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ARTICLE

Diagnostic utility of STAT6^{YE361} expression in classical Hodgkin lymphoma and related entities

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Abstract

Although the distinction of classical Hodgkin lymphoma from nodular lymphocyte predominant Hodgkin lymphoma using morphology and immunostains is straightforward in most instances, occasional cases pose diagnostic challenge. We sought to determine the utility of the novel YE361 STAT6 rabbit monoclonal antibody in Hodgkin lymphoma and diagnostically challenging B- and T-cell non-Hodgkin lymphoma entities with Hodgkin-like features. Cases from seven institutions included: 57 classical Hodgkin lymphomas (31% EBV+), 34 nodular lymphocyte predominant Hodgkin lymphomas, 34 mimicking B- and T-cell non-Hodgkin lymphomas, and 7 reactive lymphoproliferations. After review of histology, STAT6^{YE361} immunostaining was performed. The intensity and spatial localization of immunopositivity was assessed in neoplastic cells. Additional FISH for programmed death ligand-1 (*PD-L1*) was performed in one patient in paired treatment-naive and relapse biopsy tissues. Two STAT6^{YE361} immunopositive cases were examined by whole-exome sequencing after flow sorting to assess mutations in *STAT6* pathway genes. Most classical Hodgkin lymphomas showed nuclear staining for STAT6^{YE361} [46/57 cases (80%)] on Hodgkin cells. Staining was exclusively nuclear in a minority [12/46 (26%)], while dual nuclear and cytoplasmic localization was more common [34/46 (74%)]. In contrast, all nodular lymphocyte predominant Hodgkin lymphomas [0/34 (0%)] were negative for nuclear STAT6^{YE361} staining on the lymphocyte predominant cells. Within B- and T-cell non-Hodgkin lymphomas, nuclear STAT6^{YE361} was seen in: B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma, and in primary mediastinal large B-cell lymphoma. Strong *PD-L1* gene amplification was noted in the paired cHL and relapse B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma, although STAT6^{YE361} was negative in both biopsies. Whole-exome sequencing identified mutations in *B2M, XPO1,* and *ITPKB* as well *CISHP213L* (in the STAT pathway) in one classical Hodgkin lymphoma patient positive for nuclear STAT6^{YE361} although no underlying *STAT6* mutations were observed in either sample examined. STAT6^{YE361} nuclear staining has 100% positive predictive value and 85.7% negative predictive value in confirming or excluding classical Hodgkin lymphoma diagnosis in the distinction from nodular lymphocyte predominant Hodgkin lymphoma and other benign and malignant entities.

Introduction

The Hodgkin/Reed–Sternberg cells of classical Hodgkin lymphoma typically express CD30 and CD15 with weak or lost CD20, Pax5, and loss of OCT2 and/or Bob.1 reflective of the downregulation B cell and germinal center program. In contrast, the lymphocyte predominant cells of nodular lymphocyte predominant Hodgkin lymphoma express strong CD20 and Pax5 as well all other B-cell transcription factors including OCT2, BOB.1, and Bcl6, reflective of a

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preserved B-cell germinal center program, with negative CD30 and CD15. Yet, there are rare instances when Hodgkin/Reed–Sternberg cells express strong CD20 [1], or when lymphocyte predominant cells express CD15 [2] or EBV [3] despite otherwise typical immunoarchitectural features, thus creating diagnostic difficulty. Further challenges in the distinction of classical Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma are the growing number of entities with considerable histologic overlap, including EBV-related lymphoproliferative disorders [4], T-cell/histiocyte rich large B-cell lymphoma, and some cases of lymphocyte-rich classical Hodgkin lymphoma [5, 6]. In addition, improved understanding of follicular helper T-cell biology has led to the recognition of follicular variants of peripheral T-cell lymphoma, which can contain Hodgkin/Reed–Sternberg-like cells [7, 8]. Given these observations, there is a need for tissue markers that can reliably separate the two Hodgkin lymphoma subtypes and aid to distinguish true Hodgkin lymphoma from the non-Hodgkin lymphoma entities detailed above. Biomarkers with diagnostic or prognostic utility in classical Hodgkin lymphoma can broadly be categorized into tumor cell-related or microenvironment-related markers. The microenvironment of classical Hodgkin lymphoma is unique in that rare Hodgkin/Reed–Sternberg cells are capable of co-opting the peritumoral host cytokine network to its advantage. Evidence for such skewing in classical Hodgkin lymphoma, which stems from an overactive IL-13 signaling axis, was provided by Skinnider et al. who showed that Hodgkin/Reed–Sternberg cells express IL-13 and IL-13^{Ra1} in over 85% of cases, as opposed to LP cells, which were negative in all instances [9]. The expression of Th2 cytokines (IL-4, IL-5, and IL-13) leads to the accumulation of eosinophils; Th2 cells and fibroblasts are characteristics of cHL lesions. Furthermore, the resulting tumor microenvironment contributes to local suppression of Th1 cell-mediated cellular immune responses. A key downstream effector of the IL-4/IL-13 signaling axis is STAT6, which forms dimers in response to Jak2-mediated phosphorylation of the cytoplasmic domain of IL-4 and IL-13 receptors. Normally, dimerized STAT6 translocates to the nucleus influencing the transcription of its target genes. Hence, there has been an interest in examining the relevance of the JAK-STAT pathway, and specifically STAT6 in Hodgkin lymphoma as well as primary mediastinal B-cell lymphoma, both of which are unified by constitutive STAT6 phosphorylation; although in the latter, the underlying mechanism is due to *JAK2* amplification as opposed to autocrine IL-4/IL-13 signaling in cHL [10].

Consistent with overactive IL-4/IL-13 signaling in cHL, Skinnider et al. identified that 78% of cHLs demonstrated constitutively active STAT6 as demonstrated by immunohistochemistry for p-STAT6 [11]. These findings were further corroborated by subsequent studies demonstrating amplifications of *STAT6* (12q13) by array-based comparative genomic hybridization of microdissected Hodgkin/Reed–Sternberg cells [12]. Another recent study of classical Hodgkin lymphoma noted that *STAT6* mutations are seen in nearly 32–40% of classical Hodgkin lymphomas and is also the most commonly mutated gene [13, 14].

