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The prognostic significance of immune checkpoint receptor expression in patients with lymphoma: Association with disease status and clinical outcomes

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Abstract

Introduction: Little is known about the expression of immune checkpoint receptors in the peripheral blood of lymphoma patients. Herein, we assessed the expression of inhibitory checkpoint receptors, including CTLA-4, PD-1/PDL-1, LAG-3, and TIM-3 in the peripheral blood of lymphoma patients and its correlation with the clinical outcomes of patients. Therefore, 47 classical Hodgkin lymphoma (cHL), 48 non-Hodgkin lymphoma patients with diffuse large B-cell lymphoma (DLBCL) subtype, and 30 healthy controls were recruited.

Methods: The expression of inhibitory receptors was evaluated using SYBR Green real-time PCR method.

Results: CTLA-4, LAG-3, and TIM-3 genes were significantly upregulated in both cHL and DLBCL patients compared to the healthy controls. In addition, the level of these molecules was differentially expressed in cHL and DLBCL patients at different disease phases compared to the healthy controls. The CTLA-4 gene was highly expressed in newly diagnosed (ND) cHL patients compared to the relapsed ones. Relapsed DLBCL patients had significantly increased LAG-3 expression compared to patients at remission, as well as ND patients. Regarding cHL patients, high CTLA-4 expression was correlated with low lactate dehydrogenase level and better performance status, whereas the level of LAG-3 was significantly elevated in patients with poor performance status. Lower initial PD-1 expression was associated with improved disease-free survival in cHL patients.

Conclusions: Inhibitory immune checkpoint receptors are aberrantly expressed in the peripheral blood of cHL and DLBCL patients in which high LAG-3 in DLBCL patients and PD-1/LAG-3 in cHL patients are associated with relapse occurrence and worse prognosis, respectively.

KEYWORDS
Classical Hodgkin lymphoma (cHL), Diffuse large B-cell lymphoma (DLBCL), Immune checkpoint receptors, Prognosis

1 | INTRODUCTION

Lymphoma is one of the hematologic malignancies, originated from lymphoid compartments of the immune system, which is categorized into two main groups: classical Hodgkin lymphoma (cHL) and non-Hodgkin lymphoma (NHL).[1] Malignant cells in cHL are called Reed–Sternberg cells that are surrounded by different population of the immune cells, such as lymphocytes, eosinophils, fibroblasts, macrophages, and plasma cells.[2,3] NHL has different subtypes, the most common of them is diffuse large B-cell lymphoma (DLBCL) with annual incidence of 3–4 new cases per 100,000 persons in Europe.[1]

Lymphoid malignancies like cHL and NHL exploited different strategies to escape from immune cell recognition.[4,5], Impaired functional antitumor immune responses through the upregulation of the inhibitory immune checkpoint receptors is thought to be one of the key immune escape mechanisms identified in lymphoma patients, results in impressive lymphoma cell progression.[6] The most common inhibitory immune checkpoint receptors are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; CD152), lymphocyte activation gene-3 (LAG-3; CD223), T cell immunoglobulin and mucin domain-containing protein-3 (TIM-3), programmed cell death protein 1 (PD-1; CD279), and PD-1 ligand (PD-L1; CD274).[7]

CTLA-4 is expressed at the surface of activated T lymphocytes, as well as regulatory T cells (Tregs), where it interacts with the ligands CD80/CD86 on the surface of antigen-presenting cells.[8,9] These interactions provide inhibitory signals, leading to cell apoptosis, anergy, or functional exhaustion of conventional T cells, and consequently, abrogate the functional immune responses.[10]

LAG-3 is another co-inhibitory receptor, which is expressed on the surface of activated T and B cells, natural killer (NK) cells, plasmacytoid dendritic cells (DCs), and Tregs. Due to the structural LAG-3 similarity with CD4 molecule, the LAG-3 receptors bind MHC-II, albeit with higher affinity, thereby, prevent cognate cell–cell interaction.[11,12] LAG-3 is a negative regulator of T cell activation and proliferation, causes immune exhaustion in T cells, and affects the immunosuppressive function of Tregs.[13,14]

TIM-3 is an immunoregulatory molecule, which is expressed on the surface of $CD8^+$ T cells, Th1 cells, T helper 17 (Th17), Tregs, NK cells, monocytes, and DCs. It has different ligands, including Galectin-9, carcinoembryonic antigen cell adhesion molecule 1, high-mobility group box 1 , and phosphatidylserine.[15] TIM-3 suppresses the T cell responses and causes T cell exhaustion, and inhibits the expression of cytokines, such as TNF and INF-γ.[16,17]

PD-1 is an inhibitory receptor, which is expressed at the surface of T lymphocytes and it has two ligands: PD-L1 and PD-L2. Expression of PD-L1 on tumor cells and its interaction with PD-1 causes a potent immune escaping mechanism from the host T cell immunity.[18,19]

Recently, a significant breakthrough has been made by the administration of neutralizing antibodies directed against immune inhibitory receptors; also known as "immune checkpoint blockers" or "immune checkpoint inhibitors." In this regard, different antagonistic monoclonal antibodies have been widely used in clinical trials in order to overcome the immune-suppressed microenvironment observed in solid tumors and also hematologic malignancies like lymphoma, which have been associated with promising outcomes.[20–23]

There are not enough data about the expression of these inhibitory receptors in the peripheral blood of lymphoma patients and association with prognosis of patients. Therefore, in this study, we evaluated the expression of the inhibitory immune checkpoint receptors, including CTLA-4, PD-1/PD-L1, LAG-3, and TIM-3 in the peripheral blood leukocytes of lymphoma patients compared to the healthy controls and also between patients at different disease phases to investigate the association of these markers with the clinical outcome of these patients.

