Harb et al. Surgical and Experimental Pathology (2019) 2:15
https://doi.org/10.1186/s42047-019-0038-7

Surgical and Experimental Pathology

RESEARCH Open Access

Prognostic values and clinical implications of programmed cell death-ligand 1 (PD-L1), fork head transcription factor P-1 (*FOXP-1*) and signal transducer and activator of transcription-3 (*STAT-3*) expression in diffuse large B-cell lymphoma (DLBCL); an immunohistochemical study

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Abstract

Context: PD-L1 is an inhibitory ligand that functions as an essential immune checkpoint. *FOXP-1* is a member of the FOXP family. *STAT-3* plays a critical role in regulation of cell proliferation and survival. The detailed expression of the three markers together in DLBCL tissues and their prognostic value in patients with DLBCL were not fully investigated.
Aim was to assess the expression of PD-L1, *FOXP-1* and *STAT-3* in diffuse large B-cell lymphoma (DLBCL) and to correlate their expression with the pathological findings, prognostic parameters and clinical implications of patients.

Methods: PD-L1, *FOXP-1* and *STAT-3* were assessed in DLBCL tissues derived from 50 patients using immunohistochemistry. Patients were followed up for 3 years for response to therapy progression, recurrence and survival.

Results: High PD-L1 expression was associated with bone marrow involvement ($p = 0.004$), extra-nodal involvement ($p = 0.006$) and advanced stage ($p = 0.003$). High FOXP-1 expression was associated with presence of bone marrow involvement and high risk group ($p < 0.001$). High *STAT-3* expression was associated with older age of the patient ($p < 0.001$), presence of bone marrow involvement ($p = 0.002$), extra-nodal involvement ($p = 0.009$), and high risk group ($p = 0.005$). High expression of PD-L1, FOXP-1 and STAT-3 was related to poor response to therapy, poor OS rate and RFS rates ($p < 0.001$).

Conclusion: High expression of PD-L1, *FOXP-1* and *STAT-3* was related poor prognosis in DLBCL patients.

Keywords: DLBCL, PD-L1, *FOXP-1*, *STAT-3*, Immunohistochemistry, Prognosis

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Key messages

Detection of prognostic values PD-L1, *FOXP-1* and *STAT-3* expression in DLBCL.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the commonest histological sub-type of non-Hodgkin lymphomas (NHLs) which occur in adults (Liu et al. 2018). The World Health Organization (WHO) classification identified DLBCL as a lymphoid malignancy which is clinically and genetically heterogeneous that is classified into two major molecular subtypes based on gene expression pattern; germinal center B-cell-like and specifically activated B-cell-like (Sun et al. 2017). DBCL accounts for about 80 % of aggressive lymphomas (ZL et al. 2011). International Prognostic Index (IPI) is used to for prediction of its prognosis (Sehn et al. 2007), but its role is still limited (Talaulikar et al. 2009). The currently used standard chemotherapy regimen for management of DLCBL patients enhances response rates and can prolong the survival of patients but 43% of patients still fail to respond or show disease relapse or chemo-resistance. Therefore, the development of novel prognostic biomarkers which could be effectively applied to improve the classification and categorization of DLBCL patients according to severity and prognosis is considered an essential need (Liu et al. 2018). Programmed cell death-ligand 1 (PD-L1) which is a B7 family member (also known as B7-H1), is an inhibitory ligand that functions as an essential immune checkpoint which plays a role in the regulation of cellular, humoral and adaptive immune responses (LY et al. 2017). Aberrant PD-L1 expression was reported to be associated with adverse prognosis of many types of cancers (LY et al. 2017; Gao et al. 2017). Several previous studies have reported the expression of PD-L1 in lymphoma and described its relationship with DLBCL prognosis (Kiyasu et al. 2015; Westin et al. 2014). However, the prognostic role of PD-L1 expression in DLBCL is still uncertain. Fork-head box protein P-1 (*FOXP-1),* which is found to be a winged helix transcription factor, a FOXP family member and it belongs to the fork head transcription factor family. *FOXP-1* plays an important role in many biological processes, Moreover; it is considered a potential oncogene in pancreatic cancer, carcinoma of the liver, and various subtypes of B-cell non-Hodgkin lymphomas (van Keimpema et al. 2017). Several studies have evaluated the expression patterns of FOXP1 in DLBCL.

A few researchers assessed the correlations between expression of *FOXP-1* and the survival rates; however, the results remain conflicting (Yu et al. 2011). Members of Signal Transducer and Activators of Transcription (STAT) family play essential role in regulation of transcription signal transduction. Signal transducer and activator of transcription-3 *(STAT-3)* plays a critical role in regulation of cell proliferation and survival and transcription activation of angiogenesis (ZL et al. 2011). *STAT-3* is a transcription factor and an oncogene involved in cytokine growth signaling and cellular survival pathways (Silva 2004). However, the *STAT-3* expression in DLBCL tissues and its prognostic value in patients with DLBCL were not fully investigated. Previous studies have assessed the expression and prognostic values of each marker alone in DLBCL but our current study was the first one to assess the prognostic values of PD-L1, *FOXP-1* and *STAT-3* expression together in tumor cells of DLBCL patients.

The aims of this study; were to assess the expression of PD-L1, *FOXP-1* and *STAT-3* in diffuse large B-cell lymphoma (DLBCL) and to correlate their expression with the pathological findings, prognostic parameters and clinical implications of patients.

Patients and methods

For this prospective cohort study, we have included 50 patients diagnosed with DLBCL, which were surgically operated at the General Surgery Department, Oncology unit, Faculty of Medicine, Zagazig University, sent to Pathology department Faculty of Medicine, Zagazig University for processing, diagnosis, grading and staging. Patients were followed up till death or their most recent medical examination for survival, progression and recurrence of the disease after successful therapy in Clinical Oncology and Nuclear Medicine Department, in Medical Oncology Department, Faculty of Medicine, Zagazig University and in Internal Medicine Department, Hematology unit, Faculty of Medicine, Zagazig University for 3 years, from October 2015 to October 2018. Formalin fixed paraffin embedded tissue blocks of the 50 patients were retrieved from the Department of Pathology for immunohistochemical analyses. We have included all cases of DLBCL and excluded other types of lymphomas, as identified by a full panel of immunohistochemical markers. Among the excluded cases were the mediastinal large B-cell lymphomas (Med LBCL), and the EBV-positive diffuse large B-cell lymphomas (EBV-positive DLBCL), as they tend to display variable PD-L1 expression patterns and represent a different tumor type. All laboratory investigation were done at Clinical Pathology Department, Faculty of Medicine, Zagazig University.

The Lugano modification of the Ann Arbor staging system was used for pathologic staging (Cheson et al. 2014), and the World Health Organization classification was used for pathologic grading.

