ANTICANCER RESEARCH *40*: 5951-5968 (2020)
doi:10.21873/anticanres.14616

Review

EBV Positivity and Programmed Death-ligand 1 Expression in Diffuse Large B-cell Lymphoma: A Systematic Review

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Abstract. *Background/Aim: Diffuse large B-cell lymphoma (DLBCL) is the most common form of non-Hodgkin lymphoma. A systematic review to evaluate the association between Epstein-Barr Virus (EBV) and programmed death ligand-1 (PD-L1) in DLBCL biopsy was conducted. Materials and Methods: Only studies comparing EBV+ and EBV– groups were eligible following database search. Prevalence ratios were calculated for results comparison. The EBV impact on PD-L1 positivity in tumour cells and its microenvironment was analysed. Results: With 270 records screened, eleven cross-sectional studies were identified for final review. Eight studies investigated PD-L1 expression in tumour cells and found an EBV trend unlikely, while four studies found an increase in its expression in the tumour microenvironment. Nine studies showed that EBV+ cases were more commonly of non-germinal centre B-cell origin. Four studies examined genetic aberrations, but no definite consensus was reached. Conclusion: A non-EBV related mechanism is likely related to increased PD-L1 expression, with relevance to the cell of origin.*

Diffuse large B-cell lymphoma (DLBCL) is the most common form of Non-Hodgkin Lymphoma (NHL) and represents 25-45% of NHL globally (1). With annual incidence of 5-7 per 100,000 people, age is a significant factor as incidence rises to around 30 per 100,000 in those aged 65-84 years (2). DLBCL, not otherwise specified (DLBCL, NOS) accounts for 80-85% of all DLBCL cases and its diagnosis is made *via* exclusion of DLBCL subtypes with a distinct morphology or immunophenotype (3). Herein, DLBCL and DLBCL, NOS are interchangeable terms in literature unless a distinct subtype of DLBCL is specified.

DLBCL typically arises at a lymph node, although extra-nodal manifestations are also present (4). Its aetiology remains poorly understood, but changes in gene expression and mutations which promote malignant behaviour have been noted (5). DLBCL is diagnosed *via* biopsy, where tumours derived from germinal centre B-cells (GCB) have a better prognosis than those of non-GCB (N-GCB) subtype, also known as Activated B-cells (ABC) (6). Despite advancements, approximately 40% of patients relapse or are refractory (7) to standard R-CHOP treatment (Rituximab, cyclophosphamide, hydroxydoxorubicin, oncovin and prednisolone/prednisone), where the presence of Epstein-Barr Virus (EBV) appears to coincide with more severe outcomes (8).

DLBCL has undergone a major revision in the World Health Organisation (WHO) 2016 classification of lymphoid neoplasms, including renaming of "EBV-positive DLBCL of the elderly" subtype to "EBV-positive DLBCL, NOS" as younger individuals can also be affected (9). Approximately 90-95% of the world's population sustains a life-long, asymptomatic infection with EBV through saliva (10), following a primary lytic infection, where the virus avoids immune response by acquiring various latency types (11). About 5-15% of all DLBCL cases are EBV positive (EBV+) (12) and higher incidence rates are found in developing countries (13) where both immunocompetent and immunocompromised patients are affected (14).

EBV association has been increasingly analysed in articles exploring programmed cell death protein-1 (PD-1) (2q37.3 locus) and programmed death-ligand 1 (PD-L1) (9p24.1 locus). PD-1 is normally expressed on the surface of immune cells and it has the ability to negatively regulate the immune response. In cancerous state, PD-L1 present on a cancer cell

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Key Words: EBV, diffuse large b-cell lymphoma, Epstein-Barr virus positive diffuse large b-cell lymphoma, programmed death-ligand 1, tumour immunology, non-Hodgkin lymphoma, review.

binds to PD-1, thus reducing T-cell function and preventing immune response (15) leading to immune evasion. PD-1 blockade has been established as a therapy for some cancers (16), and PD-L1 has been found to be over-expressed in various DLBCL tumours, prolonging tumour progression and survival (17). The precise mechanism(s) involved in EBV– driven DLBCL carcinogenesis remain unknown (18) and up to date EBV trends related to DLBCL PD-L1 expression have not been summarised. Accordingly, a systematic review was carried out to evaluate available literature to determine whether EBV positivity has an impact on PD-L1 expression in DLBCL tumour biopsy.

Materials and Methods

This systematic review was registered with PROSPERO, an international prospective register of systematic reviews (registration ID: CRD42020183091).

Search strategy. A search of the published literature was carried out on 25th of April 2020 using Embase, LILACS, Web of Science, CINAHL, Ovid, National Cancer Institute, Cochrane Central Register of Controlled Trials (CENTRAL), EU Clinical Trials Register and ClinicalTrials.gov databases. The search terms relating to EBV ("EBV" OR "Epstein Barr Virus" OR "HHV4" OR "EBER" OR "LMP" or "EBNA"), DLBCL ("lymphoma" OR "b-cell lymphoma" OR "DLBCL" OR "diffuse large b-cell lymphoma") and PD-L1 ("PD-L1" OR "PDL1" OR "CD274" OR "programmed death-ligand 1" or OR "b7-h1" OR "PDCD1LG1") were adapted to each database, and limited to publication date of 2015 to present. In addition, article reference lists were reviewed to ensure thorough search of the literature.

Eligibility criteria. The EPICOT framework (evidence, population, intervention, comparison, outcome, timestamp) (19), was used to develop the research question and to formulate inclusion criteria to select appropriate studies. Studies were selected if they met the following requirements: 1) Discuss EBV+DLBCL and/or their extranodal manifestations, include DLBCL classified within lymphoproliferative disorder, post-transplant or immunocompromised patients. Provide analysis relevant to PD-L1 expression or therapy, whether in the tumour itself or in the tumour microenvironment; 2) Studies including minimum of ≥10 participants, 5 of which were in the EBV+ DLBCL subgroup and 5 in the control group; 3) Articles discussing tumour biopsy, whether archival or with sample obtained during study. Specific treatment(s) not required, but any intervention or study looking at genetic alterations, molecular application or drug therapies which affect PD-L1 expression to be noted; 4) Have a control group of EBV- DLBCL patients; 5) Describe EBV status identification or confirmation, accept expression and/or genetic identification of EBER, EBNA or LMP. PD-L1 identification explicitly stated and justification for positivity status provided. Offer a comparison between EBV+ and EBV– subgroups; 6) Papers with publication date 2015 onwards, with first author known, full-text article available and any conflict of interest is explicitly stated. Exclusion criteria included the following: 1) Papers discussing other distinct DLBCL subtypes or distinct clinical issues including chronic inflammation associated DLBCL or primary lymphoma of the central nervous system, addressing EBV-related B-cell neoplasms classed as: Burkitt, Hodgkin, plasmablastic or primary effusion lymphomas, discussing "gray zone" lymphomas, T-cell lymphomas or leukaemias. Papers discussing PD-1 expression only, without addressing PD-L1. Any data relating to cell culture, animal studies, case studies, editorials, abstracts; 2) Papers including patients who's EBV status was not known or where data was mixed with other population subgroups where DLBCL relevant data cannot be extracted; 3) Research describing the already established standard R-CHOP treatment for DLBCL, unless new context is added with relevance to effect on PD-L1 expression; 4) Presence of EBV+ or EBV– DLBCL group only; 5) EBV or PD-L1 Identification methods or positivity criteria not stated; 6) The articles were in non-English language.