The novel antibody for STAT6^{YE361} has epitope specificity to the C-terminus domain of the STAT6 protein, while p-STAT6 antibodies are specific only to their respective phosphorylated forms. Currently there are no studies addressing the diagnostic utility STAT6^{YE361} in classical Hodgkin lymphoma. Given this background, we sought to: determine the frequency and immunolocalization of STAT6^{YE361} staining in classical Hodgkin lymphoma and nodular lymphocyte predominant lymphoma, to compare differences in STAT6^{YE361} to p-STAT6^{YE641} staining in classical Hodgkin lymphoma, nodular lymphocyte predominant lymphoma, and select non-Hodgkin lymphoma mimicker subtypes, and to establish whether differences in immunoreactivity for non-phosphorylated STAT6 as opposed to p-STAT6^{YE641} could accurately distinguish classical Hodgkin lymphoma from non-Hodgkin lymphoma and challenging mimicker cases.

Materials and methods

Study design and patient selection

This study was undertaken at The University of Chicago Medicine with the approval of the Institutional Review Board (IRB14-1133 and IRB13-1297), and independently covered by the IRB of the participating institutions for cases submitted by them. Well-characterized cases with available formalin-fixed paraffin embedded tissues were contributed from the following institutions: University of Chicago Medicine, Henry Ford Health System, City of Hope Medical Center, Asan University, Massachusetts General Hospital, the National Cancer Institute, and Goethe-University Hospital. For list of cases examined see Table 1. The diagnosis in all cases were confirmed by two of the study authors (GV and CVS).

In addition to the whole section cases studied here, a small tissue microarray of duplicate 2 mm cores comprising 22 classical Hodgkin lymphomas and three nodular lymphocyte predominant Hodgkin lymphomas (diagnostic and few relapse) tissues of patients treated at the University of Chicago Medical Center were examined to specifically assess the reliability of staining on older paraffin tissues. The TMA tissues were obtained from the University of Chicago Hoogland Lymphoma Biorepository.

Table 1 List of cases examined for nuclear STAT6^{YE361} expression.

Entity	Number of cases
Classical Hodgkin lymphoma	57
Nodular lymphocyte predominant Hodgkin lymphoma (including variants)	34 (5)
B-cell lymphoma, unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma	8
Diffuse large B-cell lymphoma arising in nodular lymphocyte predominant Hodgkin lymphoma	5
T-cell/histiocyte rich large B-cell lymphoma (including transformations)	4 (3)
Diffuse large B-cell lymphoma, Epstein–Barr virus positive, not otherwise specified with Hodgkin and Reed/Sternberg-like cells	4
Primary mediastinal (thymic) large B-cell lymphoma	3
Peripheral T-cell lymphoma not otherwise specified with Hodgkin and Reed/Sternberg-like cells	2
B-cell posttransplant lymphoproliferative disorder	2 (2)
Anaplastic large cell lymphoma, ALK negative	1
Small lymphocytic lymphoma	1
Follicular lymphoma	1
Plasma cell myeloma	1
Reactive lymphadenitis with florid immunoblastic proliferation and follicular hyperplasia	7

Immunohistochemistry

Standard basic workup included all diagnostic antibodies: CD20, CD3, CD45, Pax5, CD30, and CD15. In addition, OCT2, CD79a, PD1, and programmed death ligand-1 (PD-L1) (Abcam, rabbit monoclonal, clone 28–8) were performed in a subset of cases. All immunohistochemical work except for p-STAT6^{Y641} staining was performed in the Clinical Immunohistochemistry Lab of the Pathology Department of University of Chicago Medicine. Immunohistochemical staining for all standard antibodies was performed on 4 µm thick tissue sections using either on the Leica Bond III or Ventana XT instruments with appropriate positive and negative controls. Sections were baked for 3 h at 60 °C in a dehydration oven.

STAT6 immunostaining

Slides were incubated with the AbCam STAT6^{YE361} primary antibody for 25 min at a 1:400 dilution. Signal amplification was accomplished by incubating with a postprimary rabbit anti-mouse IgG antibody solution for 15 min and subsequently a polymer anti-rabbit poly-HRP-IgG solution for 25 min. Endogenous peroxidase activity was blocked by incubation with a peroxidase block solution for 5 min. Signal visualization was accomplished by incubating with DAB chromogen solution for 10 min. Finally, slides were then counterstained with hematoxylin for 8 min. Immunohistochemical staining for p-STAT6^{Y641} was performed at Goethe-University Hospital, Frankfurt, Germany. A total of 13 cases (11 nodular lymphocyte predominant Hodgkin lymphomas, one T-cell/histiocyte rich large B-cell lymphoma-like transformation of nodular lymphocyte predominant Hodgkin lymphoma, and one classical Hodgkin lymphoma) with available unstained slides were studied in parallel with both STAT6 antibodies for comparison [15]. A comparison of the binding sites for both the p-STAT6^{Y641} and STAT6^{YE361} is depicted in Supplementary Fig. 1.

Assessment of immunohistochemical staining

Intensity and localization of STAT6^{YE361} and STAT6^{YE641} expression were assessed in the nuclear and cytoplasmic compartments of the neoplastic cells. Weak cytoplasmic staining for STAT6^{YE361} was identified in the background small B cells of Hodgkin lymphoma tissues, and in the follicular/mantle zone B cells of tonsillar tissue, which served as controls.

PD-L1 fluorescent in situ hybridization (FISH)

PD-L1 gene copy number alterations were determined by FISH on unstained formalin-fixed paraffin embedded tumor specimens in one case with paired diagnostic and relapse tissues. More specifically, slides were deparaffinized with Citrisolve and 100% alcohol. Pepsin protease solution was used for proteolytic pretreatment of slides. The slides were fixed and then denatured. Spectrum Orange labeled PD-L1 (CD274, Empire Genomics) probes were used together with Spectrum Aqua labeled centromere 9 reference probes (CEP9, Abbot Molecular). After overnight hybridization at 37 °C in a humidifier chamber, slides were washed and

counterstained with 2.5 mg/mL DAPI. Fluorescent microscopy was then utilized to identify whether *PD-L1* gene copy number alterations were present, and if so, the underlying mechanism (polysomy, copy gain, amplification). Thirty tumor cells per sample were analyzed. Nuclei were defined as *PD-L1* amplified if the PD-L1/CEP9 ratio was ≥3:1 or there were ≥6 PD-L1 signals; those nuclei with a PD-L1/CEP9 ratio of >1:1 but <3:1 were defined as copy gain, and those with a probe ratio of 1:1 but with more than two copies of each probe were defined as polysomic for chromosome 9. The frequency of tumor cells harboring each type of *PD-L1* copy number abnormality was assessed and tumors were considered positive for *PD-L1* copy number abnormalities if greater than 30% of nuclei exhibited alterations.