Immunohistochemical analysis

Technique of immunohistochemical staining

For immunohistochemistry (IHC) analysis, 3-mm-thick formalin fixed paraffin-embedded sections were mounted on positively charged slides. We performed de-waxing of the sections in xylene, dehydrating in ascending grades of

ethanol; we immersed the slides in methanol which contain 0.3% hydrogen peroxide for fifteen minutes to block endogenous peroxidase activity. Antigen retrieval was done for the slides by heating in an autoclave in citrated buffer (10 mM, pH 6.0) for about ninety seconds. The specimens were incubated for two hours at 37 °C with rabbit monoclonal anti PD-L1 (E1L3N) XP_ antibody (mAb) (Cell Signaling Technology, Danvers, MA, USA) 1:100 dilution, rabbit polyclonal anti-*FOXP-1* antibody 1:200 dilution (Abcam ab16645, Cambridge, UK); rabbit polyclonal anti--*STAT-3* antibody 1:100 dilution (sc-7179, Santa Cruz Biotechnology).

Evaluation of PDL-1, FOXP-1 and STAT3 immunohistochemical staining

We evaluated and assessed markers expression in tumor cells only and neglect any positivity in tumor infiltrating benign inflammatory cells. Evaluation of markers depends on the intensity and proportion of cells showing membranous staining and/or cytoplasmic staining for PD-L1, nuclear stain for *FOXP-1* and nuclear stain for *STAT-3*. The presence of subcellular localization for *STAT-3* staining in the nucleus and cytoplasm nuclear represents different cell compartments, where we included assessment of nuclear *STAT3* protein expression only as it is accepted as a marker of *STAT-3* activation and phosphorylation.

Previous history of hepatitis viral B infection was analyzed for all patients and correlated with markers expression.

The extent of markers expression was scored as follows: 0, no positive cells/high-power field; 1, fewer than 10% positive cells/ high-power field; 2, 10 to 30% positive cells/high-power field; 3, more than 30% positive cells/high power fields on average. The intensity of positive cells was scored as follows: 1, weak; 2, moderate; and 3, strong staining (ZL et al. 2011; LY et al. 2017; Kwon et al. 2016). Scores of the intensity and extent are multiplied to give the final stain score of values from 0 to 9, we used the cut off value of 3 above which is considered high expression and below which is considered low expression to facilitate statistical analysis. Each case was randomly selected at least 5 fields and quantified. Sections from adenocarcinoma of the colon were used as positive control for *PD-L1,* squamous cell carcinoma of the cervix as a positive control for *FOXP-1*, and invasive duct carcinoma of the breast as a *STAT-3* positive, while for negative controls; the primary antibodies were totally replaced by phosphate buffered saline. Both positive and negative controls were put on independent slides simultaneously. Figs. 1, 2 and 3.

Statistical analysis

The statistics were performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (Software bvba 13, Ostend, Belgium). Continuous variables were checked for normality by using Shapiro-Wilk test. Percent of categorical variables were compared using Pearson's Chi-square test or Fisher's exact test when was appropriate. Overall Survival (OS) was calculated as the duration from tumor diagnosis to death or the most new follow-up contact. Relapse Free Survival (RFS) was calculated as the time from start of treatment to date of relapse or the most recent follow-up contact that patient was known as relapse free.

Stratification of OS and RFS was done according to PDL-1, *FOXP-1* and *STAT3* expression in tumor cells.

These rates were estimated using the method of Kaplan-Meier plot, and compared using two-sided exact log-rank test. A p-value < 0.05 was considered significant.

Results

Clinicopathological results

Among the 50 patients with DLBCL there were 31 (62%) male and 19 (38%) female. All demographic data are shown in Table 1. Hepatitis B viral infection was negative in 42 (84%) of the cases; there was no association between this infection and the expression of any of the 3 markers.

Immunohistochemical results

PD-L1 expression and clinicopathological results Table 2, Fig. 1

High PD-L1 expression was present in 19 (38%) in DLBCL patients and its expression was positively correlated with older age of the patient ($p < 0.001$), presence of B symptoms, fever, weight loss, night sweating, bone marrow involvement ($p = 0.004$), bulky lymph nodes and extra-nodal involvement by the tumor ($p = 0.006$).

High expression of PD-L1 was present in 2 cases (18.2%) of stage I, 3 cases (18.8%) of stage II, 7 cases (53.8%) of stage III and 7 cases (70%) of stage IV, its expression was positively associated with advanced stage of the tumor ($p = 0.003$) and high IPI risk group ($p = 0.001$). No significant association was found between PD-L1 expression, sex of the patients or previous history of hepatitis B infection.

FOXP-1 expression and clinicopathological results Table 2, Fig. 2

High FOXP-1 expression was present in 21 (42%) in DLBCL and its expression was positively correlated with advanced age of the patient ($p < 0.001$), presence of B symptoms, presence of fever, weight loss, night seating, bone marrow involvement, bulky lymph nodes and extra-nodal involvement ($p < 0.001$).

High expression of *FOXP-1* was present in 2 cases (18.2%) of stage I, 3 cases (18.8%) of stage II, 8 cases (61%) of stage III and 8 cases (80%) of stage IV, its expression was positively associated with advanced stage of the tumor and high IPI risk group ($p < 0.001$). No significant association was found between FOXP1 expression,

