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Review Article

Diagnostic approach for classic Hodgkin lymphoma in small samples with an emphasis on PD-L1 expression and EBV harboring in tumor cells: a brief review from morphology to biology

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Classic Hodgkin lymphoma (CHL) was first described in 1832 by Thomas Hodgkin, and is characterized by a small number of Hodgkin and Reed–Sternberg cells in a rich inflammatory background. However, even in this modern era, due to the histological and biological overlap with CHL and other B-cell malignancies, including mediastinal grey zone lymphoma and other lymphomas accompanied by "Hodgkinoid cells", their discrimination is challenging and sometimes impossible. The complexity and ambiguity of the boundaries of CHL and its related diseases make the definition of CHL unresolved. Our group has studied the significance of PD-L1 expression and infection of Epstein-Barr virus (EBV) in the diagnosis of CHL, emphasizing their pathological role, clinical significance, and high reproducibility even in daily clinical practice. In this review, we summarize the diagnostic strategy of CHL and its histological lookalikes based on neoplastic PD-L1 expression and infection of EBV, and attempt a reappraisal of the definition of CHL.

Keywords: classic Hodgkin lymphoma, Epstein-Barr virus, PD-L1

INTRODUCTION

Classic Hodgkin lymphoma (CHL) is biologically heterogeneous, exemplified by several clinic-pathologic differences between nodular sclerosis (NS) and mixed cellularity (MC). It is well known that this unique lymphoma encompasses four histologic subtypes (NS, MC, lymphocyte depleted [LD], and lymphocyte-rich [LR]) and often poses differential diagnostic problems due to morphologic lookalikes of other lymphoid neoplasia/proliferation. In this modern era, small-volume tissue sampling, such as core needle biopsy, is the preferred modality for diagnosis in routine practice, but does not allow for precise sub-classification or accurate assessment of the architecture due to the limited material collected. Of note, recent advances have clearly demonstrated that PD-L1 expression in tumor cells is a highly sensitive diagnostic marker and is regarded as a defining feature of CHL.[1] EBV status is also an important prognostic indicator among the patients with CHL.[2] Given their biological significance, the presence of PD-L1 and EBV in tumor cells should provide significant information for diagnosis. The reproducibility of their detection in tumor cells also aid accurate diagnosis, especially in small-volume samples. In this review, we provide an update on the diagnostic features of CHL with an emphasis on PD-L1 expression and EBV harboring in tumor cells.

Diagnostic dilemma and future perspective on CHL in small samples

CHL accounts for around 90% of all Hodgkin lymphoma.[3] Hodgkin lymphomas (HLs) are historically defined by morphologic characteristics. CHL is characterized by tumor cells composed of mononuclear Hodgkin cells and multinucleated Reed-Stenberg (HRS) cells accompanied by a background containing a variable mixture of reactive immune cells including small lymphocytes, eosinophils, neutrophils, histiocytes, and plasma cells. HRS cells of CHL usually show loss of B-cell antigen, whereas germinal center (GC) B cells are currently regarded as the cellular origin of CHL in most cases. In the 2017 and 2022 WHO classifica-

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tions, CHL is subdivided into four 'histologic' subtypes: NSCHL, MCCHL, LDCHL, and LRCHL. It is principally recommended to sub-classify CHL as one of these four histologic subtypes. It is emphasized that the paradigm for our understanding Hodgkin lymphomas is now rapidly shifting. Of note, in the international consensus classification (2022),[4] the diagnostic title "nodular lymphocyte predominant B-cell lymphoma" (NLPBL) was recommended instead of NLPHL, with the term 'Hodgkin' being excised based on its distinctive biological and clinical features. Elaine S Jaffe clearly described that CHL is not a single disease, but likely consists of two or more entities, i.e., NSCHL and the spectrum encompassing MCCHL and LDCHL.[5] She also indicated that LRCHL is still an enigma in terms of its biology and clinical features, and in some cases even shows overlap with NLPHL/NLPBL. We recently reported that typical cases of NSCHL and EBV+ MCCHL consistently feature neoplastic PD-L1 positivity in HRS cells, which is contrasted by the lack of PD-L1 expression in tumor cells of NLPHL/NLPBL and LRCHL of our studies.[6] We believe that our observation is coincidental with that of Elaine S. Jaffe. Unfortunately, in the updated WHO classification (2022), the definitions of CHL and NLPHL remained obscure: in this content, CHL is a neoplasm derived from GC B-cells, characterized by a low fraction of tumor cells embedded in a reactive micro-environment rich in immune cells; and NLPHL is a GC-derived B-cell neoplasm composed of scattered large neoplastic B cells with multilobated nuclei (LP cells) within nodules dominated by mantle zone B cells and follicular dendritic cells (FDCs).

