Expression of the immune checkpoint molecules PD-L1 and PD-1 in EBV-associated lymphoproliferative disorders: A meta-analysis

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Received May 9, 2023; Accepted August 31, 2023

DOI: 10.3892/etm.2023.12294

Abstract. Epstein-Barr virus (EBV) has been implicated in the development of a wide range of lymphoproliferative disorders. In this process, the role of programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) has remained to be clarified. A meta-analysis of 20 studies was performed and risk ratios (RRs) with 95% confidence intervals (CIs) were used to evaluate the association between PD-L1/PD-1 expression and the status of EBV infection. The results showed that the expression level of PD-L1 in tumor cells was significantly higher in EBV^+ cases with a pooled RR of 2.26 (95% CI, 1.63-3.14; P<0.01), particularly in subtypes of diffuse large B-cell lymphoma (DLBCL) and classical Hodgkin lymphoma. Similarly, EBV infection increased the expression of PD-L1 in immune cells with a pooled RR of 2.20 (95% CI, 1.55-3.12; P<0.01). In subtypes of DLBCL and post-transplant lymphoproliferative disorder, the expression of PD-L1 in immune cells is increased in EBV^+ cases. Regarding the expression level of PD-1 in tumor-infiltrating lymphocytes (TILs), no significance was found between EBV infection and PD-1 expression, with a pooled RR of 1.10 (95% CI, 0.81-1.48; P>0.05). The present meta-analysis demonstrated that in EBV-associated lymphoproliferative disorders, EBV infection was associated with the expression level of PD-L1 in tumor cells and immune cells but was not associated with the expression of PD-1 in TILs.

Introduction

Epstein-Barr virus (EBV) is a member of the g-herpesvirus family and is classified within the lymphocryptovirus genus (1). The EBV genome contains linear double-stranded DNA of 172k base pairs (2). Primary EBV infection usually takes place during childhood and the virus subsequently undergoes an asymptomatic latency phase (2).

The occurrence of certain human malignant tumors has been closely related to EBV infection, including nasopharyngeal carcinoma and lymphoid malignancies. EBV-associated lymphoproliferative disorders have been widely clarified and are divided into B-cell and T/natural killer (NK) cell disorders (3). EBV-associated B-cell lymphoproliferative disorders include the following: i) Burkitt's lymphoma; ii) a proportion of Hodgkin lymphomas; iii) post-transplant lymphoproliferative disorders (PTLDs); iv) HIV-associated lymphoproliferative disorders; and v) other rare histotypes (3). T/NK-cell lymphoproliferative disorders that have been reported to be EBV-associated include: i) A proportion of peripheral T-cell lymphomas; ii) angioimmunoblastic T-cell lymphoma; iii) extranodal nasal type NK/T-cell lymphoma; and iv) other rare histotypes, including lymphomatoid granulomatosis, pyothorax-associated lymphoma and senile EBV-associated B-cell lymphoproliferative disorders (3).

EBV targets lymphocytes and achieves latent infection in a circular episomal form (4). Different latency patterns are recognized based on latent gene expression patterns. There are three types of latent gene expression, which have been described as latency I, II and III encoding genes: i) EBV nuclear antigen (*EBNA*)-*1*, EBV encoded RNA (*EBER*)-*1* and *EBER-2* (latency I, II and III); ii) *EBNA-2* and *EBNA-3* (latency III); and iii) latent membrane protein (*LMP*)-*1* and *LMP-2* (latency II and III) (4). Latency I is generally associated with EBV-related Burkitt's lymphoma (5,6), latency II has been associated with classical Hodgkin lymphoma (cHL) and T-cell non-Hodgkin lymphoma and latency III occurs mainly in immune-compromised individuals suffering from PTLDs and HIV-associated lymphoproliferative disorders and in lymphoblastoid cell lines (5,6).

The programmed cell death (PD)-1/PD-1 ligand 1 (PD-L1) pathway was first reported by Dong *et al* (7) in 1999. It was indicated that the PD-1/PD-L1 pathway regulates effector T-cell responses, which are considered to be involved in the negative regulation of immune responses, thus protecting tissues from immune-mediated damage (8,9). However, activation of the PD-1/PD-L1 pathway in tumor cells inhibits effector T-cell function and activates immunosuppressive regulatory T-cell function, resulting in tumor evasion of host immune surveillance (10,11). PD-L1 is expressed in tumor cells and tumor-infiltrating

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Key words: programmed cell death 1, programmed cell death ligand 1, Epstein-Barr virus, lymphoma, meta-analysis

nonmalignant cells, primarily macrophages, while PD-1 is expressed by tumor-infiltrating lymphocytes (TILs) (8).

Evidence has suggested that aberrant PD-L1 expression is associated with poor prognosis in certain types of solid cancer, such as non-small cell lung cancer, advanced melanoma and renal cell carcinoma (12-14), and cHL (15). The presence of large numbers of PD-1 expressing TILs is associated with favorable overall survival in patients with diffuse large B cell lymphoma (DLBCL) (16,17).

