Programmed cell death ligand 1 expression in aggressive pediatric non-Hodgkin lymphomas: frequency, genetic mechanisms, and clinical significance

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Abstract

Programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) are immunomodulatory molecules overexpressed in lymphomas and are promising immunotherapy targets for hematologic malignancies. However, studies of PD-1/PD-L1 overexpression and their clinical significance in aggressive pediatric non-Hodgkin lymphomas (NHL) are limited. We assessed PD-1/PD-L1 overexpression using immunohistochemistry in 68 aggressive pediatric NHL: ALK-positive anaplastic large cell lymphoma (ALK+ ALCL, n=8), Burkitt lymphoma (BL, n=27), and large B-cell lymphoma (LBCL) *de novo* LBCL, n=22 and diffuse LBCL arising as monomorphic post-transplant lymphoproliferative disorder [PTLD-DLBCL], n=11. In LBCL, correlations between PD-L1 overexpression and Epstein-Barr virus (EBV) status, cell of origin, stage, nodal status, overall survival (OS), and event-free survival (EFS) were examined. The genetic mechanisms of PD-L1 overexpression were investigated using targeted next-generation sequencing (NGS) and cytogenetic data. All ALK+ ALCL samples, 50.0% of *de novo* LBCL (11/22), 72.7% of PTLD-DLBCL (8/11), and no BL overexpressed PD-L1. Overexpressed PD-L1 correlated with EBV positivity (P=0.033) in LBCL and lower EFS in *de novo* LBCL (P=0.017). NGS of select LBCL revealed distinct somatic mutations and an ultra-hypermutated PTLD-DLBCL. Most cases with 9p24.1 copy gains overexpressed PD-L1 although some cases had no discernible genetic drivers of PD-L1 overexpression. Overexpressed PD-L1 is common in pediatric LBCL, associated with EBV positivity and 9p24.1 gains, and may have prognostic significance in *de novo* LBCL. Furthermore, diverse molecular mechanisms for PD-L1 overexpression in aggressive pediatric NHL can occur. Thus, additional studies exploring the therapeutic and prognostic significance and molecular mechanisms of PD-L1 overexpression in aggressive pediatric NHL are warranted.

Introduction

Immune checkpoint blockade has introduced a paradigm shift in cancer therapy for a variety of solid tumors and hematologic malignancies.[1-3] Programmed cell death 1 (PD-1) is a cell surface receptor in the B7/CD28 family of T-cell regulators that serves as an inhibitory co-receptor through binding of its ligands programmed cell death ligand 1 (PD-L1) and programmed cell death ligand 2 (PD-L2). PD-L1 is broadly expressed across hematopoietic cells and human tissues, PD-1 is expressed on the cell surface of B cells, T cells, and natural killer T cells, whereas PD-L2 is only expressed on dendritic cells following cytokine induction.[4,5] The PD-1/PD-L1/PD-L2 signaling axis promotes recognition of self and non-self antigens to maintain immune tolerance.[5-7] However, tumor cells and tumor microenvironment cells can aberrantly overexpress PD-L1 (e.g., macrophages) and PD-1 (e.g., lymphocytes) to induce "T-cell exhaustion" and evade immune recognition and destruction.[8-10]

In adult hematologic malignancies, PD-L1 overexpression has been reported in classical Hodgkin lymphoma (cHL),

primary mediastinal large B-cell lymphoma (PMBCL), T-cell/histiocyte-rich large B-cell lymphoma (THRLBCL), ALK-positive anaplastic large cell lymphoma (ALK+ ALCL), subsets of diffuse large B-cell lymphoma (DLBCL), and follicular lymphomas.[3,11,12] PD-1/PD-L1 overexpression is observed in approximately two thirds of adults with post-transplant lymphoproliferative disorder (PTLD) following solid organ transplantation as well as a subset of Epstein-Barr virus (EBV)-driven lymphomas (e.g., EBV-positive cHL). PD-1/PD-L1 expression in Burkitt lymphoma (BL) is typically negative.[13] Notably, a vast majority of these data were established in adult studies and may not be representative of pediatric populations.

Diverse molecular mechanisms drive PD-1/PD-L1 protein overexpression. For example, some tumors harbor high tumor mutation burden (TMB). This introduces numerous coding mutations that give rise to specific neoantigens that are presented to the cell surface and drive immune recognition and PD-1/PD-L1 overexpression.[14,15] In adult LBCL, PD-L1 overexpression results from gene rearrangements involving the 9p24.1 gene locus which contains the *CD274* (PD-L1), *PDCD1LG2* (PD-L2), and *JAK2* genes[16] or 9p24.1 copy gains and amplifications similar to cHL.[17,18] Structural variations of the *CD274* gene 3' untranslated region (3'-UTR) leading to increased transcript stabilization are also reported.[19] In EBV-associated aggressive lymphomas, PD-L1 overexpression typically occurs via latent membrane protein-1 (LMP-1)-mediated activation of the *CD274* JAK/STAT-dependent promoter or AP-1 associated enhancer activity.[20,21] In ALK+ ALCL, PD-L1 overexpression is a result of STAT3 phosphorylation and IRF4/BATF3-mediated *CD274* transcriptional upregulation.[12,22]

The anti-PD-1 immune checkpoint inhibitor pembrolizumab is a Food and Drug Admininistration (FDA)-approved therapeutic agent for relapsed/refractory adult and pediatric cHL. Another anti-PD-1 inhibitor, nivolumab, has shown promising therapeutic efficacy in early clinical trials for pediatric cHL and other PD-L1-expressing tumors.[23,24] A factor contributing to the therapeutic efficacy of immune checkpoint inhibitors is the amount of target protein on the tumor tissue and/or microenvironment cells. Yet, recent evidence suggests that additional elements may also influence the use of PD-L1 expression as a predictive and prognostic marker for immune checkpoint inhibitor therapy. These may include the immunohistochemistry (IHC) methods employed, the time point at which the assessment of this dynamic biomarker is performed, TMB status,[25,26] and other intrinsic biologic attributes such as *DUSP22* rearrangements in ALK-negative ALCL,[27] or the presence or absence of tumor infiltrating lymphocytes (TIL).[28]

