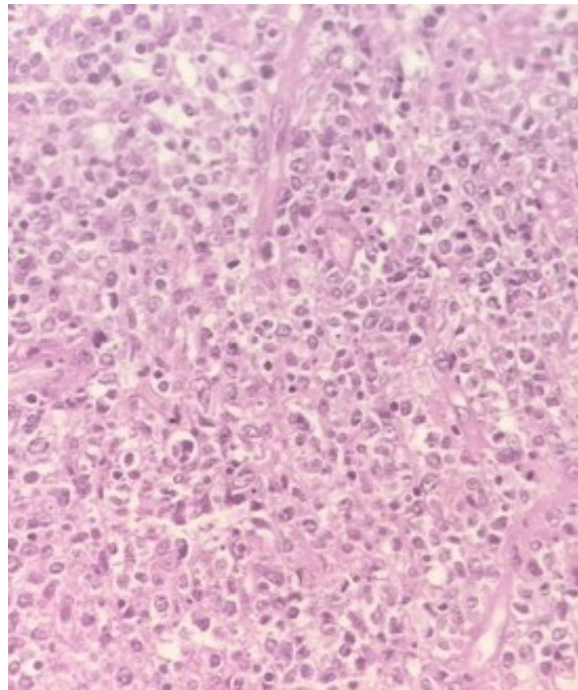
Recently a few studies evaluated the expression of checkpoint molecules and its ligands on lymphoid malignancies, including B, T, NK cells, and histiocytic neoplasms. In one comprehensive study, a total of 702 patients with various types of lymphomas and histiocytic malignancies were investigated and expression of PD-1, PD-L1, and PD-L2 was evaluated with the immunostaining method. PD-L1 was detected in 80% of anaplastic large cell lymphoma, angioimmunoblastic T-cell lymphoma, and follicular dendritic cell sarcoma; 75% of nodular lymphocyte-predominant Hodgkin lymphoma; 53% of primary mediastinal large B-cell lymphoma; 39% of extranodal NK/T cell lymphoma; 26% of peripheral T-cell lymphoma; 10% of diffuse large B-cell lymphoma; and barely in mantle, marginal zone, and small lymphocytic lymphomas (Panjwani et al., 2018). Few studies have been conducted on the PD-1/PD-L1 activation in the pathophysiology of PCNSL (Berghoff et al., 2014). We presume that immunosuppression caused by PD-1/PD-L1 activation may have an effective role in PCNSL pathobiology and so the immunotherapy against these molecules may improve patients' survival. Nevertheless, treatment with checkpoint inhibitors is costly and also associated with toxicities, moreover only some groups of patients benefit from these treatments (Atanackovic & Luetkens, 2018). Nowadays, the question is why a significant number of patients are intrinsically resistant to these therapies. To address this question, we are searching for predictive biomarkers indicating the effectiveness of these therapies. Recent studies have shown that PD-1 blockade selectively increases CD8 memory T cell numbers within the tumor microenvironment. This infiltration increases therapeutic efficacy. For example, tumor regression correlates with the increase of this population following treatment in patients with melanoma who have PD-1 CD8 positive T cells in the microenvironment of tumors

before the treatment. Tumor infiltration with PD-1 high and CD8 positive T cells has been proposed as an accurate predictor of responses to anti-PD-1 therapy in melanoma. For this reason, the evaluation of CD8 T cell infiltration in the tumor may be used as a biomarker for therapeutic efficacy (Edwards et al., 2018; Zuazo et al., 2017). We propose that the immune profile of microenvironment cells in tumors before treatment could shed light on the strategies for the efficacy of checkpoint inhibitor regimens.