EBV-infected cells that acquire alterations involving PD-1/PD-L1 are thought to effectively evade anti-EBV immune surveillance, which has been associated with immunotolerance. PD-1/PD-L1 axis checkpoint blockade may provide effective therapeutics against EBV-related lymphomas compared with conventional chemotherapy (18,19). It was also shown that PD-L1 expression was induced by LMP1 promoter activity in EBV-transformed B cells. In addition, >70% of EBV^+ cases of PTLDs express detectable PD-L1 (20).

In DLBCL, Kwon *et al* (21) observed that EBV infection may contribute to PD-L1 expression in activated B-cell type DLBCL. However, no consensus has been reached on whether EBV positivity has a definite impact on PD1/PD-L1 expression in EBV-related lymphomas and lymphoproliferative disorders. Accordingly, the present meta-analysis was carried out to elucidate the association between the PD1/PD-L1 axis and EBV infection.

Materials and methods

Search strategy and selection criteria. The present meta-analysis was performed according to the PRISMA guidelines (http://www.prisma-statement.org). Studies were identified by searching the PubMed database (https://pubmed.ncbi.nlm.nih.gov/) for articles published up to 30th June, 2022. The following keywords were searched: 'lymphoma' AND 'lymphoproliferative disorders' OR 'LPDs' AND 'Epstein-Barr virus' OR 'EBV' AND 'EBV-encoded RNA' OR 'EBER'. A reference search was also performed and article searches were restricted to literature written in English.

The inclusion criteria were as follows: i) Histopathological diagnosis of lymphomas and LPDs according to the World Health Organization classification (22), including post-transplant or immunocompromised patients; ii) detailed data sufficient to evaluate EBV status identification. Tumor cells expressing EBER, EBNA or LMP-1 confirmed by *in situ* hybridization and/or genetic identification should be considered sufficient to confirm a positive case; iii) an analysis relevant to PD-L1/PD-1 expression in tumor cells and in the lymphocytes or macrophagocytes of the tumor microenvironment, PD-L1/PD-1 identification explicitly stated and justification for positive status provided; iv) studies including a minimum of 10 participants, 5 of which were in the EBV^+ DLBCL subgroup and 5 in the control group; and v) inclusion of a control group of patients with EBV^- DLBCL, offering a comparison between EBV^+ and EBV^- subgroups.

The exclusion criteria were as follows: i) Insufficient raw data for estimating EBV and PD-L1/PD-1 identification; ii) review articles, opinion reports, conference abstracts without original data and case reports; and iii) studies not written in English. Any disagreements were resolved by consensus.

Data pooling. Data associated with clinicopathological characteristics were extracted from each of the eligible studies. The data extracted were the first author's surname and the publication year, the pathological diagnosis and the number of EBV-positive and EBV-negative cases (Table I). The expression of PD-L1 in neoplastic cells (nPD-L1) and microenvironmental PD-L1 (miPD-L1) were used for analysis. The positive expression of nPD-L1 and miPD-L1 had been detected by immunohistochemistry (IHC) using immunofluorescence staining to calculate the proportion of positive cells. nPD-L1 positivity ($nPD\text{-}L1^+$) was defined as the presence of PD-L1-positive neoplastic cells among the total tissue cellularity. miPD-L1 positivity ($miPD\text{-}L1^+$) was defined as PD-L1-positive nonmalignant cells among the total tissue cellularity. PD-1 is more commonly expressed in TILs (8) and PD-1 TIL positivity ($PD\text{-}1^+$ TILs) was defined as PD-1-positive TILs among the total tissue cellularity. However, the cut-off values to classify PD-L1 or PD-1 to be positive have no general agreement, which is one cause of heterogeneity in the present meta-analysis. The positive cases of nPD-L1, miPD-L1 and PD-1 TILs and cut-off values for IHC staining results are presented in Table I.

Statistical analysis. The meta-analysis was performed using R Studio 4.1.0 (RStudio, Inc.). In brief, effect sizes for each study were determined by calculating risk ratios (RR) and the corresponding 95% confidence interval (CI). The pooled proportions were calculated using the Mantel-Haenszel method (23). According to the recommendations provided by the Cochrane Handbook for Systematic Reviews of Interventions (https://training.cochrane.org/handbook/current/chapter-10#section-10-10-4-1), a choice of whether a common-effects or random-effects model applied should not be made through a statistical test for heterogeneity and considering that heterogeneity is always expected for the intervention effects among multiple studies, a random-effects model was employed. $P<0.05$ was considered to indicate a statistically significant difference. Publication bias was examined by funnel plots and Egger's tests.

Results

Selection and characteristics of the studies. A literature search in PubMed identified 806 relevant records for screening. Following title and abstract screening, most records were excluded for one of the following reasons: Studies not containing any human subjects, insufficient data, published in a language other than English, review articles and editorials. A total of 165 studies underwent full text screening and 17 studies met the inclusion criteria with a further three articles included through a reference search. A total of 16 studies (21,24-39) with a total of 2,396 patients were finally included in the present meta-analysis.