Studies and clinical trials assessing PD-1/PD-L1 expression have focused primarily on adult lymphomas and pediatric cHL. The prognostic significance of PD-L1 expression in adult DLBCL cohorts have shown conflicting results.[29-31] Studies investigating PD-1/PD-L1 expression in aggressive pediatric non-Hodgkin lymphomas (NHL) such as ALCL, BL, and *de novo* pediatric DLBCL are limited. Thus, we investigated PD-1/PD-L1 expression in aggressive pediatric NHL including ALK+ ALCL, BL, a pediatric cohort LBCL (consisting of *de novo* LBCL and DLBCL arising as monomorphic PTLD [PTLD-DLBCL]). In the pediatric LBCL cohort, we reviewed available cytogenetic data to assess for rearrangements involving the 9p24.1 locus and performed targeted next-generation sequencing (NGS) on selected cases to detect DNA mutations, estimate TMB, and investigate copy-number changes of the 9p24.1 locus. The impact of PD-L1 status on clinicopathologic factors and treatment outcomes in the pediatric LBCL cohort was also investigated.

Methods

Case selection and clinical data

All authors conducted the study per Institutional-approved research protocols to Drs. Curry and Fisher. Sixty-eight aggressive pediatric NHL with sufficient tissue for IHC including ALK+ ALCL (n=8), BL (n=27), and LBCL (consisting of *de novo* LBCL, n=22; PTLD-DLBCL, n=11) were identified via a pathology database search from 1997-2012. The term *de novo* LBCL was used to describe pediatric LBCL cases in our cohort arising without previous transplants or previous low-grade B-cell lymphoma. Diagnoses and further subclassification of *de novo* LBCL were rendered using the revised 4th edition 2016 World Health Organization Classification of Tumors Hematopoietic and Lymphoid Tissues.[32] Clinical and outcome data for the LBCL cohort were acquired from the electronic medical record. Staging was classified according to revised International Pediatric Non-Hodgkin Lymphoma Staging System (IPNHLSS).[33]

Immunohistochemistry

Tissue microarrays were constructed for 68 cases. PD-L1 IHC was performed using the FDA-approved Dako PD-L1 IHC 28-8 pharmDx companion diagnostic assay per manufacturer established recommendations. PD-1 IHC staining (clone MRQ-22, Cell Marque) was performed at a 1:50 dilution on an automated Leica Bond III immunostainer. All PD-1/PD-L1 IHC results were independently reviewed and scored by two hematopathologists as previously described[34] with a few modifications (*Online Supplementary Table S1*). Membranous PD-L1 staining of tumor cells and macrophages, and membranous PD-1 staining of tumor infiltrating lymphocytes (TIL) were scored for staining intensity: 0 (no staining), 1+ (weak or equivocal), 2+ (moderate), 3+ (strong). Percent positivity was recorded

as follows: the number of PD-L1-positive tumor cells out of total number of viable tumor cells (PD-L1-Tum); the area of PD-L1-positive macrophages out of total tumor area (PD-L1-Mac); number of PD-1-positive tumor infiltrating lymphocytes out of total number of tumor infiltrating lymphocytes (PD-1-TIL). The cut-offs for PD-1/PD-L1 positivity were 2+ or 3+ staining in ≥5% of tumor cells (PD-L1-Tum) or ≥20% of macrophages (PD-L1-Mac) or TIL (PD-1-TIL). LBCL cases were classified based on cell of origin into germinal center B-cell (GCB) or non-GCB phenotypes according to the Hans IHC algorithm using CD10, BCL6, and MUM1 with a 30% cut-off.[35] Epstein-Barr virus (EBV) encoded RNA (EBER) *in situ* hybridization and LMP-1 (latent membrane protein 1) staining were used to determine EBV status.

Targeted next-generation sequencing and cytogenetics

Targeted NGS mutation analysis using a custom-designed 152 gene (1.25 megabase [Mb]) panel for pediatric hematologic malignancies was performed on 13 cases of aggressive pediatric NHL (5 *de novo* LBCL not otherwise specified, 2 primary-immunodeficiency associated LBCL, 5 PTLD-DLBCL, and 1 BL) as previously reported.[36] Variants were classified as established or potential clinical significance (Tier I/II) or variants of uncertain significance (Tier III).[37] Gains of 9p24.1 were determined from copy number analysis of the targeted sequencing data. 9p24.1 rearrangements were assessed for seven LBCL cases using available cytogenetic data (*Online Supplementary Table S2*).

Statistical analysis

LBCL cases were classified as PD-L1 positive if PD-L1-Tum positive. Statistical analyses were performed using GraphPad Prism version 6.0.0. For the *de novo* LBCL and PTLD-DLBCL cohorts, correlation between PD-L1-positivity and EBV status, cell of origin, stage, and nodal status was calculated using Fisher Exact test. Overall survival (OS) was defined as the time from diagnosis to death of any cause, and event-free survival (EFS) as the time to disease progression, relapse, or death of any cause, respectively. For survival analyses, Kaplan-Meier method with log-rank test was performed. Two-sided *P*-values <0.05 were considered statistically significant.

Results

Diagnoses and clinicopathologic features

A summary of the relevant clinicopathologic features, follow-up periods, and outcomes in the study cohort is provided in Table 1. Complete details are provided in the *Online Supplementary Table S2*. A total of 68 cases were studied; eight ALK+ALCL, 27 BL, and 33 LBCL (consisting of *de novo* LBCL, n=22; PTLD-DLBCL, n=11). Patients ranged in age from 2.6 to 21.4 years (mean 10.6, median 10.8 years) with 45 males and 23 females. More than half of the cases were stage III/IV (n=41, 61.2%) and extranodal involvement was documented in a majority of cases (n=50, 73.5%).

