

## Original Article

# PD-1, FOXP3, and CSF-1R expression in patients with Hodgkin lymphoma and their prognostic value

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**Abstract:** Despite advances in the treatment of Hodgkin lymphoma (HL), there is a need for development of reliable prognostic biomarkers and improved stratification of patients for effective therapeutic intervention. The immune microenvironment is the key to HL pathophysiology. The aim of this study was to identify expression of microenvironment-related biomarkers (PD-1, FOXP3, and CSF-1R) immunohistochemically and determine their association with clinicopathological features and prognosis in HL. We found that a high number of non-HRS cells expressing CSF-1R confers inferior overall survival (OS), which is associated with the presence of Epstein-Barr virus in neoplastic cells (P=0.009). Increased FOXP3 expression conferred superior OS and progression-free survival (PFS). PD-1 expression had no significant association with OS and PFS. The combination of FOXP3 and PD-1 or CSF-1R may yield better prognostic stratification.

**Keywords:** PD-1, FOXP3, CSF-1R, Hodgkin lymphoma, prognostic

## Introduction

Hodgkin's lymphoma (HL) has a good prognosis for most patients, with long-term remission greater than 80% following conventional chemotherapy or radiotherapy-based protocols [1-3]. However, a small proportion of patients still experience relapsing or refractory disease, which eventually leads to death, and many long-term patients suffer the late effects of excessively toxic treatments [4]. About 5% to 10% of patients are resistant to initial therapy, and 10% to 30% relapse after initial remission [5]. Several diagnostic scoring systems have been proposed, including the International Prognostic Score (IPS) [6]. The IPS is still considered as the "gold standard" for assessing prognosis and has performed consistently well in independent data sets. However, it was originally designed for patients with advanced disease, with limited relevance to early-stage disease [7]. There is no consensus on the routine management of HL patients [8]. Novel biomark-

ers are needed to improve prediction of the primary treatment outcome, and decrease the mortality and treatment-related late sequelae including secondary solid tumors and end-organ dysfunction [9].

HL is characterized by the presence of Hodgkin Reed-Sternberg (HRS) cells in classical HL (cHL). The HRS cells are embedded in a heterogeneous background of non-neoplastic bystanders that include lymphocytes, macrophages, eosinophils, mast cells, plasma cells, stromal cells, fibroblasts, micro-vessels and other cells [10]. They are recruited by the HRS cells via secretion of a variety of chemokines and cytokines, which play an essential role in HL pathogenesis. HRS cells supply growth factors, inhibit anti-tumor immune responses, and are in turn perpetuated by additional factors secreted by other reactive cells [11]. Increasing interest in the bystander cells has not only contributed to a better understanding of the underlying biological characteristics of the disease but also

**Table 1.** Demographic and clinical Characteristics of the Patients (n=86)

Variable	N (%)
Gender	
Male	53 (62)
Female	33 (38)
Age (years)	
≥ 45	23 (27)
<45	63 (73)
Ann Arbor stage	
I-II	46 (55)
III-IV	39 (45)
B symptoms	
Yes	22 (26)
No	64 (74)
IPS score	
≤2	74 (86)
>2	12 (14)
LDH	
Normal	59 (69)
High	27 (31)
β2-MG	
Normal	64 (74)
High	22 (26)
Histologic subtype	
Nodular lymphocyte-predominant	13 (15)
Classical Hodgkin lymphoma	
Nodular sclerosis	37 (43)
Mixed cellularity	31 (36)
Lymphocyte-rich	4 (5)
Lymphocyte-depleted	1 (1)
Primary treatment	
Chemotherapy	42 (21)
Chemoradiotherapy	44 (79)
Response to primary therapy	
CR	31 (36)
PR	42 (49)
PD+SD	13 (15)
EBER	
Positive	37 (43)
Negative	49 (57)

enabled the identification of new prognostic biomarkers.

The macrophage appears to play a major role in tumor support [12]. Previous studies demonstrated the adverse effect of increased macrophage infiltration [13, 14]. Using gene expres-

sion and immunohistochemical (IHC) techniques, studies revealed a correlation between decreased tumor-associated macrophages (TAM) frequency and improved clinical outcome [15]. The CD4+T cells are also important in tumor pathophysiology, although functional data are lacking [10]. Several studies reported that increased numbers of cytotoxic T cells (Granzyme B+/TIA1+) were associated with worse outcome and shorter survival in HL patients. In the present study, expression of three HL microenvironment-related markers such as programmed death 1 (PD-1), a surface marker of follicular helper T-cells; regulatory T-cell marker FOXP3 [16-18] and colony-stimulating factor 1 receptor (CSF-1R), the receptor for colony-stimulating factor 1 was detected and their prognostic significance analyzed. Further, the role of Epstein-Barr virus (EBV) is poorly understood despite a clear association with this disease. Therefore, we used *in situ* hybridization (ISH) to further determine its role in tumor microenvironment.

#### Patients and methods

Between 2004 and 2013, 95 patients were recruited and diagnosed as HL from Tianjin Medical University Cancer Institute and Hospital, and Chinese PLA General Hospital, and 86 were subjected to immunohistochemical analysis. However, the final cohort showed characteristics similar to those of the original 95 patients. All primary diagnostic tumor biopsies were reviewed and reclassified according to the World Health Organization classification of tumors of the hematopoietic and lymphoid tissues [19]. The current study was performed in strict accordance with local ethical guidelines and recommendations of the Declaration of Helsinki (Seoul revision, 2008). In this retrospective study involving archived materials, no individual patient identification, or study-driven clinical interventions were performed.