Study selection and data extraction. Two independent reviewers were involved in the study selection and any disagreement was resolved through conference with a 3rd reviewer. The results were recorded in a PRISMA flow diagram (20), alongside a summary of reasons for exclusion.

An Excel data extraction tool was devised to facilitate comparisons and to aid data collection consistency. The following items were extracted: study design, ethical approval, sample type, sample size, methods of identification and measurement of EBV and PD-L1 including dilution factor and antibody source, positivity thresholds, expression values and supporting statements, significance values and statistical analysis methods. Obtained population characteristics included: age, gender, cell of origin, Lymphoma International Prognostic Index (IPI) and chemotherapy treatment.

Quality assessment. The critical Appraisal Tool for Cross-sectional Studies (AXIS) (21) was used to assess the quality of the included studies by the two reviewers. Each item was graded as "low risk" or "high risk", while insufficient information was classified as "uncertain".

Data synthesis. A narrative synthesis was carried out using tables summarising study characteristics and results of individual studies. The principal summary measures included data relevant to PD-L1 expression in tumour and tumour microenvironment alongside data related to genetic alterations. Where applicable, prevalence ratio (PR) was calculated with 95% confidence intervals to give general commonality to results, particularly where statistical analysis was not present, in order to evaluate EBV association with PD-L1 expression in DLBCL.

Results

Study selection. The database search identified 345 records for screening alongside 21 additional records (Figure 1). Following duplicate removal, 270 studies underwent title and abstract screening, where 105 records were excluded. A total of 165 studies underwent full text screening and eleven studies met the inclusion criteria for review.

Characteristics of included studies. The characteristics of the included studies are summarised in Table I. All eleven articles (22-32) were cross-sectional studies published between 2015 and 2019. Four articles originated from Japan (24, 28-30),

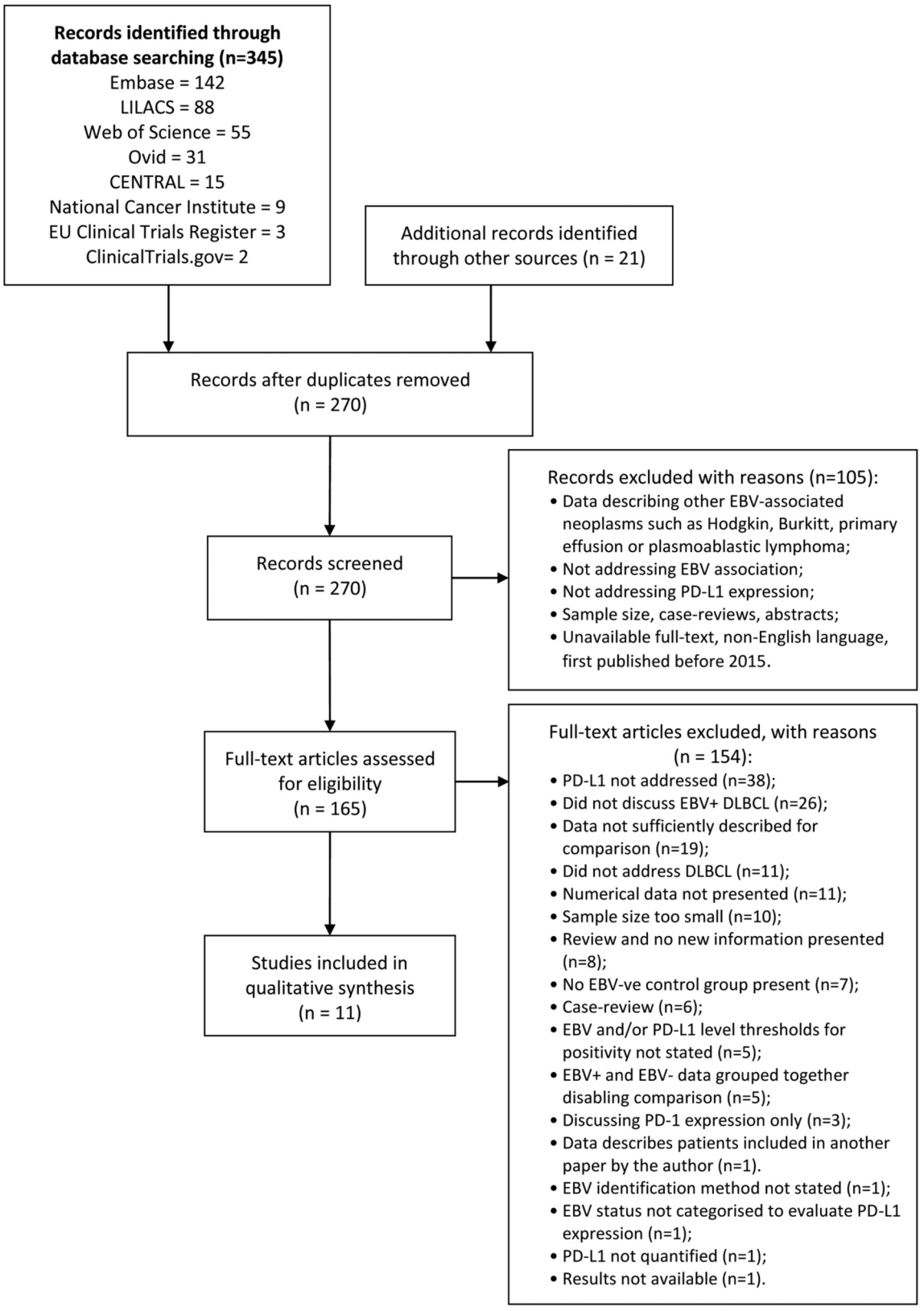

Figure 1. *PRISMA Flow Diagram (20) showing the stages of the review process, alongside lists of reasons for exclusion before the final number of included studies was reached.*