2 | PATIENTS AND METHODS

2.1 | Patients' selection

This study included 95 lymphoma patients with cHL ($n = 47$) and NHL patients with DLBCL subtype ($n = 48$), who referred to our central hospital between 2018 and 2019. Patients were at three different phases: newly diagnosed (ND) untreated patients, patients at remission phase, and the relapsed ones.

The diagnosis was confirmed by immunohistochemistry. At presentation, all staging workup, including bone marrow aspiration and biopsy, and also laboratory tests containing complete blood count, hemoglobin level (Hb), liver function test, blood urea and creatinine, erythrocyte sedimentation rate (ESR), and lactate dehydrogenase (LDH) level were examined for all patients. Patients who had previously received chemotherapy and/or corticosteroids, also HIV-positive patients, and all patients with underlying comorbid diseases were excluded from the study. The staging was performed based on the Ann-Arbor system.

Patients were treated according to the type of lymphoma, as ND cHL patients received six cycles of ABVD regimen (doxorubicin 25 mg/m^2 iv [days 1 and 15], bleomycin 10 IU/m^2 iv [days 1 and 15], vinblastine 6 mg/m^2 iv [days 1 and 15], and dacarbazine 375 mg/m^2 iv [days 1 and 15]) and three cycles of ESHAP salvage chemotherapy (etoposide 40 mg/m^2 iv over 1 h [days 1–4], methylprednisolone 500 mg iv bolus over 15 min [days 1–5], high-dose cytarabine 2000 mg/m^2 iv over 2 h [day 5], and cisplatin 25 mg/m^2 iv continuous infusion over 24 h [days 1–4]) were used for relapsed patients. The treatment protocol for ND DLBCL patients was six cycles of R-CHOP (rituximab 375 mg/m^2 iv [day 0], cyclophosphamide 750 mg/m^2 iv [day 1], hydroxydoxorubicin 50 mg/m^2 iv [day 1], vincristine 1.4 mg/m^2 iv (max 2 mg/day) [day 1], and prednisolone 40 mg/m^2 iv [days 1–5]) and also R-ESHAP salvage chemotherapy (rituximab 375 mg/m^2 iv [day 0], etoposide 40 mg/m^2 iv over 1 h [days 1–4], methylprednisolone 500 mg iv over 15 min [days 1–5], high-dose cytarabine 2000 mg/m^2 iv over 2 h [day 5], and cisplatin 25 mg/m^2 iv continuous infusion over 24 h [days 1–4]) for patients in relapsed or refractory disease phase. The response to treatment after three cycles and also at the end of the chemotherapy were evaluated for all patients. The International Prognostic Index (IPI) was determined for each patient based on the prognostic factors containing age, stage, extranodal involvement, LDH level, and also ECOG

TABLE 1 The primer sequence and PCR products for immune checkpoint receptors

Gene	Primer sequences (5'-3')	PCR product length
CTLA-4 Forward Primer	5′-TTCTTCTCTTCATCCCTGTCTTCT-3′	130 bp
CTLA-4 Reverse Primer	5′-CGGACCTCAGTGGCTTTG-3′	
PD-1 Forward Primer	5′-CCGCACGAGGGACAATAG-3′	81 bp
PD-1 Reverse Primer	5′-CCACAGAGAACACAGGCA-3′	
PD-L1 Forward Primer	5′-AGGGCATTCCAGAAAGAT-3′	118 bp
PD-L1 Reverse Primer	5′-GCTACCATACTCTACCACATA-3′	
LAG-3 Forward Primer	5′-CTTCTTGGAGCAGCAGTG-3′	133 bp
LAG-3 Reverse Primer	5′-AAAGGAGCAGAGAAAGGAC-3′	
TIM-3 Forward Primer	5′-GTCATCAAACCAGCCAAGG-3′	113 bp
TIM-3 Reverse Primer	5′-AGTGTCTGTGTCTCTGCT-3′	

performance status (PS). Thirty healthy individuals were selected as the control group who had no previous history of autoimmune and inflammatory diseases, as well as human malignancies.

2.2 | Sample collection

Five milliliter of peripheral blood was collected in ethylenediaminetetraacetic acid-treated tubes from all patients and healthy control group. For ND and relapsed patients, blood sampling was performed at the time of patients' admission before starting medical treatment. The peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-hypaque (Inno-Train, Germany) density gradient centrifugation (2800 rpm for 20 min) and cells were washed twice with phosphate-buffered saline prior to RNA extraction.

2.3 | RNA isolation and cDNA synthesis

Total RNA was extracted from PBMCs using Trizol solution (Invitrogen, USA), according to manufacturer's instructions. The purity of extracted RNA was assessed as OD 260/280 by Nanodrop (ThermoFisher, USA). Then, cDNA synthesis was performed using Primescript^{TM} RT reagent kit (Takara, Japan), according to its protocol in the T100 thermocycler (Bio-Rad Laboratories, USA).