Clinical and follow-up data were obtained from clinical records including histopathological subtype, age, gender, presence or absence of B symptoms, clinical stage according to the Cotswolds modification of the Ann Arbor staging system [20], lactate dehydrogenase level, EBV status, and primary treatment. Most patients were treated with four to eight cycles of ABVD (adriamycin, bleomycin, vinblastine, and dacar-

## PD-1, FOXP3, CSF-1R, and Hodgkin lymphoma

**Table 2.** Correlation Between CSF-1R, PD-1 and FOXP3 and Clinical Variables

Characteristic	CSF-1R		P value	FOXP3		P value	PD-1		P value
	NO.	Expression (%)		NO.	Expression (%)		NO.	Expression (%)	
	Low (<30%) (n=49)	High (≥ 30%) (n=37)		Low (<25%) (n=41)	High (≥ 25%) (n=45)		Positive (≥ 20%) (n=22)	Negative (<20%) (n=64)	
<b>Gender</b>									
Male	32	21	0.503	23	30	0.377	11	42	0.213
Female	17	16		18	15		11	22	
<b>Age (years)</b>									
≥ 45	8	15	0.015	13	10	0.341	8	15	0.270
<45	41	22		28	35		14	49	
<b>Ann Arbor stage</b>									
I-II	32	14	0.010	20	26	0.388	12	34	1.000
III-IV	16	23		21	18		10	29	
<b>B symptoms</b>									
Yes	12	10	0.808	13	9	0.229	16	48	1.000
No	37	27		28	36		6	16	
<b>IPS score</b>									
≤2	45	27	0.115	35	39	1.000	20	54	0.723
>2	4	29		6	6		2	10	
<b>LDH</b>									
Normal	38	21	0.060	30	29	0.487	13	46	0.295
High	11	16		11	16		9	18	
<b>β2-MG</b>									
Normal	38	26	0.465	31	33	1.000	14	50	0.256
High	11	11		10	12		8	14	
<b>EBER</b>									
Positive	15	15	0.009	23	14	0.029	12	25	0.223
Negative	34	22		18	31		10	39	

**Table 3.** Correlation between CSF-1R, PD-1, and FOXP3 expression

Characteristic	CSF-1R	Expression	P
	Low (n=49)	High (n=37)	
<b>PD-1 expression</b>			
Positive (n=22)	35 (71.4)	29 (78.4)	0.618
Negative (n=64)	14 (28.6)	8 (21.6)	
<b>FOXP3 expression</b>			
Low (n=41)	20 (40.8)	21 (56.8)	0.191
High (n=45)	29 (59.2)	16 (43.2)	

bazine) chemotherapy. Additional radiotherapy was administered in cases of pre-therapeutic bulky or localized residual masses. Relapsed or refractory patients received either salvage chemotherapy or high-dose chemotherapy with autologous stem-cell transplantation.

### *Immunohistochemistry and in situ hybridization*

Tissue blocks of untreated patients were obtained from the Department of Pathology at two hospitals. Paraffin-embedded lymph node specimens of 86 patients were available. Immunohistochemical staining for CSF-1R, PD-1 and FOXP3 was performed as described below. Briefly, 5-μm-thick sections were transferred onto poly-L-lysine-coated adhesive slides and dried at 65°C for 90 min. After standard heat-induced epitope retrieval for 2 to 3 min in citrate buffer (pH 7.4), the samples were incubated with antibodies against PD-1 (dilution 1:50; R&D Systems, Germany), FOXP3 (dilution 1:100; Abcam, UK), and CSF-1R (dilution 1:100; Abcam, UK) overnight at 4°C. The sections were incubated with biotinylated anti-goat and

**Table 4.** Analysis Overall Survival and Progression-Free-Survival

Covariate	OS		PFS	
	HR (95% CI)	P value	HR (95% CI)	P value
<b>Univariate analysis</b>				
Age, ≥ 45 vs. <45	1.040 (1.019-1.062)	<0.001	0.419 (0.204-0.858)	0.017
Ann Arbor stage, I-II vs. III-IV	2.131 (1.067-4.255)	0.032	1.147 (0.577-2.279)	0.695
IPS score, ≤2 vs. >2	4.489 (1.847-10.909)	0.001	1.343 (0.463-3.894)	0.587
β2-MG, Normal vs. High	2.550 (1.213-5.361)	0.014	1.245 (0.562-2.759)	0.589
EBER, Positive vs. Negative	2.785 (1.367-5.672)	0.005	2.605 (1.309-5.184)	0.006
B symptoms, Yes vs. No	1.431 (0.663-3.090)	0.361	2.381 (1.186-4.778)	0.015
CSF-1R, Low vs. High	2.018 (1.015-4.013)	0.045	1.336 (0.679-2.262)	0.401
PD-1, Positive vs. Negative	1.826 (0.882-3.780)	0.105	1.210 (0.564-2.597)	0.624
FOXP3, Low vs. High	0.196 (0.084-0.453)	<0.001	0.209 (0.097-0.451)	<0.001
<b>Multivariate analysis</b>				
Age, ≥ 45 vs. <45	5.615 (2.512-12.553)	0.002	2.250 (1.073-4.715)	0.027
FOXP3, Low vs. High	0.262 (0.108-0.634)	0.002	0.253 (0.114-0.561)	0.001

anti-mouse/rabbit immunoglobulins, and DAB was used as a substrate.