Table I. *Characteristics of individual included studies.*

First author, Year of publication	Country	Study type/ Sample type	Sample size (EBV+/ EBV–)	EBV identification method	PD-L1 identification	PD-L1 antibody (dilution)	Source	PD-L1 expression definitions and/or cut-off value(s)	Patient characteristics		Ethics
Anastasiadou, 2019 (22)	United States	Cross-sectional survey/ DLBCL	27 (11/16) where 9 (6/3) clinical samples further quantita tively tested for % positivity	EBER ISH using PNA probe/ Fluorescein and FITC/HRP (DAKO, Glostrup, Denmark).	IHC	E1L3N (dilution not known)	Cell Signaling Technology	PD-L1+ when stain detected in membrane, cytoplasm, or Golgi area. +1 intensity was partial membrane staining, +2 was moderate and complete staining and +3 is intense and complete membrane staining. For quantitative estimation, number of PD-L1+ cells was determined using the Aperio software IHC Membrane v1 and the IHC Membrane Image Analysis algorithm for score 2-3 membrane staining, where %PD-L1+ was calculated by counting positive cells in three squared areas measuring 50000 μm^2.	EBV+ (11) GCB: 0 N-GCB: 11 (100%)	EBV– (16) GCB: 10 (63%) N-GCB: 6 (37%)	Written informed consent obtained from all patients involved in the study. Approved by the Institute's ethics review board.
Jiang, 2019 (23)	China	Cross-sectional survey/ Histological confirmation of *de novo* DLBCL, not otherwise specified, in compliance with WHO classification.	113 (30/83)	EBER ISH	IHC	ab205921 (28-8) (1:200)	Abcam, Cambridge, MA	IHC score was given as range 0-300 where: percentage of positive cells (0 to 100, recorded in the increment by 5%) was multiplied by mean intensity (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining).	EBV+ (30) Age: 61 (23-84) M:F 22:8 IPI 0-2: 12 (40%) IPI 3-5: 18 (60%) Immuno-suppression: 1(3%) GCB 3 (10%) N-GCB 27 (90%) "Overall, the two groups do not have significant differences in gender, age, IPI, bulky disease, and immunosuppression status. Consistent with previous reports, EBV+ DLBCL patients were predominantly of non-GCB subtype (27/30, 90%) compared to EBV-DLBCL (44/83, 53%, p=0.001)"	EBV– (83) Age: 60 (23-86) M:F 50:33 IPI 0-2: 24 (29%) IPI 3-5: 59 (74%) Immuno-suppression: 3 (4%) GCB 39 (47%) N-GCB 44 (53%)	Approved by the ethical committee of Fudan University Shanghai Cancer Centre.

Table I. *Continued*

Table I. *Continued*

First author, Year of publication	Country	Study type/ Sample type	Sample size (EBV+/ EBV–)	EBV identification method	PD-L1 identification	PD-L1 antibody (dilution)	Source	PD-L1 expression definitions and/or cut-off value(s)	Patient characteristics		Ethics
Kataoka, 2019 (24)	Japan	Cross-sectional survey/ DLBCL	75 (27/48)	Gene reads mapped to the EBV genome (NC_007605) with regard to mapped human reference genome (GRCh37); cut-off value: ratio of 0.00015%. Value was set to match EBV+ based on EBER ISH (Bond Ready-to -Use probe, Leica Biosystems, Wetzlar, Germany) and IHC for LMP-1 (CS1-4, Dako) of samples.	Target capture sequencing	E1J2J (dilution not known); SP142 (dilution not known)	Cell Signaling Technology, Beverly, MA, USA; Spring Bioscience, Fremont, CA, USA)	Target capture sequencing for CD274 (PD-L1), where additional probes for 1999 SNPs were designed to calculate genomic copy numbers. Somatic mutations underwent further filtering and mapping errors were removed by visual inspection. Same principle applied to SVs and CNAs. TCGA data set: to evaluate the copy number of PD-L1, the level 3 segmented copy number data (Affymetrix Genome-Wide Human SNP Array 6.0) were downloaded from the TCGA data portal.	N/A - patient characteristics not provided		Approved by the institutional ethics committees of the Graduate School of Medicine, Kyoto University, and other participating institutes.
Keane, 2019 (25)	Australia	Cross-sectional survey/ Histological DLBCL, excluding follicular lymphoma IIIB, transformed lymphoma or immuno-suppression-related lymphoma.	433 (30/403), of those 390 (26/364) where included in EBER-digital cohort	EBER ISH, EBER-digital, nCounter platform (NanoString™) on EBER-1, EBER-2 and LMP-1. EBER-1 <30 gene count considered as background level (EBV–).	Gene quantification using nCounter platform, NanoString™	Not identified	Not identified	Gene count	EBV+ (30) Age: 66.7 (38.5-90) Age <60: 21 (72%) M:F 15:8 IPI 0-2: 12 (45%) IPI 3-5: 15 (55%) GCB: 16 (69%) N-GCB: 7 (31%) R-CHOP: 27 (90%) Other treatment: 3 (10%) "EBV+ cases did not differ by IPI or any of IPI parameters (chi-squared test) including age category >60 years of age. However, patients with EBV+ DLBCL were older than EBV- cases (p=0.018)." "28% of EBV+ tumours occurred in patients ≤60 years, […] whilst patients with EBV+ DLBCL were significantly older, it does not preclude the possibility of EBV+ DLBCL occurring in younger patients"	EBV– (403) Age: 61 (18-89.95) Age <60: 215 (58%) M:F 177:150 IPI 0-2: 194(56%) IPI 3-5: 150 (44%) GCB: 174 (61%) N-GCB: 80 (48%) R-CHOP: 355 (83%) Other treatment: 68 (17%)	Approved by Metro South (Brisbane) Ethics Committee.

Table I. *Continued*

Table I. *Continued*

First author, Year of publication	Country	Study type/ Sample type	Sample size (EBV+/ EBV–)	EBV identification method	PD-L1 identification	PD-L1 antibody (dilution)	Source	PD-L1 expression definitions and/or cut-off value(s)	Patient characteristics		Ethics
Kinch, 2019 (26)	Sweden	Cross-sectional survey/ Post-transplant DLBCL	46 (26/20)	EBER ISH; expression of LMP-1 was determined by IHC in routine diagnostics.	IHC	E1L3N (1:75)	Cell Signaling Technology, Danvers, MA	PD-L1+ when moderate or strong staining seen in ≥5% of tumour cells, graded into ≥5-<30% or ≥30% group. mPD-L1+ defined as ≥20% of the total tissue cellularity showing moderate or strong staining of PD-L1 in PD-L1- tumour cell cases.	EBV+ (26) GCB: 1 (4%) N-GCB: 25 (96%)	EBV– (20) GCB: 8 (40%) N-GCB: 11 (55%) Unknown type: 1 (1%)	Approved by the Regional Ethical Review Board in Uppsala, Sweden; in accordance with the Declaration of Helsinki and the Swedish Ethical Review Act.
Veloza, 2019 (27)	Spain	Cross-sectional survey/ Post-transplant DLBCL	37 (21/16); of those 25 (13/12) in FISH analysis	EBER ISH with fluorescein-labelled probes (INFORM EBER; Ventana). IHC to further determine EBV latency types: LMP-1 (clone CS1-CS5; Agilent) and EBNA-2 (clone PE2; Abcam, Cambridge, UK), BZLF1/ZEBRA lytic gene. Case considered positive for EBV replication when >2 positive cells were seen.	IHC; FISH	IHC: SP263 (dilution not known); DAK-PAX5 (dilution not known; CD68 clone PG-M1 (dilution not known). FISH: 145kb probe mapping to the 9p24.1 region including PD-L1 locus (Spectrum Orange-Red-labelled probe SureFISH chr9: 5348900–5494553)	IHC: Ventana, Tucson, AZ, USA; Agilent, Santa Clara, CA, USA; Agilent, Santa Clara, CA, USA; FISH: Agilent, Santa Clara, CA, USA	PD-L1+: moderate or strong staining seen in ≥5% of tumour cells; PD-L1+ TAM: moderate to strong staining in ≥20% of CD68-positive cells; FISH for PD-L1 CNAs: cases classified according to the highest observed level of PD-L1 alteration, where cases with PD-L1 copy gains lacked amplification, and cases with PD-L1 polysomy lacked copy gains and amplification.	EBV+ (21) Age: 56 (26-78) GCB: 1 (5%) N-GCB: 17 (81%) Unknown type: 3 (15%) R: 3 CHOP: 3 R-CHOP: 6 Methotrexate: 1	EBV– (16) Age: 63.5(42-78) GCB: 9 (56%) N-GCB: 5 (31%) Unknown type: 2 (13%) R: 7 CHOP: 0 R-CHOP: 6 Methotrexate: 1	Performed according to Hospital Clinic of Barcelona and Hospital Universitari de Bellvitge ethics committee guidelines.
Ishikawa, 2018a (28)	Japan	Cross-sectional survey/ Gastric DLBCL (gDLBCL)	240 (25/215); of those 54 (14/40) cases examined for PD-L1 expression	EBER ISH; cases considered EBER-positive when nuclear expression observed in ≥80% of tumour cell	IHC	SP142 (1:50), E1J2J (1:50)	Spring Bioscience, Cell Signaling Technology	PD-L1+ when moderate or strong staining seen in >5% of cells mPD-L1+ when among total tissue cellularity, ≥20% of cells had moderate/strong staining in the membrane or cytoplasm, including non-malignant cells.	EBV+ (25) Age: 69 (37-85) Age <60: 7 (28%) M:F 12:13 IPI 3-5: 7(28%) GCB: 8/24 (33%) N-GCB: 16 (67%) R-c treatment: 12 (48%) "We found no significant difference	EBV– (215) Age: 67 (32-89) Age <60: 63 (29%) M:F 124:91 IPI 3-5: 62 (29%) GCB: 79/208 (37%) N-GCB: 129 (62%) R-c treatment: 144 (67%)	Approved by the Institutional Review Board of Nagoya University.