Never-the-less, a precise sub-classification is not always possible when the biopsy material inadequate, such as in the case of core needle biopsy. However, small-volume biopsy sampling is now the most favored clinical approach for node-based neoplastic diseases including CHL. Indeed, the 2022 WHO classification also described that the increasing trend to use core needle biopsies makes CHL diagnosis challenging due to the scarcity and uneven distribution of HRS cells, although most descriptions of the CHL histopathology have been made on the findings of whole node biopsies. In 2016, Roemer *et al.* indicated that *PD-L1* and *PD-L2* genetic alteration, which result in neoplastic PD-L1 expression in tumor cells, is a defining feature of CHL. An increasing number of the studies also provide additional support to the assertion that neoplastic PD-L1 expression in tumor cells is a highly sensitive diagnostic marker for the discrimination of CHL from other diagnostic targets, i.e., morphologic CHL look-alikes. In this diagnostic process, the association or not of large B-cells with EBV is also critical for our approach, because EBV-positive and –negative CHLs constitute quite distinct differential diagnostic lists. These situations led us to re-assess CHL from the morphologic basis to the biology, i.e., the high-frequent PD-L1 genetic alteration resulting in neoplastic PD-L1 overexpression and the EBV harboring (or not) in HRS cells, especially in small samples. Given the clinicopathological differences between CHL and its look-alikes in extranodal sites, CHL is hypothetically assumed to be a lymphoid organ (LN, thymus and spleen)-sited, immune escape-associated, lymphoid cell-driven (B-cell origin in most at least) neoplasm. From these standpoints, we propose a diagnostic strategy combing PD-L1 expression and EBV infection in HRS cells (Table 1).

Genetic alterations of *PD-L1*

Repeatedly, the most frequent genetic alteration of CHL is copy number gain of 9p24.1 at the locus that includes *PD-L1/L2* and *JAK2,* constituting up to 97% of all CHL.[1] Copy number gain of *PD-L1/L2* increases its transcripts in HRS cells,[7] and copy number gain of *JAK2,* leading to constitutive activation of JAK/STAT signals, also induces neoplastic PD-L1 expression in HRS cells.[8] In a minority of cases of CHL (4/200, 5%), unbalanced translocations involving 9p24.1 have been found, and these might upregulate PD-L1 expression by stabilizing *PD-L1* mRNA.[9] The inactivating mutation of the Beta 2 microglobulin gene (*B2M*), one of the most prevalent gene mutations in CHL (up to 40%), also contributes to escape from immune surveillance by $CD8^+$ T cells by limiting expression of major histocompatibility complex class I (MHC class I) on the cell surface.[10,11] These immune-evasion-associated genetic alterations are more frequently observed in EBV+ CHL than in EBV$^-$CHL, and presumably inhibit the T cell response to the virus-derived antigen of EBV+ CHL tumor cells.[11]

PD-L1 Immunostaining

In almost all CHL cases, HRS cells express CD30 and CD15, historically traditional, but not specific, diagnostic markers for CHL.[3,12,13] PD-L1 is a novel diagnostic marker of CHL, and its expression is observed in 73~96% of the CHL.[1,14-17] PD-L1 immunostaining is now also highlighted to be helpful for recognizing neoplastic cells in small biopsy samples.[17] Its expression in HRS cells indicates the significance of immune evasion in the tumorigenesis. More importantly, blocking the PD-1/PD-L1 axis has been proven to be an efficient therapeutic approach for CHL,[18,19] and PD-L1 expression level is associated with a good response to PD-1/PD-L1 inhibitors.[20] The prevalence of PD-L1 expression is largely dependent on the histologic subtype. Almost all NSCHL cases show PD-L1 expression in neoplastic cells, whereas other histological subtypes express PD-L1 much less frequently, especially in LRCHL.[6,21] The PD-L1 expression rate if neoplastic cells may be affected by the antibody clone used in studies.[22] SP142 has been reported to have lower sensitivity compared with other clones such as 22C3, 28-8, and SP263. However, our group and Menter *et al.* showed staining concordance between SP142 and E1L3N clones.[23,24] These antibodies' binding affinity are also affected by binding epitope and assay protocol.[22] It is notable that the recognition sites of SP142 and E1L3N can be genetically impaired by structural variations involving *PD-L1* 3′-UTR in non-Hodgkin lymphomas, and the prevalence of such genetic alterations in classic Hodgkin lymphoma has not yet been elucidated.[25] Some subtypes of classic Hodgkin lymphoma, such as syncytial variant Hodgkin lymphoma, show heteroge-