Patients in the studies had a histologically confirmed diagnosis of lymphoma subtypes, with 11 articles on DLBCL comprising 1,936 patients (21,24-32,39), 5 articles on cHL including 236 patients (33-37), 3 articles on PTLDs comprising 147 patients (25,27,28) and 1 article on plasmablastic lymphoma (PBL) including 77 patients (38). According to the cut-off values, the included articles described the IHC

Table I. Main characteristics of the eligible studies.

First author, year	Pathology	Features	EBV status	Cases	Tumor cells	Immune cells		Cut off		(Refs.)
					nPD-L1$^+$ cases, n (%)	miPD-L1$^+$ cases, n (%)	PD-1$^+$TILs cases, n (%)	nPD-L1 positive cells, %	miPD-L1 positive cells, %	
Kataoka, 2019	DLBCL	/	i) +; ii) -	i) 27; ii) 48	i) 5 (19); ii) 1 (2)	/	/	5	/	(39)
Kiyasu, 2015	DLBCL	/	+; -	i) 114; ii) 1139	i) 22 (19)[a]; ii) 110 (9.6)[a]	i) 37 (32); ii) 135 (12)	i) 58 (0-802)[a]; ii) 19 (0-802)[a]	30	20	(24)
Chen, 2013	DLBCL	Elderly, HIV-associated	+; -	i) 16; ii) 66	i) 16 (100); ii) 7 (11)	i) 16 (100); ii) 9 (14)	/	5	20	(25)
Anastasiadou, 2019	DLBCL	/	+; -	/	i) (84); ii) (28)	/	/	NA	/	(26)
Kinch, 2019	DLBCL	PTLD	+; -	i) 27; ii) 20	i) 18 (67); ii) 8 (40)	/	/	5	/	(27)
Veloza, 2019	DLBCL	PTLD	+; -	i) 21; ii) 16	i) 18 (86); ii) 6 (38)	i) 20 (95); ii) 8 (50)	i) 3/13 (23); ii) 5/12 (42)	5	20	(28)
Ishikawa, 2018	DLBCL	Primary gastric DLBCL	+; -	i) 25; ii) 215	i) 0/14 (0); ii) 0/40 (0)	i) 12/14 (86); ii) 17/40 (43)	/	5	20	(29)
Ishikawa, 2018	DLBCL	Primary intestinal DLBCL	+; -	i) 10; ii) 52	i) 2 (20); ii) 1 (2)	i) 8/8 (100); ii) 31/48 (65)	/	5	20	(30)
Quan, 2015	DLBCL	/	+; -	i) 7; ii) 20	i) 5 (71); ii) 8 (40)	/	/	using FCM[a]	/	(31)
Cohen, 2017	DLBCL	/	+; -	/	/	/	i) (15.70); ii) (14.90)	/	/	(32)
Kwon, 2016	DLBCL	/	+; -	i) 6; ii) 107	i) 4 (66.7); ii) 9 (8.4)	i) 4 (66.7); ii) 10 (9.3)	i) 1 (16.7) ii) 27/103; (26.2)	NA	NA	(21)
Sakakibara, 2018	cHL	/	+; -	i) 11; ii) 16	i) 11 (100); ii) 8 (50)	i) 11 (100); ii) 11 (69)	/	5	20	(33)
Paydas, 2015	cHL	/	+; -	i) 40; ii) 47	i) 8 (20); ii) 10 (21)	/	i) 10 (25); ii) 8 (17)	5	20	(34)
Ozturk, 2020	cHL	/	+; -	i) 15; ii) 21	i) 11 (73); ii) 4 (19)	/	i) 8 (53); ii) 12 (57)	80	5	(35)
Antel, 2021	cHL	44% HIV positive	+; -	i) 39; ii) 38	i) 23 (59); ii) 17 (45)	/	/	50	/	(36)

Table I. Continued.

First author, year	Pathology	Features	EBV status	Cases	Tumor cells	Immune cells		Cut off		(Refs.)
					nPD-L1$^+$ cases, n (%)	miPD-L1$^+$ cases, n (%)	PD-1$^+$TILs cases, n (%)	nPD-L1 positive cells, %	miPD-L1 positive cells, %	
Kohno, 2020	cHL	MTX-LPD	+; -	i) 8; ii) 1	i) 7 (87); ii) 1 (100)	/	/	NA	/	(37)
Chen, 2013	PTLD	/	+; -	i) 10; ii) 7	i) 6 (60); ii) 4 (57)	i) 7 (70); ii) 4 (57)	/	5	20	(25)
Kinch, 2019	PTLD	/	+; -	i) 43; ii) 37	i) 24 (56); ii) 16 (43)	/	/	5	/	(27)
Veloza, 2019	PTLD	/	+; -	i) 34; ii) 16	i) 23 (68); ii) 7 (44)	i) 29 (85); ii) 8 (50)	i) 8/25 (32); ii) 5/12 (42)	5	20	(28)
Laurent, 2016	PBL	/	+; -	i) 39; ii) 38	i) 7/9 (78); ii) 1/2 (50)	i) 16/23 (70); ii) 8/18 (44)	i) 14/18 (78); ii) 6/14 (43)	NA	NA	(38)