Table I. *Continued*

Table I. *Continued*

First author, Year of publication	Country	Study type/ Sample type	Sample size (EBV+/ EBV–)	EBV identification method	PD-L1 identification	PD-L1 antibody (dilution)	Source	PD-L1 expression definitions and/or cut-off value(s)	Patient characteristics		Ethics
									in clinicopathological findings between the EBV+ and EBV– gDLBCL subgroups, except for the endoscopic features"		
Ishikawa, 2018b (29)	Japan	Cross-sectional study/ Intestinal DLBCL including EBV+, *de novo* CD5+ and DLBCL-NOS	62 (10/52)	EBER ISH; cases considered EBER-positive when nuclear expression observed in ≥80% of tumour cell	IHC	SP142 (1:50)	Spring Bioscience	PD-L1+ when moderate or strong staining seen in >5% of tumour cells, mPD-L1+ mPD-L1+ when among total tissue cellularity, ≥20% of cells had moderate/strong staining in the membrane or cytoplasm, including non-malignant cells.	EBV+ (10) Age: 74 (47-82) Age <60: 2 (20%) M:F 4:6 IPI 3-5: 6 (67%) GCB: 1 (10%) N-GCB: 9 (90%) R-c treatment: 9 (90%)	EBV– (52) Age: 67 (15-88) Age <60: 14 (27%) M:F 38:14 IPI 3-5: 8/47 (17%) GCB: 21/50 (42%) N-GCB: 29/50 (58%) R-c treatment: 42/49 (86%)	Approved by the Institutional Review Board of Nagoya University.
									"EBV+ […] surprisingly, seven of these were related with treated lymphoma-associated (peripheral T-cell lymphoma [n=2], classic Hodgkin lymphoma [n=2]) or iatrogenic immunodeficiency (methotrexate [n=1], infliximab [n=1], and tacrolimus [n=1]. The other one also had a synchronous gastric carcinoma, while the remaining two had no episode suggestive of immunodeficiency in their lifestyle analysis. This prompted us to reexamine the presence of events related to immunodeficiency among EBV– iDLBCL cases, but none were found."		
Kiyasu, 2015 (30)	Japan	Cross-sectional survey/ Newly diagnosed, untreated DLBCL NOS and DLBCL of elderly	1181 (90/1091)* *Figure excludes other DLBCL types presented in study	EBER ISH with a fluorescein conjugated EBV peptide nucleic acid probe kit (Dakocytomation, Glostrup, Denmark)	IHC, double staining with PAX5	Anti PD-L1: ab174838 (EPR1161) (1:200); Anti-PAX5: BC/24 (1:50)	Abcam; Biocare Medical, Concord, CA	PD-L1+ when ≥30% of cells showed distinct membranous and/or cytoplasmic staining. mPD-L1+ when ≥20% non-malignant cells among total tissue cellularity expressed positivity in PD-L1-DLBCL.	N/A (patient characteristics mixed with other DLBCL subtypes)		Approved by the Research Ethics Committee of Kurume University in accordance with the Declaration of Helsinki.
Quan, 2015 (31)	China	Cross-sectional survey/ Previously untreated	27 (7/20)	EBER ISH where >20% EBER+ tumour cells; IHC to further	Flow cytometry	Antibody not specified (dilution not known), FITC-A used.	Cannot determine	Flow cytometry values	EBV+ (7) Age median: 67 Age <60: 2 (29%) Age >60: 5 (71%) M:F 5:2	EBV– (20) Age median: 54 Age <60: 13 (46%) Age >60: 7 (54%) M:F 11:9	Approved by the Clinical Research Ethics Committee of The Cancer

Table I. *Continued*

Table I. *Continued*

First author, Year of publication	Country	Study type/ Sample type	Sample size (EBV+/ EBV–)	EBV identification method	PD-L1 identification	PD-L1 antibody (dilution)	Source	PD-L1 expression definitions and/or cut-off value(s)	Patient characteristics		Ethics
		DLBCL patients		determine EBV latency types. LMP1 (CS1-4, Dako, Germany), LMP2A (15F9, AbCam, UK), EBNA2 (PE2, AbCam, UK), EBNA3A (ab16126, Abcam, UK).					IPI 0-2: 2 (39%) IPI 3-5: 5 (71%) GCB: not reported N-GCB: not reported	IPI 0-2: 13 (65%) IPI 3-5: 7(35%) GCB: 8/20 (40%) N-GCB: 12/20 (60%)	Hospital of Harbin Medical University (Harbin, China); according to the Declaration of Helsinki and the study protocol.
Yoon, 2015 (32)	South Korea	Cross-sectional survey/ DLBCL of elderly	36 (13/23); of those 28 (8/20) involved in microarray gene expression, 16 (8/8) analysed by MIP assay and gene microarray.	EBER ISH, cases showing >50% EBER+ enrolled in the study	Gene copy number analysis and expression profiling	Microarray gene expression profiling: WG-DASL assay, using 200 ng of RNA PCR: Genomic regions of PDCD1LG2 were PCR amplified using TaqMan probes and primers: Hs01115641_cn and Hs05087845_cn	Illumina, Hayward, CA Applied Biosystems, Carlsbad, CA	DNA copy number analysis was performed using the OncoScan™ FFPE Express 2.0 Services of Affymetrix. Functional annotations with an enrichment score of >1.0, a *p*-value <0.01, and a false-discovery rate (FDR) ≤10% were considered biologically interesting. Microarray gene expression profiling using the significant analysis of microarrays program (33) with at least two-fold changes and an estimated FDR of ≤10%. Array Comparative Genomic Hybridization (CGH) Validation: following PCR amplification, estimated copy number calculated using the (–)ΔΔCT method, where CT=cycle thresholds. Values >2.5 were considered copy number gains.	EBV+ (13) Age: 70 (50-86) M:F 8:5 GCB: 0 (0%) N-CGB: 13 (100%) IPI 0-2: 2 (15%) IPI 3-5: 9 (70%) IPI unknown: 2 (15%)	EBV+ (23) Age: 61(35-82) M:F 15:8 GCB: 0 (0%) N-CGB: 23 (100%) IPI 0-2: 12 (52%) IPI 3-5: 10 (43%) IPI unknown: 1 (4%)	Approved by the institutional review board in accordance with the Declaration of Helsinki.