Table 1. Differential diagnosis of CHL and its lookalikes

Diagnosis	AITL/FHTL with HRS-like large B-cells	Age-related EBV+ B-LPD, i.e., EBV+ DLBCL		CHL						
		Young (<45 years)	Elderly	EBV+GZL	EBV+ MCCHL	CHL in patients with immune deficiency/ dysregulation	NSCHL	LDCHL, syncytial variant	EBV- MCCHL	LRCHL
Clinical presentation	Lymphadenopathy	Lymphadenopathy	Lymphadenopathy; extranodal mass	Lymphadenopathy	Lymphadenopathy		Lymphadenopathy	Lymphadenopathy	Lymphadenopathy	Lymphadenopathy
EBV	+	+		+	+	+	-	Variable (25%)	-	-
PD-L1 IHC	- (5%)	+ (77%)	-/+ (11%)	+ (70%)	+ (100%)	+ (89%)	+ (100%)	Heterogeneous†	- (0%)	- (0%)
Other	EBV+ large cells: CD20+/-, CD30+, PAX5+	CD20+, CD30+, PAX5+ (often strong), OCT2+, BOB1+		CD20+, PAX5+/-, CD30+, CD15+, OCT2+/-, BOB1+/-		CD20-/+, PAX5+(dim), CD30+, CD15+, BOB1-, OCT2-				
PD-1+ rosetting T cells	+	-		-	-	-	-	-	NA	-
Cytology	Clear cells; immunoblasts, HRS-like cells; inflammatory background	TCRBCL-like appearance	Sheets of large lymphoma cells with HRS-like morphology; geographic necrosis	Mononuclear Hodgkin cells; rich inflammatory background		HRS and HRS-like cells, including lacunar cells; rich inflammatory background				

Diagnosis	NLPHL/ NLPBL/ TCRBCL	EBV+ NLPHL	FHTL mimicking LRCHL*	MGZL		PMBCL	ALK+ ALCL, HL-like	DLBCL, anaplastic variant
				CHL-like	PMBL-like			
Clinical presentation	Lymphadenopathy	Lymphadenopathy	Lymphadenopathy	Mediastinal mass		Mediastinal mass		Lymphadenopathy
EBV	-	+		-		-	-	-
PD-L1 IHC	- (0%)	uncertain	-	NA		+ ~ - (50%)	+ (76%)	- (0%)
Other	CD20+, PAX5+, CD30-, CD15-, BOB1+, OCT2+		Large cells: CD20-/+, PAX5+(dim), CD30+, CD15+, BOB1-, OCT2-	CD20+, PAX5+, CD30+, CD15+/-, OCT2+/-, BOB1+/-	CD20-/+, PAX5-/+, CD30+, CD15+/-, OCT2-/+, BOB1-/+	CD20+, PAX5+, CD30+, CD15+/-, CD23+, OCT2+, BOB1+, MUM1+	CD2-, PAX5-, CD30+, CD15-, ALK+, granzyme B+	CD20+, PAX5+, CD30+, CD15-, OCT2+, BOB1+
PD-1+ rosetting T cells	+		-	-		-	-	-
Cytology	Scattered large cells, including popcorn cells; rich inflammatory background			HRS and HRS-like cells, including lacunar cells; rich inflammatory background	Sheets of large lymphoma cells with HRS-like morphology	Sheets of large lymphoma cells; sclerosis	HRS-like cells, including hallmark cells; rich inflammatory background	Sheets of large lymphoma cells with HRS-like morphology; sinusoidal pattern

Abbreviations: AITL/FHTL, angioimmunoblastic T-cell lymphoma/follicular helper T-cell lymphoma; HRS, Hodgkin and Reed-Sternberg; age-related EBV+ B-LPD, age-related EBV-associated B-cell lymphoproliferative disorder; DLBCL, diffuse large B-cell lymphoma; CHL, Classic Hodgkin lymphoma; GZL, gray zone lymphoma; MCCHL, mixed cellularity classic Hodgkin lymphoma; NSCHL, nodular sclerosis classic Hodgkin lymphoma; LDCHL, lymphocyte depletion classic Hodgkin lymphoma; LRCHL, lymphocyte-rich classic Hodgkin lymphoma; NLPHL/NLPBL/TCRBCL, nodular lymphcyte predominant Hodgkin lymphoma/nodular lymphocyte predominant B-cell lymphoma/T-cell/histiocyte-rich large B-cell lymphoma; MGZL, mediastinal grayzone lymphoma; PMBL, primary mediastinal large B-cell lymphoma; ALK; ALCL, HL-like, anaplastic lymphoma kinase+ anaplastic large-cell lymphoma, Hodgkin lymphoma-like; and NA, not available

*The literature is limited, consisting of single case reports.