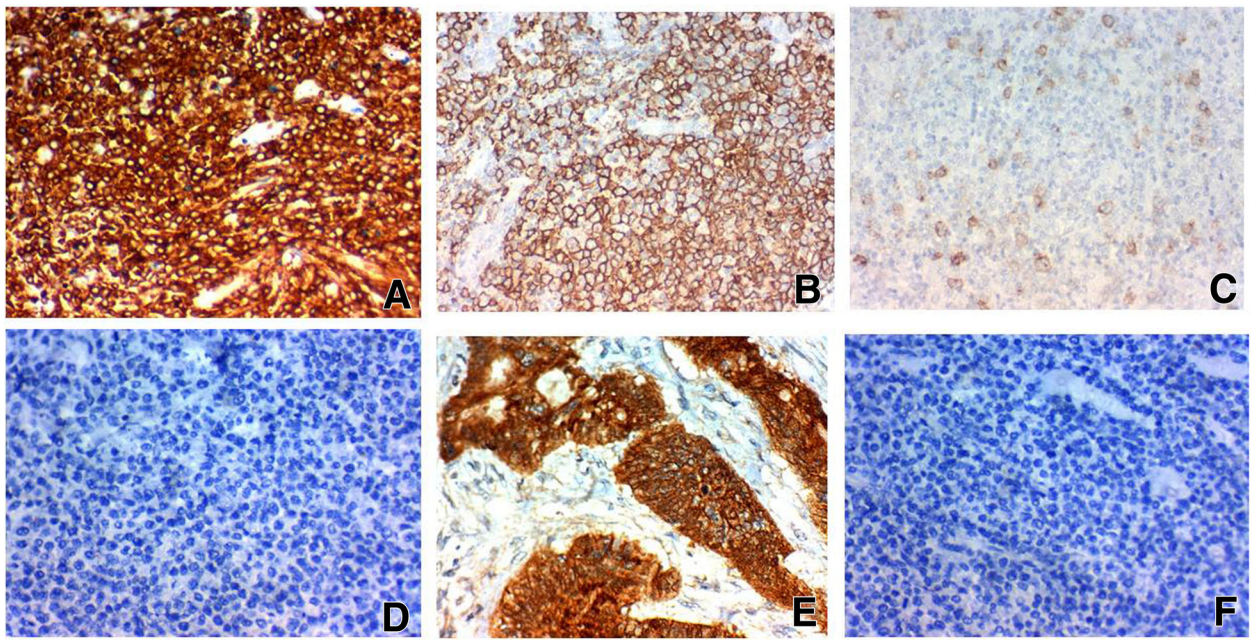

Fig. 1 Immunohistochemical expression of Programmed cell death-ligand 1 (PD-L1) (**a**) high cytoplasmic and membranous PD-L1 expression in DLBCL; stage IV × 400 (**b**) high cytoplasmic and membranous PD-L1 expression in DLBCL; stage III × 400 (**c**) low cytoplasmic and membranous PD-L1 expression in DLBCL; stage II × 400 (**d**) negative cytoplasmic and membranous PD-L1 expression in DLBCL; stage I × 400. (**e**) Positive cytoplasmic and membranous PD-L1 expression in sections from adenocarcinoma of the colon as appositive control of the marker × 400. (**f**) Negative control by replacement of the primary anti-PD-L1 antibody by phosphate buffered saline × 400

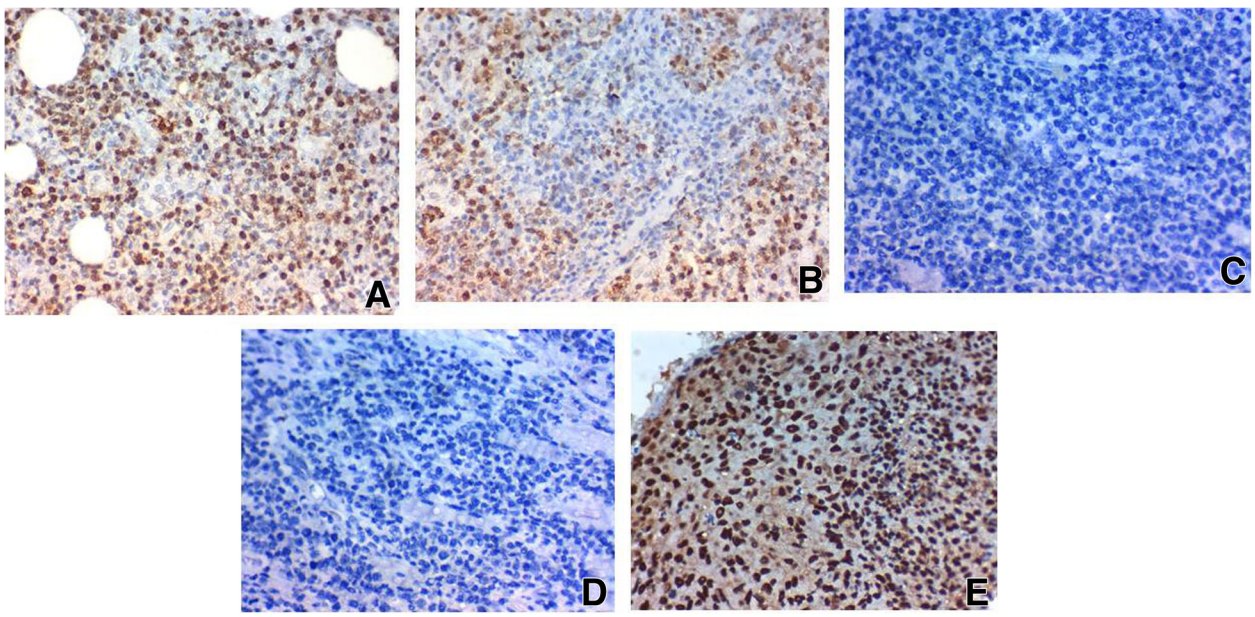

Fig. 2 Immunohistochemical expression of Fork head transcription factor (FOXP-1); (**a**) High nuclear *FOXP-1* expression in DLBCL stage IV × 400 (**b**) High nuclear *FOXP1* expression in DLBCL stage III × 400 (**c**) negative nuclear *FOXP-1* expression in DLBCL stage I × 400. (**d**) Positive nuclear *FOXP-1* expression in sections from squamous cell carcinoma of the cervix as appositive control of the marker × 400 (**e**) Negative control by replacement of the primary anti-*FOXP-1* antibody by phosphate buffered saline × 400