Of the patients with LBCL, the *de novo* LBCL cohort age ranged from 4.2 to 18.0 years (mean 11.6, median 11.3 years), with an equal number of males (n=11) and females (n=11). Diagnoses included DLBCL not otherwise specified (DLBCL-NOS, n=14), PMBCL (n=3), DLBCL with IGHα-MYC rearrangement (n=2), primary immunodeficiency-associated LBCL (PIA-LBCL) arising in Ataxia-Telangiectasia (n=2), and T-cell/histiocyte-rich LBCL (THRLBCL, n=1); one PIA-LBCL was subclassified further as THRLBCL. Two *de novo* LBCL (9.1%) were EBV+ and 15 (68.2%) were classified as germinal center B-cell (GCB) phenotype including 10 DLBCL-NOS (71.4%). *De novo* LBCL patients were treated according to various COG and POG protocols for pediatric B-cell NHL without Rituximab and three patients received bone marrow transplant (BMT) for relapsed/refractory disease (*Online Supplementary Table S2*). The follow-up period ranged from 4.2 to 253.9 months (mean 91.4, median 88.4 months).

Of the patients with LBCL, the PTLD-DLBCL cohort age ranged from 3.4 to 21.4 years (mean 12.9, median 15.9 years). Disease occurred after lung (n=5), bone marrow (n=2), renal (n=2), liver (n=1), or heart (n=1) transplantation. Ten PTLD-DLBCL (90.9%) were EBV+ and two (22.2%) were classified as GCB. PTLD-DLBCL patients were treated according to various regimens that all included Rituximab (*Online Supplementary Table S2*). The follow up period ranged from 0.6 to 265.9 months (mean 61.1, median 50.0 months).

PD-1/PD-L1 immunohistochemistry

PD-L1 immunoreactivity in tumor cells (PD-L1-Tum) and macrophages (PD-L1-Mac), and PD-1 immunoreactivity in tumor infiltrating lymphocytes (PD-1-TIL) were assessed in the ALK+ ALCL, BL, and LBCL cohorts. A summary of the PD-1/PD-L1 IHC results is provided in Table 2. Representative IHC staining is shown in Figure 1.

PD-L1-Mac and PD-1-TIL

No ALK+ ALCL cases showed PD-L1 staining in macrophages. In the BL cohort, PD-L1-Mac positivity was observed in five cases (range, 30-60%), but none of the PD-L1-Mac-positive BL demonstrated positive PD-L1-Tum staining. PD-L1-Mac positivity was seen in two DLBCL-NOS (20% each) and one PIA-LBCL that was classified as TCHRBCL (70%) although neither PD-L1-Mac-positive DLBCL-NOS demonstrated positive PD-L1-Tum staining. PD-1-TIL positivity was observed in four cases (2 ALK+ ALCL, 1 BL, and 1 THRLBCL), and all cases occurred with concomitant PD-L1-Tum positivity.

Table 1. Summary of study cohort (N=68).

Diagnoses (count)	Median age in years (range)	M | F, N	Stage III/IV, N	Site of involvement, N (%)	
				Nodal	Extranodal
ALK+ ALCL (8)	12.7 (6.7-15.8)	6 | 2	5 (62.5%)	6 (75.0%)	2 (25.0%)
BL (27)	8.4 (2.6-18.5)	24 | 3	16 (59.3%)	2 (7.4%)	25 (92.6%)
LBCL (33) *De novo* (22) PTLD-DLBCL (11)	11.7 (3.4-21.4) 11.6 (4.2-18.0) 12.9 (3.4-21.4)	15 | 18 11 | 11 4 | 7	20 (62.5%)* 13 (61.9%)* 7 (63.6%)	10 (30.3%) 6 (27.3%) 4 (36.4%)	23 (69.7%) 16 (72.7%) 7 (36.6%)
Totals (68)	10.6 (2.6-21.4)	45 | 23	41 (61.2%)*	18 (26.5%)	50 (73.5%)
LBCL classification		**Count (%)**	**GCB, N (%)**		**EBV+, N (%)**
De novo LBCL DLBCL-NOS PMBCL THRLBCL DLBCL with IGHα-MYC PIA-LBCL^		22 (66.7%) 14 3 1 2 2	15/22 (68.2%) 10/14 (71.4%) 1 1 2 1		2/22 (9.1%) 2/14 (14.3%) 0 0 0 0
PTLD-DLBCL		11 (33.3%)	2/9 (22.2%)†		10/11 (90.9%)$
Totals		33 (100.0%)	17/31 (54.8%)†		12/33 (36.4%)$
LBCL events		**All LBCL (33)**	**De novo (22)**		**PTLD-DLBCL (11)**
Months follow-up (range)		80.7 (0.6-265.9)	91.4 (4.2-253.9)		61.1 (0.6-265.9)
Outcome, N (% cohort) NED REL, NED REL, DOD REF, DOD DOD DOC (transplant)		 21 (63.6%) 4 (12.1%) 2 (6.1%) 1 (3.0%) 4 (12.1%) 1 (3.0%)	 15 (68.2%) 3 (13.6%) 2 (9.1%) 1 (4.5%) 1 (4.5%) -		 6 (54.5%) 1 (9.1%) - - 3 (27.3%) 1 (9.1%)

*Staging was scored using International Pediatric Non-Hodgkin Lymphoma Staging System (IPNHLSS) criteria[32] and data were unavailable for 1 *de novo* PIA-LBCL. ^Both PIA-LBCL occurred in Ataxia-Telangiectasia patients. One PIA-LBCL was further subclassified as THRLBCL. †Cell of origin (GCB *vs.* non-GCB) classification for 2 cases of PTLD-DLBCL was unsuccessful due to insufficient tissue. $One EBV+ PTLD-DLBCL was positive for EBER (Epstein-Barr virus encoded RNA) only and negative for LMP-1 (latent membrane protein 1). ALK+ ALCL: ALK-positive anaplastic large cell lymphoma; BL: Burkitt lymphoma; DOC: died of complications; DOD: died of disease; DLBCL: diffuse large B-cell lymphoma; EBV+: Epstein-Barr virus positive; F: female; GCB: germinal center B-cell phenotype; LBCL: large B-cell lymphoma; M: male; NED: no evidence of disease; NOS: not otherwise specified; PIA: primary immunodeficiency-associated; PTLD: post-transplant lymphoproliferative disorder; PMBCL: primary mediastinal large B-cell lymphoma; REF: refractory; REL: relapsed; THRLBCL: T-cell/histocyte-rich large B-cell lymphoma.