2.4 | SYBR Green real-time PCR

Real-time PCR amplification was performed using SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus) (Takara) and designed primers specific for each genes in an iQ5 thermocycler (Bio-Rad Laboratories, USA). The specific forward and reverse primers for CTLA-4, PD-1/PD-L1, LAG-3, and TIM-3 genes were designed using AlleleID version 6, Oligo software version 7, and Molecular Beacon software and rechecked with BLAST program. The GAPDH was used as the housekeeping gene and expression of the CTLA-4, PD-1/PD-L1, LAG-3, and TIM-3 genes was normalized to that. Primer sequences and product length have been shown in Table 1. Finally, the relative expression of targeted genes was calculated using $2^{-\Delta\Delta CT}$ method.

2.5 | Statistical analysis

All statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) version 22.0. Quantitative variables were described as mean±standard deviation (SD) and median. In order to compare the relative expression of CTLA-4, PD-1/PD-L1, LAG-3, and TIM-3 genes between two groups of patients, the Student's *t*-test and Mann–Whitney U test were used for parametric and nonparametric data distribution, respectively. For comparing of more than two groups, ANOVA and Kruskal–Wallis test were used. Pearson's correlation test was performed to evaluate the association between quantitative variables. The survival curve was plotted, using the Kaplan–Mayer method and log-rank test. The overall survival (OR) was calculated from the time of entry to the end of the study when patients were alive irrespective to the present or absent of the disease. Disease-free survival (DFS) was defined from the time of entry to the end of the study when patients survived without any signs or symptoms of disease recurrence. p Value<0.05 was considered as statistically significant.

3 | RESULTS

This study included 47 cHL and 48 DLBCL patients at different disease phases, as well as 30 healthy controls. The mean age of patients was 38.68±13.08 (range 23–83 years) and 50.14±14.9 (range 23–82 years) for cHL and DLBCL patients, respectively, and 40±12.4 (range 22–73 years) for healthy controls. The laboratory and clinical data of all patients at remission, as well as the new case and the relapsed ones were collected at the time when the blood sampling was taken from them. The laboratory and clinical data of patients are summarized in Table 2.

3.1 | Change in the expression of the immune checkpoint receptors in lymphoma patients

For calculation of gene expression, the expression of the CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes was normalized to the GAPDH

TABLE 2 The laboratory and clinical characteristics of lymphoma patients

Variable	cHL patients ($n = 47$)	NHL patients ($n = 48$)
Age (years)		
Mean±SD	38.68±13.08	50.14±14.9
Range (years)	23–83	23–82
Sex		
Male (%)	31 (65.9)	24 (50)
Female (%)	16 (34.1)	24 (50)
Laboratory data	**mean±SD**	**mean±SD**
WBC count ($\times 10^3$/ml)	6.36±2.81	5.84±2.14
Platelet count ($\times 10^3$/ml)	228±91.14	225±74.35
Hb level (g/dl)	12.85±2.71	12.54±2.13
ESR (mm/h)	27.89±4.91	25.56±4.71
LDH (U/L)	323.92±127.06	317.5±126.34
$CD4^+$ T cells (%)	31.22±10	29.67±13.23
$CD4^+IFN\text{-}\gamma^+IL\text{-}4^-$ Th1 (%)	41.29±17.51	37.17±15.79
$CD4^+IFN\text{-}\gamma^-IL\text{-}4^+$ Th2 (%)	1.79±1.05	2.05±1.26
$CD4^+IL\text{-}17^+$ Th17 (%)	1.16±.39	1.12±.39
$CD4^+CD25^+FOXP3^+$ Treg (%)	1.42±.63	1.48±.59
Clinical characteristics	**Number (%)**	
Disease status		
New case	10 (21.3)	11 (22.9)
Remission	24 (51.1)	30 (62.5)
Relapse	13 (27.6)	7 (14.6)
B symptom		
Yes	28 (59.6)	28 (58.3)
No	19 (40.4)	20 (41.7)
Nodal involvement		
Nodal involvement	42 (89.4)	30 (62.5)
Extra-nodal involvement	0 (0)	5 (10.4)
Nodal+Extra-nodal involvement	5 (10.6)	13 (27.1)
Stage		
I–II	21 (44.7)	28 (58.3)
III–IV and others[a]	26 (55.3)	20 (41.7)
Performance status (ECOG)		
<2	27 (57.4)	20 (41.7)
≥2	20 (42.6)	28 (58.3)
IPI (score)		
Low-intermediate (0–2)	43 (91.5)	43 (89.5)
High (3–4)	4 (8.5)	5 (10.4)

Abbreviations: WBC, white blood cells; Hb, hemoglobin; ESR, erythrocyte sedimentation rate; LDH, lactate dehydrogenase; IPI score, International prognostic index score.

[a]Patients with only extranodal involvement.