[a]In this study, the nPD-L1$^+$ result is obtained through FCM, while all others were obtained using IHC. NA or /, not available; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; PD-L1, programmed cell death 1 ligand 1; nPD-L1, PD-L1 in neoplastic cells; miPD-L1, microenvironmental PD-L1; TILs, tumor-infiltrating lymphocytes; DLBCL, diffuse large B-cell lymphoma; cHL, classical Hodgkin lymphoma; PTLD, post-transplant lymphoproliferative disorders; PBL, plasmablastic lymphoma; MTX, methotrexate; LPD, lymphoproliferative disease; FCM, flow cytometry; IHC, immunohistochemistry.

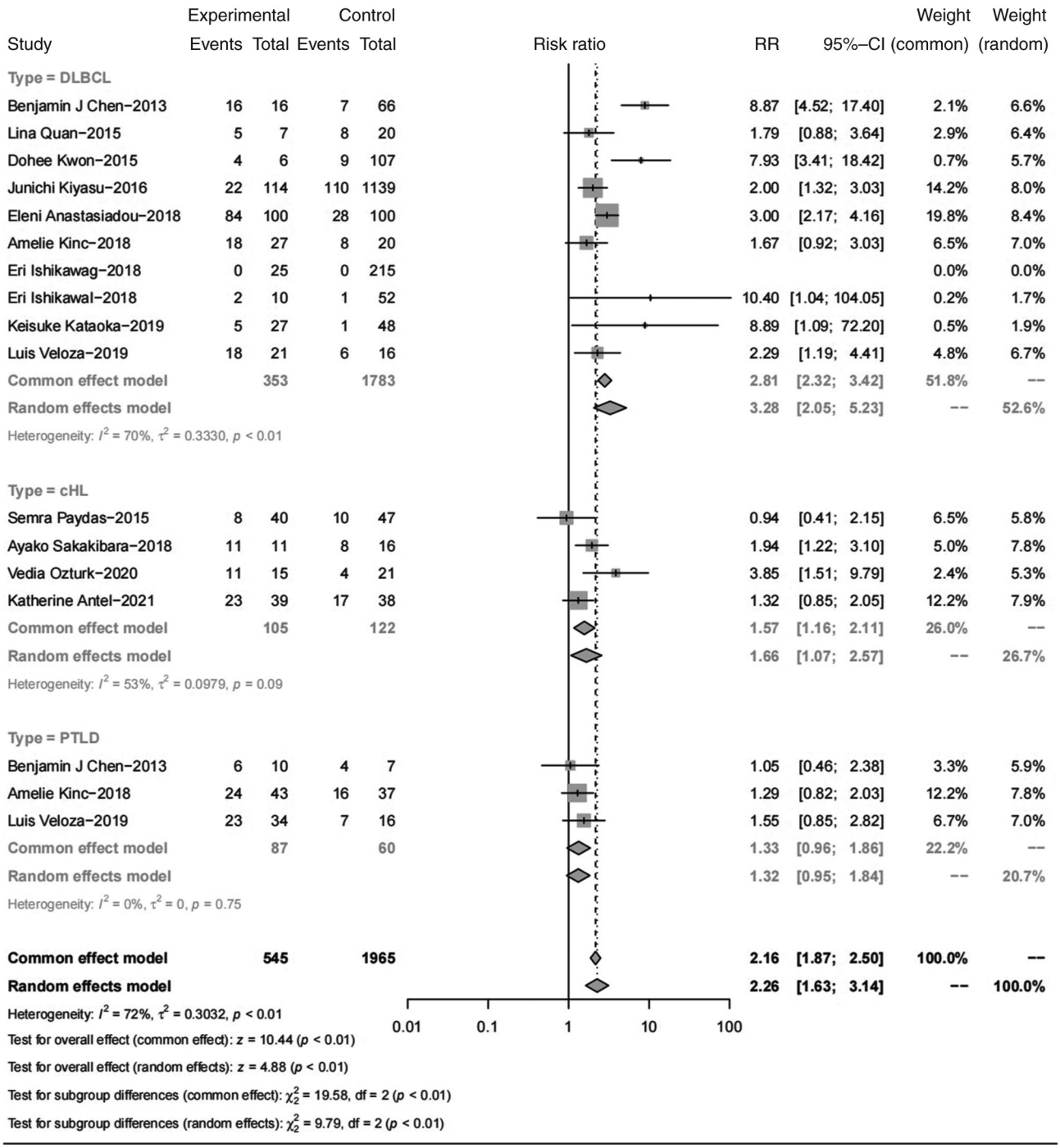

Figure 1. Forest plot of the RR for nPD-L1 positive proportion between EBV$^+$ and EBV$^-$ lymphomas. nPD-L1, programmed cell death 1 ligand 1 in neoplastic cells; EBV, Epstein-Barr virus; DLBCL, diffuse large B-cell lymphoma; df, degrees of freedom; cHL, classical Hodgkin lymphoma; PTLD, post-transplant lymphoproliferative disorders; RR, relative risk; CI, confidence interval.

results of the immune checkpoint molecules PD-L1 and PD-1. In addition, all studies were retrospective and reported positive and negative cases of EBV infection and immune checkpoint molecules. The main characteristics of the eligible studies are summarized in Table I.