Table 2. Summary of PD-1/PD-L1 immunohistochemistry results.

Diagnoses (count)	PD-L1-Tum ≥5%* N (%)	Range %	PD-L1-Mac ≥20%* N (%)	Range %	PD-1-TIL ≥20%* N (%)	Range %
ALK+ ALCL (8)	8 (100.0%)	30-100%	0 0.0%	.	2 25.0%	20%
BL (27)	0 (0.0%)	.	5 18.5%	30-60%	0 0.0%	.
LBCL (33)	19 (57.6%)	30-100%	3 9.1%	20-70%	1 3.0%	20%
De novo LBCL (22) DLBCL-NOS (14) PMBCL (3) THRLBCL (1) DLBCL with IGHα-MYC (2) PIA-LBCL (2)	11 (50.0%) 4/14 (28.6%) 3 1 1 2	 50-60% 50-90% 100% 30% 30-100%	3 13.6% 2 0 0 0 1	 20% . . . 70%	1 4.5% 0 0 1 0 0	 . . 20% . .
PTLD-DLBCL (11)	8 (72.7%)	40-100%	0 0.0%	.	0 0.0%^	.

*Cases were considered PD-L1-Tum positive if PD-L1 membranous expression on tumor cells was ≥5%, PD-L1-Mac positive if PD-L1 membranous expression on macrophages was ≥20%, and PD-1-TIL positive if ≥20% tumor infiltrating lymphocytes showed membranous PD-1 staining (*Online Supplementary Table S1*). ^Scoring PD-1 expression on tumor infiltrating lymphocytes (PD-1-TIL) was unsuccessful for one PTLD-DLBCL case due to insufficient tissue. ALK+ ALCL: ALK-positive anaplastic large cell lymphoma; BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma; LBCL: large B-cell lymphoma; Mac: macrophages; NOS: not otherwise specified; PIA: primary immunodeficiency-associated; PD-L1-Mac: PD-L1 expression on macrophages; PMBCL: primary mediastinal large B-cell lymphoma; PTLD: post-transplant lymphoproliferative disorder; THRLBCL: T-cell/histocyte-rich large B-cell lymphoma; TIL: tumor-infiltrating lymphocyte.

PD-L1-Tum

As expected, all eight (100.0%) of the ALK+ ALCL cases showed positive PD-L1-Tum staining (range, 30-100%) and none of the 27 BL cases (0.0%). Overall, PD-L1-Tum positivity ranging from 30-100% was detected in 19 LBCL (57.6%) and 11 *de novo* LBCL (50.0%) including four DLBCL-NOS (28.6%). Eight PTLD-DLBCL (72.7%) showed PD-L1-Tum-positive staining ranging from 40-100%. In our cohort, none of the PD-L1-Tum-positive cases demonstrated less than 30% membranous positivity despite a 5% threshold for positivity. Three *de novo* LBCL cases showed a cytoplasmic PD-L1 staining pattern that was considered PD-L1-negative, and three LBCL showed essentially similar PD-L1 staining intensity and percent positivity on initial and relapsed/refractory lesions (data not shown).

PD-L1 expression in pediatric large B-cell lymphomas

LBCL cases were recorded as positive for PD-L1 expression only if PD-L1-Tum positivity was observed and subsequent statistical analyses focused on PD-L1-Tum staining status only (Table 3). PD-L1-Tum positivity significantly correlated with EBV+ LBCL (P=0.033). There were no statistically significant differences between PD-