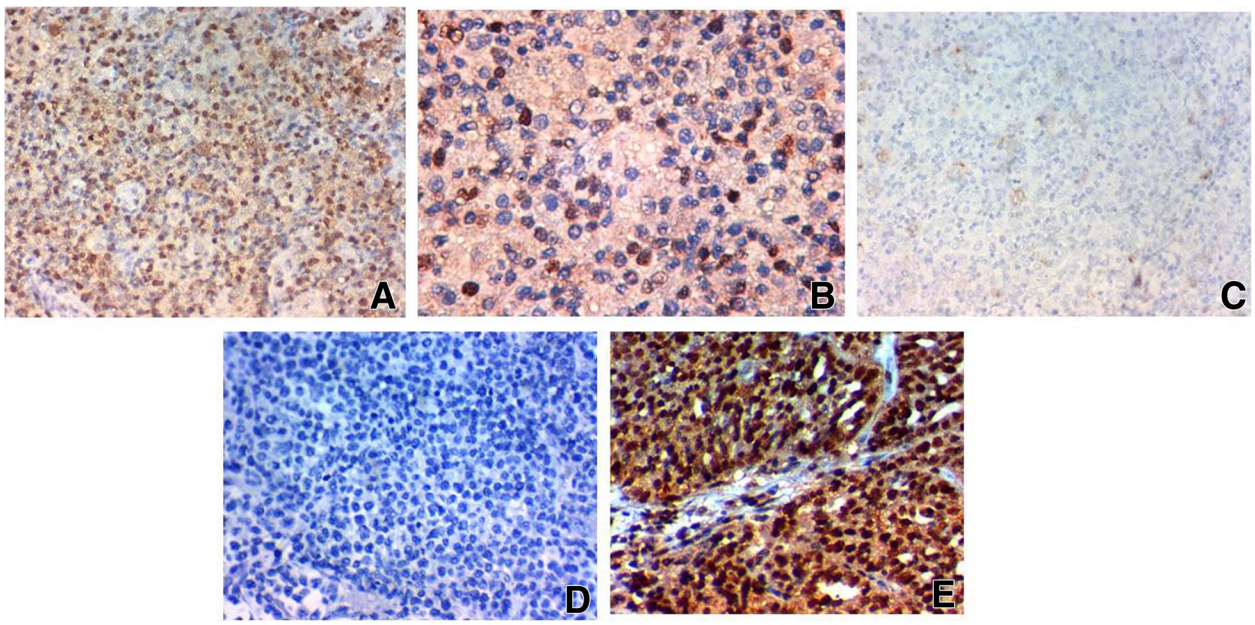

Fig. 3 Immunohistochemical expression of Signal transducer and activator of transcription-3 (STAT-3); (**a**) High nuclear *STAT-3* expression in DLBCL stage IV × 400 (**b**) High nuclear *STAT-3* expression in DLBCL stage III × 400 (**c**) negative nuclear STAT-3 expression in DLBCL stage I × 400 (**d**) positive nuclear *STAT-3* expression in sections from invasive duct carcinoma of the breast as appositive control of the marker × 400 (**e**) Negative control by replacement of the primary anti- *STAT-3* antibody by phosphate buffered saline × 400

sex of the patients or previous history of hepatitis B infection.

STAT-3 expression and clinicopathological results Table 2, Fig. 3

High *STAT-3* expression was present in 23 (46%) in DLBCL and its expression was positively correlated with advanced age of the patient ($p < 0.001$), presence of B symptoms, presence of fever, weight loss, night seating, bone marrow involvement ($p = 0.002$), bulky lymph nodes and extra-nodal involvement ($p = 0.009$),

High expression of *STAT-3* was present in 2 cases (18.2%) of stage I, 5 cases (31.2%) of stage II, 9 cases (69.2%) of stage III and 7 cases (70%) of stage IV, its expression was positively associated with advanced stage of the tumor ($p = 0.003$) and high IPI risk group ($p = 0.005$). No significant association was found between *STAT-3* expression, sex of the patients or previous history of hepatitis B infection.

Images for both positive and negative control of the three markers are all included in with figures of their corresponding markers.

Follow-up and survival results Table 3, Fig. 4

The detailed treatment regimens were taken according to the clinical stage and prognostic group. Chemotherapy, radiotherapy and/or combined modalities are described in Table 1 and we correlated the response of therapy to markers expression, we found that high expression of PD-L1, *FOXP-1* and *STAT-3* was related to absent response to chemotherapy, higher incidence of resistance to first line chemotherapy ($p = 0.13$, 0.030, 0.044 respectively) higher incidence of disease progression poor OS rate and RFS rates ($p < 0.001$).

We have found a positive association between PD-L1 and *FOXP-1*; Phi coefficient = + 0.753, PD-L1 and *STAT-3*; Phi coefficient = + 0.766, *FOXP1* and *STAT 3*; Phi coefficient = + 0.515 ($p < 0.001$).

Discussion

It has been suggested that immunotherapy directed against PD-1/PD-L1 axis could be combined with targeted therapy for adequate management and improvement of prognosis of patients' with different malignancies including DLBCL (Goodman et al. 2017; Jelinek et al. 2017; Ok and Young 2017). In the current study we have chosen to assess the expression of PD-L1 in tissues of such malignant lymphoid tumor, we assessed its expression in all cases of DLBCL and exclude assessing its level in Med LBCL, and in EBV-positive cases as such subtypes tend to display variable PD-L1 expression patterns and are considered a different tumor type.

We have proved that PD-L1 is overexpressed in tumor cells of DLBCL patients and its expression is related to poor prognosis and dismal patients' outcome which was similarly, results of many previous reports (ZL et al. 2011; Gravelle et al. 2017; Jo et al. 2017; Xing et al. 2016). Moreover, we proved that, PD-L1 was found to

Table 1 Clinicopathological features, PDL1, FOXP1, STAT3 expression and outcome of our patients

Parameters	All studied patients (*N* = 50)		Parameters	All studied patients (N = 50)	
	No.	%		No.	%
Age			PD-L1		
< 40 years	8	16%	Low	31	62%
40–60 years	20	40%	High	19	38%
61–74 years	17	34%			
≥ 75 years	5	10%			
Sex			FOXP-1		
Male	31	62%	Low	29	58%
Female	19	38%	High	21	42%
History of Hepatitis B			STAT-3		
Absent	42	84%	Low	27	54%
Present	8	16%	High	23	46%
B symptoms			Received regimen		
Absent	31	62%	No	2	4%
Present	19	38%	CVP	7	14%
Fever			RCVP	8	16%
Absent	31	62%	CHOP	18	36%
Present	19	38%	RCHOP	15	30%
Weight loss			Number of cycles		
Absent	31	62%	4 cycles	7	14%
Present	19	38%	4–6 cycles	23	46%
Night sweating			6–8 cycles	20	40%
Absent	31	62%			
Present	19	38%			
ECOG Ps			Involved field RT		
ECOG 1	37	74%	No	28	56%
ECOG 2–4	13	26%	Yes	22	44%
Bulky nodes			Dose		
Absent	23	46%	No	28	56%
Present	27	54%	30Gy	9	18%
Extranodal involvement			36Gy	6	12%
Absent	23	46%	40Gy	7	14%
Present	27	54%			
Bone marrow involvement			Response		
Absent	31	62%	PD	3	6%
Present	19	38%	SD	2	4%
LDH			PR	3	6%
≤ ULN	20	40%	CR	42	84%
> ULN - <3xULN	14	28%	NR	5	10%
> 3xULN	16	32%	OAR	45	90%
Stage			Follow-up months		
Stage I	11	22%	Mean ± SD	28.82	±8.98
Stage II	16	32%	Median Range	35	(10–36)

Table 1 Clinicopathological features, PDL1, FOXP1, STAT3 expression and outcome of our patients *(Continued)*

Parameters	All studied patients (*N* = 50)		Parameters	All studied patients (N = 50)	
	No.	%		No.	%
Stage III	13	26%	Relapse	(*N* = 42)	
Stage IV	10	20%	Absent	28	66.7%
IPI risk group			Present	14	34%
Low	22	44%	Mortality		
Low – Intermediate	5	10%	Alive	33	66%
High – Intermediate	7	14%	Died	17	34%
High	16	32%			