The positive index was estimated by counting the number of positive cells in five randomly selected high power fields (HPF) at 40×10 magnification. For FOXP3+ quantification, the results were considered high when more than 25% of the cells were positive among the total cells. Among the percentage of non-HRS cells expressing CSF-1R, we selected more than 30% of CSF-1R-positive cells as the cut-off value for defining high-and low-CSF-1R groups. Cases stained with anti-PD-1 were scored according to the intensity of cytoplasmic and/or membranous positivity. It was considered positive when more than 20% of all cell population was stained.

*In situ* hybridization analysis for Epstein-Barr virus encoded RNA (EBER) was performed. EBER was considered as positive in case of dark-blue nuclear staining.

*Statistical analysis*

Overall survival (OS) was defined as the interval between the date of diagnosis and death from any cause. The follow-up of living patients (with or without events) was censored at their last follow-up date. Progression-free survival (PFS) was defined as the interval between the date of treatment and the date of disease progression, relapse, or death from any cause. Cumulative OS and PFS were analyzed by Kaplan-Meier method, and comparisons were made using

the log-rank test. Multivariate prognostic analyses were performed for OS and PFS using the Cox proportional hazards regression model. All *p* values were two-sided, and a *p* value <0.05 was considered as significant. Statistical analyses were performed using the SPSS 17.0.

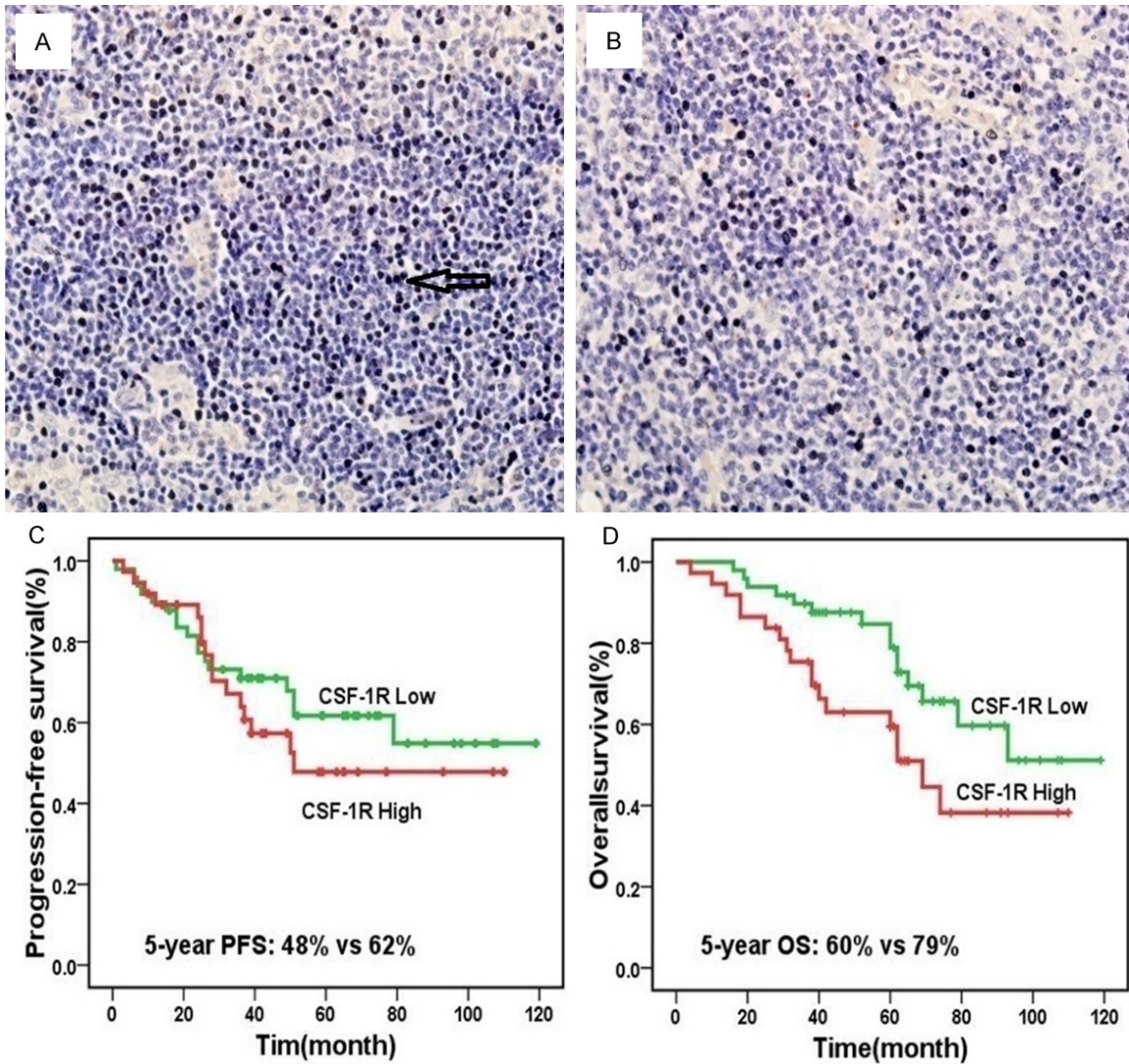
**Results**

*Patient characteristics*

The main clinical and histopathological characteristics are summarized in **Table 1**. The median age of HL patients was 31.5 years (ranging from 7 to 82 years) and 53 patients were male (61.6%). Most patients were diagnosed with advanced disease (stages III and IV) (n=39; 45.3%) and systemic B symptoms (n=22; 25.6%). Approximately 43% of patients had EBV infection (EBER-positive). The estimated 5-year OS and PFS were 75.6% and 54.2%, respectively.