Whole-exome sequencing

Two EBV-negative classical Hodgkin lymphoma cases were examined by whole-exome sequencing. Whole-exome libraries were prepared from genomic DNAs of FACS-purified Hodgkin/Reed–Sternberg cells (CD3− CD19− CD30+ CD40+), from two classical Hodgkin lymphoma patients, and matched T cells (CD19− CD3+ CD4+ CD8+) as normal controls, using SureSelectXT Human All Exon V5 kit (Agilent Technologies, Santa Clara, CA). The prepared whole-exome libraries were sequenced by 100-bp paired-end reads on HiSeq2500 Sequencer (Illumina, San Diego, CA). Sequence alignment and mutation calling were performed using our in-house pipelines described previously [16]. Briefly, the sequence reads were mapped to the human reference genome GRCh37/hg19 using Burrows–Wheeler Aligner (v0.7.10) [17]. Possible PCR duplicates were removed using Picard tool (http://broadinstitute.github.io/picard/), and reads with a mapping quality of <30 and with mismatches of more than 5% of sequence reads were also excluded. Somatic variants (single-nucleotide variations, and insertion/deletions) were called with the following parameters, (i) base quality of ≥15, (ii) sequence depth of ≥10, (iii) variant depth of ≥2, (iv) variant frequency in tumor of ≥10%, (v) variant frequency in normal of <2%, and (vi) Fisher *P* value of <0.05. SNVs and indels were annotated based on RefGene using ANNOVAR [18].

Identification and analysis of mutated genes of interest

We performed reactome pathway analysis to identify the relevant mutated genes that affected biological processes and pathways of interest. Genomic analysis was conducted with the Catalogue of Somatic Mutations in Cancer (http://cancer.sanger.ac.uk), a database system that collects somatic mutation in all forms of human cancer from a variety of public sources [19] and with algorithm Protein variation effect analyzer (ProVEAN). This computational tool provides a functional prediction for amino acid substitution and in-frame insertion and deletion change on protein variants. The alignment-based score defines the variants as neutral or deleterious. The PROVEAN web server (http://provean.jcvi.org) can provide precomputed prediction for large sets of genome-wide nucleotide or amino acid variants for both human and mouse [20].

Data and statistical analysis

Data were kept on secure university servers and analyzed using basic descriptive statistical methods in STATA11 (College Station, TX).

Results

STAT6^{YE361} expression in normal lymphoid tissue

In normal lymphoid tissues that included a total of seven cases (four reactive tonsils, and three reactive nodes including one with florid immunoblastic proliferation), STAT6^{YE361} staining was typically observed as granular cytoplasmic/perinuclear staining in mantle zone lymphocytes. Occasional diffuse cytoplasmic staining was also observed in histiocytes and immunoblasts. Nuclear expression of STAT6^{YE361} was not observed in normal lymphoid tissues (Fig. 1).

STAT6^{YE361} in classical Hodgkin lymphoma tissues

Fifty-seven cases of classical Hodgkin lymphoma were analyzed [subtypes: 38 nodular sclerosis, six mixed cellularity, five not otherwise specified, four interfollicular, two lymphocyte rich, and two classical Hodgkin lymphoma-Richter transformations of chronic lymphocytic leukemia]. The mean age was 40.1 years; the male-to-female ratio was 1.5. Nuclear expression of STAT6^{YE361} was observed in 46/57 (81%) classical Hodgkin lymphomas (Fig. 2a–d). Positivity was defined by at least a subset of the Hodgkin/Reed–Sternberg cells (>5%) showing nuclear expression exceeding the intensity of cytoplasmic staining seen in background lymphocytes. Generally, classical Hodgkin lymphoma cases positive for nuclear STAT6^{YE361} contained variable numbers of positive Hodgkin/Reed–Sternberg cells (from 10 to 100%). Nuclear expression of STAT6^{YE361} was most optimal in well-fixed specimens from recent biopsies. In poorly fixed specimens, positive Hodgkin/Reed–Sternberg cells were best identified at the periphery of the biopsy material. Among classical Hodgkin lymphoma cases with nuclear positivity, expression of STAT6^{YE361} was exclusively nuclear in 12/46 (26%) cases, while dual nuclear and

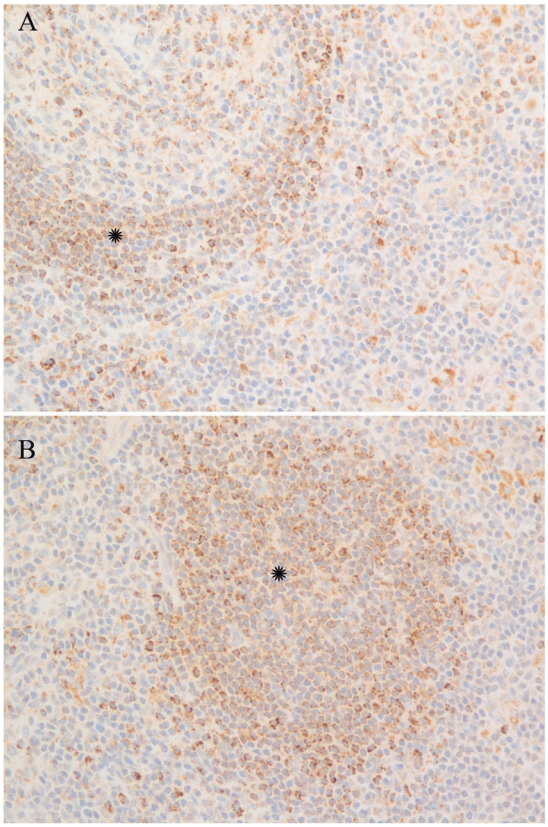

Fig. 1 Typical STAT6^{YE361} staining pattern in reactive nodal tissues: STAT6^{YE361}. a Mantle zone lymphocytes and primary follicle lymphocytes **b** staining for STAT6^{YE361} with granular cytoplasmic staining (*). Note that paracortical T-cell areas are negative. Germinal center centrocytes are notable for patchy granular cytoplasmic staining.