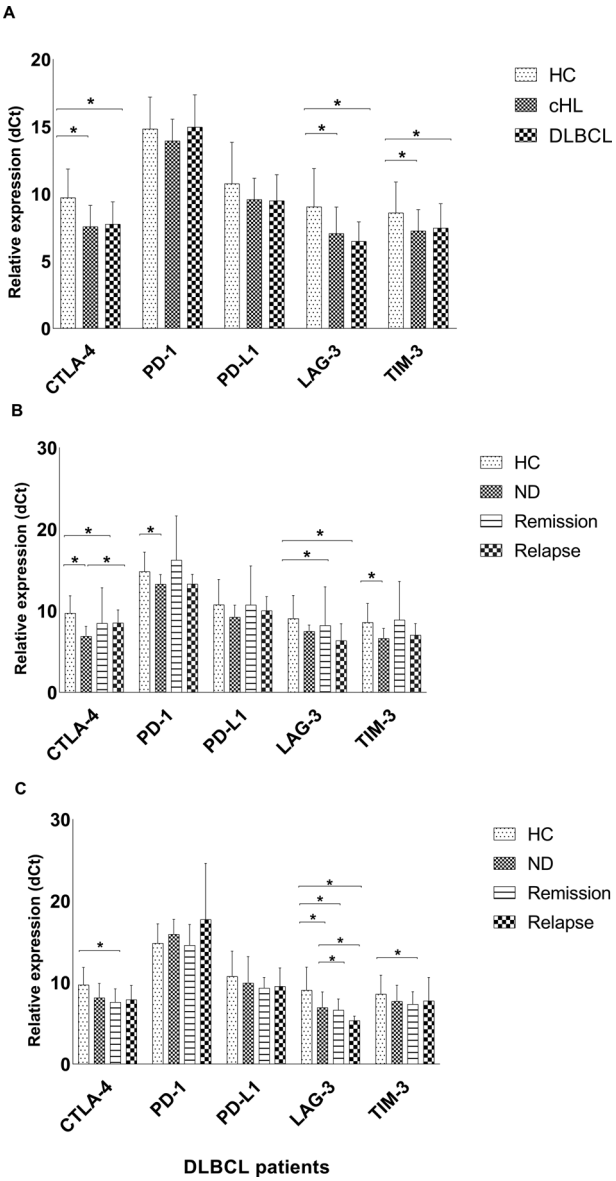

FIGURE 1 Expression of CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes in healthy controls (HCs) and cHL and DLBCL patients at different disease phases. The graph is created by GraphPad Prism version 8. Abbreviations: cHL, classical Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; HCs, healthy controls; ND, newly diagnosed. * p-Value<.05

housekeeping gene by subtracting GAPDH Ct from Ct value of these genes and was defined as ΔCT (dCt). Then, the dCt of all genes was compared between lymphoma patients and healthy control group, as well as between patients at different phases of the disease.

Analysis of our results showed that the mean expression of the CTLA-4 gene was significantly higher in cHL patients compared to the healthy controls (7.56±1.58 vs. 9.72±2.12; *p<.001). In addition, the mean expression of the LAG-3 and TIM-3 genes significantly increased in cHL patients compared to the healthy controls (7.06±1.95 vs. 9.05±2.84; *p = .003 and 7.26±1.56 vs. 8.59±2.29; *p = .015, respectively) (Figure 1A).

Comparison of cHL patients at different disease phases demonstrated that CTLA-4, PD-1, and TIM-3 genes were expressed at the higher level in ND cHL patients compared to the healthy controls (6.88±1.19 *vs.* 9.72±2.12; *p<.001, 13.26±1.19 *vs.* 14.83±2.36; *p = .032, and 6.63±1.2 *vs.* 8.59±2.29; *p = .008, respectively) (Figure 1B). Also, cHL patients at remission had higher level of CTLA-4 and LAG-3 expression than healthy controls (7.52±1.58 *vs.* 9.72±2.12; *p = .001 and 7.16±2.29 *vs.* 9.05±2.84; *p = .018). In addition, relapsed cHL patients expressed higher LAG-3 compared to the normal individuals (6.27±2.2 *vs.* 9.05±2.84; *p = .018). The expression of PD-1, PD-L1, LAG-3, and TIM-3 genes was not significantly different between cHL patients at different disease phases (p>.05). However, the expression of the CTLA-4 gene was significantly higher in ND patients compared to the relapsed ones (median 6.85% *vs.* 8.39%; *p = .042) (Figure 1B).

Similar to the cHL patients, the mean expression of CTLA-4, LAG-3, and TIM-3 was significantly elevated in DLBCL patients compared to the healthy individuals (7.75±1.65 *vs.* 9.72±2.12; *p<.001, 6.48±1.44 *vs.* 9.05±2.84; *p<.001, and 7.47±1.79 *vs.* 8.59±2.29; *p = .035, respectively) (Figure 1C).

The expression of LAG-3 was significantly higher in ND DLBCL patients in comparison to the healthy control group (6.92±1.92 *vs.* 9.05±2.84; *p = .044). Also, DLBCL patients at remission phase had significantly increased level of CTLA-4, LAG-3, and TIM-3 compared to the normal group (7.58±1.64 *vs.* 9.72±2.12; *p<.001, 6.62±1.35 *vs.* 9.05±2.84; *p = .001, and 7.34±1.53 *vs.* 8.59±2.29; *p = .01, respectively). Also, the expression of LAG-3 mRNA was higher in relapsed DLBCL patients than healthy controls (5.33±.53 *vs.* 9.05±2.84; *p = .001).