EBER: Epstein–Barr virus-encoded small RNA; EBNA: EBV nuclear antigen; FDR: False discovery rate; LMP: Latent membrane protein; M:F: male:female.

two from China (23, 31), and five articles originated from each of the following countries: United States (22), Australia (25), Sweden (26), Spain (27), and South Korea (32). Although all papers addressed DLBCL, there was a variation in its assortment, with two papers referring to 'DLBCL' only (22, 24) while others further specified the DLBCL sample type. Two papers (31, 32) referred to 'DLBCL of the elderly' due to publication date of 2015, which occurred before the WHO 2016 classification update. All studies used existing records to retrospectively select DLBCL participants. Out of 2,277 cases described with patient characteristics, 2,044 samples were PD-L1 tested including 272 EBV+ and 1,772 EBV– cases. Sample size varied between 27-1181 participants. Ten studies addressed EBV+ cohort with n=7-30 patients, and one had an n=90 cohort (30). The EBV– cohort ranged from n=16-1091, with two studies having an n>100 control cohort (25, 28).

Patient characteristics were described to varied degree, and most were not equally distributed within and between studies. Three articles (25, 28-29) addressed all relevant characteristics relating to age, gender, cell of origin, IPI and chemotherapy treatment, none were provided within one study (24), and in one (30) the characteristics were mixed with other lymphomas, hence, were not included in this review. When cell of origin proportions were combined from nine studies addressing this parameter (22-23, 25-29, 31, 32) where 850 samples were addressed, the overall proportion for EBV+ cases was 0.19 GCB and 0.81 N-GCB, while EBV– cases was 0.50 GCB and 0.50 N-GCB. Seven studies addressed age (23, 25, 27-29, 31, 32), and the median age for EBV+ groups ranged from 56 to 74 while for EBV– cases it ranged from 54 to 67; patients with an age of <60 years were included in four studies (25, 28-29, 31). Gender was addressed by six studies (23, 25, 28-29, 31, 32) where 805 samples were distinguished (466 males and 339 females), with overall male to female ratio (M:F) of 1.37. Of the six studies addressing IPI (23, 25, 28-29, 31, 32), EBV+ cases were of higher proportion in four out of six studies (25, 29, 31, 32) looking at IPI 3-5, with one study noting marginal difference in proportion between EBV+ and EBV– cases (28). Four studies specified chemotherapy approach (27, 29-31) relating to the patient characteristics and therapies were predominantly of Rituximab-containing nature.

EBV-encoded small RNAs (EBER) *in situ* hybridization (ISH) was applied in eleven studies, while two also applied genetic analysis (24-25). Overall, seven studies examined PD-L1+ expression *via* immunohistochemistry (IHC) staining (22-23, 26-30), four papers applied genetic analysis (24-25, 27, 32) and one utilised flow cytometry (31). A combination of IHC and genetic analysis was used in one study (27).

Risk of bias assessment. Quality assessment was based on the AXIS tool (21) with exclusion of questions 3, 13 and 14 which did not apply to the participants as non-responders were not associated with any of the studies.

The quality and clarity of reporting relating to various parameters of AXIS including design, measures and conclusions were uniform across multiple studies. Study design was appropriate in all articles, however, due to relative rarity of EBV+ DLBCL, sample size justification was not provided in seven studies (22, 24, 27-29, 31, 32), while four stated small sample size (23, 25-26, 30). All studies defined their population and had an appropriate sample frame. One study did not address its aims/objectives (23) while the participant selection process was not explained in sufficient detail in three studies (22, 31, 32), omitting details such as dates of retrospective record inclusion. Appropriate measures were incorporated in all studies and they were all measured correctly, with adequate data and methods, whether in main text or supplementary data. The statistical significance criterion was not applied to two studies (26, 30) as statistical analysis relevant to DLBCL was mixed with other lymphoma types, while another two (25, 27) did not address the reasons behind missing results. Study limitations were not addressed in three studies (24, 31, 32), whereas only three stated no funding or conflict of interest (28-30). Overall, all studies included ethical approval and were judged as low risk for selective outcome reporting as links between funding sources and flaws in reporting were not identified.

Results of individual studies. Table of results was compiled before undertaking thematic analysis. The analysis incorporated data relevant to PD-L1 expression in tumour cell, tumour microenvironment and genetic alterations. Alongside this, a trend regarding cell of origin type in EBV+ and EBV– cases was investigated.

EBV status and PD-L1 positivity

Tumour cells. Eight studies addressed PD-L1 expression on tumour cells (22-23, 26-31) (Table II) where two provided statistical analysis (23, 29). Seven articles (22-23, 26-27, 29-31) found that EBV+ showed an increase in PD-L1, and one detected no PD-L1 expression (28). Importantly, in six papers where PR was applied, only three had PR>1 with 95%CI (22, 27, 30), while three papers included PR=1 in CI (26, 29, 31).

Four articles (26-29) utilised 5% threshold for positivity, and three found an increase in PD-L1+ on EBV+ biopsies. One paper provided *p*-value: 0.072 (29), although this included PR=1 and a statement that PD-L1 and EBV were independent factors. Two other papers (26, 27) also showed PR>1 where one included PR=1 in CI (29), while another found that PD-L1 was not expressed on any EBV+/– tumour cells (28). Another study (30) utilised percentage expression with >30% threshold and found that PD-L1+ was expressed in EBV+ cases with PR 1.75 (1.04, 2.93).

Table II. *PD-L1+ expression on tumour cells based on staining patterns in eight included studies.*