cytoplasmic expression was present in 34/46 (74%) cases. Exclusive cytoplasmic expression of STAT6^{YE361} was present in only 6/57 (9%) cases. Nuclear STAT6^{YE361} expression did not correlate with either EBV status (by in situ hybridization) or with classical Hodgkin lymphoma subtype. Of the two cases of classical Hodgkin lymphoma-Richter transformation of chronic lymphocyte leukemia cases, one was positive for nuclear STAT6^{YE361}, while the other was negative. Two classical Hodgkin lymphomas expressing strong STAT6^{YE361} were also strongly positive for PD-L1. Seventeen of 22 (77%) classical Hodgkin lymphomas on tissue microarray were positive for nuclear STAT6^{YE361}. Notably, three of the five negative cases were from paraffin tissues that were at least 8 years old.

STAT6^{YE361} in nodular lymphocyte predominant Hodgkin lymphoma

Thirty-four cases of nodular lymphocyte predominant Hodgkin lymphoma were analyzed. The mean age was 41.0 years; the male-to-female ratio was 6.0. No cases of nodular lymphocyte predominant Hodgkin lymphoma showed nuclear expression of STAT6^{YE361} but most showed weak-to-moderate cytoplasmic expression [25/34 (74%)] (Fig. 2e–h). All three nodular lymphocyte predominant Hodgkin lymphoma cases included in the tissue microarray were negative for STAT6^{YE361}. Background T cells were negative in these cases providing good internal negative controls. See Supplementary Fig. 2 for relative bar graph of Hodgkin lymphoma cases for expression of STAT6^{YE361}.

Diagnostically challenging cases with classical Hodgkin lymphoma-like features

In addition, a series of diagnostically challenging cases with classical Hodgkin lymphoma-like morphologic features were analyzed. In cases of B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma, nuclear STAT6^{YE361} expression was identified in the large neoplastic cells of in one of eight cases examined while only one of three cases of primary mediastinal large B-cell lymphoma stained positive. The single STAT6^{YE361} positive case of cases of B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma occurred in a 53-year-old male in whom STAT6^{YE361} was examined in two consecutive biopsies. The initial biopsy demonstrated a sheet-like proliferation of large B cells with Hodgkin-like cytomorphology showing weak CD20 and Pax5 but with negative CD30 and CD15. STAT6^{YE361} demonstrated strong nuclear reactivity in the large cells. There was poor response to CHOP-R since a diagnosis of large B-cell lymphoma was made initially at the outside institution. The subsequent biopsy a year later was notable for identical cytomorphology and immunophenotype with respect to both STAT6 antibodies except for strong and uniform CD30 supporting closer relationship with classical Hodgkin lymphoma in the subsequent biopsy. However second review of both materials after the relapse biopsy allowed designation as B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma. STAT6^{YE361} expression was not observed in the malignant cells of: EBV positive diffuse large B-cell lymphoma with Hodgkin/Reed–Sternberg-like cells (four cases), T-cell/histiocyte rich large B-cell lymphoma-like transformation of nodular lymphocyte predominant Hodgkin lymphoma (three cases), T-cell/histiocyte rich large B-cell lymphoma (one case), peripheral T-cell lymphoma not otherwise specified with Hodgkin/Reed–Sternberg-like cells (two cases) [Fig. 3: case 1], one case of nodular lymphocyte predominant Hodgkin lymphoma with Hodgkin/Reed–Sternberg-like cells [Fig. 3: case 2], and nor in single cases of anaplastic large cell lymphoma, small lymphocytic leukemia, follicular