The mRNA of CTLA-4, PD-1, PD-L1, and TIM-3 genes did not differentially expressed between DLBCL patients at different phases (p>.05). Despite that, the LAG-3 expression was significantly increased in relapsed patients compared to those at remission (median 5.3 *vs.* 6.54; *p = .028), as well as ND ones (median 5.3 *vs.* 6.25; *p = .03) (Figure 1C).

There was a positive correlation between expression of CTLA-4 with PD-L1 (r = .707, *p<.001) and TIM-3 gene (r = .603, *p = .001), PD-1 with PD-L1 (r = .512, *p = .006), TIM-3 with PD-1 (r = .601, *p = .001) and PD-L1 gene (r = .532, *p = .004) in cHL patients. Regarding DLBCL patients, a positive association was observed between expression of CTLA-4 with PD-1 (r = .659, *p<.001), PD-L1 (r = .401,*p = .025), LAG-3 (r = .403, *p = .02), and TIM-3 (r = .746, *p<.001), between PD-1 and PD-L1 (r = .558,*p = .002), and TIM-3 with PD-1 (r = .73, *p<.001), PD-L1 (r = .7 and *p<.001), and LAG-3 gene (r = .396 and *p = .023).

3.2 | The relationship between immune checkpoint receptor expression and clinical and laboratory characteristics

The correlation between expression of the CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes and laboratory data, including white blood cell (WBC) and platelet count, serum Hb, ESR, and LDH level, was evaluated. The results showed that there was a negative association between the CTLA-4 gene expression and LDH level in cHL patients (r = −.37 and *p = .037). No significant correlation was observed between the PD-1, PDL-1, LAG-3, and TIM-3 gene expression and these laboratory parameters (p>.05).

In DLBCL patients, there was no significant correlation between the CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 gene expression and laboratory parameters like WBC and platelet count, serum Hb, ESR, and LDH level (p>.05).

Moreover, the expression of CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes was assessed regarding main clinical features of lymphoma patients, including B symptom, disease stages, nodal/extranodal involvement, and the two key prognostic factors: IPI score and PS (Figure 2). Accordingly, there was no significant change in the PD-1, PD-L1, and TIM-3 expression regarding main clinical features of lymphoma patients, including B symptom, disease stages, nodal/extranodal involvement, and prognostic factors like IPI score and PS in both cHL and DLBCL groups (Figure 2). However, the expression of CTLA-4 was significantly higher in cHL patients with PS<2 (good performance) compared to those patients with PS≥2 (poor performance) (median 6.85 *vs.* 8.34, respectively; *p = .012) (Figure 2). Conversely, the expression of LAG-3 was significantly decreased in cHL patients with PS<2 compared to those patients with PS≥2 (median 7.52% *vs.* 5.86%, respectively; *p = .024) (Figure 2A).

In addition, the expression of CTLA-4, PD-L1, LAG-3, and TIM-3 genes was not different among cHL patients who entered complete remission (also known as good responders) compared to those patients who did not (also known as poor responders) (p>.05; Figure 3A). However, cHL patients who achieved CR had decreased level of the PD-1 mRNA compared to those cHL patients who did not enter CR (median 13.85 *vs.* 12.32; *p = .016) (Figure 3A).

The expression of immune checkpoint receptors was not different between patients who survived compared to those who died at the end of study in both cHL and DLBCL patients (p>.05) (Figure 3).

3.3 | Change in the expression of the PD-1 inhibitory receptor can improve DFS in cHL patients

The relationship between initial expression level of CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 inhibitory receptors and OS and DFS was evaluated, and survival curve was plotted using Kaplan–Mayer survival analysis and log-rank test.

For this purpose, patients were categorized into two groups based on the median value for dCT of CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes (group 1 ≤median and group 2 >median) and then, the OS and DFS were compared between these two groups. The results showed that the initial expression level of the CTLA-4, PD-L1, LAG-3, and TIM-3 inhibitory receptors had no impact on OS and DFS in both cHL and DLBCL patients (p>.05). However, those cHL patients who expressed lower level of PD-1 mRNA at the beginning of the study (dPD-1>13.63) had significantly improved DFS rate compared to those cHL patients with high PD-1 expression (dPD-1≤13.63) (100% *vs.* 76.9%; *p = .04) (Figure 4).