†Not expressed in the confluent sheet of tumor cells.

neous PD-L1 expression in neoplastic cells, whose biological backgrounds remain elusive.[26] Under these circumstances, there is no consensus on the cutoff value for PD-L1 expression and which antibodies should be used. In our studies, we used an SP142 antibody for PD-L1 expression. The diagnostic efficacy of SP142 was also recently documented in a review article by Sakakibara *et al.* (Table 1).[27]

EBV infection

The prevalence of EBV positivity varies greatly depending on the histologic subtype of CHLs. EBV infection is most frequently detected in up to 75% and 65% of MCCHL and LDCHL cases, respectively.[28] NSCHL shows a relatively low range of EBV-positivity (10–25%), and around half of LRCHL cases have EBV infection.[29] Intriguingly, NSCHL in the elderly has a higher frequency of EBV positivity (40%) than NSCHL in the young (10%), suggesting that EBV+ NSCHL may be more associated with immunosenescence.[30] EBV infection may play a substitute role in genetically altered intracellular pathways, as EBV+ CHL have a much lower number of somatic mutations compared with EBV-CHL.[11,31] For example, EBV+ CHL cases express EBV-encoded latent membrane protein 1 (LMP1), and LMP1 interacts with tumor necrosis factor receptor-associated factors and activates NF-κB signaling.[32] As a consequence, EBV+ CHL is less reliant on genetic aberrations inducing constitutive active NF-κB signaling.[33] In addition, another EBV-coded latent membrane protein, LMP2a, mimics B-cell receptor (BCR) signaling, presumably rescuing BCR-deficient B-cells in the course of CHL development.[34]

PD-L1 overexpression is mediated by EBV-latent membrane protein 1 (LMP1), which activates the transcription factor AP-1, and JAK/STAT and NF-κB pathways,[8,35] besides structural variations involving *CD274 (PD-L1)/ PDCD1LG2 (PD-L2)* described above.[25] Interestingly, Nicoale *et al.* reported neoplastic PD-L1 expression in 95% of nodal EBV+ DLBCL young patients with a unique pattern of T-cell/histiocyte-rich large B-cells.[36] In contrast to this, Takahara *et al.* recently reported a much lower frequency (11%) of neoplastic PD-L1 expression among elderly patients, suggesting that immune escape may be more important in younger patients than in older patients.[23] The discrepancy in the positive percentages of neoplastic PD-L1 expression among patients with EBV+ DLBCL has been highlighted by others, and should be clarified in the future.[37,38]

Cellular origin of Hodgkin lymphoma

The cellular origin of CHL has been controversial for a long time, because HRS cells of CHL have a morphology and immunophenotype that does not fit any type of immune cells. Genetic analysis has revealed that HRS cells have a clonally rearranged Immunoglobulin (IG) gene in almost all CHL cases.[39] Furthermore, a significant number of HRS cells have a somatic hypermutation in the IG gene locus.[39,40] In around 30% of cases, IG gene rearrangements were rendered nonfunctional by the introduction of stop codons, deletions, or destructive promoter mutations generated within the GC.[40] In the other cases, HRS cells frequently lack Ig gene transcription ability due to functional defects in the Ig gene regulatory elements.[39] These genetic analyses indicate that HRS cells originate from GC cells capable of surviving without BCR signaling.[41] It should be noted that a minority of CHL cases have clonal TCR gene rearrangement and/or express T-cell markers.[42] Moreover, Karube *et al.* also recently reported adult T-cell leukemia/lymphoma with HTLV-1-infected Hodgkin and Reed-Sternberg-like cells.[43] Additionally, these HRS-like cells also share the neoplastic PD-L1 expression beyond their non-B-cell phenotype. Whether these minority cases represent T-cell derived Hodgkin lymphoma, T-cell lymphomas mimicking CHL, or 'B-cell' lymphomas with aberrant phenotypic/genetic changes still needs to be researched, although it might be dependent on our definition of "Hodgkin neoplasm". Tsuyama *et al.* more recently reported unique cases of Hodgkinoid histiocytosis with CHL-like morphologic features and CD30 and PD-L1 expression in Hodgkinoid histiocytes.[44] This diagnostic term ,"Hodgkinoid", was created by Tsuyama *et al.* in order to avoid confusion with CHL, which is now defined as GC-derived B-cell neoplasm in the 2022 WHO classification. An international consensus should be achieved for these issues.