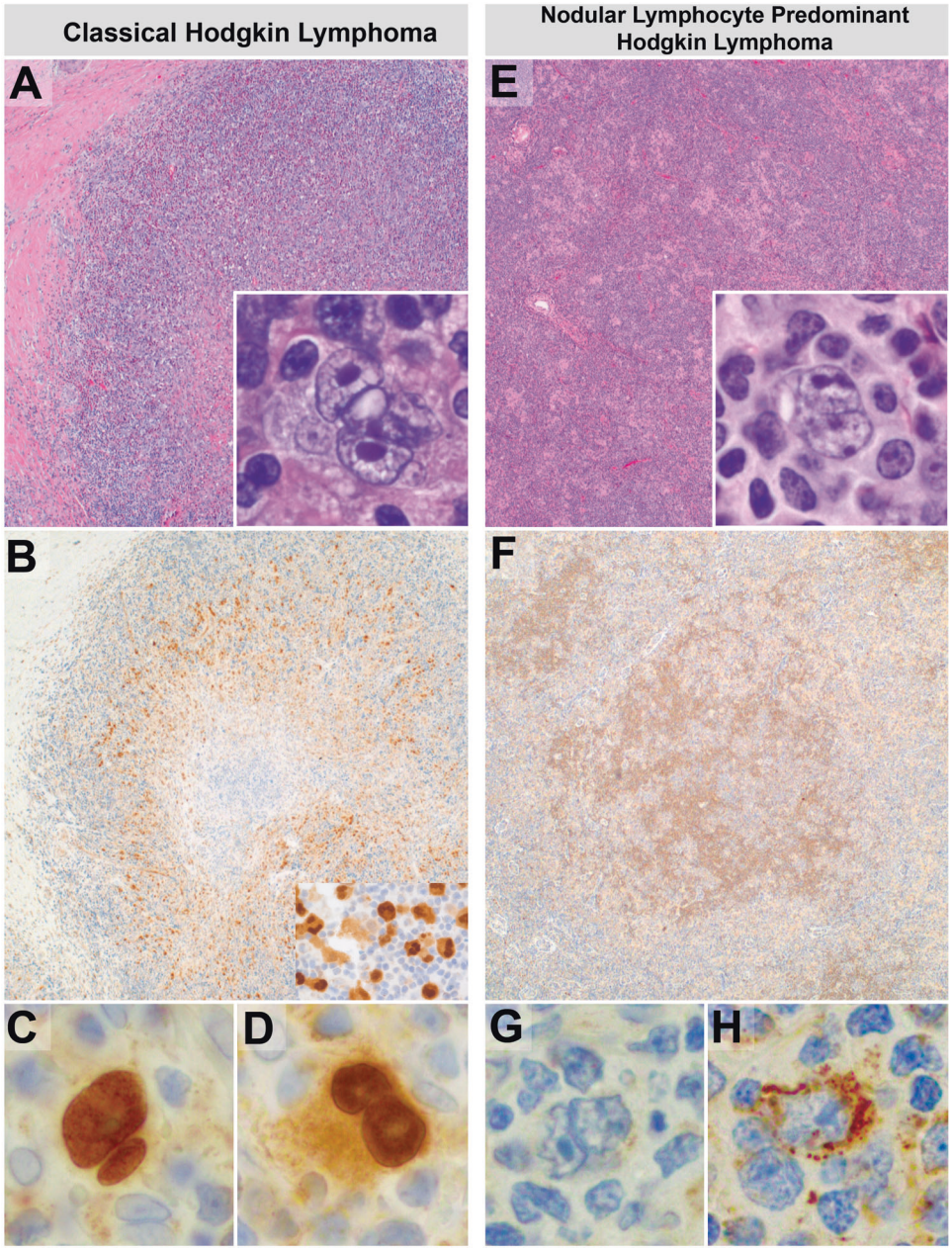

Fig. 2 Typical STAT6^{YE361} staining patterns in classical Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma. A typical case of classical Hodgkin lymphoma, nodular sclerosis subtype [**a** cHL, H&E 4× and 100× (inset)]. The Hodgkin/Reed–Sternberg cells are highlighted by STAT6^{YE361} [**b** cHL, STAT6^{YE361}, 4× and 40x (inset)] by moderate/strong nuclear expression of STAT6^{YE361} with variable cytoplasmic staining [**c**, **d** classical Hodgkin lymphoma, STAT6^{YE361} 100×]. In contrast, a typical case of nodular lymphocyte predominant Hodgkin lymphoma [**e** H&E, 4× and 100× (inset)] shows the proliferating weak/moderate cytoplasmic staining in background small lymphocytes [**f** nodular lymphocyte predominant Hodgkin lymphoma, STAT6^{YE361}, 4×]; however, the lymphocyte predominant cells are consistently negative for nuclear STAT6^{YE361}, showing only variable cytoplasmic staining [**g**, **h** nodular lymphocyte predominant Hodgkin lymphoma, STAT6^{YE361}, 100x].

lymphoma, plasma cell myeloma, or acute lymphadenitis with florid immunoblastic proliferation. A case of classical Hodgkin lymphoma-type posttransplant lymphoproliferative disorder was also negative for nuclear STAT6^{YE361}.

Comparison of STAT6^{YE361} and p-STAT6^{YE641}

It has been previously shown that a subset of nodular lymphocyte predominant Hodgkin lymphoma cases (18/49; 37%) contains lymphocyte predominant cells with distinct nuclear expression of p-STAT6^{YE641} [21]. Since some antibodies may lack specificity for the phosphorylated forms of the protein of interest, we asked if the STAT6^{YE361} antibody showed reduced sensitivity for the phosphorylated form by directly comparing the STAT6^{YE361} antibody with the p-STAT6^{YE641} antibody in selected cases of nodular lymphocyte predominant Hodgkin lymphoma. To determine if lymphocyte predominant cells express nuclear STAT6^{YE361} at a level below the threshold of detection for the STAT6^{YE361} antibody, 12 cases of nodular lymphocyte predominant Hodgkin lymphoma with available unstained material were selected and analyzed for immunohistochemical expression of STAT6^{YE361} and p-STAT6^{YE641}. Lymphocyte predominant cells showed cytoplasmic positivity for STAT6^{YE641} in a subset of the selected cases [3/12 (25%)]. However, none of the nodular lymphocyte predominant Hodgkin lymphoma cases [0/12, (0%)] showed