Categorical variables were expressed as number (percentage)
Continuous variables were expressed as mean ± SD & median (range)

be an independent predictor for poor OS, additionally; PD-L1 expression in tumor cells was associated with advanced clinical stage as it was expressed in stages; III and IV more than stages; I and II, demonstrating its role as an adverse prognostic factor in DLBCL. Kiyasu et al., showed results also similar to ours; PD-L1 expression level was positively correlated with the poor clinical outcomes of patients with DLBCL (Kiyasu et al. 2015). Fang et al., add to the previous results that low PD-L1 expression in DLBCL tissues is associated with favorable prognosis of some cases of such malignancy (Fang et al. 2017). In the Hu et al., study have found that PD-L1 expression in tumor cells was significantly associated with poor prognosis (ZL et al. 2011). We also found that PD-L1 expression in tumor cells was associated with resistance to first line chemotherapy, similarly, Hu et al., showed that PD-L1 expression is associated with chemoresistance (ZL et al. 2011). DLBCL have several pathways to escape from the antitumor immunity which is essential in these aggressive lymphomas which explain the reason of PD-L 1 overexpression and association with poor prognosis in such malignancy (Song et al. 2016). The mechanisms explaining the relationship between increased PD-L1 expression and poor prognosis of DLBCL patients are still not clear (ZL et al. 2011). The activation of Janus kinase *(JAK)/ STAT3* signaling increased the constitutive expression of PD-L1 in DLBCL and leads to poor outcome (ZL et al. 2011; Green et al. 2010). So, immunotherapies that are targeting the *JAK/STAT3* signaling pathway might benefit patients with such aggressive subtype of disease. Most DLBCL patients are cured with standard chemo-immunotherapy but about 30% still die of due to disease progression which needed a novel targeted therapy to improve their outcome (Juárez-Salcedoa et al. 2017). The first published experience with check point inhibition in DLBCL was with ipilimumab (anti-CTL4 antibody) (Ansell et al. 2009). It was found that the successful uses of PD1/PD-L1 block-age in solid tumors with advanced stages generally, and in DLBCL particularly, have established a new era in oncologic therapeutics by altering one of the hallmarks of cancer; the tumor immune evasion. But, the principles of PD1/PD-L1mediated immune evasion and checkpoint inhibition cannot be generalized on all lymphomas, as they have relatively different natural history. So in the current study we have specified results on DLBCL which is an example of aggressive high grade lymphomas which have high proliferative activity and they can be cured with intensive courses of combined chemo-immuno-therapy regimens. Graveled et al., (Gravelle et al. 2017) reviewed the results of previous studies about PD-L1 expression in lymphomas and have concluded similar results to us and, that hence PD-L1 overexpression is associated with the poorest prognosis in several types of aggressive NHLs; namely DLBCL, so, monoclonal antibodies which are selectively blocking the PD-1/PD-L1 axis could preserve tumor infiltrating lymphocytes (TILs) from exhaustion and could promote antitumor immunity as an effective therapeutic strategy for NHL generally and DLBCL particularly. Moreover the cell surface expression of PD-1/PD-L1 is considered a critical determinant for the identification of DLBCL patients who will be eligible for immune checkpoint blockade therapies. To add to results of our study we assessed another marker which is realted to disturbed tumor immunity; FOXP-1, as it was found that dys-regulation of *FOXP-1* expression is seen in tissues of many tumors (Yu et al. 2011).

Many previous researches regarding FOXP-1 expression have been limited to nodal DLBCL. But, few data were available regarding the FOXP-1 expression in extra-nodal DLBCL. Our current study was the first one which investigated the expression of *FOXP1* in DLBCL which occurred both in lymph nodes and in extra-nodal sites, and we proved that high expression of *FOXP-1* in nuclei of tumor cells of patients with DLBCL was associated with poor pathological criteria as advanced patients stage, poor patients outcome, poor survival rates and

Table 2 Correlation between clinicopathological features, PDL-1, FOXP-1, STAT-3 expression in our patients

Characteristics	All	PD-L1		*p*-value	FOXP-1		*p*-value	STAT-3		*p*-value
	(N = 50)	Low (*N* = 31)	High (*N* = 19)		Low (*N* = 29)	High (*N* = 21)		Low (*N* = 27)	High (*N* = 23)	
	No. (%)	No. (%)	No. (%)		No. (%)	No. (%)		No. (%)	No. (%)	
Age										
< 40 years	8 (16%)	8 (100%)	0 (0%)	< 0.001[b]	8 (100%)	0 (0%)	< 0.001[b]	6 (75%)	2 (25%)	0.003[b]
40–60 years	20 (40%)	16 (80%)	4 (20%)		16 (80%)	4 (20%)		14 (70%)	6 (30%)	
61–74 years	17 (34%)	6 (35.5%)	11 (64.7%)		4 (23.5%)	13 (76.5%)		7 (41.2%)	10 (58.8%)	
≥ 75 years	5 (10%)	1 (20%)	4 (80%)		1 (20%)	4 (80%)		0 (0%)	5 (100%)	
Sex										
Male	31 (62%)	20 (64.5%)	11 (35.5%)	0.640[a]	19 (61.3%)	12 (38.7%)	0.547[a]	19 (61.3%)	12 (38.7%)	0.186[a]
Female	19 (38%)	11 (57.9%)	8 (42.1%)		10 (52.6%)	9 (47.4%)		8 (42.1%)	11 (57.9%)	
History of Hepatitis B										
Absent	42 (84%)	27 (64.3%)	15 (35.7%)	0.459[a]	27 (64.3%)	15 (35.7%)	0.056[a]	25 (59.5%)	17 (40.5%)	0.121[a]
Present	8 (16%)	4 (50%)	4 (50%)		2 (25%)	6 (75%)		2 (25%)	6 (75%)	
B symptoms										
Absent	31 (62%)	24 (77.4%)	7 (22.6%)	0.004[a]	24 (77.4%)	7 (22.6%)	< 0.001[a]	22 (71%)	9 (29%)	0.002[a]
Present	19 (38%)	7 (36.8%)	12 (63.2%)		5 (26.3%)	14 (73.7%)		5 (26.3%)	14 (73.7%)	
Fever										
Absent	31 (62%)	24 (77.4%)	7 (22.6%)	0.004[a]	24 (77.4%)	7 (22.6%)	< 0.001[a]	22 (71%)	9 (29%)	0.002[a]
Present	19 (38%)	7 (36.8%)	12 (63.2%)		5 (26.3%)	14 (73.7%)		5 (26.3%)	14 (73.7%)	
Weight loss										
Absent	31 (62%)	24 (77.4%)	7 (22.6%)	0.004[a]	24 (77.4%)	7 (22.6%)	< 0.001[a]	22 (71%)	9 (29%)	0.002[a]
Present	19 (38%)	7 (36.8%)	12 (63.2%)		5 (26.3%)	14 (73.7%)		5 (26.3%)	14 (73.7%)	
Night sweating										
Absent	31 (62%)	24 (77.4%)	7 (22.6%)	0.004[a]	24 (77.4%)	7 (22.6%)	< 0.001[a]	22 (71%)	9 (29%)	0.002[a]
Present	19 (38%)	7 (36.8%)	12 (63.2%)		5 (26.3%)	14 (73.7%)		5 (26.3%)	14 (73.7%)	
ECOG Ps										
ECOG 1	37 (74%)	27 (73%)	10 (27%)	0.018[a]	27 (73%)	10 (27%)	< 0.001[a]	23 (62.2%)	14 (37.8%)	0.051[a]
ECOG 2–4	13 (26%)	4 (30.8%)	9 (69.2%)		2 (15.4%)	11 (84.6%)		4 (30.8%)	9 (69.2%)	
Bulky nodes										
Absent	23 (46%)	19 (82.6%)	4 (17.4%)	0.006[a]	19 (82.6%)	4 (17.4%)	0.001[a]	17 (73.9%)	6 (26.1%)	0.009[a]
Present	27 (54%)	12 (44.4%)	15 (55.6%)		10 (37%)	17 (63%)		10 (37%)	17 (63%)	
Extranodal involvement										
Absent	23 (46%)	19 (82.6%)	4 (17.4%)	0.006[a]	19 (82.6%)	4 (17.4%)	0.001[a]	17 (73.9%)	6 (26.1%)	0.009[a]
Present	27 (54%)	12 (44.4%)	15 (55.6%)		10 (37%)	17 (63%)		10 (37%)	17 (63%)	
Bone marrow involvement										
Absent	31 (62%)	24 (77.4%)	7 (22.6%)	0.004[a]	24 (77.4%)	7 (22.6%)	< 0.001[a]	22 (71%)	9 (29%)	0.002[a]
Present	19 (38%)	7 (36.8%)	12 (63.2%)		5 (26.3%)	14 (73.7%)		5 (26.3%)	14 (73.7%)	
LDH										
≤ ULN	20 (40%)	18 (90%)	2 (10%)	< 0.001[b]	18 (90%)	2 (10%)	< 0.001[b]	16 (80%)	4 (20%)	0.003[b]
> ULN - <3xULN	14 (28%)	9 (64.3%)	5 (35.7%)		8 (57.1%)	6 (42.9%)		6 (42.9%)	8 (57.1%)	
> 3xULN	16 (32%)	4 (25%)	12 (75%)		3 (18.8%)	13 (81.2%)		5 (31.2%)	11 (68.8%)	
Stage										
Stage I	11 (22%)	9 (81.8%)	2 (18.2%)	0.003[b]	9 (81.8%)	2 (18.2%)	0.001[b]	9 (81.8%)	2 (18.2%)	0.003[b]
Stage II	16 (32%)	13 (81.2%)	3 (18.8%)		13 (81.2%)	3 (18.8%)		11 (68.8%)	5 (31.2%)	