## 2. Materials and Methods

In this retrospective cross-sectional study, formalin-fixed and paraffin-embedded tumor tissue of surgical resections or stereotactic biopsies of 71 patients with PCNSL between 2004 and 2018 were selected from archives of Shiraz, and Tehran medical university hospitals, Shiraz and Tehran cities, Iran. The patients with a history of systemic lymphoma, immune-deficiency, and under corticosteroid therapy before biopsy (for tumor shrinkage following corticosteroid therapy) were excluded from the study. Three tissue microarrays are made from duplicate or triplicate 1-mm cores of blocks. Histomorphological subtyping was performed on hematoxylin and eosin (H&E) sections according to the fourth edition of the WHO classification of hematopoietic and lymphoid neoplasms. Lymphoma cells may be scarce or compact and accumulate in perivascular spaces (Figure 1).

Immunophenotyping, clinical, imaging, and demographic data were filled in the prepared table. The following antibodies were used after antigen retrieval according to the manufacturer's instructions: CD20 (clone L26, Master Diagnostica, Spain), CD10 (clone 56C6, Master Diagnostica, Spain), BCL6 (clone LN22, Master Diagnostica, Spain), MUM1 (clone MUM1p, Master Diagnostica, Spain), PD-1 (clone NAT105, Master Diagnostica, Spain), PD-L1 (clone CAL10, Master Diagnostica, Spain), CD3 (clone EP41, Master Diagnostica, Spain), CD4 (clone EP204, Master Diagnostica, Spain), CD8 (clone SP16, Master Diagnostica, Spain), and Ki-67 (clone SP6, Master Diagnostica, Spain). The cell of origin was determined by estimating protein expression in tumoral cells using IHC in 30% cut-off for CD10, BCL6, MUM1 based on the Hans algorithm (Jaffe, Arber, Campo, Harris, & Quintanilla-Fend, 2016). We used the placenta for PD-L1 and lymph node for PD-1 as positive controls. We evaluated the expression of PD-1 and or PDL-1 on tumor cells, Tumor-Infiltrating Lymphocytes (TILs), and Tumor-Associated Macrophages (TAMs). Immunohistochemistry was evaluated semi-quantitatively with a light microscope by two authors who were blinded to any clinical data on a multi-headed microscope. For both PD-1 and PD-L1, strong, and fully membranous immunoreactivity on more than ~ 5% of any cell type was considered positive (Figure 2). The cut-off

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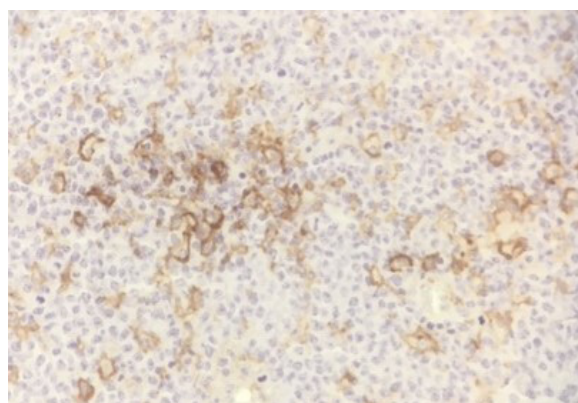
**Figure 1.** Primary diffuse large B cell lymphoma of CNS. Proliferation of tumor cells as solid and some accumulation in perivascular space. X400

point of 5% was chosen based on previous research (Berghoff et al., 2014). The density of PD-1 and PD-L1 positive cells should be evaluated in non-necrotic tissue areas and are scored as follows: low density, scattered cells; moderate density, loose but widespread infiltration; high density, dense and widespread infiltration (Berghoff et al., 2014). For the quantification of intratumoral cytotoxic lymphocytes, we quantified anti-CD8 immunostained cells in a total area of 1 mm<sup>2</sup>. For this purpose, we counted 4 microscopic fields (x400) (each sized 0.25 mm<sup>2</sup>) showing non-necrotic compact tumor infiltrates. In each field, all anti-CD8 immunostained cells were counted manually. Finally, the median number of infiltrating CD8 cytotoxic lymphocytes was calculated and the patients

were classified as two groups of higher and lower than the median. The patients were also classified into two groups according to Ki-67 immunostaining proliferation rate to equal or more than 70% and less than 70% mitosis (Preusser et al., 2010).