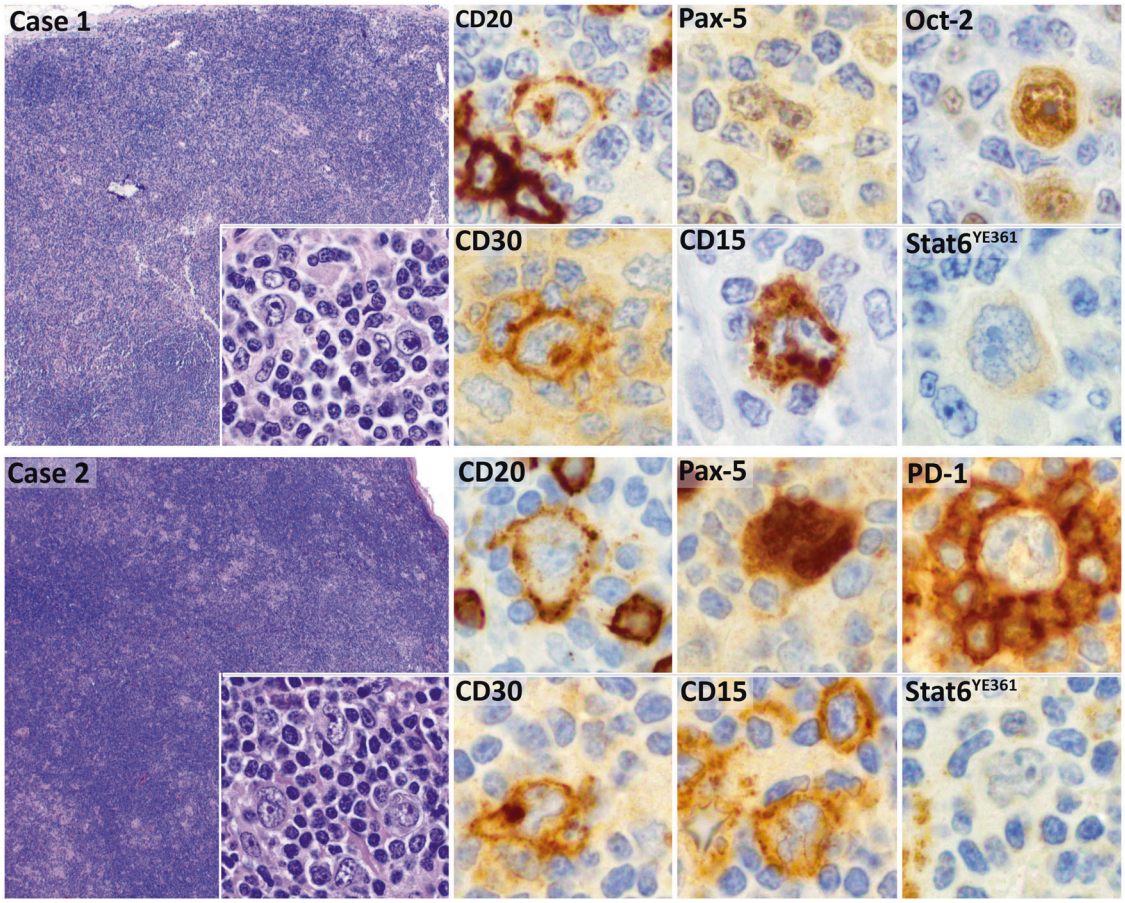

Fig. 3 Examples of challenging cases mimicking classical Hodgkin lymphoma. Case 1 (follicular variant of peripheral T-cell lymphoma with Hodgkin/Reed–Sternberg-like cells): this case of follicular T-cell lymphoma was initially thought to be classical Hodgkin lymphoma due to an effacing nodular pattern and scattered Hodgkin/Reed–Sternberg-like cells (CD20+/−, Pax5+, OCT2+, CD30+, and CD15+). However, the majority of the proliferation is small atypical T cells (CD3+, CD4+, CD5+, CD7+, and PD1+), which had a clonal rearrangement of *TCR* gamma. STAT6^{YE361} was negative in the Hodgkin/Reed–Sternberg-like cells, allowing for classification as follicular variant of peripheral T-cell lymphoma. Case 2 (nodular lymphocyte predominant Hodgkin lymphoma with aberrant immunophenotypic features): this case showed an effacing nodular proliferation containing scattered large neoplastic cells, some of which showed aberrant immunophenotypic features (CD20+, Pax5+, CD30+, and CD15+) mimicking Hodgkin/Reed–Sternberg cells; however, STAT6^{YE361} was also negative, supporting closer relationship with nodular lymphocyte predominant Hodgkin lymphoma over classical Hodgkin lymphoma.

nuclear expression of STAT6^{YE361}. One case also contained an area of T-cell/histiocyte rich large B-cell lymphoma-like large cell transformation and both components were negative for nuclear STAT6^{YE361} and p-STAT6^{YE641}.

In addition, one previously published case of coexisting and clonally identical classical Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma were similarly analyzed for expression of both markers [22]. The classical Hodgkin lymphoma component showed nuclear expression of both STAT6^{YE361} and p-STAT6^{YE641}, while the nodular lymphocyte predominant Hodgkin lymphoma component showed no nuclear staining with either STAT6 antibodies (Fig. 4). In this unique case, both STAT6 antibodies were concordant, although there was increased cytoplasmic staining of background lymphocytes and histiocytes seen in STAT6^{YE361} compared with p-STAT6^{YE641}. STAT6^{YE361} nuclear staining has 100% positive predictive value and 85.7% negative predictive value in confirming or excluding classical Hodgkin lymphoma diagnosis in the distinction from nodular lymphocyte predominant Hodgkin lymphoma and other benign and malignant entities affording better discrimination supporting its diagnostic utility in challenging cases.

PD-L1 FISH on primary classical Hodgkin lymphoma and relapsed (gray zone lymphoma) tissues

Due to the relevance of unifying 9p24.1 amplifications and/or aberrations in JAK/STAT pathway resulting in PD-L1 amplifications, we examined STAT6 expression in one PD-L1 amplified classical Hodgkin lymphoma. This patient had classical Hodgkin lymphoma at diagnosis (treated with doxorubicin, bleomycin, vinblastine, and dacarbazine) and

