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STATUS OF (PD-L1) EXPRESSION IN HODGKIN LYMPHOMAIN A SET OF IRAQI PATIENTS

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ABSTRACT

Introduction: Hodgkin Lymphoma is a hematolymphoid malignancy that can affect any age group worldwide. Both genders can be affected by Hodgkin lymphoma with slight predilection for male gender. Involvement by Hodgkin lymphoma range from single node to wide spread disease. Although it has a successful story to response to conventional therapies, there still a proportion of patients who are refractory to primary therapy and those who relapse after prolong courses of therapy. The aim of study is to Show PD-L1 expression in Iraqi Hodgkin lymphoma. PD-L1 expression and Hodgkin lymphoma clinicopathologic status should be correlated. PD-L1 expression in primary and refractory/relapsed patients. **Method:** This cross-sectional study gathered thirty paraffin blocks from thirty Hodgkin lymphoma patients from the histo-pathological lab. Two 5-µm portions per block. One segment was stained with hematoxylin and eosin and the other with monoclonal rabbit anti-Human PD-L1 E1L3N[R] antibody. The positive threshold was 1%, hence the case was termed negative when <1% of tumour cells expressed PD-L1, low when 1-49%, and high when > 50%. Negative, weak, moderate, and severe staining were rated. **Results:** 90% of patients had positive PD-L1 and 10% had negative. PD-L1 expression was high (73.3%) and low (16.7%) in those staining positively. PD-L1 staining correlated with age ($P < 0.05$), whereas gender, Hodgkin lymphoma histologic subtypes, clinical stage, initial disease, refractory/relapsed condition, and tumour cell CD30 and CD15 expression did not. **Conclusion:** Most Hodgkin Lymphoma tumour cells expressed PD-L1. Older people expressed more PD-L1, but other clinicopathologic markers did not correlate. Novel PD-L1 medication should provide equivalent results in initial disease and refractory/relapsed patients.

KEYWORDS: (PD-L1) Expression, Hodgkin Lymphoma, Iraqi Patients.

INTRODUCTION

Reed–Sternberg cells are seen amid a "characteristic backdrop" of reactive inflammatory cells and fibrosis in Hodgkin lymphoma.[1] It causes 79,990 cases globally. Men have a 1.1 per 100,000 worldwide incidence rate, whereas women have 0.8 per 100,000. The illness killed 15,770 men and 10,397 women in 2018, a substantially lower rate.[2] Iraq ranks 9th in incidence with 650 cases and 168 fatalities. In 2018, both genders had 1.9 incidence and 0.64 fatality per 100,000[3] 547 instances with 2.88% incidence rate in men and women in 2016 (295 in men and 252 in women).[4] Hodgkin lymphoma starts in a node or chain of nodes and extends to anatomically adjoining lymphoid tissues, splenic disease, hepatic disease, the marrow, and other tissues.[1] Reactive lymphocytes, macrophages, and granulocytes, which make up over 90% of the tumor's cellularity, are released by Reed-Sternberg cells.[5] Nodular lymphocyte predominates HL (NLPHL) and traditional HL are classified based on the immunophenotype, morphology, and cellular background of the neoplastic cells. NLPHL and CHL have few neoplastic cells and a robust inflammatory backdrop of T lymphocytes.[6] Classical form of Hodgkin lymphoma (CHL), the most prevalent lymphoma subtype in Western children and young adults, accounting for 15% to 25% of all lymphomas.[7] It is categorised into four types: nodular sclerosis, lymphocyte rich, mixed cellularity, and lymphocyte deficient based on locations of involvement, clinical characteristics, growth pattern, fibrosis, cellular background, tumour cell atypia, and EBV infection.[1] 90% of HLs are classic and 10% or fewer are NLPHL, with a peak incidence in 15-35-year-olds and a second peak in late life for subtypes other than nodular