Association between EBV infection and PD-L1 expression in tumor cells. PD-L1 expression in tumor cells was significantly higher in EBV$^+$ lymphomas than in EBV$^-$ lymphomas, with a pooled RR of 2.26 (95% CI, 1.63-3.14; P<0.01; Fig. 1). Specifically, nPD-L1 expression was higher in patients with EBV$^+$ DLBCLs than in those with EBV$^-$ DLBCLs (RR=3.28; 95% CI, 2.05-5.23). This result was similar in cHLs, as nPD-L1 was higher in EBV$^+$ cases than in EBV$^-$ cases (RR=1.66; 95% CI, 1.07-2.57). In PTLDs, nPD-L1 expression showed no significant increase in EBV$^+$ cases, with an RR of 1.32 (95% CI, 0.95-1.84).

Association between EBV infection and PD-L1 expression in immune cells. The PD-L1 expression of immune cells in the tumor microenvironment was compared between EBV$^+$ and EBV$^-$ lymphomas (Fig. 2). Statistically, EBV infection

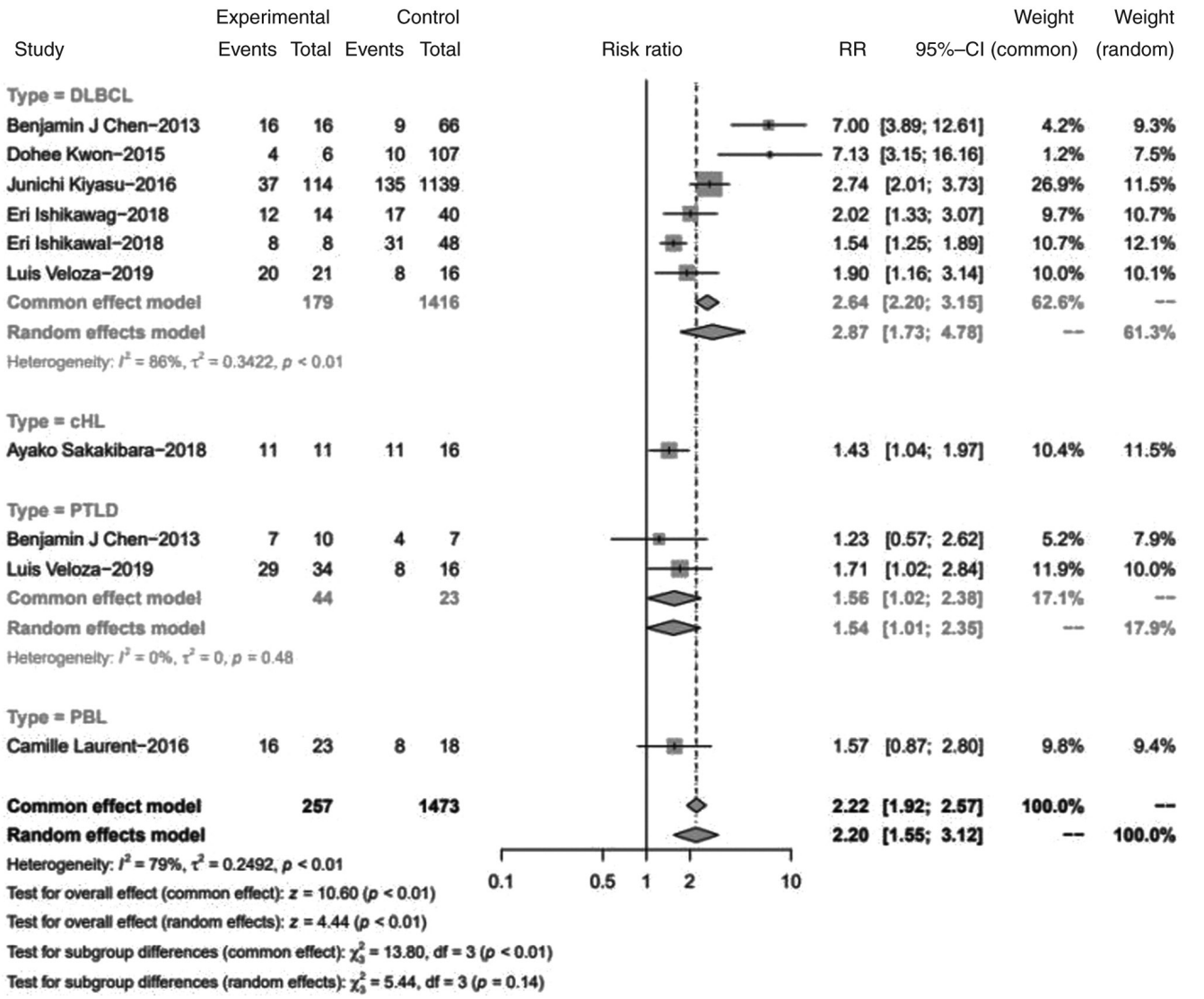

Figure 2. Forest plot of the RR for miPD-L1 positive proportion between EBV$^+$ and EBV$^-$ lymphomas. miPD-L1, microenvironmental PD-L1; EBV, Epstein-Barr virus; DLBCL, diffuse large B-cell lymphoma; df, degrees of freedom; cHL, classical Hodgkin lymphoma; PTLD, post-transplant lymphoproliferative disorders; RR, relative risk; CI, confidence interval; PBL, plasmablastic lymphoma.