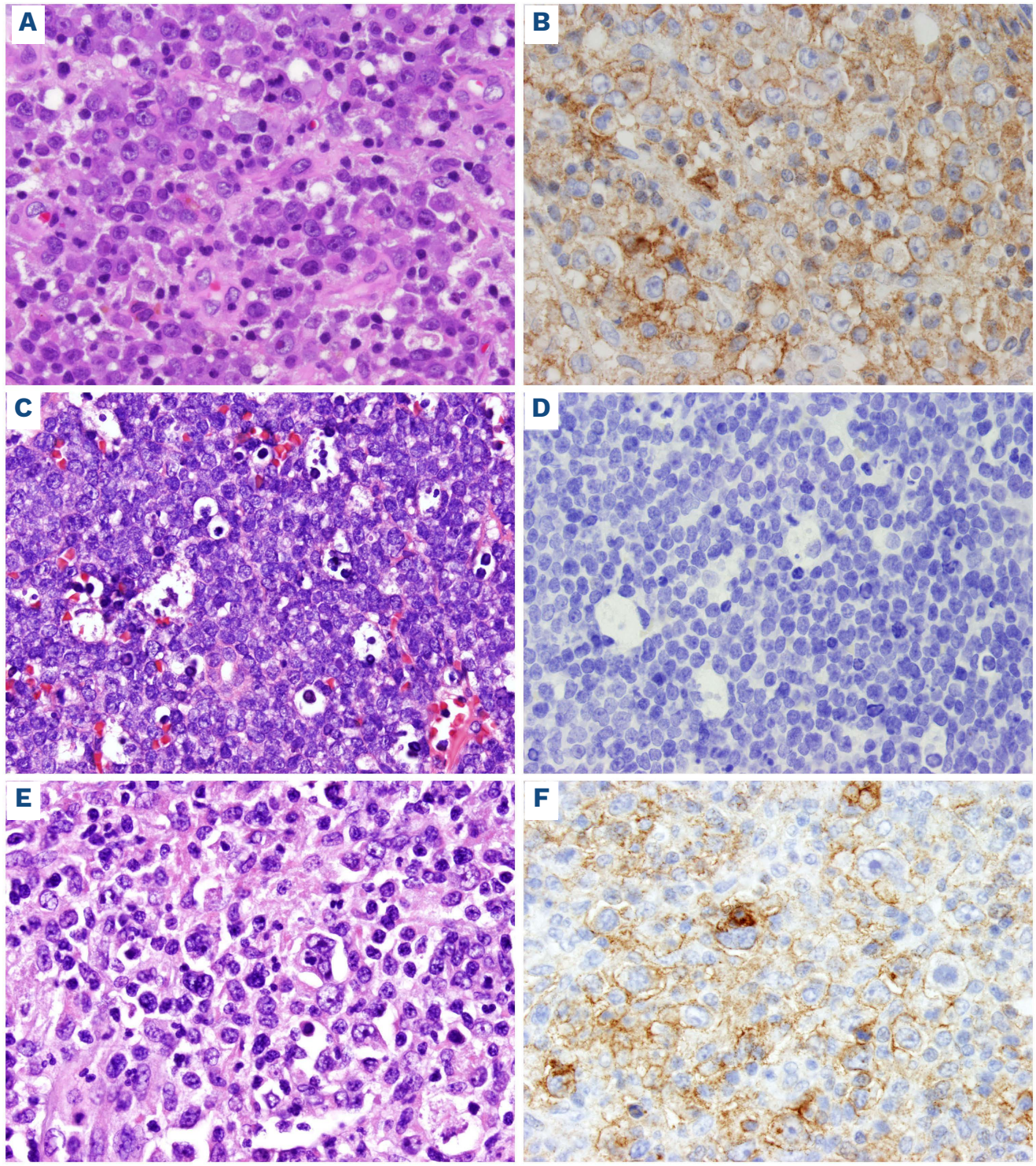

Figure 1. PD-L1 immunohistochemical stains in representative case of pediatric aggressive non-Hodgkin lymphomas. (A and B) ALK-positive anaplastic large cell lymphoma (ALK+ ALCL) showed tumor cells admixed with tumor microenvironment cells (lymphocytes and macrophages). Nearly all tumor cells show PD-L1 expression with moderate to strong staining intensity (2+ to 3+). (C and D) Burkitt lymphoma showed predominantly intermediate-sized lymphoma cells admixed with macrophages. PD-L1 showed no staining. (E and F) Epstein-Barr virus-postive diffuse large B-cell lymphoma not otherwise specified (EBV+ DLBCL-NOS) showed large lymphoma cells and PD-L1 overexpression in 50% of tumor cells with moderate staining intensity (2+). (A to F magnification 400x).

L1-Tum staining and cell of origin, stage, or site of involvement in either the total cohort or within the *de novo* or PTLD-DLBCL subsets. The correlation between PD-L1-Tum and OS and EFS was also examined (Figure 2). PD-L1-Tum did not correlate with OS in the total LBCL cohort or within the *de novo* or PTLD-DLBCL subsets. Likewise, PD-L1-Tum was not significantly associated with EFS in the total LBCL cohort or in the PTLD-DLBCL subset. However, PD-L1 overexpression significantly correlated with lower EFS in the *de novo* LBCL subset (*P*=0.017). Neither OS nor EFS could be calculated in the DLBCL-NOS subset due to small sample size (n=14) and number of events.

Targeted next-generation sequencing

In order to investigate the molecular mechanisms of PD-L1 positivity in a subset of aggressive pediatric NHL, targeted NGS to assess for 9p24.1 copy number and TMB was performed on 12 LBCL (7 *de novo* LBCL [5 DLBCL-NOS, 2 PIA-LBCL] and 5 PTLD-DLBCL) and one BL. Cytogenetic data to assess for the presence of 9p24.1 gene rearrangements were reviewed for seven LBCL (5 *de novo* LBCL [4 DLBCL-NOS, 1 DLBCL with IGH@-MYC] and 2 PTLD-DLBCL). Characteristic somatic *ARID1A* and *TP53* and germline *ATM* mutations were detected in the BL[38] and PIA-LBCL samples, respectively. One PTLD-DLBCL harbored an *ARID1A* mutation, another PTLD-DLBCL harbored

Table 3. Summary of PD-L1 statistical analyses for correlation study in large B-cell lymphoma cohort.

LBCL cohort	Count	EBV+ / EBV-	GCB / non-GCB*	Stage I-II / III*	Nodal / Extranodal
De novo LBCL cohort					
PD-L1-Tum POS	11	2 / 9	7 / 4	3 / 7	2 / 9
PD-L1-Tum NEG	11	0 / 11	8 / 3	5 / 6	4 / 7
P-value	-	0.476	1.000	0.659	0.635
PTLD-DLBCL cohort					
PD-L1-Tum POS	8	8 / 0	1 / 6	4 / 4	4 / 4
PD-L1-Tum NEG	3	2 / 1	1 / 1	0 / 3	0 / 3
P-value	-	0.273	0.417	0.236	0.236
Total cohort					
PD-L1-Tum POS	19	10 / 9	8 / 10	7 / 11	6 / 13
PD-L1-Tum NEG	14	2 / 12	9 / 4	5 / 9	4 / 10
P-value	-	**0.033**	0.275	1.000	1.000

*Cell of origin classification for 2 cases of PTLD-DLBCL was unsuccessful due to insufficient tissue. Staging was unavailable for 1 PIA-LBCL. LBCL: large B-cell lymphoma; EBV: Epstein-Barr virus; GCB: germinal center B-cell phenotype; PD-L1-Tum: PD-L1 expression on tumor cells; PTLD-DLBCL: diffuse large B-cell lymphoma arising as post-transplant lymphoproliferative disorder; POS: positive; NEG: negative.