*CSF-1R, PD-1, and FOXP3 expression in HL tissue*

Correlation between CSF-1R, PD-1, and FOXP3 and clinical variables are summarized in **Table 2**. Patients' tumor samples were classified into a low-CSF-1R group expressing low number (<30%) of CSF-1R-positive non-HRS cells, and a high-CSF-1R group with 30% or more cells expressing CSF-1R. Compared with the low-CSF-1R group (n=49), the high-CSF-1R group (n=37) included more patients who were older ≥ 45 years) (40.5% vs. 16.3%, P=0.015), with



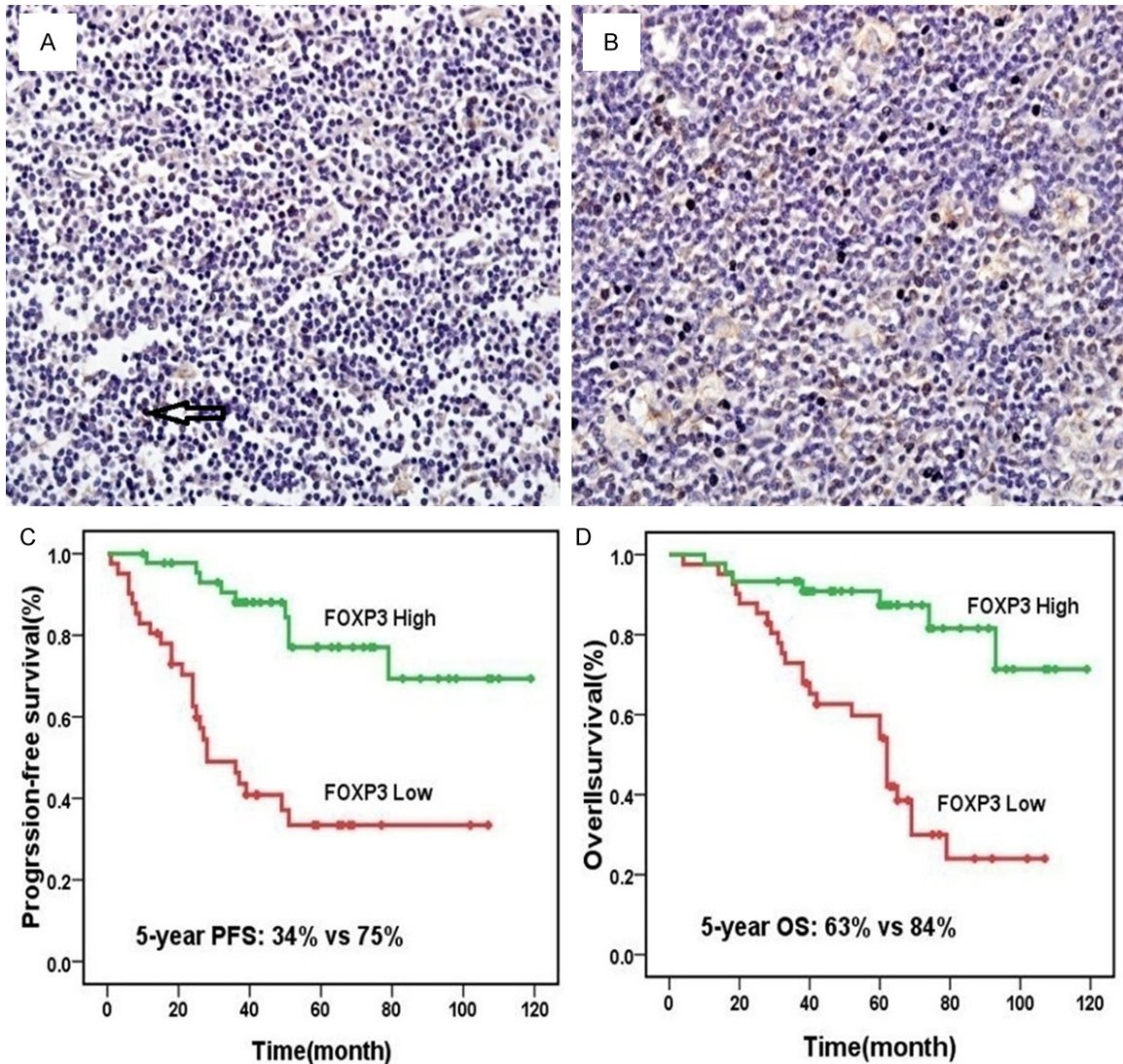
**Figure 1.** CSF-1 re-expression in non-HRS cells and prognosis, high (A) and low expression (B) of CSF-1R in Hodgkin Lymphoma (magnification  $\times 40$ ); Patients with a low number of CSF-1R-positive cells had better progression-free survival than those with high number ( $P=0.401$ ) (C); a high number of CSF-1R-positive cells led to significantly worse overall survival compared with low numbers ( $P=0.045$ ) (D).

advanced clinical stage (III/IV) (62.2% vs. 32.7%,  $P=0.010$ ) and EBER positivity (40.5% vs. 30.6%,  $P=0.009$ ). Patients were similarly divided into high and low groups according to their FOXP3 expression. The high-FOXP3 group ( $n=45$ ) included more patients who had EBER positivity (56.1% vs. 31.1%,  $P=0.029$ ) than the low-FOXP3 group ( $n=41$ ). However, PD-1 expression was not associated with any of the clinical variables.

