CD44H and CD44V6 Expression in Different Subtypes of Hodgkin Lymphoma

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CD44 is a broadly distributed family of cell surface glycoproteins. The expression of CD44H has been documented in both Hodgkin lymphoma and non-Hodgkin lymphoma. CD44V6 has been associated with more aggressive behavior in non-Hodgkin lymphoma, but such a correlation has not been established in Hodgkin lymphoma. In addition, the utility of CD44 and CD44V6 in the subclassification of Hodgkin lymphoma in paraffin-embedded tissues has not previously been evaluated. The current study included formalin- or methacarn-fixed, paraffin-embedded tissue specimens from 42 patients with Hodgkin lymphoma (25 nodular sclerosis, three interfollicular, four lymphocyte-rich classic Hodgkin, six lymphocyte predominant, and four mixed cellularity). The clinical stage of the study population at initial presentation ranged from stage IA to IVB. Evaluation of CD44H and CD44V6 (Novocastra) was performed by ABC immunoperoxidase technique after heat-induced epitope retrieval. In the six cases of lymphocyte predominant Hodgkin, the neoplastic cells lacked reactivity with CD44H reminiscent of their normal germinal center counterparts. On the other hand, classic Hodgkin lymphoma showed variable membranous and Golgi reactivity in the neoplastic cells in all cases irrespective of disease stage at presentation. In all cases, the neoplastic cells lacked reactivity with CD44V6 except for three (one lymphocyte predominant, one interfollicular, and one nodular sclerosis), all of which represented recurrent cases. In conclusion, CD44 evaluation is useful in the distinction between lymphocyte predominant and classic Hodgkin lymphoma. The presence of CD44H expression has no relation to the clinical stage of the disease at presentation or recurrence. CD44V6 is detected in a minority of cases irrespective of the histologic subtype and its presence may be associated with recurrence. There was no correlation between disease stage at presentation and the expression of CD44V6.

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CD44 is a broadly distributed family of cell surface glycoproteins that are now generally believed to be cell adhesion molecules with proposed functions in extracellular matrix binding, cell migration, lymphopoiesis, and lymphocyte homing (1–3). Previous studies have concluded that CD44 represents a single gene, located on the short arm of chromosome 11 in humans (4). At least 18 CD44 transcripts have been described to date, a heterogeneity resulting from alternative splicing of 12 out of the 19 exons (5). The common form of the CD44 (CD44H) molecule is ubiquitously expressed in epithelial and mesenchymal tissues, whereas the different isoforms generated by alternative RNA splicing are found in a very restricted distribution (6, 7). As an example, whereas CD44H is expressed on fibroblasts and endothelial cells, CD44 variants are not expressed on these same cells (6).

Lymphocyte circulation and homing properties are dependent on the expression of cell surface receptors (8, 9), which vary during both lymphocyte maturation and activation (10–12). Cortical thymocytes and germinal center B-cells have a lower level of expression of CD44 or Hermes-1 compared with mature B and T lymphocytes which express the highest level of these surface antigens. The expression of CD44 in non-Hodgkin lymphoma has been addressed by Picker *et al.* (13). In their series, most subtypes of lymphoma recapitulated a pattern of reactivity seen in their non-neoplastic counterparts, *i.e.*, the lymphomas of follicular origin and thymic cortical origin lacked or weakly expressed CD44. Multiple authors have addressed the expression of CD44V6 in non-Hodgkin lymphoma with some studies finding a correlation between expres-

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sion of this variant and aggressive biological behavior (14, 15). Inagaki *et al.* (14) found correlation between CD44V6 expression and both B symptoms and high-risk group as defined by the International Prognostic Index. A study by Ristamaki *et al.* (15) found an association between CD44V6 expression and unfavorable, recurrence-free survival with a 72-month follow-up.

Hodgkin lymphoma is a heterogeneous entity that encompasses several subtypes having different histologic findings, immunophenotypic features, and biological potential. In particular, lymphocyte predominant (LP) Hodgkin lymphoma, has a distinct morphologic, immunophenotypic, and biological profile that warrants its separation from other subtypes of classic Hodgkin lymphoma (CHL) (16). The L&H cells of LP have been found to express CD45 (leukocyte common antigen) in the majority of the cases as well as CD20 (17–19). On the other hand, Reed-Sternberg (RS) cells present in CHL express CD20 in a minority of cases and in a focal, weak pattern, compared with LP neoplastic L&H cells (17, 18). However, L&H cells usually lack expression of CD30 and CD15, which are expressed on RS cells and variants in CHL (20–22). Although the origin of the L&H cells in LP has been confirmed to be a germinal center B cell (23, 24), the origin of RS cells in CHL is less agreed upon. Recent evidence, however, strongly suggests they are also of B cell origin (24, 25).