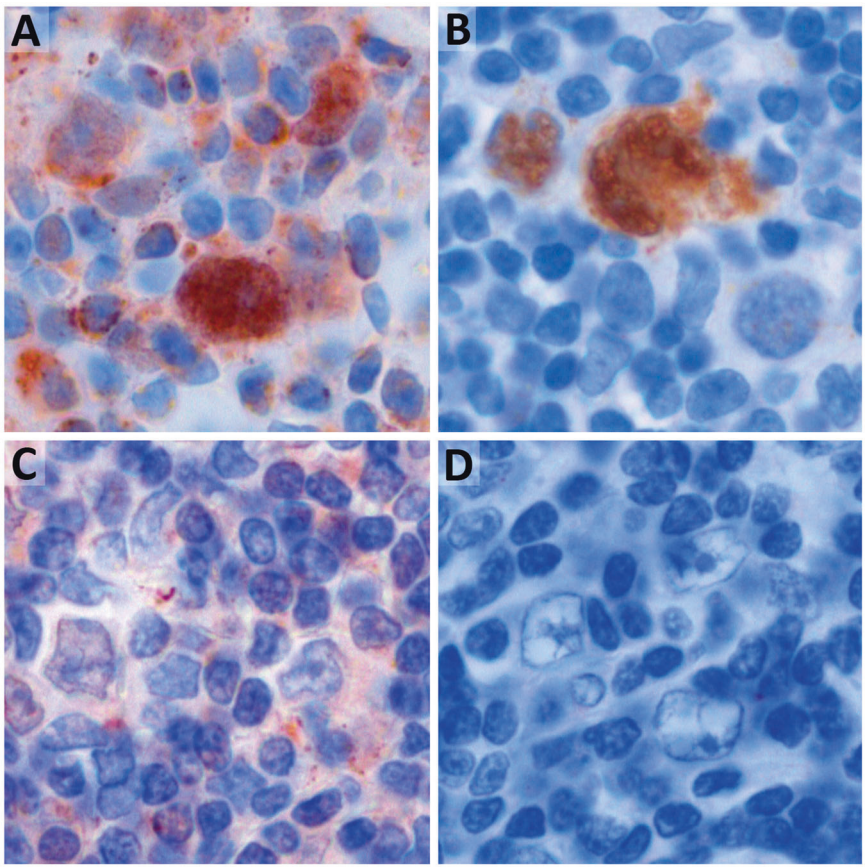

Fig. 4 Comparison of STAT6^{YE361} and p-STAT6^{YE641} in a case of coexisting and clonally identical classical Hodgkin lymphoma and nodular lymphocyte predominant Hodgkin lymphoma. The classical Hodgkin lymphoma component showed Hodgkin/Reed–Sternberg cells with convincing nuclear expression of both STAT6^{YE361} (**a** 100×) and STAT6^{YE641} (**b** 100×), while the nodular lymphocyte predominant Hodgkin lymphoma component showed lymphocyte predominant cells that were negative for both STAT6^{YE361} (**c** 100×) and STAT6^{YE641} (**d** 100×).

relapsed as a gray zone lymphoma 4 years later. Both biopsies were examined for PD-L1 via FISH to see the relationship between STAT6^{YE361} expression and PD-L1 via FISH and immunostaining. Interestingly, both biopsies were negative for STAT6^{YE361}. The diagnostic classical Hodgkin lymphoma tissue was strongly PD-L1 positive in most Hodgkin/Reed–Sternberg cells, while the CD20 positive gray zone lymphoma relapse tissue was also positive for PD-L1 by immunohistochemistry. By FISH analysis, the initial classical Hodgkin lymphoma tissue showed a high degree of PD-L1 gene amplification with up to ten PD-L1 gene copies per cell. The subsequent gray zone lymphoma tissue showed a relative copy gain of PD-L1 of 3–4 copies in a number of cells but was not as highly amplified as the initial classical Hodgkin lymphoma tissue (see Fig. 5). These findings suggest that the PD-L1 driven alterations are not dependent on upstream STAT6 activation.

Whole-exome sequencing data on two classical Hodgkin lymphoma patients

To assess for possible underlying mutations in *STAT6* in cases expressing STAT6 by immunohistochemistry, two cases with nuclear STAT6^{YE361} expression, one representing a relapse tissue (case 1), and the other a treatment-naive tissue (case 2) of a patient with advanced stage, were examined by flow sorting and whole-exome sequencing. Case 1 showed variable nuclear and cytoplasmic STAT6^{YE361} expression in a significant fraction of cells, while case 2 showed isolated nuclear staining in 50% of Hodgkin/Reed–Sternberg cells.

Using whole-exome sequencing on both biopsy samples, we achieved an average of 79% proportion of nucleotides > Phred quality score 30 of total bases, according to the base calling accuracy (range, 69–89%). After filtering, 110 somatic single-nucleotide variants and insert/deletions were identified. G > A/G > T/C > A/T > C transitions were the most common nucleotide substitutions observed.

Consistent with published reports, somatic mutations were identified in: *B2M* (Stopgain), *XPO1* p.E571K, and *ITPKB* p.A61V. *CISH* p.P213L (Provean score −7.829) were identified in one of the two patients without evidence of *STAT6* mutations (Supplementary Fig. 3). Some of these mutations including the *CISH* mutations determined have been previously described in JAK/STAT signaling [23].

Discussion

Proper distinction of classical Hodgkin lymphoma from the numerous entities, which morphologically and

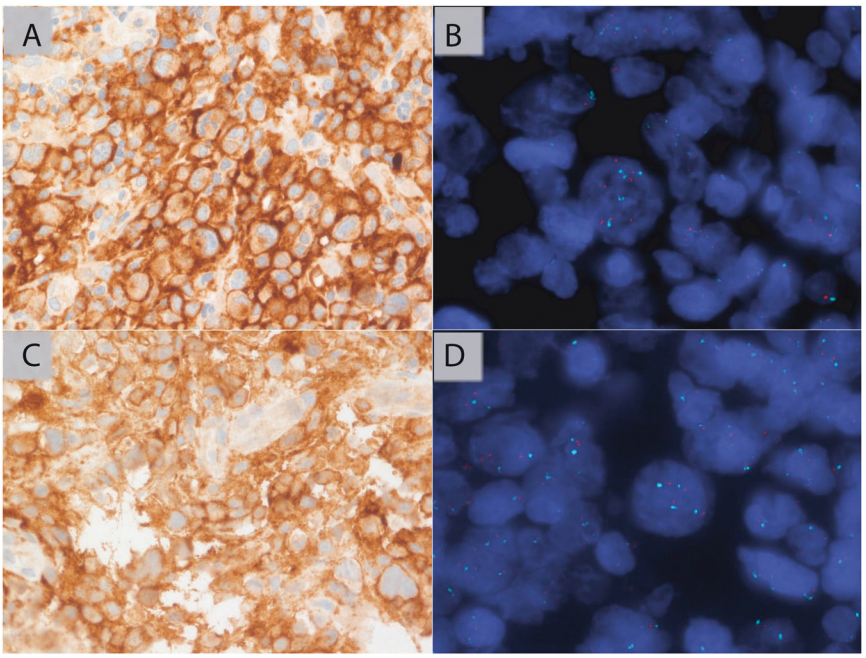

Fig. 5 PD-L1 IHC and FISH in diagnostic classical Hodgkin lymphoma tissue and relapsed gray zone lymphoma tissue. Both diagnostic (**a**) and relapse tissues (**c**) are positive for PD-L1 via IHC with evidence of amplification of PD-L1 locus (orange-red) in the diagnostic classical Hodgkin lymphoma (**b** ten copies/cell) while there was relative copy number gain in the relapse gray zone lymphoma tissue (**d** three to four copies/cell). Centromeric chromosome 9 probe is colored aqua. The patient responded well to nivolumab therapy and went on to undergo consolidative autologous stem cell transplantation.

immunophenotypically border with classical Hodgkin lymphoma, is of significant clinical importance in the upfront and relapsed settings. Chemotherapy with doxorubicin, bleomycin, vinblastine, and dacarbazine remains the standard approach for most classical Hodgkin lymphoma patients [24]. Ever since the initial descriptions of primary mediastinal large B-cell lymphoma and subsequent descriptions of mediastinal gray zone lymphomas as a missing link [25], we have gained significant understanding of the clinical, histologic, and more recently, genetic overlap between these three entities [26, 27].