We analyzed the relationship between CSF-1R, PD-1, and FOXP3, and found no correlation between CSF-1R expression in non-HRS cells and PD-1 expression ( $P=0.618$ ). There was also no

significant correlation between CSF-1R expression in non-HRS cells and FOXP3 expression ( $P=0.191$ ) as shown in **Table 3**.

Univariate analysis revealed that age  $\geq 45$  years ( $P<0.01$ ), advanced stage III/IV ( $P=0.032$ ), IPS $>2$  score ( $P=0.001$ ), high  $\beta 2$ -MG levels ( $P=0.014$ ), and EBER-positive status ( $P=0.005$ ) were significant predictors of shorter OS, whereas age  $\geq 45$  years ( $P=0.017$ ), B system-positive ( $P=0.015$ ) and EBER-positive status (0.006) were significant factors associated with shorter PFS. Multivariate analysis revealed that older age ( $\geq 45$  years) and elevated FOXP3 expression were independent prognostic mark-



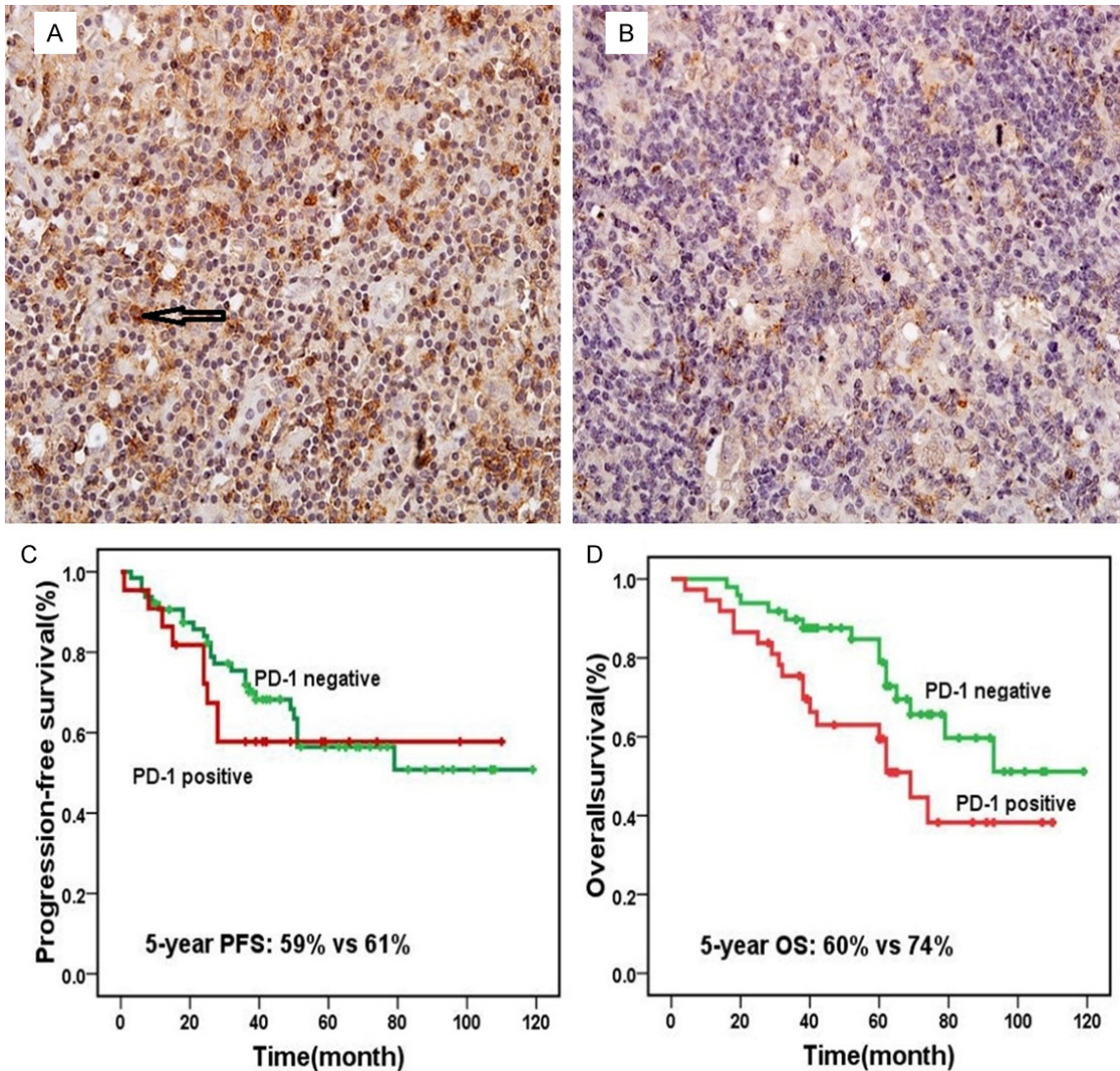
**Figure 2.** FOXP3 expression and prognosis, high (A) and low expression (B) of FOXP3 in Hodgkin Lymphoma (magnification  $\times 40$ ); Patients with a high number of FOXP3-positive cells had better progression-free survival than those with low number ( $P < 0.001$ ) (C); a low number of FOXP3-positive cells led to significantly worse overall survival compared with high numbers ( $P < 0.001$ ) (D).

ers for PFS and OS ( $P = 0.027$  and  $P = 0.001$ ) as shown in **Table 4**.