The expression of CD44 variants in Hodgkin lymphoma has only been evaluated for the nodular sclerosis subtype by Beham-Schmid *et al.* (26). The authors found a correlation between the expression of CD44V10 and recurrence or initial bone marrow involvement, whereas correlation was not found between these two prognostic parameters and CD44V6 expression. Another study by Ellis *et al.* (27) evaluated the expression of CD44H only in frozen sections of 18 cases of CHL. The latter study did not evaluate the antigen expression in lymphocyte predominant Hodgkin lymphoma. In that study, the authors have documented the expression of CD44H on the neoplastic cells in all 18 cases (strong reactivity in 17 cases and focal reactivity in only one case) of CHL included in their series. No correlation with disease stage or recurrence was done in that study. In the current study, we assessed the expression of CD44H and CD44v6 by the neoplastic cells in LP and various subtypes of CHL in the setting of formalin-fixed, paraffin-embedded tissues to determine their utility in the subclassification of Hodgkin lymphoma. The relationship between the expression of these antigens and recurrence as well as the clinical stage of the disease at presentation was also evaluated.

MATERIALS AND METHODS

Case Selection

Representative sections of 10% neutral buffered formalin- or methacarn- (absolute methanol combined with chloroform and acetic acid) fixed, paraffin-embedded tissue were obtained from 42 different patients. The cases included specimens from the archived files at the University of Washington Department of Pathology and cases referred to our consultation service. These cases of Hodgkin lymphoma included 25 nodular sclerosis (NS), three interfollicular (IF) (28), four lymphocyte-rich classic Hodgkin (LRCH), six lymphocyte predominant (LP), and four mixed cellularity (MC) subtypes. The interfollicular Hodgkin cases were classified as such due to their lack of features that are diagnostic to any subtype of Hodgkin lymphoma as defined both in the WHO and the REAL classifications (29, 30). In addition, all cases of IF Hodgkin presented as interfollicular involvement of the lymph nodes with maintained follicular architecture as was described by Doggett, Colby, and Dorfman (28). As for other Hodgkin lymphoma subtypes, all hematoxylin- and eosin- (H&E) stained sections on each case were reviewed by the two authors to confirm the diagnoses. The cases were subclassified using both the WHO and REAL classification criteria (29, 30), which essentially represent the Rye modification of Lukes and Butler Classification of Hodgkin lymphoma (31). The distinction of CHL from LP was confirmed by immunocytochemistry using CD45, CD20, CD30, and CD15 in every case. The diagnosis of the entity of lymphocyte rich CHL was based on the criteria identified in both the WHO and REAL classification (29, 30) and confirmed by immunohistochemistry.

Immunohistochemistry

Analyses were performed using an avidin-biotin complex immunoperoxidase technique. Four-μm tissue sections were cut and placed on electrostatically charged slides and heated to 60° C for 15 min. The tissues were then deparaffinized, rehydrated, and incubated in 3% hydrogen peroxide for 5 min to block endogenous peroxidase and then washed in dH_2O. Heat-induced epitope retrieval was performed by microwaving for 18 min in 10-mM citrate buffer at pH 6.0. Sections were held in hot buffer for 20 min. The sections were then washed in PBS, at which point the monoclonal primary antibodies CD44 (Novocastra, clone F10–44-2) and CD44V6 (Novocastra, clone VFF-7) were applied for 45 min at room temperature at different dilutions for methacarn- and formalin-fixed tissues (Table 1). Sections were then washed in PBS and a biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, 1:200) was applied for 25

TABLE 1. The Antibody Clones and Dilutions Used for Immunohistochemistry

Antibody	Clone	Dilution
CD44	F1044-2	1:1000 (F), 1:2000 (M)
CD44V6	VFF-7	1:100 (F and M)

F, formalin, M, methacarn.

min. Again the sections were washed in PBS and an avidin-biotin complex (Vector Laboratories, 1:100) was applied for an additional 25-min incubation. Diaminobenzidine tetrahydrochloride enhanced with 1% $FeCl_3$ was used for the chromogen brown reaction product, followed by hematoxylin counterstaining, dehydration, and coverslipping.