Prior work by Skinnider et al. demonstrated the central role of IL-13 signaling, which serves as the primary activator of STAT6 downstream in Hodgkin/Reed–Sternberg cells [9, 11].

In the latter study, the authors examined p-STAT6^{Y641} with demonstration of frequent nuclear staining (nearly 80% of cases) in classical Hodgkin lymphoma (more so in nodular sclerosis subtype). Four other nodular lymphocyte predominant Hodgkin lymphomas, eight diffuse large B-cell lymphomas, and five peripheral T-cell lymphoma not otherwise specified cases were negative while EBV-LMP1 status was not correlated with expression of STAT6 [11]. These results are in congruence with our findings with the STAT6^{YE361} demonstrating specificity of nuclear staining restricted to classical Hodgkin lymphoma compared with a larger group of nodular lymphocyte predominant Hodgkin lymphomas, as well as other B- and T-cell non-Hodgkin lymphomas examined.

Two earlier studies since then have examined p-STAT6 expression in nodular lymphocyte predominant Hodgkin lymphoma and demonstrated positivity rates of 37% using the p-STAT6^{Y641} antibody [28] and 49% [15]. The former study originated from the German Hodgkin study group, further noted that p-STAT6 did not have any prognostic relevance in nodular lymphocyte predominant Hodgkin lymphoma. Evidence from in vitro studies in classical Hodgkin lymphoma cell lines implicate underlying constitutive STAT6 activation, which is responsible for the IL-4/13 induced latent membrane protein-1 expression independent of EBV nuclear antigen-2 [29].

Recent studies indicate that *STAT6* mutations are recurrently present in up to 40% of classical Hodgkin lymphoma. Therefore, we examined for mutations in *STAT6* in two nuclear STAT6^{YE361} positive classical Hodgkin lymphoma cases. One of these biopsies was a relapse tissue (case 1), while the other was a treatment-naive tissue (case 2) in a patient with advanced stage disease. We identified a deleterious mutation in the *CISH* gene. Although anecdotal, these findings imply the possible effect of novel mutation *P213L* in *CISH* on JAK/STAT signaling and congruent with the work of Ritz et al. that nuclear p-STAT6 expression was not correlated with presence of an underlying *STAT6* mutation [30].

The results of our comparison of nodular lymphocyte predominant Hodgkin lymphoma cases with both the STAT6^{YE361} and p-STAT6^{YE641} antibodies support enhanced diagnostic specificity for STAT6^{YE361} in distinguishing classical Hodgkin lymphoma from nodular

lymphocyte predominant Hodgkin lymphoma. We believe this may be explained by reduced sensitivity for the phosphorylated form and preferential staining for total nuclear and cytoplasmic STAT6 with the YE361 antibody. Compared with STAT6^{YE641}, our experience is that STAT6^{YE361} shows higher background staining, a finding that has been previously described in the context of follicular lymphoma [31]. The STAT6^{YE361} antibody performed best in well-fixed tissues from recent biopsies, and showed some reduced sensitivity in central poorly fixed areas of large tissues, particularly in older specimens as noted on our tissue microarray cases that were older (>5 years old). Hence, the age and quality of tissue should be taken into account when using STAT6^{YE361} in the diagnostic clinical setting. Even within well-fixed areas, there is variability in the number of positive cells with some cases showing uniform strong staining in all Hodgkin/Reed–Sternberg cells to some cases with only a fraction staining positive. Whether differential expression within classical Hodgkin lymphoma cases may have prognostic relevance independent of the *STAT6* mutation status remains to be seen.

Indeed, recent in vitro studies note that blocking *STAT6* activation in Hodgkin lymphoma using IL-4 and IL-13 blocking antibodies sensitizes to classical chemotherapy. Hence, testing for and quantifying STAT6 expression routinely (on well-fixed tissues) may be prognostically relevant [32] in predicting response to check point inhibitors recently demonstrating immense promise in refractory classical Hodgkin lymphoma with uniform 9p24 amplifications implicating PD-L1 [33]. Of note, the extended 9p24.1 region also includes *JAK2* and the resulting *JAK2* amplifications has been shown to be associated with increased JAK2 protein expression with resulting PD-L1 transcription and enhanced sensitivity to *JAK2* inhibitors in in vitro models [34]. However, in the one case of classical Hodgkin lymphoma with relapse as a gray zone lymphoma, there was only minimal expression of STAT6 by immunohistochemistry in both diagnostic and relapse tissues despite strong PD-L1 expression by immunohistochemistry and amplification by FISH indicating that STAT6 phosphorylation is not directly correlated with PD-L1 expression. Equally exciting are recent in vitro studies demonstrating reduction of p-STAT6 in Hodgkin lymphoma cell lines with combinations of *JAK2* inhibitor, ruxolitinib with the anti-CD30 conjugate brentuximab vedotin [35]. In light of these data, STAT6 status by immunohistochemistry at diagnosis and relapse would perhaps help stratify these patient subsets with unifying 9p24 amplifications. Both classical Hodgkin lymphoma cases with nuclear STAT6^{YE361} were positive for PD-L1 by immunohistochemistry although more cases would need to be examined for both markers to assess the prognostic utility in conjunction.

In conclusion, nuclear expression of STAT6^{YE361} is useful in supporting a classical Hodgkin lymphoma phenotype, which is useful in the distinction of cases intermediate between lymphocyte-rich classical Hodgkin lymphoma, and nodular lymphocyte predominant Hodgkin lymphoma and in B-cell lymphoma unclassifiable with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma with high positive (100%) and negative (85.7%) predictive value as compared with the STAT6 ^{YE641} antibody.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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