Table 2 Correlation between clinicopathological features, PDL-1, FOXP-1, STAT-3 expression in our patients *(Continued)*

Characteristics	All	PD-L1		*p*-value	FOXP-1		*p*-value	STAT-3		*p*-value
	(N = 50)	Low (*N* = 31)	High (*N* = 19)		Low (*N* = 29)	High (*N* = 21)		Low (*N* = 27)	High (*N* = 23)	
	No. (%)	No. (%)	No. (%)		No. (%)	No. (%)		No. (%)	No. (%)	
Stage III	13 (26%)	6 (46.2%)	7 (53.8%)		5 (38.5%)	8 (61.5%)		4 (30.8%)	9 (69.2%)	
Stage IV	10 (20%)	3 (30%)	7 (70%)		2 (20%)	8 (80%)		3 (30%)	7 (70%)	
IPI risk group										
Low	22 (44%)	19 (86.4%)	3 (13.6%)	0.001[b]	19 (86.4%)	3 (13.6%)	< 0.001[b]	17 (77.3%)	5 (22.7%)	0.005[b]
Low – Intermediate	5 (10%)	3 (60%)	2 (40%)		3 (60%)	2 (40%)		3 (60%)	2 (40%)	
High – Intermediate	7 (14%)	3 (42.9%)	4 (57.1%)		3 (42.9%)	4 (57.1%)		1 (14.3%)	6 (85.7%)	
High	16 (32%)	6 (37.5%)	10 (62.5%)		4 (25%)	12 (75%)		6 (37.5%)	10 (62.5%)	
PD-L1										
Low	31 (62%)				27 (87.1%)	4 (12.9%)	< 0.001[a]	26 (83.9%)	5 (16.1%)	< 0.001[a]
High	19 (38%)				2 (10.5%)	17 (89.5%)		1 (5.3%)	18 (94.7%)	
FOXP1										
Low	29 (58%)	27 (93.1%)	2 (6.9%)	< 0.001[a]				22 (75.9%)	7 (24.1%)	< 0.001[a]
High	50 (42%)	4 (19%)	17 (81%)					5 (23.8%)	16 (76.2%)	
STAT3										
Low	27 (54%)	26 (96.3%)	1 (3.7%)	< 0.001[a]	22 (81.5%)	5 (18.5%)	< 0.001[a]			
High	23 (46%)	5 (21.7%)	18 (78.3%)		7 (30.4%)	16 (69.6%)				

Categorical variables were expressed as number (percentage); [a] Chi-square test; [b] Chi-square test for trend; $p < 0.05$ is significant

resistance to chemotherapy in addition to positive association with PD-L1 expression in tumor cells. Similarly Yu et al., showed that FOXP-1 was, expressed both in nodal and extra-nodal DLBCLs and they concluded that *FOXP-1* is involved in the progression of both nodal and extra-nodal DLBCL, but the mechanisms its function could be different (Yu et al. 2011), moreover our study demonstrates that evaluation of *FOXP-1* tissue protein expression in DLBCL was of greater prognostic importance regarding worse OS rate that was similar to results of previous studies (Bellas et al. 2014; Banham et al. 2005; Barrans et al. 2004; Sagaert et al. 2006; Hoefnagel et al. 2006; Kodama et al. 2005), while others found that tissue protein expression of *FOXP-1* had no effect on pathological criteria, clinical outcomes, or survival rates (Davis et al. 2001; Lam et al. 2008), such apparent discrepancy might be explained by different criteria in detecting antigen expression and different cutoff values (Barrans et al. 2004). Sagaert et al. found that increased tissue protein expression of FOXP-1 is a significant predictor of unfavorable clinical outcome in MALT lymphoma which is at risk of transforming into aggressive subtypes of DLBCLs (Sagaert et al. 2006). There are many mechanisms which may be incriminated in FOXP-1 deregulation in DLBCL. Primarily, there is a reported significant association between the high FOXP-1 expression and high proliferation rates, between the high FOXP1 expression MYC overexpression in DLBCL tissues (Bellas et al. 2014). Additionally, *FOXP-1* was associated with the expression of Bcl2 (Xing et al. 2016), and in plethora of patients with DLBCL, Bcl2 up-regulation may be mediated through NF-κB pathway that is continuously expressed and has an essential role in its pathogenesis, progression and poor prognosis (Davis et al. 2001). As we have found a positive correlation between PD-L1 expression and *FOXP-1* expression and as both markers were associated with poor prognosis in DLBCL patients, so there is a role of immune dysfunction in such malignancy and both agents may be used as novel prognostic parameters of such cancer as proved by findings of our study. Due to conflicting results of previous studies regarding the prognostic role of both markers, we assessed the prognostic role of a third marker with them to strengthen the assessment of their prognostic role in DLBCL patients that is *STAT-3*. Up to our knowledge, it is the first study that assessed those three markers expression in DLBCL tissues using immunohistochemistry. Previous studies proved that DLBCL had the highest level of *STAT-3* mRNA and such expression is associated with dismal outcome of those patients (Sun et al. 2017; Lam et al. 2008; Scuto et al. 2011). In the study, we investigated the tissue protein expression level of STAT-3 in DLBCL and we have proved that high expression is associated with advanced stage, dismal patients' outcome and poor survival rates. Our study and ZL et al., demonstrated the possibility of using immunohistochemistry to detect tissue protein *STAT-3*