sclerosis.[6] NLPHL varies from CHL histopathologically and clinically. NLPHL patients usually have cervical or inguinal involvement, early clinical stage, and minimal poor prognostic markers.[8] As early as 1966, elevated antibody titers to Epstein-Barr virus (EBV) antigens in Hodgkin lymphoma patients compared to other lymphomas suggested an infectious agent.[9] Acute EBV infection or infectious mononucleosis is a risk factor for HL because EBV protects germinal centre B cells from apoptosis.[10] Prognostic variables and illness stage determine HL treatment.[11] Early-stage HL patients receive combined chemotherapy and radiation treatment, whereas advanced-stage patients receive chemotherapy alone.[12] Despite the high cure rate of HL with first therapy, 5–10% of patients are primary refractory and 10–20% will recur.[13] Relapsed Hodgkin lymphoma (HL) patients get high-dose chemotherapy (HDCT) and autologous stem cell transplantation (ASCT).[14] However, such groups had a dismal prognosis with less than 50% chance of CR and median overall survival of 2 years.[15] For such individuals, a better knowledge of immune regulatory networks and immunophysiologic interactions in the tumour microenvironment led to the development of innovative medications to treat HL, including immune checkpoint inhibitors.[16] PD-L1 preferentially interacts to its receptor, Programmed cell death protein 1 (PD-1), a 288-amino-acid transmembrane protein encoded by the PDCD1 gene on chromosome 2q37.3 and expressed on immune-related lymphocytes such T cells, B cells, and myeloid cells.[17] Immune tolerance—negative selection of auto reactive cells in main (central tolerance) and secondary lymphoid organs—requires the PD-1/PD-L1 pathway (peripheral tolerance).[18] Reed–Sternberg cells shape the tumour microenvironment to induce immune tolerance and counteract immune tumour rejection in classic Hodgkin lymphoma by expressing many immune checkpoint ligands, including PD-L, which exhaust infiltrating cytotoxic and type 1 helper T-cell subsets.[19] In sick lymph nodes, tumor-associated macrophages (TAMs) produce high amounts of PD-L1/PD-L2.[20] This may explain why typical Hodgkin lymphoma patients with more TAMs had shorter survival.[21] In 2016, the FDA approved Nivolumab, a humanised, IgG4 kappa monoclonal antibody that disrupts the interaction between programmed death receptor-1 (PD-1) and its ligands, PD-L1 and PD-L2.[22] The aim of study is to focus on the widespread occurrence of PD-L1 expression in a sample of Iraqis with Hodgkin lymphoma. Evaluate how PD-L1 expression levels relate to patients' clinicopathologic characteristics in cases of Hodgkin lymphoma. Examination of PD-L1 expression in individuals with initial illness and those with refractory/relapsed disease.

METHOD

This is a cross sectional study that was carried out in the Laboratory Department of Al-Hussain medical city in Karbala during the period from (Nov. 2019- Sep. 2020). **Inclusion criteria:** Cases already diagnosed as Hodgkin Lymphoma by H&E and Reed Sternberg cells' markers in both node excisional biopsy or core needle types from primary nodal disease. Cases with available clinical data including: age, gender, subtype, stage, and primary/relapse state. **Exclusion criteria:** Biopsies with insufficient pathological material. Biopsies with poor quality of pathological material. 45 cases of Hodgkin lymphoma were collected from the histo- pathological lab in AL-Hussain medical city in Karbala, as well as many other private labs. 15 cases of them was discarded not met our inclusion criteria needed to accomplish this study. The remaining thirty cases that meet our criteria were (13 females and 17 males). Information regarding stage of cases obtained from department of oncology in Al-Hussain medical city. **Control Group: Positive control:** We use placental tissue as positive control. **Negative control:** Sections without treatment with primary antibody (PD-L1) were considered as negative control for each set of slides. PathnSitu ready to use antibodies are used. The primary antibody applied to the tissue sections and incubated at room temperature for 30 minutes. Cells labeled with this Antibody display membrane staining. As positive control, the antibody labels the placenta and lung squamous cell carcinoma. PD-L1 immunohistochemical results were very sensitive to the circumstances of the staining procedures. The used staining kit was (PathnSitu PolyExcel detection system, PathnSitu, UK) and according to the manufacturer's instructions. The criteria for positive immunohistochemical reaction of PD-L1is of membranous as indicated by the manufacturer literature. Anti-PD-L1 immunostaining was only evaluated in field ×20 on tumor cells (Hodgkin cells, Reed-Sternberg) and with respect to the global tumor cellularity, and was classified as membranous, partial, or total. When samples allowed, 100 tumor cells were required to assess the PD-L1+ cell count. Labeling was classified as: Negative: when PD-L1 expression < 1% of tumor cells. Low: when PD-L1 was expressed in 1-49% of tumor cells. High: when expression was ≥ 50% of tumor cells.[23] The positivity threshold was 1%.[24] Staining intensity: 0: no staining, +1: weak or equivocal staining, +2: moderate staining, +3: strong staining.[25] Each immunohistochemically stained slide was firstly scanned under light microscope to detect and score the positive expression for PD-L1 and to choose the fields that reflect the best overall marker expression in each slide. Then the microscopic sections were captured by using Olympus Dp72 microscope camera. Statistical Analysis of all results were preceded by the help of SPSS statistical package at level of significance alpha=0.05 to find (P value).