Diagnostic approach in routine pathologic practice

It is well known that HRS cells are diagnostic hallmarks of CHL, but are not specific, because RS-like cells are often found in other non-Hodgkin lymphomas and lymphoproliferative disorders.[45] A precise histologic sub-classification is very challenging in the setting of small biopsy specimens. Even if a diagnosis of CHL without further subtype classification can be made, we recommend to assess the presence or absence of neoplastic PD-L1 positivity and/or EBV harboring in HRS cells and their lookalikes, which can be delineated into 4 subgroups; i.e., PD-L1+ EBV-, PD-L1+EBV+, PD-L1-EBV-, and PD-L1-EBV+ large cell subtypes.

In our routine practice, neoplastic PD-L1+ EBV- cases correspond approximately to NSCHL. Nodular growth pattern and the presence of fibrosis are regarded as defining morphologic features of NSCHL, but may be also be obscured in small samples. Lacunar cells are also characteristic of NSCHL and typically have small, but not large, nucleoli, which may ironically be difficult to identify as HRS cells.[45] Differential diagnosis includes mediastinal grey zone lymphoma and ALK+ anaplastic large cell lymphoma (ALCL) (especially with a Hodgkin-like pattern), which share the neoplastic PD-L1+ EBV-negative large cells. Their differential diagnostic approaches were well discussed in a recent review by Elaine S. Jaffe's group.[5] Cytotoxic molecule (CM)-positive CHL in our studies usually shows neoplastic PD-L1 expression (unpublished data).[46] This combination of PD-L1 and CM positivity may be shared by ALK-negative ALCL, except for cases with *DUSP22* rearrangement which lack CM expression.[47] As another diagnostic pitfall, the syncytial variant of CHL may be accompanied by reduced expression of PD-L1 in tumor cells.[26,48]

Conversely, three cases of nodal diffuse large B-cell lymphoma with neoplastic PD-L1 positivity, but without EBV association, have been reported by Kohno *et al.*[49]

Neoplastic PD-L1+EBV+ CHL is almost equivalent to EBV+ MC and LD subtypes with prototypic HRS cells characterized by large distinct nucleoli and embedded in a reactive immune cell-rich background. Never-the-less, EBV+ MCCHL poses differential diagnostic problems from EBV+DLBCL, especially in young patients[36] and EBV+ grey zone lymphoma.[50] Interestingly, the latter two were characterized by strong expression of B-cell markers and a greater number of mononuclear tumor cells often with a T-cell/histiocyte-rich large B-cell lymphoma-like pattern, although their patients' age distributions are quite distinct. Some exceptional cases of EBV+ nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) have been reported, but in which PD-L1 information was not available.[51] Notably, neoplastic PD-L1+EBV+ CHL may be accompanied by primary extranodal disease among immunocompromised patients, and should be distinguished from EBV+ mucocutaneous ulcers, which usually lack PD-L1 positivity.[52] Neoplastic PD-L1+EBV+ CHL-type lesions may be seen post-transplant and in other iatrogenic lymphoproliferative disorders, and detailed information of the patients' background should be requested.

Neoplastic PD-L1-EBV- HL includes NLPHL, LRCHL, and MCCHL without EBV association in our preliminary studies. As is well-known, differential diagnosis based on morphology alone in small-volume samples is highly challenging or impossible among NLPHL, LRCHL, and T-cell/histiocyte-rich large B-cell lymphoma. An adequate tissue biopsy is recommended, if necessary. Anaplastic variant of DLBCL also possesses the same phenotype as PD-L1-EBV- type tumor cells, but is characterized by sheet-like proliferation of large cells and/or HRS-like cells, which is distinct from ordinal CHL.[53]

Neoplastic PD-L1-EBV+ HRS-like cells are frequently encountered as bystander cells linked with reactivation of EBV in normal B-cells, but are hardly seen in our studies of CHL. Indeed, Kume *et al.* also recently reported that all EBV+ CHL cases consistently expressed PD-L1 in tumor cells.[54] It is emphasized that EBV+ HRS-like cells, which are often accompanied by nodal peripheral T-cell lymphoma of mainly follicular helper T-cell phenotype, i.e., angioimmunoblastic T-cell lymphoma in many, generally lack immunohistochemical PD-L1 positivity, which was first highlighted by Eladl *et al.* in 2017.[55]

CONCLUSION

Diagnosis of CHL is challenging in small-volume samples. However, our knowledge/understanding of CHLs' biology is now rapidly expanding. This will prompt us to redefine the diagnosis CHL from morphology to biology based. Future discussion is warranted.

CONFLICT OF INTEREST

There is no conflict of interest.

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