*Prognostic significance of CSF-1R, PD-1, FOXP3, and EBER expression*

OS was significantly different between low-CSF-1R and high-CSF-1R groups ( $P = 0.045$ ) with 5-year OS of 78.9% and 59.5%, respectively. No significant differences were found in PFS between low- and high-CSF-1R in non-HRS cell group (**Figure 1**). Patients in the low-FOXP3 group had lower 5-year PFS and OS rates than those in the high-FOXP3 group (34.1% vs. 75.3%,  $P < 0.001$ ; 62.9% vs. 83.6%,  $P < 0.001$ ,

respectively) (**Figure 2**). There was no significant difference in OS and PFS between the positive-PD-1 and the negative-PD-1 group (**Figure 3**). Patients in the EBER-positive group had lower 5-year PFS rates (37.1% vs. 69.2%,  $P = 0.006$ ) and lower 5-year OS rates (55.6% vs. 83.1%,  $P = 0.005$ ) than those in the EBER-negative group (**Figure 4**). Based on PD-1 status and FOXP3 expression, all the patients were divided into three groups: high FOXP3 and PD-1-negative; FOXP3 and PD-1 discordant; and low FOXP3 and PD-1-positive, with significant differences in PFS and OS ( $P = 0.002$  and  $P < 0.001$ , respectively). Furthermore, the FOXP3 and



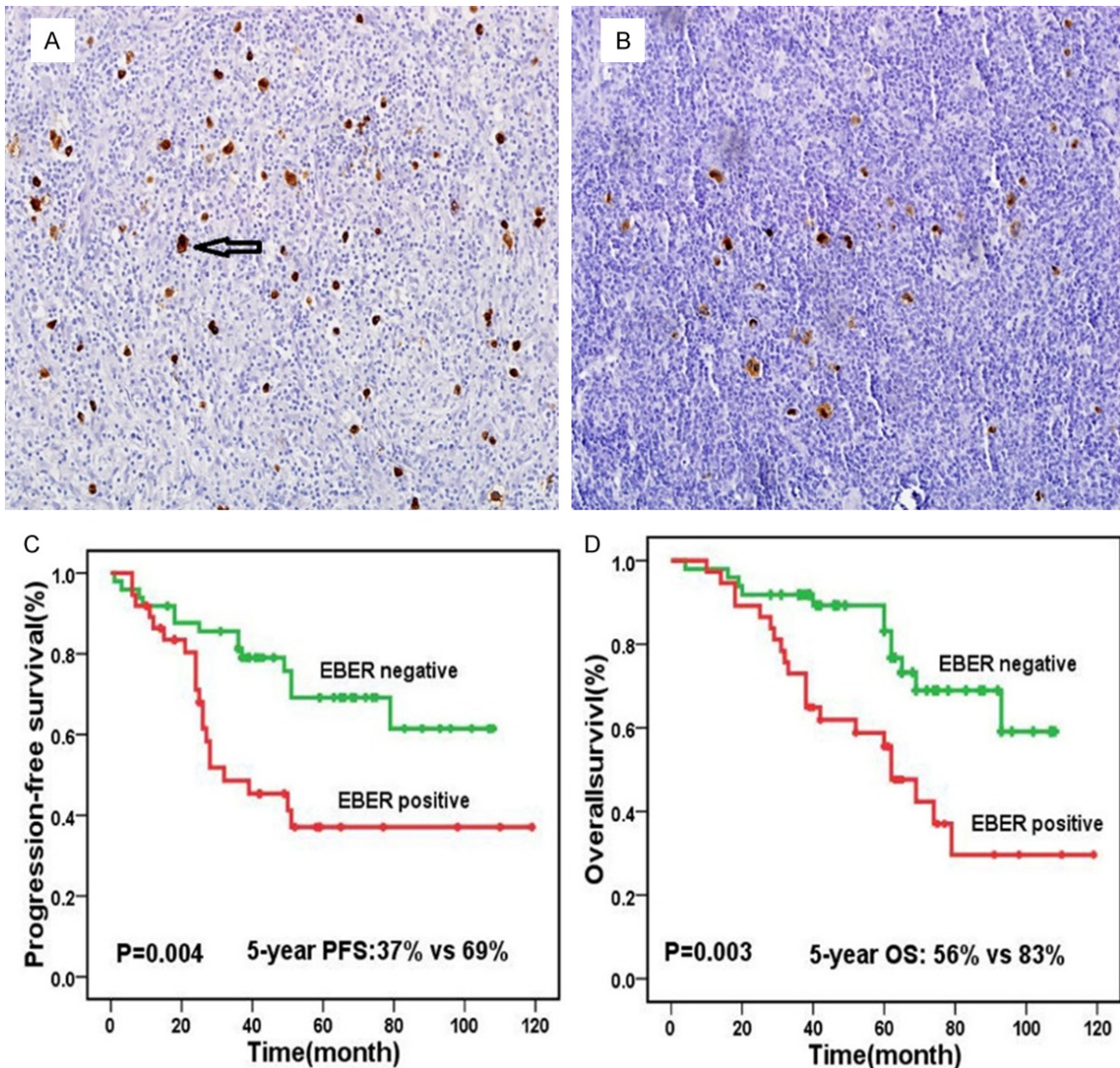
**Figure 3.** PD-1 expression and prognosis, positive (A) and negative (B) expression of PD-1 in Hodgkin Lymphoma (magnification  $\times 40$ ); Patients with PD-1-positive had no significant difference progression free survival than patients with PD-1-negative ( $P=0.624$ ) (C); PD-1-positive led to significantly worse overall survival compared with PD-1-negative ( $P=0.105$ ) (D).