increased the expression of PD-L1 in immune cells with a pooled RR of 2.20 (95% CI, 1.55-3.12; P<0.01). Specifically, miPD-L1 expression was higher in EBV$^+$ DLBCLs than in EBV$^-$ DLBCLs (RR=2.87; 95% CI, 1.73-4.78). In PTLD, a similar result of PD-L1 expression increasing in immune cells of EBV$^+$ cases was observed, with an RR of 1.54 (95% CI, 1.01-2.35).

Association between EBV infection and PD-1 expression in TILs. It was found that PD-1 expression in TILs was not associated with EBV infection, with a pooled RR of 1.10 (95% CI, 0.81-1.48; P>0.05). Specifically, in the DLBCL and cHL subtypes, the expression of PD-1 TILs showed no discrimination between EBV$^+$ and EBV$^-$ cases, with RRs of 0.89 (95% CI, 0.52-1.53) and 1.09 (95% CI, 0.67-1.78) (Fig. 3).

Publication bias. The funnel plots revealed that the RR analyses of nPD-L1, miPD-L1 and PD-1 TILs may have publication bias and heterogeneity (Fig. 4).

Discussion

EBV-associated lymphomas and lymphoproliferative diseases are rare but are often malignant and largely resistant to current chemotherapeutic regimens. Given their association with the oncogenic virus and an 'immune privileged' milieu, they are attractive targets for immune-based therapies (40). Certain virus-associated solid cancers were reported to induce PD-L1 expression (41-43) and anti-PD-1 and anti-PD-L1 blockades have resulted in durable clinical responses in various types of cancer (44,45). However, the efficacy of such immune-targeted therapies in EBV-associated lymphomas and LPDs has not been fully elucidated. In the present study, it was demonstrated that EBV infection may have certain effects on the antitumor immune response in EBV-associated lymphomas through a mechanism of increasing PD-L1 expression in tumor cells and TILs.

Several studies have uncovered the functional mechanism of PD-L1 in EBV$^+$ lymphomas. Green *et al* (20) identified an