First author, Year of publication	PD-L1 expression by population		Statistical value	Prevalence ratio (CI)	Further comments
	EBV+	EBV–			
Anastasiadou, 2019 (22)	PD-L1+: 100% (11/11) Non-GCB quantitative analysis: % PD-L1+ cells: 91.8 (78-100); where EBNA2+ latency III=99.3% and EBNA2– latency II=84.3%	PD-L1+: 69% (11/16) Non-GCB quantitative analysis: % PD-L1+ cells: 28 (19-35)	N/A For EBV+, EBNA2+/ EBNA2– % total PD-L1-positive cells *p*-value=0.0125	PD-L1+: 1.45 (1.05, 2.02)	N/A
	% of strong intensity score (2-3): 45.3% (where EBNA2+ latency III=70%, EBNA2– latency II=20.7%)	% of strong intensity score (2-3): 1.34%	For EBV+, EBNA2+/ EBNA2– % cells with strong staining intensity *p*-value=0.0040		
Jiang, 2019 (23)	Median PD-L1 score 110 (range=0-300)	Median PD-L1 score 80 (range=0-279)	*p*-value=0.006	N/A	"… found that EBV infection is associated with over-expression of PD-L1 in our cohort" "Interestingly, although PD-L1 was expressed heterogeneously in both EBV– and EBV+ DLBCL, the level of PD-L1 in EBV+ DLBCL was much higher than that in EBV– DLBCL"
Kinch, 2019 (26)	PD-L1+: 17/26 (65%)	PD-L1+: 8/20 (40%)	N/A	PD-L1+: 1.63 (0.89, 2.99)	N/A
Veloza, 2019 (27)	PD-L1+: 18/21 (86%)	PD-L1+: 6/16 (38%)	N/A	PD-L1+: 2.29 (1.19, 4.41)	N/A
Ishikawa, 2018a (28)	PD-L1+: 0/14 (0%)	PD-L1+: 0/40 (0%)	N/A	N/A	"none of EBV+ gDLBCL cases expressed PD-L1 on tumor cells, although LMP1 was positive in 7 of 14 EBV+ cases evaluated."
Ishikawa, 2018b (29)	PD-L1+: 2/10 (20%)	PD-L1+: 1/49 (2%)	PD-L1+ *p*-value=0.072	PD-L1+: 9.8 (0.98, 97.96)	"EBER positivity and PD-L1 expression (≥5%) are independent prognostic factors for poor survival"
Kiyasu, 2015 (30)	PD-L1+: 14/90(15.6%)	PD-L1+: 97/1091 (8.9%)	N/A	PD-L1+: 1.75 (1.04, 2.93)	N/A
Quan, 2015 (31)	PD-L1+: 5/7 (71.4%); flow cytometry peak count at 300 ($10^{4.5}$ FITC-A)	PD-L1+ 8/20 (40%) where: GCB PD-L1+: 0/8; flow cytometry peak count at 40 ($10^{2.5}$ FITC-A). N-GCB PD-L1+: 8/12 (66.7%); flow cytometry peak count at 200 ($10^{3.75}$ FITC-A)	N/A	Combined (GCB, n-GCB), PD-L1+: 1.78 (0.88, 3.64)	"PD-L1 expression in DLBCL occurs almost exclusively in tumors of non-germinal center origin and EBV positive DLBCL."

N/A: Not applicable.

Table III. *PD-L1+ expression in the tumor microenvironment based on staining patterns in five included studies.*

First author, Year of publication	PD-L1 expression by population		Statistical value	Prevalence ratio (CI)	Further comments
	EBV+	EBV–			
Kinch, 2019 (26)	mPD-L1: 0	mPD-L1: 0	N/A	N/A	"In PD-L1 negative cases, the microenvironment of the tumor was assessed for an expression of PD-L1 exceeding 20% of the cells […] but this was not observed" "there was no association between expression of intratumoral Tregs and PD-1 or its ligands"
Veloza, 2019 (27)	tPD-L1+: 20/21 (95%)	tPD-L1+: 8/16 (50%)	N/A	tPD-L1+: 1.90 (1.16, 3.14)	N/A
Ishikawa, 2018a (28)	mPD-L1+: 12/14 (86%)	mPD-L1+: 17/40 (43%)	mPD-L1 *p*-value: 0.006	mPD-L1+: 2.02 (1.33, 3.07)	"Histological results showed that PD-L1 was detected in microenvironmental immune cells at a significantly higher rate" "Patients with EBV+ gDLBCL commonly exhibited microenvironmental PD-L1 expression and showed a significantly worse prognosis than subjects with EBV– gDLBCL"
Ishikawa, 2018b (29)	mPD-L1: 8/8 (100%)	mPD-L1: 31/48 (65%)	mPD-L1 *p*-value: 0.09	mPD-L1+: 1.55 (1.26, 1.90)	N/A
Kiyasu, 2015 (30)	mPD-L1+:30/76 (55.6%)	mPD-L1+: 124/994 (12.5%)	N/A	mPD-L1+: 3.16 (2.29, 4.37)	N/A

tPD-L1+: Tumor associated macrophage positivity; mPD-L1+: microenvironment positivity; N/A: not applicable.

Two articles (22, 23) utilised a modified score based on expression values, where IHC score (23) showed higher detection in EBV+ cases with a statically significant value. The other papers (22) identified PR 1.45 (1.05, 2.02) for EBV+ irrespective of the staining score, where EBV latency had higher %PD-L1+ expression and higher staining intensity in EBNA2+ cases, *p*-values: 0.0125 and 0.004, respectively. Using flow cytometry, another study (31) stated that PD-L1+ expression was higher in EBV+ cases, PR 1.78 (0.88, 3.64), but specified EBV and cell of origin as separate factors.

Tumour microenvironment. Five papers examined PD-L1 expression in relation to the tumour microenvironment (26-30) and four (27-30) found an EBV association with PR>1, whilst one paper (31) identified no PD-L1 positive cases in the microenvironment of either subgroup (Table III).

All papers adopted a 20% threshold for positivity. Of those, four analysed total tissue cellularity (26, 28-30), where two which did not specify the cellularity further found an association in EBV+ cases, at *p*-values of 0.09 (29) and 0.006 (28), respectively. One paper further specified the total tissue cellularity to non-malignant cells only (30), finding PR 3.16

Table IV. *Cell of origin commentary, cell type comparison and relation to PD-L1 expression in nine included studies.*

First author, Year of publication	Cell type commentary	Cell of origin type				PD-L1 expression relating to EBV+	
		GCB		N-GCB			
		EBV+	EBV–	EBV+	EBV–		
Anastasiadou, 2019 (22)	"suggest that EBV+ non-GC DLBCLs have slightly higher PD-L1 expression than those non-GC DLBCLs without the virus." "number of cells with high staining intensity (+2, +3) […] was significantly higher in EBNA2+ ABC DLBCLs in comparison with EBNA2– cases"	0 (0%)	10 (63%)	11 (100%)	6 (37%)	PR PD-L1+: 1.45 (1.05, 2.02) N-GCB quantitative analysis: % PD-L1+ cells: 91.8 (78-100); where EBNA2+ latency III=99.3% and EBNA2– latency II=84.3%; EBNA2+/EBNA2– *p*-value=0.0125	N-GCB quantitative analysis: % PD-L1+ cells: 28 (19-35) % of strong intensity score (2-3): 1.34%
						% of strong intensity score (2-3): 45.3% (where EBNA2+latency III=70%,EBNA2– latency II=20.7%); EBNA2+/EBNA2– *p*-value=0.0040	
Jiang, 2019 (23)	N/A	3 (10%)	39 (47%)	27 (90%)	44 (53%)	*p*-value: 0.006	
Keane, 2019 (25)	N/A	16 (69%)	174 (61%)	7 (31%)	80 (48%)	Gene count *p*-value <0.0001	
Kinch, 2019 (26)	N/A	1 (4%)	8 (40%)	25 (96%)	11 (55%)	PR PD-L1+: 1.63 (0.89, 2.99) mPD-L1+: N/A	
Veloza, 2019 (27)	"EBV-positive DLBCL cases were more commonly non-GCB than GCB (94% *versus* 6%, *p*=0.001)"	1 (5%)	9 (56%)	17 (81%)	5 (31%)	PR PD-L1+: 2.29 (1.19, 4.41) PR tPD-L1+: 1.90 (1.16, 3.14)	
Ishikawa, 2018a (28)	N/A	8 (33%)	79 (37%)	16 (67%)	129 (62%)	PR PD-L1+: N/A PR mPD-L1: 2.02 (1.33, 3.07)	
Ishikawa, 2018b (29)	"Most of our cases (90%) of EBV+ iDLBCL had non-GCB immunophenotype, which may be related to their adverse outcome"	1 (10%)	21 (42%)	9 (90%)	29 (58%)	PR PD-L1+: 9.8 (0.98, 97.96) PR mPD-L1: 1.55 (1.26, 1.90)	
Quan, 2015 (31)	"PD-L1 expression in DLBCL occurs almost exclusively in tumors of non-germinal center origin and EBV positive DLBCL."	Not reported	8 (40%)	Not reported	12 (60%)	Combined (GCB, n-GCB) PD-L1+: 1.78 (0.88, 3.64)	
Yoon, 2015 (32)	N/A	0 (0%)	0 (0%)	13 (100%)	23 (100%)	9q24.1 copy number gain: 38.5% *vs*. 24% EBV–	

N/A: Not applicable.