CSF-1R expression revealed a positive impact on PFS and OS ( $P=0.001$  and  $P<0.001$ , respectively) (Figure 5).

### Discussion

Hodgkin lymphoma (HL) is characterized by massive reactive infiltrates surrounding HRS cells. Several studies reported that tumor microenvironment was positively related to HL prognosis [10]. Lymphoma-associated macrophages may have a prognostic role in several lymphoproliferative disorders, including cHL. High expression of the macrophage/monocyte-

related antigens CD68 and CD163 correlates with adverse outcomes and the presence of Epstein-Barr virus in the tumor cell population in classical HL [13]. CSF-1 is the most pleiotropic of the macrophage growth factors, stimulating the survival, proliferation and differentiation of mononuclear phagocytes and promoting the spread and motility of macrophages [21]. Furthermore, CSF-1R expression in the peri-tumoral area is associated with poor prognosis in leiomyosarcoma, prostate cancer [22, 23] and cHL [24, 25]. In cHL groups, CSF1/CSF1R signaling plays an essential role in the reciprocal crosstalk between tumor HRS cells



**Figure 4.** EBER expression and prognosis, positive (A) and negative (B) expression of EBER in Hodgkin Lymphoma (magnification  $\times 40$ ); Patients with EBER-positive had worse progression free survival than those with EBER-negative ( $P=0.006$ ) (C); EBER-positive led to significantly worse overall survival compared with EBER-negative ( $P=0.005$ ) (D).

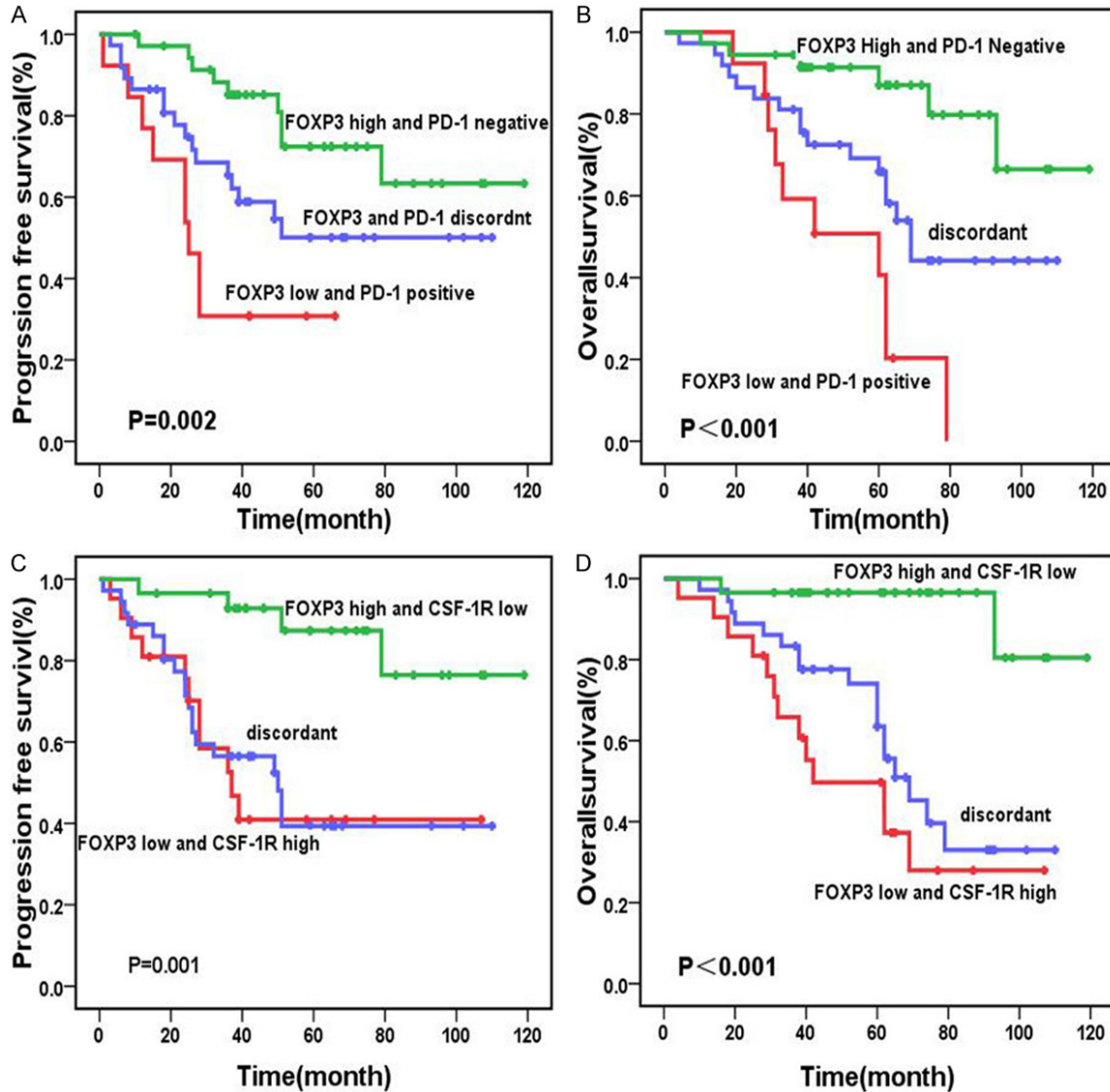
and microenvironment. Previous studies revealed anti-tumor activity following disruption of CSF1/CSF1R signaling, probably due to inhibition of the recruitment and activation of TAMs [26]. In this study, we validated that high CSF-1R expression in non-HRS cells led to an inferior prognosis in HL. In addition, we also found that expression of CSF-1R was associated with EBER status. Although no positive relationship was observed between CSF-1R expression and FOXP3 expression, the combination of CSF-1R and FOXP3 expression was predictive of survival.