RESULTS

Thirty patients diagnosed with Hodgkin lymphoma were included in this study with an age range from 2.5 to 70 years old with a mean age = 33 years. As the incidence of HL is known to have bimodal peaks, we set the age of 40 as a cut-point for age subgroup analysis. 21(70%) of patients were under forty and 9 (30%) were 40 years and

above. Out of thirty cases involved, 17 cases were male (56.7%) and 13 cases were female (43.3%). All cases were biopsied by nodal excision or core biopsy specimens. Four histological subtypes were found in this study: 15 (50%) were of nodular sclerosis Hodgkin lymphoma, 13(43.3%) were of mixed cellularity HL, 1 (3.3%) was of lymphocyte rich HL and 1(3.3%) lymphocyte depleted HL. According to tumor stage, no case was in stage I, 13cases (43.3%) were in stage II, 11cases (36.7%) were stage III and 6 cases (20%) were stage IV. Patients were divided into two groups according whether the biopsies were performed for first time diagnosis of HL 24cases (80%) or they performed to confirm refractory or relapse state of HL 6 cases (20%). Out of 30 cases involved in this study 29 cases (96.7%) were CD30 positive, while one case (3.3%) was negative for this marker.CD15 expression was positive in 25 cases (83.3%) and negative in 5 cases (16.7%). The positive PD-L1 immunohistochemical staining in the tumor cells was membranous, according to the Kit manufacturer. PD-L1 IHC expression was reported as positive staining in 27(90%) out of 30 cases of Hodgkin Lymphoma with only 3(10%) of cases were negative. Regarding the 27 positive cases, 22 (73.3%) cases show high expression level while 5(16.7%) cases show low level of expression. Intensity of PD-L1 expression by tumor cells was variable, 12(40%) cases show +3 expressions, 11(36.7%) cases show +2 expression, 4(13.3%) cases show +1 expression and 3(10%) cases show negative results. As show in table 1.

Table 1: distribution of patients according to study variables.

variables		frequency	percentage
Age group (years)	< 40	21	70
	≥ 40	9	30
Histological types	nodular sclerosis Hodgkin lymphoma	15	50
	mixed cellularity HL	13	43.3
	lymphocyte rich HL	1	3.3
	lymphocyte depleted HL	1	3.3
stages	1	13	43.3
	2	11	36.7
	3	6	20
Gender	Males	17	56.7
	Females	13	43.3
presented cases	first time diagnosis	24	80
	refractory or relapse	6	20
CD30	Positive	29	96.7
	Negative	1	3.3
CD15	Positive	25	83.3
	Negative	5	16.7
PD-L1 expression	Negative	3	10
	Low	5	16.7
	High	22	73.3
PD-L1 expression	Negative	3	10
	Mild	4	13.3
	Moderate	11	36.7
	Strong	12	40

Patients aged less than 40yrs show18 cases (60%) out of total positive whereas all patients aged 40 years and above show high level of PD-L1 expression. Calculated P value <0.05 (higher level of PD-L1 expression among older age group patients) (Table 2).