(2.29, 4.37) for EBV+ cases. This article considered samples which were already established as PD-L1- in tumour cells, an approach also undertaken by another study (26). However, the latter identified no patients with PD-L1 positivity, and also commented on the lack of association between PD-L1 and intratumoural regulatory T-cells irrespective of the EBV status.

One paper (30) remarked on a significantly higher rate of PD-L1 detection in microenvironmental immune cells

Table V. *Frequency of genetic aberrations relevant to PD-L1 expression in EBV+ and EBV- cases in four included studies.*

First author, Year of publication	PD-L1 expression by population		Statistical value	Prevalence ratio (CI)	Further comments
	EBV+	EBV–			
Kataoka, 2019 (24)	PD-L1/L2 Genetic aberrations frequency: 5/27	PD-L1/L2 Genetic aberrations frequency: 1/48	*p*-value: <0.05. 'The observation was confirmed by examining DLBCL cases in the TCGA cohort (27% *vs*. 5%, *p*=0.07)'	Genetic aberration 8.9 (1.09, 72.20)	"Although the numbers of mutations and CNAs were comparable between EBV– and + DLBCLs, EBV+ DLBCLs had a larger number of SVs than EBV- tumors', targeted sequencing *p*-value <0.05, RNA-sequencing + SNP array (TCGA) *p*-value=0.07" "There were no differences in genetic alterations between EBV+ DLBCLs with and without PD-L1/PD-L2 genetic alterations"
Keane, 2019 (25)	Gene count: 364 (31-760)* *Values interpreted from graph	Gene count: 82 (4-1536)* *Values interpreted from graph	*p*-value <0.0001	N/A	"LMP1, an EBV-related oncogene with immunomodulatory properties, showed significant correlations with PD-L1 (r=0.49, *p*=0.014)."
Veloza, 2019 (27)	PD-L1 CNA: 10/13 (77%)	PD-L1 CNA: 8/12 (67%)	'CNAs in a variable proportion of cells, regardless of EBV status (*p*=0.6)'	N/A	FISH: "Moreover, the number of cells with PD-L1 CNAs did not differ between EBV-positive and E BV-negative cases [residual PD-L1 disomies: 76±standard deviation (SD) 16 *versus* 87±SD 14, respectively, *p*=0.1].", "high percentages of residual PD-L1 disomies were observed in most cases suggests that EBV may play a major role in PD-L1 up-regulation in PTLD, whereas PD-L1 CNAs may further increase PD-L1 expression in variable numbers of neoplastic cells in EBV+ cases" "most EBV- DLBCL cases with PD-L1+ TCs showed frequent PD-L1 CNAs, which indicates that genetic alterations in the 9p24.1 locus may have an important role in modulating PD-L1 expression in some EBV– PTLD cases. However, EBV– PD-L1+ TCs without PD-L1 CNAs were also found, which suggests that additional pathways of activation could be involved."
Yoon, 2015 (32)	9q24.1 copy number gain: 38.5% PDCD1LG2 gene overexpression noted in EBV+: 24.47% PDCD1LG2 enrichment score identified at 3.74	9q24.1 copy number gain: 25%	*p*-value <0.001; FDR <0.001 *p*-value: 0.003, FDR: 3.86%.	N/A	"The overall frequency of genomic alterations was lower in EBV+DLBCL than it was in EBV–DLBCL. Thirteen recurrent copy number aberrations (>30%) were observed in EBV+DLBCL, whereas EBV-DLBCL showed 24 chromosomal regions with recurrent aberrations."

N/A: Not applicable.

without further evidence. Another study (27) examined this in relation to tumour associated macrophages (TAM), noting an EBV+ related increase in PD-L1+ at PR 1.90 (1.16, 3.14).

Cell of origin subtype. Overall, nine studies characterised cell of origin (22-23, 25-29, 31, 32) (Table IV). Six papers (22-23, 26-29) noted EBV+ N-GCB majority, one study found N-GCB was higher in EBV– cases (25), one did not identify any cases with GCB origin (32), and one did not report EBV+ cell origin (31). Five papers reported origin without further analysis (23, 25-26, 28, 32) and among those, three reported ≥90% of N-GCB in EBV+ cases (23, 26, 32), whilst another had a 67% majority (28). There was only one study where N-GCB was a minority at 31% (25).

In four papers reporting origin in detail (22, 27, 29, 31), two found that EBV+ DLBCL cases were more commonly N-GCB, at 94% with p=0.001 (27) and 90%, respectively (29). EBV+ cases were exclusively N-GCB in one study (22), showing increased PD-L1+ cells (91% *vs*. 28%). The fourth paper (31) reported that PD-L1 expression appeared more commonly in N-GCB subtype and EBV+ cases, but EBV+ cell of origin was not reported. When the overall cell origin proportions were combined, EBV+ cases were 0.19:0.81 GCB:N-GCB, while EBV– cases were 0.5:0.5 GCB:N-GCB.

In relation to PD-L1, five papers (22-23, 26-27, 29) reported an increase in PD-L1 in tumour cell when EBV+ N-GCB cases were greater in number, although two of those included PR=1 (26, 29). One of the two papers (29) showed PR>1 increase in environment, consistent with two other papers reporting on microenvironment and cell type (27, 28).

EBV status and PD-L1 genetic aberrations. Four studies (24-25, 27, 32) examined genetic aberrations relating to PD-L1 and 9q24.1 (Table V). All studies noted an increase, but only two concluded that EBV+ harbours significant change (24, 25). One paper (25) showed an EBV-related increase in frequency of PD-L1 gene count, where latent membrane protein 1 (LMP1) showed significant correlations. Another paper (24) described an increase in frequency of PD-L1/PD-L2 structural variation (SV) in EBV+ cases, although SV type was not specified. Interestingly, it noted that EBV+ cases did not show differences in other genetic aberrations irrespective of the PD-L1/PD-L2 alteration, while another study (32) stated that other genetic aberrations where lower in EBV+ cases.

In contrast, despite noting that PD-L1 copy number alterations (CNAs) were detected in more EBV+ cases, another study (27) found PD-L1 CNAs in variable proportion of cells regardless of EBV status. In addition, no significant difference was found between disomies of EBV+/– cases, although it was noted that with high percentage of residual disomies, EBV may play a role in PD-L1 up-regulation in post-transplant lymphoproliferative disorder (PTLD). One study (32) found a 9q24.1 copy number gain in EBV+, where it was reported that the PD-L2 gene (*PDCD1LG2*) located on 9q24.1 was also overexpressed in EBV+ cases.