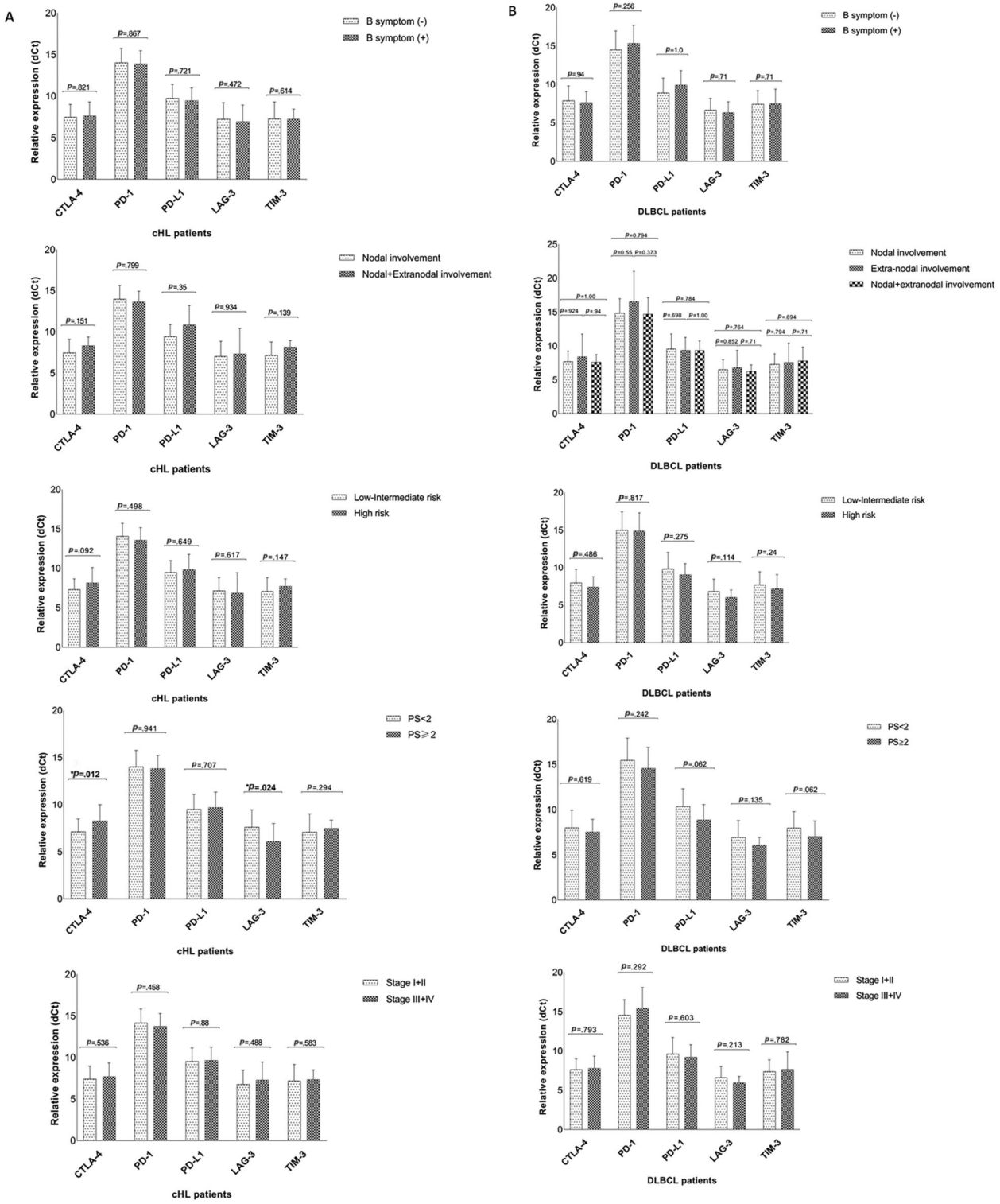

FIGURE 2 The relationship between CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes and clinical parameters in cHL (A) and DLBCL (B) patients. The graph is created by GraphPad Prism version 8. Abbreviations: cHL, classical Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; PS, performance status. * *p*-Value<.05

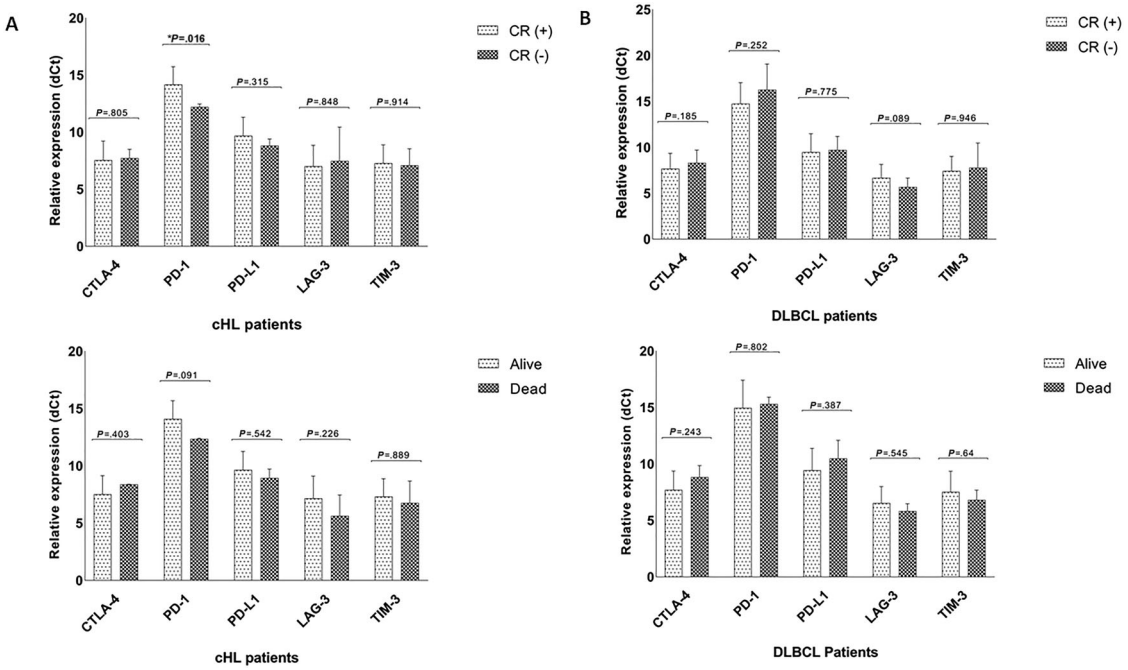

FIGURE 3 The relationship between expression of the CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes and complete remission rate (CR) and survival in cHL (A) and DLBCL (B) patients. The graph is created by GraphPad Prism version 8. Abbreviations: cHL, classical Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; CR (+), patients who entered complete remission (CR); CR (–), patients who did not enter complete remission (CR). * *p*-Value<.05