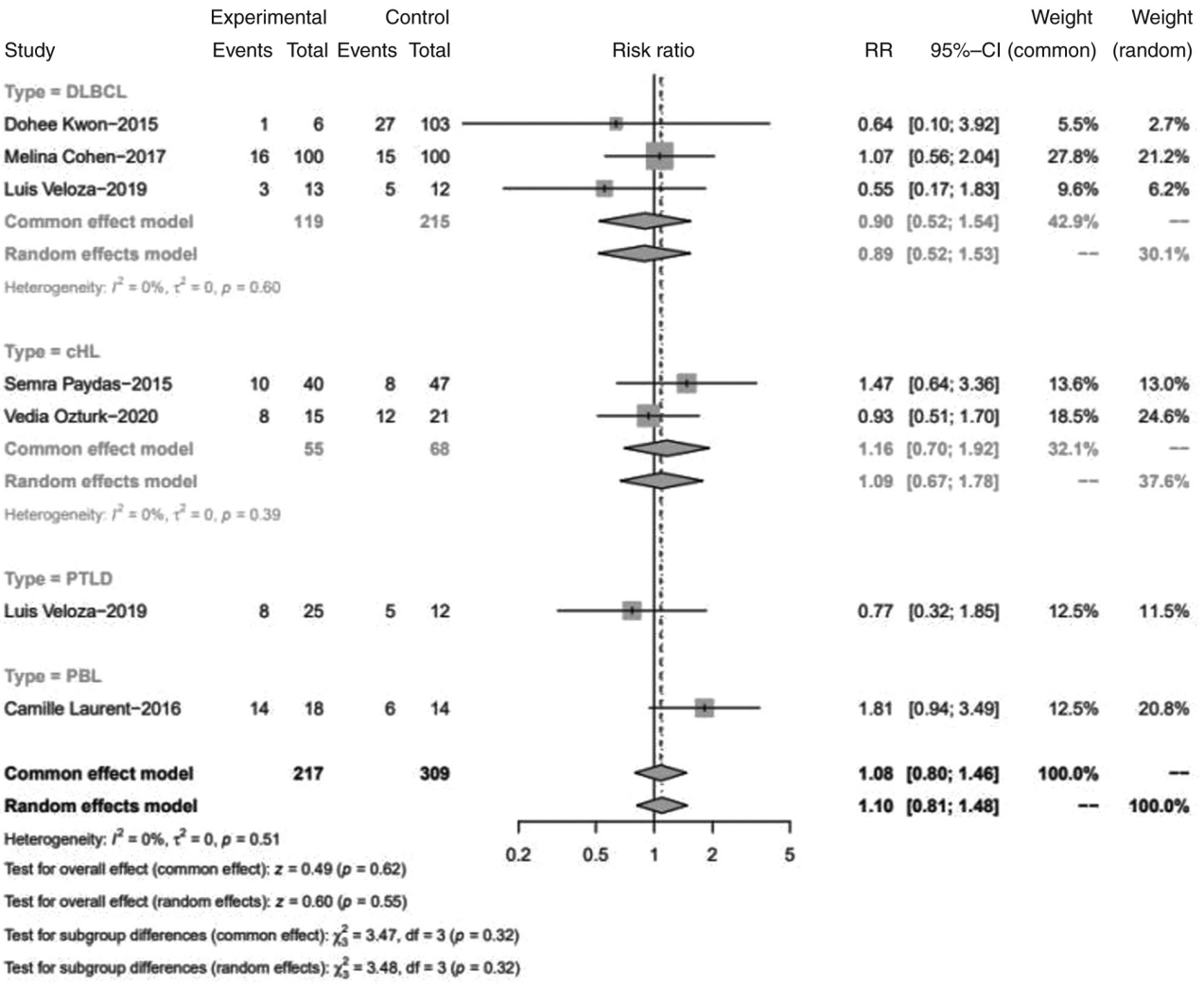

Figure 3. Forest plot of the RR for PD-L1 TILs positive proportion between EBV$^+$ and EBV$^-$ lymphomas. PD-L1, programmed cell death 1; TILs, tumor-infiltrating lymphocytes; EBV, Epstein-Barr virus; DLBCL, diffuse large B-cell lymphoma; df, degrees of freedom; cHL, classical Hodgkin lymphoma; PTLD, post-transplant lymphoproliferative disorders; RR, relative risk; CI, confidence interval; PBL, plasmablastic lymphoma.

activating protein-1 (AP-1)-responsive enhancer in the PD-L1 gene. Using EBV-transformed B cells, it was further demonstrated that the expression of EBV-encoded LMP-1 promotes PD-L1 expression through both AP-1 signaling and JAK-STAT signaling activity. Quan *et al* (31) also found that the antitumor immune effects of PD-1 blockade are more effective in EBV$^+$ DLBCL than in EBV$^-$ DLBCL. The results of the aforementioned studies suggest that PD-1 blockade may restore T-cell exhaustion and immune escape, resulting in more efficacious immunotherapy treatment for EBV$^+$ DLBCL.

Barzyk and Sheriff (46) performed a systematic review, which included 11 studies, to evaluate the association of EBV with PD-L1 expression in DLBCL. A narrative synthesis was conducted using table summarization and concluded that a non-EBV related mechanism is likely related to increased PD-L1 expression, with relevance to the cell of origin. In the present study, statistical methods were used to analyze the effect of EBV infection on the expression of immunomodulatory molecules in EBV-associated lymphomas. Statistically significant results based on abstracted data from 20 studies suggested that antitumor immunity appears to have an important role in these virus-associated lymphomas. The increased expression of PD-L1 in tumor cells and the tumor microenvironment may be a mechanism contributing to the pathogenesis of EBV$^+$ lymphomas. Further research is needed to elucidate the intrinsic molecular mechanism of antitumor immunity in EBV infection.

In the process of collating data for the present study, it was noticed that the expression of CD30 probably has relevance to EBV virus infection. Therefore, a meta-analysis was performed in the present study to determine whether such an association existed. The results indicated increased CD30 expression in EBV$^+$ DLBCL cases compared to EBV$^-$ cases, with statistical significance (RR=2.36; 95% CI, 1.60-3.47; P<0.01; Fig. S1). In a review of the molecular biology of Hodgkin's lymphoma, the author proposed that the occurrence of Hodgkin's lymphoma is responsible for constitutive NF-κB activation, which is induced by CD30 overexpression, EBV LMP-1, and factors of immune evasion (47). The findings of the present study also showed the probable relevance of the increased expression of CD30 and EBV infection in the development of EBV$^+$ DLBCL, but this still requires further exploration.