Table 3 Correlation between PD-L1, *FOXP-1* and *STAT-3* expression and outcome of our patients

Characteristics	All	PD-L1		*p*-value	FOXP-1		*p*-value	STAT-3		*p*-value
	(N = 50)	Low (N = 31)	High (N = 19)		Low (N = 29)	High (N = 21)		Low (N = 27)	High (N = 23)	
	No. (%)	No. (%)	No. (%)		No. (%)	No. (%)		No. (%)	No. (%)	
Response										
PD	3 (6%)	1 (3.2%)	2 (10.5%)	0.013[a]	1 (3.4%)	2 (9.5%)	0.030[a]	0 (0%)	3 (13%)	0.044[a]
SD	2 (10.5%)	0 (0%)	2 (10.5%)		0 (0%)	2 (9.5%)		1 (3.7%)	1 (4.3%)	
PR	3 (6%)	0 (0%)	3 (15.8%)		0 (0%)	3 (14.3%)		0 (0%)	3 (13%)	
CR	42 (84%)	30 (96.8%)	12 (63.2%)		]28 (96.6%)	14 (66.7%)		26 (96.3%)	16 (69.6%)	
NR	5 (10%)	1 (3.2%)	4 (21.1%)	0.062[a]	1 (3.4%)	4 (19%)	0.148[a]	1 (3.7%)	4 (17.4%)	0.467[a]
OAR	45 (90%)	30 (96.8%)	15 (78.9%)		28 (96.6%)	17 (81%)		26 (96.3%)	19 (82.6%)	
Relapse	(N = 42)	(*N* = 30)	(*N* = 12)		(*N* = 28)	(*N* = 14)		(*N* = 26)	(*N* = 16)	
Absent	28 (66.7%)	26 (86.7%)	2 (16.7%)	< 0.001[a]	27 (96.4%)	1 (7.1%)	< 0.001[a]	22 (84.6%)	6 (37.5%)	0.002[a]
Present	14 (33.3%)	4 (13.3%)	10 (83.3%)		1 (3.6%)	13 (92.9%)		4 (15.4%)	10 (62.5%)	
RFS										
Mean (months)	30.11	33.72	20.35	< 0.001[b]	35.81	18.42	< 0.001[b]	35.81	18.42	< 0.001[b]
(95%CI)	(27.27–32.95)	(31.42–36.03)	(15.42–25.27)		(35.45–36.17)	(14.32–22.52)		(35.45–36.17)	(14.32–22.52)	
1 year RFS	90.5%	96.7%	75%		100%	71.4%		96.2%	81.3%	
2 year RFS	73.6%	90%	30%		100%	21.4%		88.5%	48.6%	
3 year RFS	66.1%	86.5%	10%		96.3%	7.1%		84.4%	34.7%	
Mortality	(N = 50)	(N = 31)	(N = 19)		(N = 29)	(N = 21)		(N = 27)	(N = 23)	
Alive	33 (66%)	29 (93.5%)	4 (21.1%)	< 0.001[a]	29 (100%)	4 (19%)	< 0.001[a]	24 (88.9%)	9 (39.1%)	< 0.001[a]
Died	17 (34%)	2 (6.5%)	15 (78.9%)		0 (0%)	17 (81%)		3 (11.1%)	14 (60.9%)	
OS										
Mean (months)	29.78	34.77	21.18	< 0.001[b]	36	21.09	< 0.001[b]	33.92	24.80	< 0.001[b]
(95%CI)	(27.23–32.34)	(33.12–36.42)	(17.37–24.99)			(17.51–24.68)		(31.70–36.14)	(20.72–28.89)	
1 year OS	92%	100%	78.9%		100%	81%		100%	82.6%	
2 year OS	69.8%	93.5%	29.6%		100%	28.6%		88.9%	46.8%	
3 year OS	65.7%	93.5%	17.8%		100%	19%		88.9%	37.5%	

Continuous variables were expressed as mean (95%CI); categorical variables were expressed as number (percentage); [a] Chi-square test; [b] Log rank test; $p < 0.05$ is significant

expression in routine pathologic specimens that might enable us to identify DLBCL cases with poor clinical outcome, and subsequently guides us to make more intensive treatment for those patients (ZL et al. 2011). Additionally, we have shown that high nuclear expression of *STAT-3* in DLBCL was correlated with poor overall survival rate, especially in patients receiving CHOP regimen, which was similar to results of; Sun et al., ZL et al and Huang et al., who found similar results and that targeting the *STAT-3* pathway might lead to reverse CHOP resistance in patients with DLBCL (Sun et al. 2017; ZL et al. 2011; Huang et al. 2013).

Lam et al. reported that DLBCL had higher level of *STAT3* mRNA and protein levels as detected with immunohistochemistry and such high levels were associated with poor outcome and with inferior overall survival (Lam et al. 2008). Such association is explained by that STAT-3 regulates the expression of plethora of genes like, survivin, bcl-xl, mcl-1which modulate cell survival, and proliferation, *c-myc, cyclin D1, p21, cyclin E* and matrix metalloproteinase-9 which control malignant cells invasion and metastasis (Ding et al. 2008), vascular endothelial growth factor which control angiogenesis (Dechow et al. 2004). Moreover, STAT-3 found to suppress the anti-tumor immune responses (Baran-Marszak et al. 2010; Kujawski et al. 2008; Kortylewski et al. 2009; Wang et al. 2009) and could regulate many cancer-promoting inflammatory mediators that could initiate and promote ontogenesis, genetic and epigenetic changes in cancer cells (Yin et al. 2011; Ok et al. 2014).