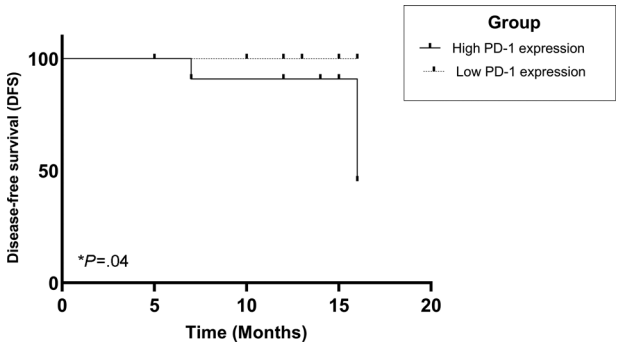

FIGURE 4 Kaplan–Meier plots for disease-free survival (DFS) based on the PD-1 expression level in cHL patients. The median PD-1 dCt = 13.63 was considered as the cutoff value and those cHL patients with PD-1 dCt≤13.63 and >13.63 were known as patients with high and low PD-1 expression, respectively. The graph is created by GraphPad Prism version 8. * *p*-Value<.05

4 | DISCUSSION

The mechanisms by which cancer cells escape from antitumor immune response have been well described for solid tumors. Despite sharing various mechanisms with solid tumors to induce tolerogenic state in the host, hematologic malignancies like leukemia and lymphoma have a unique feature of immune evasion strategies to escape from immune surveillance.[4] Increased expression of immune regulatory receptors (also known as inhibitory immune checkpoint receptors) on the tumor cells itself, as well as on tumor-infiltrating lymphocytes (TILs) serves as one of the main strategies exploited by lymphoma cells to escape from immune recognition.[4,5,24,25] Despite these findings, there are not enough data on the expression of these receptors in the circulatory leukocytes and association with the clinical outcome in lymphoma patients.

In this study, we evaluated the expression of CTLA-4, PD-1, PD-L1, LAG-3, and TIM-3 genes in PBMCs of cHL and DLBCL patients in comparison with healthy controls, as well as between patients at different disease phases. We also studied whether change in the expression of these genes was associated with laboratory data and clinical outcome of these patients. Our results revealed that the expression of CTLA-4, LAG-3, and TIM-3 genes was upregulated in both cHL and DLBCL patients than healthy controls. In case of cHL patients, the ND patients had higher level of CTLA-4, PD-1, and TIM-3, whereas patients at remission had elevated level of CTLA-4 and LAG-3 gene expression compared to the healthy controls. For DLBCL patients, ND patients had higher expression of LAG-3, while patients at remission phase had increased level of CTLA-4, LAG-3, and TIM-3 genes compared to the healthy controls. The expression of LAG-3 gene was significantly higher in relapsed patients compared to the healthy control group in both cHL and DLBCL patients. Moreover, the expression of CTLA-4 was significantly increased in the peripheral blood of ND cHL patients compared to the relapse ones. Also, we found that in DLBCL patients, the expression of LAG-3 was significantly augmented in relapsed cHL patients compared to those at remission phase and also ND patients.

There are various reports on the expression of immune checkpoint receptors on tumor cells, as well as TILs in lymphoma patients. Previous studies have shown that the expression of immune checkpoint

receptors, such as PD-L1, TIM-3, and LAG-3, was increased in HL patients compared to the control group, which causes immune evasion.[26–28] In a study by Chen et al. on tissue sections and TILs of ND DLBCL patients, the higher expression of TIM3 receptor on tumor cells (about 40%) compared to the PD-1 and LAG-3 inhibitory receptor was found, while PD-1 and LAG-3 were broadly expressed on TILs.[29] They also revealed that patients with high TIM-3 expression had inferior 4-year progression-free survival and OS.[29] In a study by Liao et al. in 14 cases with peripheral T-cell lymphomas and eight with NK/T-cell lymphomas, aberrant expression of the peripheral blood PD-1, CTLA-4, BTLA, LAG-3, TIM-3, and TIGIT was shown in comparison to the healthy controls.[30]

Another important finding of our study was that increased initial expression of the PD-1 gene was associated with poor prognosis like lower CR rate and inferior DFS in cHL patients. Data on the prognostic significance of the PD-1 expression in the peripheral blood of cHL patients are lacking. However, different studies have shown that high PD-1 expression on the TILs is associated with poor outcome of the cHL patients.[31–33]