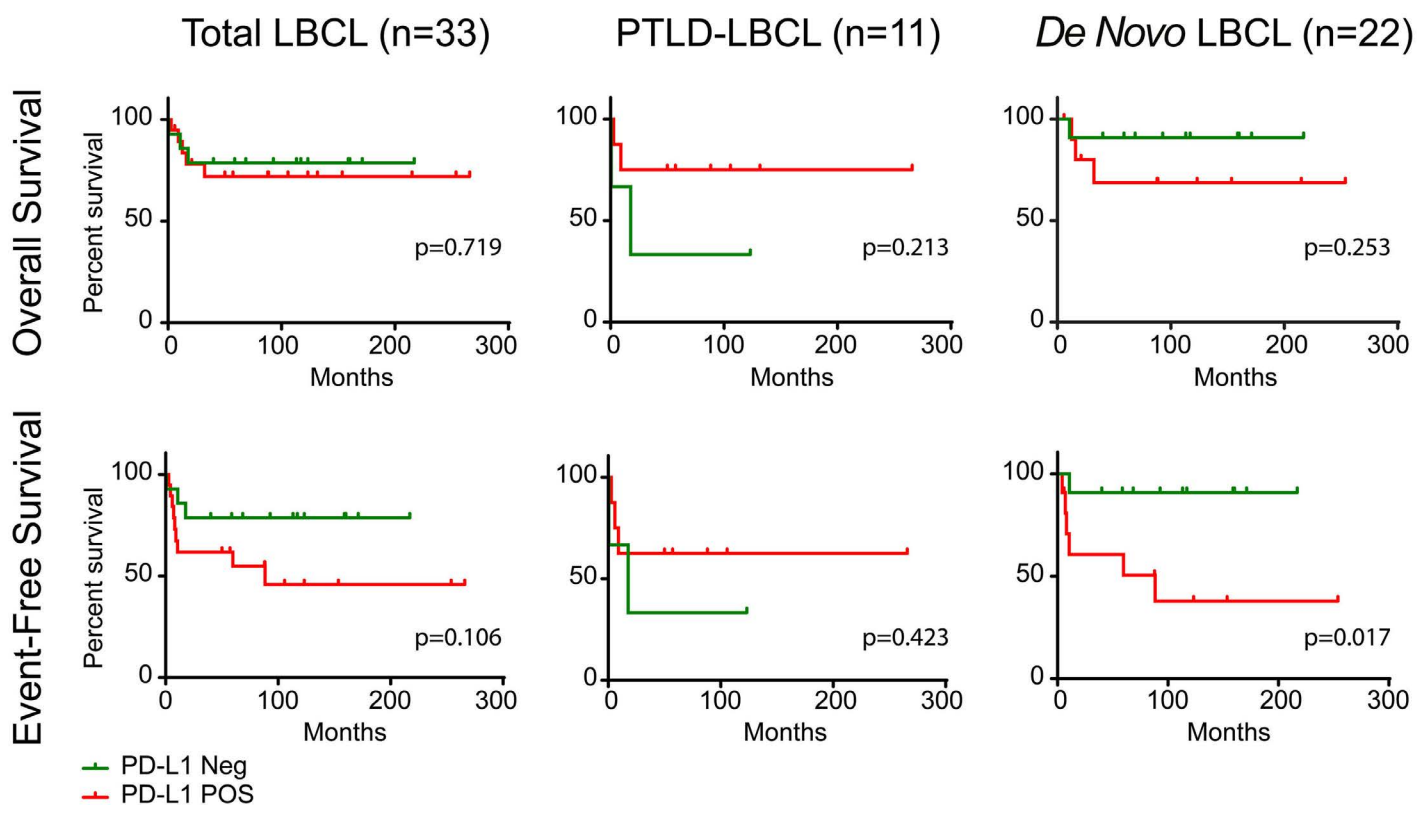

Figure 2. Kaplan-Meier survival curves. Overall survival (OS) and event-free survival (EFS) curves for PD-L1 staining in the total large B-cell lymphoma (LBCL), *de novo* LBCL, and diffuse LBCL arising as monomorphic post-transplant lymphoproliferative disorder (PTLD-DLBCL) cohorts.

DDX3X, *ID3*, and two *TP53* co-mutations, and two DLBCL-NOS cases showed mutations in *CCND3* alone, and *BCOR*, *CCND3*, and *U2AF1* co-mutations, respectively. The results are summarized in Table 4 and the *Online Supplementary Table S3*.

PD-L1-positive LBCL showed EBV positivity (n=2), copy gains of the 9p24.1 locus (n=1), or both (n=1). One PD-L1-positive PIA-LBCL had no discernible mechanism for PD-L1 overexpression and no 9p24.1 rearrangements were observed in any case. EBV positivity was observed in one PTLD-DLBCL and DLBCL-NOS without PD-L1 overexpression. Interestingly, one PTLD-DLBCL (LBCL-29) harbored an estimated TMB of 180 mutations per megabase (Mb), which is considered an "ultra-hypermutated" tumor (>100 mutations/Mb).[39] Pathogenic mutations in *ASXL1*, *CDKN2A*, *CREBBP*, *FBXW7* (n=2), *KMT2C*, *KMT2D*, *KRAS*, *TP53* (n=2), and *ZFHX3* as well as 104 coding or splicing variants of uncertain significance were detected in this tumor (*Online Supplementary Table S3*). Notably, this case did not show PD-L1-Tum positivity.