### 3. Results

We studied 71 confirmed cases of PCNSL, 42 men and 29 women, aged between 7 and 81 years (mean age: 55.6 years and median age: 58 years). Most of the tumors were located in the supratentorial region (55.71%). Locations of tumors were as follows: frontal lobe, 14.71%;



**Figure 2.** Fully membranous immunoreactivity of PDL-1 by immunohistochemistry. X400

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temporal lobe, 11.71%; parietal lobe, 5.71%; occipital lobe, 1.71%; multiple lesions of the cortex, 3.71%; periventricular area, 3.71%; cerebellum, 5.71%; spinal cord, 8.71%; meninges, 3.71%; multiple lesions of brain lobes, 5.71%; and brain NOS, 13.71%. About 57.71% of tumors showed non-germinal center B cell origin. The proliferation rate was between 10% and 95%; in 48 patients it was equal or more than 70%; in 23 patients, it was less than 70%. Infiltration of CD8 positive T cells were less than 10/mm<sup>2</sup> to 1400/mm<sup>2</sup> with the median of 35/mm<sup>2</sup>. About 16.71% showed PD-1 expression on TIL while PD-L1 expression on TIL, TAM, and or tumor cells were 42.71% (Table 1).

We used the t test for the evaluation of the relationship between age and expression of PD-1/PD-L1. There was no association between age and PD-1/PD-L1 expression. The Chi-square test was used for checking the relationship between expression of PD-1/PD-L1 with gender, location, cell of

origin, proliferation rate, and CD8 positive T-cell infiltration. There was also no association between PD-1 expression and gender or cell of origin. PD-1 expression decreased in tumors with a high proliferation rate but statistically, this association was not significant. No association was determined between PD-L1 expression and gender, cell of origin, or proliferation rate, but a highly significant difference was observed between the infiltration of CD8 positive T cells between two groups of PD-1/PD-L1 positive and negative (Tables 2 and 3).

#### 4. Discussion

Primary CNS lymphoma is a rare lymphoma with an aggressive clinical course and poor response to current therapies. Biologic studies of PCNSL are challenging because of the rarity of the disease. So little is known about the role of immune checkpoints in the pathogenesis of PCNSL and impact on tumor aggressive course (Berghoff et al., 2014; Chapuy et al., 2016; Nayak et al., 2017; Panjwani et al., 2018;

**Table 1.** Summary of the patients' characteristics and results of immunohistochemical analyses

Parameters		No. (%)
Age at operation	<60 y	37 (52.1)
	≥60 y	34 (47.9)
Localization	Frontal	14 (19.7)
	Temporal	11 (15.5)
	Parietal	5 (7)
	Occipital	1 (1.4)
	Multiple lesion of cortex	3 (4.2)
	Periventricular	3 (4.2)
	Cerebellum	5 (7)
	Spinal cord	8 (11.2)
	Meninges	3 (4.2)
	Multifocal	5 (7)
Cell of origin	Brain NOS	13 (18.3)
	GC (Germinal Cell) type	14 (19.7)
Gender	ABC (Activated B-cell) type	57 (80.3)
	Male	42 (59.2)
Proliferation rate	Female	29 (40.8)
	<70%	23 (32.4)
PD-1 expression	≥70%	48 (67.6)
	Negative	55 (77.5)
PD-L1 expression	Positive	16 (22.5)
	Negative	29 (40.9)
Number of intra tumoral cytotoxic lymphocytes	Positive	42 (59.1)
	<median	36 (50.7)
	≥median	35 (49.3)

**Table 2.** Association of PD-1 with demographic data, proliferation rate, cell of origin and CD8 positive T-cell infiltration