Upregulation of the PD-1 significantly attenuates TCR signaling and has been known as a hallmark of "T cell exhaustion" phenotype, which is a common feature observed in hematologic malignancies, including chronic myeloid leukemia (CML), DLBCL, follicular lymphoma, as well as cHL.[34] In cHL patients, a high accumulation of the PD-1$^+$ and PD-L1$^+$ leukocytes in the tumor milieu renders infiltrated lymphocytes especially CD8$^+$ T cells into the anergic state within tumor microenvironment, thus might abrogate the effector function of CD8$^+$ T cell subsets.[34,35] Therefore, the abnormal expression of the PD-1 along with overexpression of its ligand PD-L1, a unique phenomenon which occurs in about 95% of cHL cases, may explain that dysregulated PD-1/PD-L1 axis might be responsible for defective antitumor immune responses in cHL patients. In this regard, two types of the Food and Drug Administration (FDA)-approved PD-1 blocking antibodies (anti-PD-1), including Nivolumab and Pembrolizumab, have been applied especially in relapsed/refractory cHL patients to overcome the immunosuppressive microenvironment in these patients, which has demonstrated a remarkable efficiency.[22–24]

We also found that in cHL patients, increased CTLA-4 expression was associated with lower LDH level and better performance status (PS<2) of patients, while elevated level of LAG-3 was associated with poor performance status (PS≥2) of patients.

The reason for association of the high CTLA-4 expression with lower LDH level and good PS of cHL patients in our study is unclear. However, it might be attributed to the CTLA-4 expression on the surface of Tregs and the possible beneficial role of Tregs in the eradication of lymphoma cells. Interestingly, recent studies have shown that in contrast to the solid tumors, elevated peripheral blood Tregs might be associated with favorable outcome in lymphoid malignancies like cHL and NHL.[36–38] In this regard, it has been proposed that activated Tregs can directly kill lymphoma cells by Granzyme B and Fas/FasL-mediated mechanisms.[39–41] Accordingly, the analysis of CTLA-4 expression in the peripheral blood Tregs, as well as effector T cells in these patients might be helpful to elucidate the definite role of CTLA-4 expression in the pathogenesis and clinical outcomes of lymphoma patients.

In addition, in contrast to solid tumors, clinical trials with anti-CTLA-4 antibodies, ipilimumab, have been associated with modest efficacy in hematological cancers and only a small number of patients have demonstrated durable responses.[42,43] Generally, it appears that the effect of CTLA-4 blockade on the restoration of anticancer immunity in hematological malignancies might be partial and not promising, associated with limited efficacy in these diseases. One possible explanation for these results might be due to the fact that CTLA-4 blockade might abrogate the positive role of Tregs in lymphoid cancers. This assumption needs to be verified by more experimental studies.

The LAG-3 is another checkpoint receptor expressed at low level on resting Tregs, but is upregulated on the surface of activated CD4$^+$ and CD8$^+$ T cells, NK cells, B cells, as well as Tregs (both natural nTregs and induced iTregs).[20,21,44] Given the higher upregulation level of LAG-3 on activated CD8$^+$ T cells than CD4$^+$ T cells, it is not surprising that like PD-1, LAG-3 can directly modulate effector function of CD8$^+$ T cell especially against cancer cells.[20,21,44]

According to the higher LAG-3 expression in our cHL patients with poor performance status (PS≥2), it is possible that the high LAG-3 expression in these patients might be somehow attributed to the defective expression of LAG-3 molecule in CD8$^+$ T cell subsets. Therefore, evaluation of LAG-3 expression on T cell subsets; both CD4$^+$ and CD8$^+$ T cell populations can be useful to clarify this assumption.

In addition, evaluation of the expression of CTLA-4, PD-1, and LAG-3 receptors, as well as other coinhibitory markers on tumor cells and TILs within tumor microenvironment might provide useful knowledge about the clinical significance of these receptors in lymphoid malignancies. Moreover, increasing the number of patients especially ND and relapsed ones along with continuous follow up of these patients for possible change in the expression of inhibitory, as well as activatory immune checkpoint receptors is also recommended.

Regarding the clinical significance of the PD-1 and LAG-3 in our patients, it is tempting that those patients with high PD-1 and LAG-3 expression may benefit from single and/or even dual inhibitors for PD-1 and LAG-3 to improve their clinical outcomes and thereby, reducing relapse rate among them.

5 | CONCLUSION

This is the first report describing that the inhibitory immune checkpoint receptors are aberrantly expressed in the peripheral blood of cHL and DLBCL patients in high LAG-3 in DLBCL patients and PD-1/LAG-3 in cHL patients is associated with relapse occurrence and worse prognosis and poor PS, respectively. Therefore, based on the expression of specific immune checkpoint receptors, lymphoma patients can benefit from the administration of its immune checkpoint blockers as a targeted immunotherapeutic approach. Study of larger population along with histopathologic examination of tumors, as well as the adjacent immune cells and its correlation with the peripheral blood expression of these receptors can be helpful to elucidate the clinical

significance of these inhibitory markers in the outcome of lymphoma patients.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHORS' CONTRIBUTION

Shokrgozar N. contributed to performing the research and drafting the manuscript. Dehghani M. contributed to study design and interpretation of data. Golmoghaddam H. and Moghadam M. contributed to analysis and interpretation of data. Rezaei N. and Moayed V. provided essential contribution for blood sampling and data collection. Arandi N. contributed to study design, analysis, and interpretation of data, writing paper, and performing the research.

ETHICAL APPROVAL

This study was performed according to the ethical standards of the local Ethics Committee of Shiraz University of Medical Sciences and in compliance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences (No. IR.SUMS.REC.1397.856).

INFORMED CONSENT

Informed consent was obtained from all patients for being included in the study.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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