Discussion

Pembrolizumab is an FDA-approved PD-1 inhibitor for refractory childhood cHL. Likewise, the anti-PD-1 immune checkpoint inhibitor nivolumab has shown promising therapeutic efficacy in early phase clinical trials for pediatric lymphomas that overexpress PD-L1.[23,24] Thus, defining PD-L1 staining patterns and cut-off positivity in aggressive pediatric NHLs using the 28-8 clone antibody, the complementary IHC diagnostic for nivolumab, is of potential clinical significance.

In this study, we defined positive PD-L1 staining as membranous staining of 5% of total viable tumor cells (PD-L1-Tum). We observed positive PD-L1-Tum staining in all eight ALK+ ALCL and none of the 27 BL consistent with previous reports.[20,40] PD-L1-Tum positivity was observed in a ma-

Table 4. Summary of next-generation sequencing and 9p24.1 locus results.

Sample	Diagnosis	PD-L1	EBV+	GCB *versus* non-GCB	9p24.1 rearrangements	9p24.1 gains	Tier I/II Mutations	VAF
BL-21	BL	Neg	Neg	.	N/A	Not Detected	*ARID1A* p.Y1679X *TP53* p.R175H	0.32 0.75
LBCL-14*	PIA-LBCL^	Pos	Neg	non-GCB	N/A	Detected	*ATM* p.S1924X[$]	0.52
LBCL-9*	PIA-LBCL	Pos	Neg	non-GCB	Not Detected	Not Detected	*ATM* p.W2638X[$] *ATM* p.Q1970X[$]	0.43 0.52
LBCL-27	PTLD-DLBCL	Pos	Pos	non-GCB	N/A	Detected	*ARID1A* p.Q172X	0.40
LBCL-30	PTLD-DLBCL	Pos	Pos	non-GCB	N/A	Not Detected	None Detected	.
LBCL-32	PTLD-DLBCL	Pos	Pos	non-GCB	Not Detected	Not Detected	None Detected	.
LBCL-26	PTLD-DLBCL	Neg	Pos	N/A	N/A	Not Detected	*DDX3X* c.1171-2A>G *ID3* p.L70Q *TP53* p.I50fs *TP53* p.R175H	0.71 0.43 0.26 0.35
LBCL-17*	DLBCL-NOS	Neg	Neg	non-GCB	Not Detected	Not Detected	None Detected	.
LBCL-16*	DLBCL-NOS	Neg	Neg	GCB	Not Detected	Not Detected	CCND3 p.R271fs	0.17
LBCL-12*	DLBCL-NOS	Neg	Neg	GCB	N/A	Not Detected	None Detected	.
LBCL-13*	DLBCL-NOS	Neg	Neg	GCB	N/A	Not Detected	*BCOR* p.N1459S *CCND3* p.R271fs *U2AF1* p.S34F	0.89 0.41 0.45
LBCL-3*	DLBCL-NOS	Neg	Pos	GCB	N/A	Not Detected	None Detected	.
LBCL-29[†]	PTLD-DLBCL	Neg	Neg	GCB	Not Detected	Not Detected	11 total, high TMB[†]	0.43

*Denotes *de novo* LBCL. ^Further subclassified as T-cell/histiocyte-rich large B-cell lymphoma: [†]Ultra-hypermutated tumor with ~180 mutations per megabase (Mb). A complete list of mutations is provided in the *Online Supplementary Table S3*. [$]Known germline. BL: Burkitt lymphoma; DLBCL: diffuse large B-cell lymphoma; GCB: germinal center B-cell phenotype; LBCL: large B-cell lymphoma; N/A: not available; Neg: negative; Pos: positive; PTLD: post-transplant lymphoproliferative disorder; TMB: tumor mutation burden; VAF: variant allele fraction.

jority of LBCL including 50.0% of *de novo* LBCL. The percentage of PD-L1 positive DLBCL-NOS cases in our pediatric cohort (28.6%) was slightly higher than two recent adult studies in which positive PD-L1 staining was reported in approximately 10-15% of DLBCL-NOS cases.[29,31] The frequency of PD-L1 positive pediatric PTLD-DLBCL cases (72.7%) was similar to other pediatric and adult PTLD-DLBCL cohorts that reported approximately 60-80% positivity.[13,34,41]

Despite defining 5% as the threshold for PD-L1-positivity, all PD-L1-positive cases in our cohort showed at least 30% positive membranous staining. Kiyasu *et al.*[29] employed a similar cut-off in adult DLBCL and reported PD-L1 positivity ranges from 30-100% while observing a marked decrease in staining below the 30% threshold. Additionally, we detected minimal PD-L1-Mac staining or PD-1-TIL staining consistent with other pediatric studies that reported low levels of PD-1-positivity.[34] Together, the data support a potential role for PD-L1 testing in pediatric aggressive NHL, including ALK+ ALCL, *de novo* LBCL, and PTLD-DLCBL. Furthermore, our data suggest that a positivity threshold of 30% is suitable when assessing pediatric aggressive NHL for PD-L1 staining of tumor cells.

We correlated LBCL PD-L1-Tum staining with multiple clinicopathologic features although the small number of cases precluded comprehensive statistical analyses. There was no statistically significant correlation between stage, nodal status, cell of origin, or OS in the total LBCL cohort, or when separated into *de novo* LBCL and PTLD-DLBCL. PD-L1-Tum staining correlated with EBV positivity in our study for the entire LBCL cohort, but not with PTLD-DLBCL cohort alone. Although PTLD-DLBCL comprised a large portion of the EBV+ LBCL samples in our cohort, the grouping of EBV+ *de novo* LBCL and PTLD-LBCL is not necessarily representative of clinical practice. Nonetheless, correlation between EBV positivity and PD-L1 overexpression in pediatric LBCL was previously reported; Nicolae *et al.*[42] reported a strong correlation in patients younger than 45 years of age between EBV+ and PD-L1 expression in LBCL, particularly in THRBCL and non-GCB DLBCL, which was recapitulated here. Furthermore, LBCL classified as intrinsic (oncogenic) induction of TME correlated with EBV positivity suggesting that EBV is a potent regulator of PD-L1 in pediatric LBCL.