Discussion

The systematic review included eleven cross-sectional studies and to the authors' knowledge, this was the first systematic review which aimed to determine whether EBV impacts PD-L1 expression in DLBCL tumour biopsy. There was considerable variability in the methodologies of published literature and although staining thresholds seemed more consistent, it is apparent that investigation of PD-L1 expression requires a degree of standardisation to distinguish definite DLBCL trends. However, the review was able to identify several research avenues to explore.

EBV+ is unlikely to have an impact on PD-L1+ in tumour cells as despite seven out of eight papers finding an apparent increase (22-23, 27-29, 31, 32), PR overlap at 95%CI was noted. Particularly in cases where 5% threshold was applied giving commonality to results (26-29), values indicated that there may be no difference as some PR>1 had CI close to 1.0. Importantly, as this is an arbitrary cut-off value where higher thresholds may show definite trends (34), and as EBV-related PD-L1 expression has been identified in epithelial malignancies (35), this result should not be negated, and should be further investigated using higher positivity thresholds. Due to small sample sizes for EBV+ cohorts, it is likely that patients experience PD-L1 related changes, however, this does not appear to be an exclusive feature of EBV+ patients. As such, there is a need to identify the non-EBV mechanism of this phenomenon, although EBV latency may be a contributing factor in disease progression.

One of the most interesting trends indicated that EBV+ is associated with increased PD-L1 expression in tumour microenvironment. Although taken with caution due to limited number of studies, the PR values suggest that this may be a common finding (36). However, as the 20% threshold was applied to various parameters of the total tissue cellularity, it is possible that this result arose due to selection or confirmation bias (34). Moreover, the use of different antibodies and dilution factors could account for the observed patterns due to false-positives as selected dilutions were not justified (37). Taking into consideration that EBV relationship to PD-L1 in tumour cell is unlikely, it is apparent that selected patients might benefit from targeted anti-PD-1 therapies as the 20% threshold relating PD-L1 to tumour microenvironment appears to show a more predominant EBV link to PD-L1 expression. However, as this feature is not solely found in EBV+ patients, this finding further supports the notion that a non-EBV related mechanism operates leading to PD-L1 increase.

It is well understood that N-GCB DLBCL is associated with worse outcomes (38). A pattern regarding PD-L1 increase in N-GCB was found across several studies (39) suggesting that those of N-GCB cell type may benefit from introduction of PD-L1 therapy irrespective of EBV status. However, EBV has been linked to increased pSTAT3 expression in N-GCB (40), highlighting that this behaviour is likely cell-type dependant, where EBV+ may have an enhancing effect on PD-L1. EBV has been stipulated to up-regulate PD-L1 *via* a direct effect on JUN-B signalling cascade *via* LMP-1 (41-43) or *via* indirect up-regulation of inflammatory cytokines (44-46), with effects on JAK/STAT signalling pathway in Hodgkin and NHL (47). As five articles in this review related N-GCB to an increase in PD-L1 expression (22-23, 26-27, 29), although with variable PR, this result indicates that EBV+ patients may have greater likelihood of PD-L1+ and may need treatment revaluation. This includes possible addition of anti-PD-L1 medication such as Pembrolizumab (48) to selected patients considering that various trials involving R-CHOP combinations have not yielded positive effects (49). The analysis of cell type with focus on N-GCB and its impact on PD-L1 should thus be prioritised in future research to enable appropriate immunotherapeutic strategy and risk stratification in DLBCL. Moreover, more studies should utilise genetic approaches in addition to methods such as IHC, thus enabling the assessment of whether PD-L1+ and EBV+ are independent prognostic factors considering the direct and indirect involvement of EBV, particularly in relation to N-GCB potency and worse prognostic outcomes. Regarding patient characteristics, it has been reassuring to see inclusion of younger patients following the WHO classification update, where aside from N-GCB, no other correlations were identified.

Considering genetic aberrations, findings similar to those identified in this review were noted in PTLD-DLBCL (50). However, due to mixed methods and commentary, no consensus can be appreciated despite PD-L1 aberrations appearing to increase in EBV+. Various studies link 9p24.1 locus alteration (43, 47, 51) and the JAK/STAT pathway (42, 52) as a contributing factor to PD-L1 overexpression irrespective of the EBV status, as identified in this review. This supports the notion that EBV is not a sole deterministic factor (53), but likely an enhancer of PD-L1. As such, it is important to determine the level of contribution of EBV to PD-L1 genetic aberrations and JAK/STAT through the indirect mechanisms. Of note, EBV+ may also explain the relatively small gain of genetic aberrations identified in DLBCL in some studies (54), as often EBV status is not addressed and such cases may in fact be EBV+, affecting PD-L1 expression *via* the direct pathway.

Limitations. The review included a comprehensive search of literature, where stringent inclusion criteria ensured that studies which described relevant methodology in sufficient detail were included in the synthesis. However, a substantial amount of literature did not describe methodology to sufficient standard and multiple results were presented in conference abstracts. As these articles were not suitable for inclusion in this review and only English language papers were included, publication bias may have been introduced. Importantly, EBER status was considered sufficient for EBV identification irrespective of threshold, however experts agree that >50% of malignant cells should be EBER positive (55) and this could have impacted the data. Similarly, thresholds for PD-L1+ and mPD-L1+ need standardisation, as overall meta-analysis was not possible due to diversity of PD-L1 and EBV measures. Despite application of PR and CI to provide commonality to data comparison, variability in thresholds meant that overall, the predictive capability of this parameter was less reliable.

It is important to note that gene expression does not equate to molecular expression and studies where staining was not used to clarify results offer limited picture into impact of a genetic aberration. Furthermore, due to relative rarity of EBV+ DLBCL, sample sizes were not justified in multiple studies and this could have caused data inaccuracies.

Implications for future research. One of the most important factors identified from this review is the need to standardise reporting and thresholds, perhaps at higher levels (34), to enable meta-analysis of identified trends. More studies should utilise a dual approach with staining and genetics to clarify whether EBV-related changes affect PD-L1 expression and this must be related to cell of origin type, which appears to be more indicative of PD-L1 relationship.

Conclusion

With increased number of EBV+ cases identified as N-GCB, this systematic review indicates that cell of origin is of relevance to DLBCL, where a link or enhancement of PD-L1 pathway *via* EBV may be of significance. EBV+ does not seem to be the sole factor determining PD-L1+ despite noting increase in PD-L1 in tumour microenvironment as EBV– patients also show PD-L1+, and it is likely that another mechanism is involved. Although this needs to be further explored due to small sample size, it is apparent that the protocols require standardisation to confirm trends through large-scale studies before any anti-PD-L1 treatment application is considered.

Conflicts of Interest

The Authors declare no potential conflicts of interest. The review has no funding source.

Authors' Contributions

GA Barzyk (study concept and design, data collection, data analysis and interpretation, manuscript preparation and final approval), V Sheriff (study design, data collection, data analysis and interpretation, manuscript preparation and final approval).

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Received August 5, 2020
Revised August 24, 2020
Accepted August 25, 2020