Table (2): Immunohistochemical expression of PD-L1 in regards to patients' age.

			level of PD-L1 expression			Total	P value
			negative	low	high		
Age	<40 years	Count	3	5	13	21	0.047
		% of Total	10.00%	16.70%	43.30%	70.00%	
	≥ 40 years	Count	0	0	9	9	
		% of Total	0.00%	0.00%	30.00%	30.00%	
Total		Count	3	5	22	30	
		% of Total	10.00%	16.70%	73.30%	100.00%	

PD-L1 immunohistochemical positive expression revealed that 15(50%) of cases are of male gender while

12(40%) of cases was of female gender. No significant difference (P>0.05) were seen between the two gender groups (Table 3).

Table (3): Immunohistochemical expression of PD-L1 in regards to patients' gender.

			level of PD-L1 expression			Total	p value
			negative	low	high		
Gender of patient	male	Count	2	2	13	17	0.9
		% of Total	6.7%	6.7%	43.3%	56.7%	
	female	Count	1	3	9	13	
		% of Total	3.3%	10.0%	30.0%	43.3%	
Total		Count	3	5	22	30	
		% of Total	10.0%	16.7%	73.3%	100.0%	

Regarding the histological type, the positive immunohistochemical staining was recorded as follows: 15 (50%) cases out of total positive cases were seen in nodular sclerosis type. The mixed cellularity type recorded 10 cases (33.3%) out of total 27positive cases, and each of lymphocyte depleted and lymphocytes rich type recorded 1 cases (3.3%) for each one with p value > 0.05 (Table 4).

Table (4): Immunohistochemical expression of PD-L1in regards to the histological type of Hodgkin lymphoma.

			level of PD-L1 expression			Total	P value
			Negative	low	high		
Histologic Subtype	NSCHL	Count	0	2	13	15	0.197
		% of Total	0.0%	6.7%	43.3%	50.0%	
	MCCHL	Count	3	2	8	13	
		% of Total	10.0%	6.7%	26.7%	43.3%	
	LRCHL	Count	0	1	0	1	
		% of Total	0.0%	3.3%	0.0%	3.3%	
	LDCHL	Count	0	0	1	1	
		% of Total	0.0%	0.0%	3.3%	3.3%	
Total		Count	3	5	22	30	
		% of Total	10.0%	16.7%	73.3%	100%	

PD-L1immunohistochemical expression reported in 12 (40%) out of total positive was in stage II cases, 9 (30%) out of total positive was in stage III cases & was positive in all 6 (20%) cases of stage IV disease. There is no significant between level of expression and stage of disease (p value >0.05) (Table 5).

Table (5): Immunohistochemical expression of PD-L1 in regards to the stage of Hodgkin Lymphoma.

			level of PD-L1 expression			Total	p value
			negative	low	high		
stage	II	Count	1	1	11	13	0.843
		% of Total	3.3%	3.3%	36.7%	43.3%	
	III	Count	2	3	6	11	
		% of Total	6.7%	10.0%	20.0%	36.7%	
	IV	Count	0	1	5	6	
		% of Total	0.0%	3.3%	16.7%	20.0%	
Total		Count	3	5	22	30	
		% of Total	10.0%	16.7%	73.3%	100%	

PD-L1 expression reported in 21(70%) cases out of total positive was reported in patient with first time diagnosis with HL, while all 6 cases with refractory/relapsed HL show positive staining with PD-L1 which represent (20%) of total positive cases. P value was >0.05 indicating no significant difference between two groups (Table 6).