The present study had certain limitations. The funnel plot estimates suggested substantial statistical heterogeneity. No

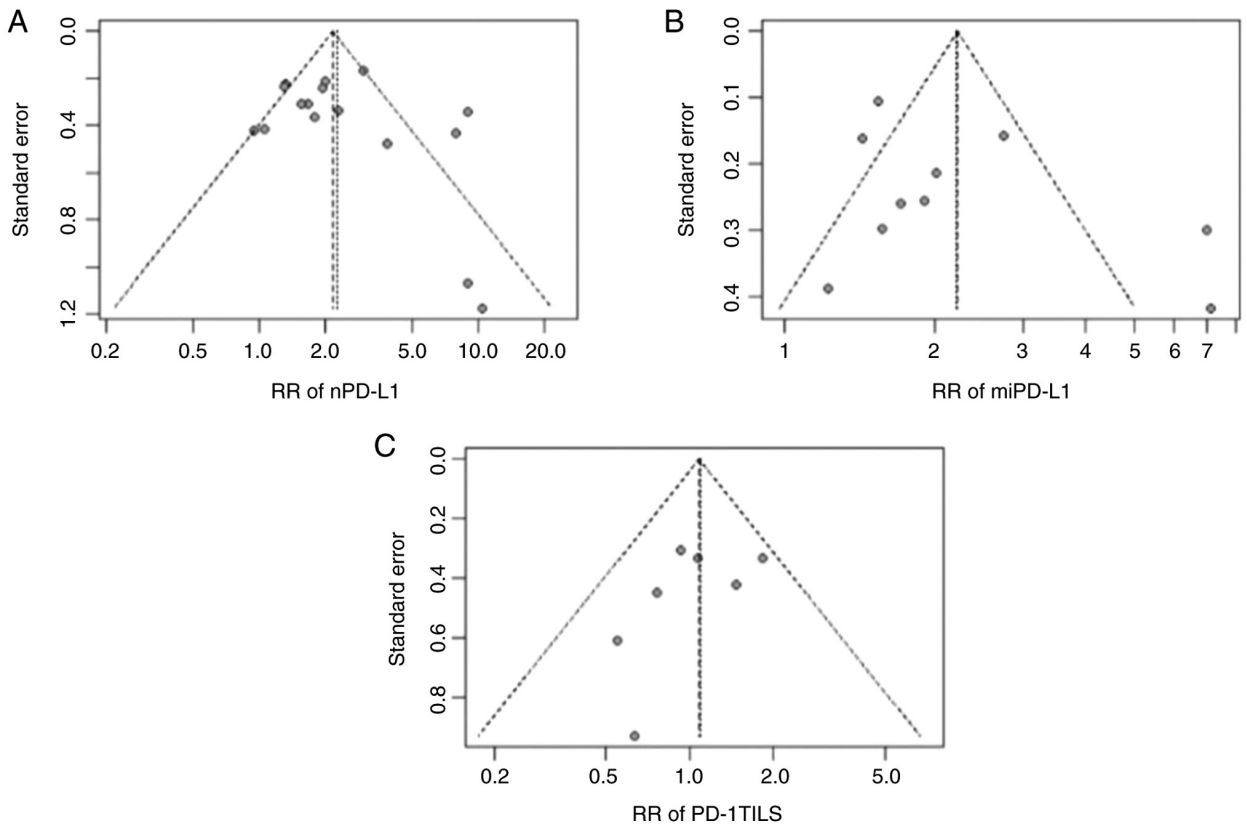

Figure 4. A funnel plot was constructed to visualize potential publication bias for the RR analysis of (A) nPD-L1, (B) miPD-L1 and (C) PD-1 TILs positive proportion in EBV^+ and EBV^- cases. EBV, Epstein-Barr virus; PD-1, programmed cell death 1; PD-L1, PD-1 ligand 1; nPD-L1, PD-L1 in neoplastic cells; miPD-L1, microenvironmental PD-L1; TILs, tumor-infiltrating lymphocytes.

sensitivity analysis or meta-regression analysis was performed to determine which factors affected the results. Potentially, the following aspects have been present. First, the detection and determination of $PD\text{-}L1^+$ and $PD\text{-}1^+$ expression require standardization. In general, the threshold for nPD-L1 positivity is ≥5% of the tumor cell population showing 2+ or 3+ membrane staining for IHC, while miPD-L1 is considered positive when ≥20% of tumor-infiltrating immune cells show 2+ or 3+ membrane or cytoplasmic staining. The positive thresholds of nPD-L1 and miPD-L1 are different in two articles (23,34), as shown in Table I. While most studies used IHC methods to detect PD-L1 expression, one article adopted the flow cytometry method (30). As another limitation, the small size of included articles may have limited the strength of the evidence in the present study. The insufficient number of cases of PBL and PTLD subtypes made it impracticable to conduct a meta-analysis.

In conclusion, EBV involvement is a distinctive subtype of lymphoma and the present systematic review indicates that enhancement of the PD-1/PD-L1 pathway in tumor cells and the tumor microenvironment may be a potential mechanism in the development of EBV-associated lymphatic diseases. The impact of EBV infection on immune-mediated damage and the efficacy of immune-targeted therapies in these EBV-positive diseases need to be further explored.

Acknowledgements

The authors would like to thank Professor Wangjian Zhang (Department of Medical Statistics, School of Public Health, Sun Yat-sen University, Guangzhou, China) for his methodological guidance.

Funding

The study was supported by The National Natural Science Foundation of China (grant no. 82000146).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JY performed data analysis and wrote the manuscript. SJ was involved in the acquisition of data and analysis. XY performed analyses and obtained the funding. HD was involved in the conception and design of the study. JY and HD confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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