Variables			Patients With PD-1 (+) Cells (n = 16)	Patients With PD-1 (-) Cells (n = 55)	P
Male		42	9	33	0.788
Female		29	7	22	
Germinal cell type		14	2	12	0.727
Activated B-cell type		57	11	46	
Proliferation rate	< 70	23	9	14	0.063
	≥ 70	48	7	41	
Number of intratumoral cytotoxic lymphocytes	< median	36	4	32	0.019
	≥ median	35	12	23	

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**Table 3.** Association of PD-L1 with demographic data, proliferation rate, cell of origin and CD8 positive T-cell infiltration

Variables			Patients With PD-L1 (+) Cells (n = 42)	Patients With PD-L1 (-) Cells (n=29)	P
Male		42	24	18	0.678
Female		29	18	11	
Germinal cell type		14	5	9	0.211
Activated B-cell type		57	25	32	
Proliferation rate	< 70%	23	9	14	0.862
	≥ 70%	48	20	28	
Number of intratumoral cytotoxic lymphocytes	< median	36	14	22	0.000
	≥ median	35	28	7	

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Yang & Liu, 2017). The expression of PD-1 and PD-L1 has recently been found in several lymphoma types (Berghoff et al., 2014). PD-L1 expression was detected in a high percentage of anaplastic large cell lymphoma, angioimmunoblastic T-cell lymphoma, follicular dendritic cell sarcoma, nodular lymphocyte-predominant Hodgkin lymphoma, primary mediastinal large B-cell lymphoma, only 10% of diffuse large B-cell lymphoma, and barely in mantle, marginal zone, and small lymphocytic lymphomas (Panjwani et al., 2018). In a study by Berghoff et al., PD-1 and or PDL1 were seen on tumor cells, TILs, or TAMs, TAMs in a total of 18/20(90%) of cases, only 10% of PCNSL cases (n=2/20) were found to have PD-L1+ tumor cells. PD-L1 expression on intratumoral TAMs was found in 4/20 (20%) tumors and 12/20 (60%) cases; intratumoral PD-1 positive TIL was present (Berghoff et al., 2014). A study on PCNSL by Miyasato Y demonstrated no PD-L1 expression by immunohistochemistry on lymphoma cells while PD-L1 expression on TAMs was seen in 3 out of 5 cases (Miyasato et al., 2018). In another study by Melissa Alame et al. in France, PD-L1 protein expression by the neoplastic B cells was observed in 17.5% (10/57) of PCNSL cases (Alame et al., 2019). In this study, we found 29% (16/71) PD-1 expression on TILs and TAMs, as well as, 76.4% (42/71) PD-L1 expression on TILs, TAMs, and tumor cells in PCNSL. Also, 27 out of 71 patients were neg-

ative for both PD-1 and PD-L1. Nevertheless, checkpoint inhibitors therapies are also associated with side effects and are expensive. Besides, only some groups of patients benefit from these treatments (Atanackovic & Luetkens, 2018). It remains largely unknown why a significant number of patients are intrinsically resistant to these therapies and limited studies considered predictive biomarkers for these therapies. A few studies observed that CD8 T cell infiltration may be used as a biomarker for therapeutic efficacy in some solid tumors (Edwards et al., 2018; Zuazo et al., 2017). In this study, infiltration of CD8 positive T cells were also evaluated by immunostaining as a message for informing strategies for immune checkpoint blockade therapies.

Our study offers novel insight into the immunosuppressive identity of checkpoint molecules in PCNSL and may open new therapeutic recommendations in this aggressive lymphoma type. However, in all cases, some variants, including alterations in background inflammatory cells probably affect the therapeutic efficacy and expected outcomes.

## Ethical Considerations

### Compliance with ethical guidelines

This article does not contain any studies with direct human participants' involvement by any of the authors.

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### Authors' contributions

All authors contributed equally in preparing all parts of the research.

### Conflict of interest

The authors declared no conflict of interest.

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