Table (6): immunohistochemical expression of PD-L1 in regards to the patients' group in Hodgkin Lymphoma.							
			level of PD-L1 expression			Total	p value
			negative	low	high		
Patients' group	Primary diagnosis	Count	3	3	18	24	0.894
		% of Total	10.0%	10.0%	60.0%	80.0%	
	refractory/ relapse	Count	0	2	4	6	
		% of Total	0.0%	6.7%	13.3%	20.0%	
Total		Count	3	5	22	30	
		% of Total	10.0%	16.7%	73.3%	100.0%	

Regarding CD30 expression by tumor cells, PD-L1 expression in 26(86.7%) out of total positive cases were in cases which show immune- reactivity to CD30 marker. Calculated P value was > 0.05 indicate no significant association between CD30 & PD-L1 expression by tumor cells. In other hand PD-L1 expression in 23 (76.7%) out of total positive cases were in cases which show immune-reactivity to CD15 marker. No significant correlation between CD15& PD-L1 expression by tumor cells with P value > 0.05 (Table 7).

Table (7): Immunohistochemical expression of PD-L1 in regards to CD30 & CD15 expression by tumor cells:							
			level of PD-L1 expression			Total	p value
			negative	low	High		
expression of CD30 by RS cells	+ve	Count	3	4	22	29	0.344
		% of Total	10.0%	13.3%	73.3%	96.7%	
	-ve	Count	0	1	0	1	
		% of Total	0.0%	3.3%	0.0%	3.3%	
Total		Count	3	5	22	30	
		% of Total	10.0%	16.7%	73.3%	100.0%	
expression of CD15 by RS cells	+ve	Count	2	4	19	25	0.402
		% of total	6.7%	13.3%	63.3%	83.3%	
	-ve	Count	1	1	3	5	
		% of total	3.3%	3.3%	10%	16.7%	
Total		Count	3	5	22	30	
		% of total	10.0%	16.7%	73.3%	100.0%	

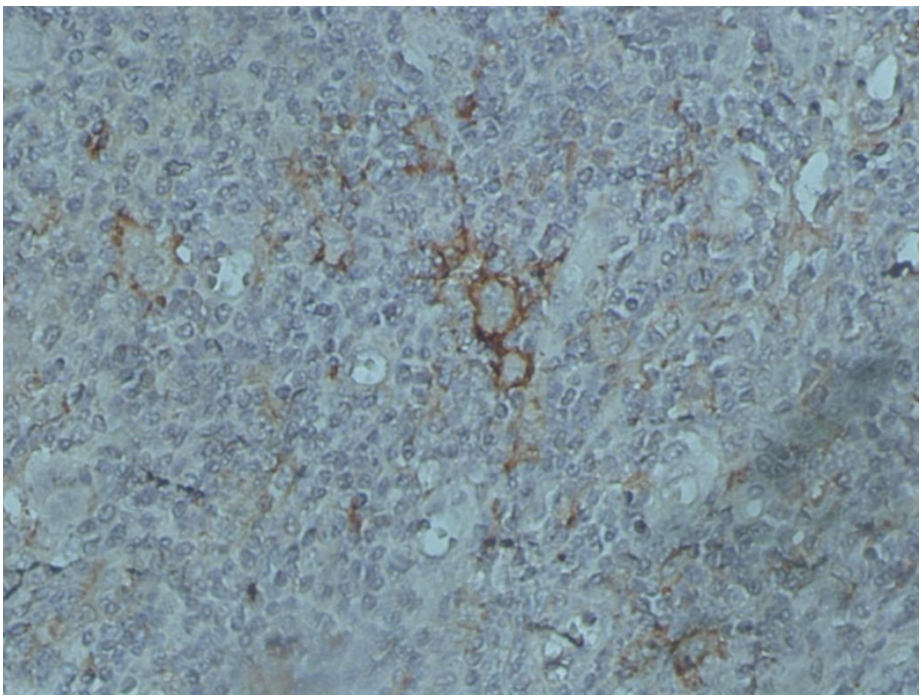

Figure (1): PD-L1 immunohistochemistry staining in Hodgkin Lymphoma(+3) score (40X).