T cells play a vital role in the immune system, especially in the tumor microenvironment.

Therefore, in this study, we selected PD-1 and FOXP3 as two important CD4+T cells in the microenvironment-related biomarkers. PD-1, expressed on T cells after activation, is one of the most important immune checkpoints, which mediate immune suppression [27]. The PD-1/PD-L axis is very important in tumor pathogenesis. Prognostic significance of PD-1 or PD-L1 expression has been evaluated in many solid tumors, including lymphoma [28]. PD-1 signaling results in “T-cell exhaustion”, which is essential to the pathogenesis of HL [29]. Increased number of PD-1 (+) tumor infiltrating lymphocytes have been found to be an independent negative prognostic factor for survival in HL patients [30]. Another study involv-



PD-1, FOXP3, CSF-1R, and Hodgkin lymphoma



**Figure 5.** Comparison of survival rates according to FOXP3/PD-1 and FOXP3/CSF-1R expression: Patients with a low number of FOXP3-positive cells and PD-1 positive cells showed significantly worse overall survival than patients with other expression patterns ( $P<0.001$ ) (A). Patients with a high number of FOXP3-positive and PD-1 negative cells manifested significantly better progression-free survival than those with other expression patterns ( $P=0.002$ ) (B). Patients with a low number of FOXP3-positive cells and high number of CSF-1R showed significantly worse overall survival than patients with the other expression patterns ( $P<0.001$ ) (C). Patients with a high number of FOXP3-positive and low number of CSF-1R manifested significantly better progression-free survival than those with the other expression patterns ( $P=0.001$ ) (D).

ing cHL found that macrophages constitute the major source of PD-L1. Macrophage-mediated upregulation of PD-1/PD-L1 signaling axis and suppression of anti-tumor immunity are critical factors inpatient prognosis. In our study, although OS was found to be shorter in PD-1-positive subgroups, there was no significant difference. Paydas S et al. [31] concluded that PD-1 and PD-L1 co-expression is associated with significantly shorter disease-free survival (DFS) and OS when compared with those with-

out PD expression. Furthermore, we found that EBV was involved in nearly 43% of HLs. EBV-positive status was related to CSF-1R and FOXP3 expression. Studies evaluating the association between EBV and PD-1/PD-L1 yielded complex results. EBV infection has been implicated in PD ligand expression. EBV-encoded LMP1 promoted PD-L1 expression in tumor cells [32]. However, in another HL study, they found that EBV status does not predict the presence or absence of PD-L1 expression [33].

In this study, we did not find an important relationship between EBV and PD-1.

FOXP3, representing the regulatory T cells (Tregs), plays a critical role in the modulation of immune response, particularly suppression of tumor-associated antigen-reactive lymphocytes [34]. Increased number of Tregs was correlated with unfavorable prognosis in non-small cell lung cancer [35] and ovarian carcinoma [36]. However, data regarding the importance of Tregs in the prognosis of cHL remain controversial. A few studies have shown that Tregs are a positive prognostic marker in cHL [16, 18]. However, Schreck et al. [37] showed that Tregs negatively influenced the prognosis with a high risk of relapse. In the present study, we found that high FOXP3 expression confers superior PFS and OS. We also found that the presence of EBV in HRS cells correlated with increased FOXP3 expression, which is consistent with other studies [38]. EBV-specific cytotoxic T cells (CTLs) are unable to eliminate EBV-infected tumor cells *in vivo* [39] due to an increase in the recruitment of Tregs, which play an essential role in controlling autoimmunity and inhibit the development of successful anti-tumoral immune response. Further, the expression of EBNA-1 in HL cells mediates upregulation of CCL20, facilitating migration of natural Tregs into the tumor [40]. To the best of our knowledge, our study is the first of its kind to report that the prognostic role played by the combination of FOXP3 and CSF-1R or PD-1 in the survival of HL patients, indicating a reciprocal relationship between microenvironment biomarkers [41]. Expression of PD-1, FOXP3 and CSF-1R requires further evaluation in a larger group of patients with HL. These biomarkers could facilitate diagnosis and predict treatment response in patients with HL.

In conclusion, our data show that high levels of FOXP3 expression are associated with superior survival. Increased CSF-1R level in non-HRS confers worse survival in HL. Further, the combination of FOXP3 and PD-1 or CSF-1R expression may improve prognostic stratification. The three biomarkers in tumor microenvironment represent new strategies for the diagnosis, prognosis and treatment of HL.

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#### Disclosure of conflict of interest

None.

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