We have found a positive association between expression of PD-L1 and *FOXP-1* in DLBCL which is similar to results of Anastasiadou et al. that found similar results in NSCLC (Anastasiadou et al. 2018). We found a positive association between PD-L1 and *STAT-3* expression in

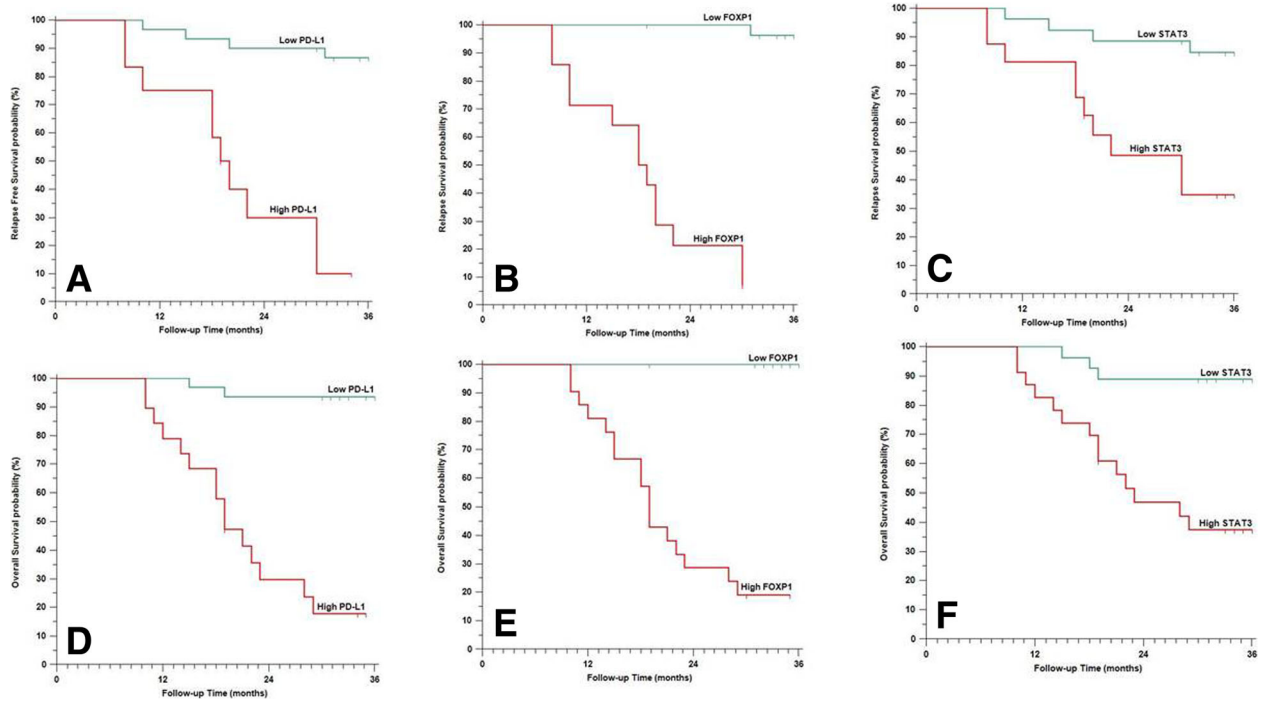

Fig. 4 Kaplan Meier plot (**a**) Relapse Free Survival Stratified by PD-L1, (**b**) Relapse Free Survival Stratified by *FOXP-1* (**c**) Relapse Free Survival Stratified by STAT3. (**d**) Overall Survival Stratified by PD-L1, (**e**) Overall Survival Stratified by FOXP1 (**f**) Overall Survival Stratified by *STAT-3*

DLBCL which was similar to results of Song et al., that performed novel functional and structural characterization of *STAT-3* activating mutations and detected a regulatory role of *STAT-3* activation in PD-L1 expression and both of them is associated with poor prognosis (Song et al. 2018). Targeting *STAT-3* could have a synergistic effect in immune checkpoint blockade therapy. To our knowledge, we are the first study that assessed the association between PD-L1, *FOXP-1* and *STAT-3* tissue protein expression in DLBCL patients.

Previous studies assessed each two markers together in lymphoma and other solid tumors (Song et al. 2018; Atsaves et al. 2017; Kataoka et al. 2016; Bu et al. 2017), but our findings regarding their expression in DLBCL were novel and detect positive correlations between the PD-L1, *FOXP-1* and *STAT-3* and all are associated with poor prognosis and dismal outcome of patients.

Conclusion

We have evaluated the expression of PD-L1, *FOXP-1* and *STAT-3* in tissues of DLBCL patients and we have correlated their expression with pathological, clinical and follow-up data of patients and we have found a positive association between their expression and poor pathological criteria, advanced clinical stage, poor response to therapy and dismal outcome of patients. Our results point to the possibility of using such markers as novel prognostic markers of such malignancy which might help the better classification of patients and improve DLBCL patients' prognosis.

Limitations of the work and recommendations

The limitations of our study are; the small number of included cases in addition to difficulty in separating neoplastic cell from infiltrating reactive cells during immunohistochemical evaluation of markers expression so we have recommended performing a large scale study on a huge number of patients using different methods of assessment as genetic studies to prove our results and improve the findings.

It remains unclear whether high levels of expression of PD-L1, *FOXP-1* and *STAT-3* plays a key role that is associated with the clinical outcome of DLBCL patients, so further integration of genomic and clinical data is needed to deepen our understanding of their roles in DLBCL.

Abbreviations

CR: Complete response; DLBCL: Diffue large B-cell lymphoma; EBV-positive DLBCL: EBV-positive diffuse large B-cell lymphomas; *FOXP-1*: Forkhead transcription factor; IHC: For immunohistochemistry; IPI: International Prognostic Index; Med LBCL: Mediastinal large B-cell lymphomas; NR: No response; OAR: Overall response; OS: Overall Survival; PD: Progressive disease; PD-L1: Programmed cell death-ligand 1; PR: Partial response; RFS: Relapse Free Survival; SD: Stable disease; *STAT-3*: Signal transducer and activator of transcription-3

Acknowledgements

Not applicable

Funding
No funds were received

Availability of data and materials
All data are available and acquired by the authors from Zagazig University, Faculty of Medicine, Departments

Authors' contributions
All authors contributed in idea design, data collection, writing, statistical analysis and revesion of the manuscript before publication. All authors read and approved the final manuscript.

Authors' information
not applicable

Ethics approval and consent to participate
The study complied with the guidelines of the local ethics committee of Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Consent for publication
Were taken from all the contributing authors

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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Received: 8 January 2019 Accepted: 23 April 2019
Published online: 01 June 2019

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