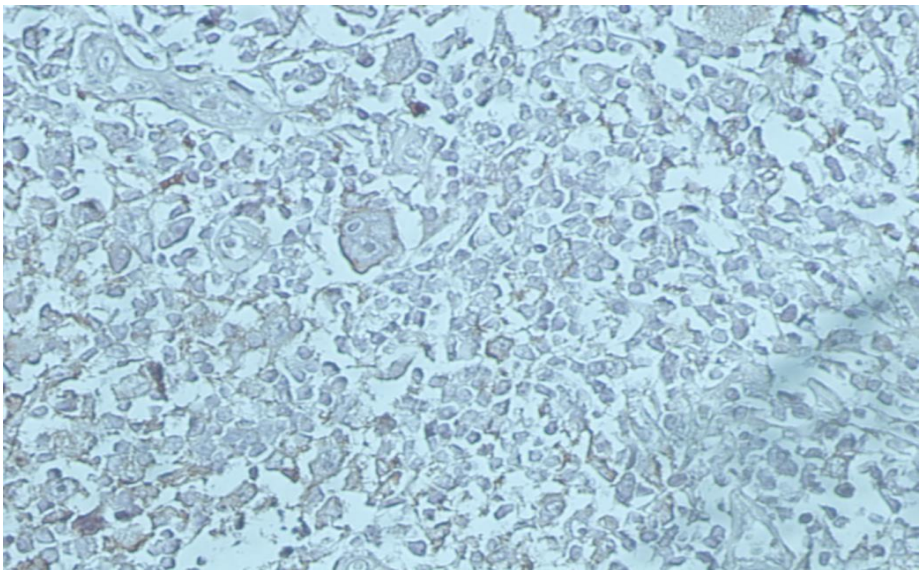

Figure (4.14): PD-L1 immunohistochemistry staining in Hodgkin Lymphoma (+1) score (40X).

DISCUSSION

Male to female ratio in this study was (5.7:4.3), this is somewhat similar to **Hollander et al 2018**[26], with ratio (63.4:36.6) % but differ from that of **Ozturk 2020,**[27] with ratio (36.1:63.9). The detected PD-L1 expression in tumor cells was 90% (27cases out of 30) with 73.3% of them show high level of expression. Researches that study the level of PD-L1 expression in Hodgkin lymphoma show variable results. **Menter et al 2016** who study the diagnostic and prognostic value of PD-L1 in HL and B cell lymphoma, found that PD-L1 was expressed in 70% of classic Hodgkin lymphoma case.[28] **Panjwani et al 2018** found that PD-L1 was expressed in 82% of 63 cases of CHL involved in his study.[29] **Dilly-Feldis et al 2019** showed that all 42 cases involved in his study was PD-L1 positive and majority show high level of expression.[23] **O'Malley D et al 2019** involve 12 case of CHL in his study and PD-L1 was positive in 50% of the cases[24] this variation in results among different studies could be due differences in number of cases, various methods with variable cutoffs, clone of PD-L1 used, type of antibody used or scoring system performed. Also the differences could be attributed to EBV infection, constitutive AP activity[27] and the 9p24.1 amplification in the tumor cells.[30] Regarding the expression of PD-L1 among different age group, all patient aged 40years and above express high level of PD-L1 by tumor cells (>50%of tumor cells), on other hand 18 patient aged under 40 show PD-L1 expression with P value <0.05 indicating significant association between increasing age and level of PD-L1 expression. This analogue to results obtained by **Tanaka et al 2018**, all cases aged 40 years or older showed reactivity to PD-L1 antibody in more than 70% of tumor cells.[31] The expression of PD-L1 among gender was (50% of total positive) in male and (40% of total positive) in female, with p value > 0.05 reflecting absence of difference between two genders. Regarding the expression of PD-L1 in subtypes of HL, all NSCHL (100%), 10 out of 13 MCCHL (76.9%), and each of two LRCHL and LDCHL show expression of PD-L1. There was no significant difference between level of expression and histologic subtypes p value >0.05. This is going with diverse results obtained by different researchers. **Tanaka et al 2018,** showed that PD-L1 expression in MCCHL is 100%[31], **Chen et al 2013,** detect PD-L1 in (84%) cases of NSCHL, (88%) cases of MCCHL[25], while **Kohno et al 2020,** show 89% of PD-L1 in his CHL cases which was all resembling nodular sclerosis type[32] such variable may be due to different positivity cut offs considered by different researchers. Speaking about stage, 43.3% of cases were stage II which show 40% of total PD-L1 positivity, 36.7% were stage III, 20% were stage IV which show which show 30% & 20% of total PD-L1 positive results respectively. The calculated P value was > 0.05 indicating that the expressed level of PD-L1 by RS cells unrelated to the clinical stage of HL. This goes with the result obtained by **Volarics et al 2020;** there was no significant association between PD-L1 expression pattern and clinical stage[33] in other hand **Ozturk et al 2020,** reported that the presence of advanced stage disease related to PD-L1 positivity in Reed Sternberg cells.[27] There were two groups of patients in regard to whether the biopsy was for primary diagnosis of Hodgkin lymphoma (24) cases and those for refractory/relapsed disease (6) cases. PD-L1expression was 70% (of total positive) for primary diagnosis cases and 20% (of total positive) for the other group with P value was >0.05 reflecting the absence of association, despite the fact that all cases in the second group show PD-L1 positivity (2 cases low, 4 cases high PD-L1). However, there was a small number of patients with refractory/relapsed disease and unfortunately their biopsies at time of primary diagnosis were lacked, so it was not possible to establish a comparison between the two statuses and to detect whether the positivity of PD-L1 in the second group is primary or it treatment related. **Vranic et al 2016,** studied PD-L1 expression in refractory lymphomas of both B & T cells lineage and