In two previous adult LBCL studies in which a higher threshold for tumor cell positivity was utilized (30%), PD-L1 positivity correlated with inferior OS.[29,31] Using a *de facto* cut-off of 30% in our study, PD-L1 staining correlated with reduced EFS in *de novo* LBCL, although no statistical significance in OS was observed. However, studies investigating the correlation between PD-L1 positivity and survival in adult DLBCL are confounded by multiple variables such as the antibody clone employed, IHC scoring methodology adopted, and/or non-uniform positivity thresholds.[29-31,43] We acknowledge that conclusions regarding OS and EFS are limited due to the retrospective nature of our study, the small numbers of LBCL subtypes, and non-uniform treatment regimens, particularly pediatric intensive treatment regimens without rituximab for *de novo* LBCL treatment. Also, the grouping of diagnostic entities that have distinct clinicopathologic and therapeutic considerations (e.g., PMBCL) restricts the generalization of the statistical findings. Nonetheless, the negative correlation between PD-L1 and EFS in pediatric *de novo* LBCL was observed, and additional studies are warranted to establish PD-L1-Tum as a negative prognostic marker in clinical pediatric practice.

CD274 gene overexpression and increased PD-L1 protein translation can occur through a variety of mechanisms such as increased *CD274* transcription via promoter activation or *CD274* copy gains, amplifications, and gene rearrangements.[17,18,20,21] In our study, PD-L1 staining was observed most frequently in cases with EBV positivity and 9p24.1 copy gains. However, in two EBV+ cases, one *de novo* LBCL and one PTLD-DLBCL, no PD-L1 staining was observed. This is not unexpected, as EBV-associated *CD274* gene regulation requires complex interplay between transcriptional elements, microRNA, and post-transcriptional modifications.[44,45] Also, in one PD-L1-positive PIA-LBCL case no 9p24.1 copy gains or rearrangements were observed. Thus, it is possible that this case represented a false-negative 9p24.1 result; comprehensive molecular assessments of *CD274* 3'-UTR structural variations and gene rearrangements[46] not detectable by karyotype analysis were not performed.

Interestingly, we encountered an EBV-negative PTLD-DLBCL with no PD-L1 positivity with an estimated TMB of 180 mutations/Mb consistent with an "ultra-hypermutated" tumor (>100 mutations/Mb). The high TMB was not attributable to somatic hypermutation; all mutations were detected in coding exons and not in immunoglobulin heavy chain and light chain variable region genes.[47] The accuracy of TMB assessment is proportional to the number of bases sequenced so smaller targeted panels can lead to imprecise TMB scores. However, recent studies suggest that highly accurate TMB assessments can be obtained with targeted panels larger than 1.0 Mb.[48,49] In this study, we utilized a targeted sequencing panel that captures and sequences approximately 1.25 million individual bases (1.25 Mb) so we conclude that our TMB estimates are reasonably accurate. Hypermutation is rare in pediatric tumors; however when detected, it often occurs in the setting of congenital mismatch repair deficiency (CMMRD) syndromes and/or acquisition of somatic *POLE/POLD1* mutations[39] but genetic testing for CMMRD or *POLE/POLD1* somatic mutation testing was not performed in this case. Despite the lack of PD-L1 positivity in this case, emerging data suggest that either PD-L1

staining or high TMB should be considered positive biomarkers for response to immune checkpoint inhibitor therapy.[50] Collectively, our data indicate that EBV and 9p24.1 copy gains are key regulators of PD-L1 overexpression in a subset of pediatric LBCL, particularly in PTLD-DLBCL, which is consistent with other studies in adult patients.[40] Assessment of TMB may identify some patients that may benefit from immune checkpoint inhibitor therapy in the absence of PD-L1-Tum staining.

The genomic landscape of pediatric LBCL has not been extensively studied, but in adults, mutations in DLCBL tend to cluster in pathways that regulate cell signaling and growth, B-cell development, and nucleic acid transcription and translation.[51] Our sequencing analysis revealed similar somatic pathogenic mutations regulators of the cell cycle, transcription, and DNA repair (*CCND3*, *DDX3X*, *ID3*, *TP53*), and mRNA splicing (*U2AF1*), but also identified mutations in epigenetic modifiers (*ARID1A*, *BCOR*). There did not appear to be significant differences in the types of mutations encountered in GCB *versus* non-GCB cases or differences in mutational profiles between *de novo* LBCL or PTLD-DLBCL. However, only 152 genes were sequenced in our study and the panel did not include several genes that were recently reported to cluster in discreet LBCL subtypes (e.g., *B2M*, *CD79B*, *CIITA*, *GNAI2*, and *SGK1*).[52, 53] Thus, the limited gene content and small number of cases hinders a definitive differential assessment of mutational signatures.

In conclusion, as one of the largest pediatric series to date, we showed that 30% cut-off for membranous PD-L1 staining in the tumor cells (PD-L1-Tum) is an appropriate threshold to assess for PD-L1 positivity and correlated with EBV positivity in LCBL and lower EFS in *de novo* LBCL. Also, EBV and copy gains of 9p24.1 appear to regulate PD-L1 overexpression in a subset of cases and rare cases of pediatric NHL may demonstrate high TMB highlighting a potential new biomarker in this disease. Additional studies exploring the therapeutic and prognostic significance, molecular mechanisms, and host environments that affect PD-1/PD-L1 staining in aggressive pediatric NHL are warranted.

Disclosures

No conflicts of interest to disclose.

Contributions

KEF and CVC designed the study, wrote, edited the manuscript, and supervised the project. LSF, AMC, AMM, and BYM performed experiments and collected and analyzed data. JLC, ANM, DLT, and KYK assisted in data collection and provided clinical expertise.

Data sharing statement

Data sharing is subject to Institutional policies governing clinical research data.

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