his study revealed that nearly all classic Hodgkin Lymphoma exhibit diffuse & strong PD-L1 positivity on tumor cells.[34] **Paydas** has performed two consecutive researches **2015**, **2018** focusing on changes in PD-L1 expression in primary and relapsing CHL, he concluded that PD-L1 immunoreactivity after treatment are detected in the majority of cases, and suggested checking the expressions of programmed death pathway biomarkers in tissue samples from relapse cases.[35] He also suggested that checkpoint blockade therapy must be considered in earlier treatment steps of Hodgkin lymphoma cases before other ineffective & too toxic chemotherapeutic agents.[36] **Hollander et al 2018,** had reach to similar results comparing primary and relapse biopsies of 87 cases of CHL and he thought that high level of PD-L1 expression in relapse state is most likely due to primary treatment with chemotherapy and radiotherapy.[26] In this study there was an attempt to correlate the expression of PD-L1 by RS cells with expression of surface CD30 & CD15. PD-L1 expression in 26 (86.7%) out of total positive cases were in cases which show immune-reactivity to CD30 marker and 23 (76.7%) out of total positive cases were in cases which show immune-reactivity to CD15 marker. No significant correlation detected and p value >0.05 for both. These two correlations are not discussed yet, except for recent study by **Tiemann et al 2020,** PD- L1 immunoreactivity was detected in CD30 positive cells in Hodgkin lymphoma but not in CD30 Positive tumor cells in angioimmunoblastic T cell lymphoma.[37]

CONCLUSION

The majority of classical Hodgkin lymphoma tumour cells express PD-L1. Patients over the age of 65 had greater levels of PD-L1 expression in their tumour cells, but there is no correlation between PD-L1 expression and other patient characteristics such as gender, clinical stage, initial disease, refractory/relapsed status, histologic subtype, or CD30 expression. Due to the similarity in expression profile between patients with primary disease and those with refractory/relapsed disease, it is possible that patients receiving primary, secondary, or tertiary therapy with immune checkpoint inhibitors directed against the PD1/PD-L1 pathway will all experience similar responses.

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