Evaluation of Immunohistochemistry

The reactivity with both CD44 and CD44V6 was detected predominantly as membranous pattern with occasional Golgi or diffuse cytoplasmic staining. The degree of reactivity with the antibody was graded semiquantitatively into focal (less than 25%), variable (25 to 75%), and uniform (more than 75%) based on an estimate of the percentage of the neoplastic cells staining positively. The staining intensity in different cases did not show enough variability to warrant grading of the intensity of reactivity. The specificity of the immunohistochemical stains in each case was confirmed by a concomitant run with a negative control. The consistency and quality of staining were confirmed by the presence of positive internal controls and uniform reactivity with a known positive external control matched to both methods of fixation.

RESULTS

Results with CD44H

The expression of CD44H in normal lymphoid tissue was evaluated on tonsils with reactive lymphoid hyperplasia. The antibody highlighted the mantle zone and interfollicular areas, whereas the germinal centers lacked expression of the surface antigen. The histiocytes and both follicular and interdigitating dendritic reticulum cells showed uniform reactivity with CD44 in a membranous pattern and occasionally in a Golgi or diffuse cytoplasmic pattern (Fig. 1).

The RS cells and variants reacted positively with CD44H in all subtypes of CHL evaluated, whereas the L&H variants in the lymphocyte predominant subtype were consistently negative (Fig. 2) (Table 2). The number of positive cells was variable from case to case and ranged from variable reactivity to uniform staining. The background lymphocytes in all cases showed variable membranous reactivity with the antibody.

Results with CD44V6

Contrary to the standard or hematopoietic form of CD44 (CD44H), the splice variant V6 (CD44V6) reacted with only rare background lymphocytes (Fig. 2). Only three cases showed positive reactivity of the neoplastic cells with the antibody (Fig. 3), and all of them represented recurrences (one LP, one IF, and one NS). In the recurrent LP Hodgkin case, the reactivity with CD44V6 was detected both in the initial presentation and the tumor that recurred 5 years later. In the NS case, CD44V6 was positive in the initial diagnostic material but the

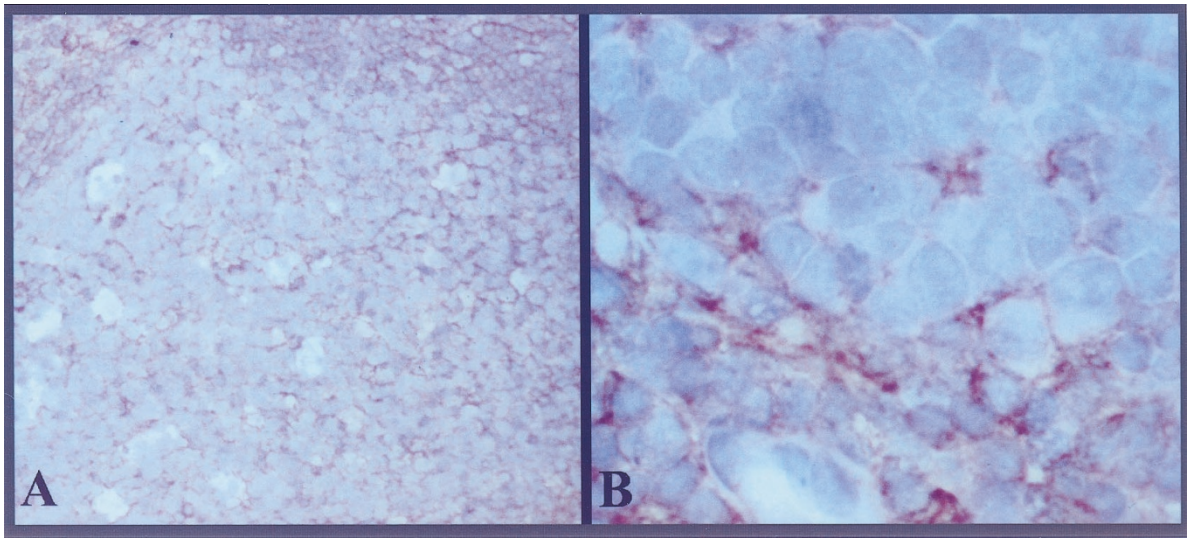

FIGURE 1. A, low-power view (10×) of a tonsil with reactive lymphoid hyperplasia, showing uniform reactivity with CD44H in the mantle zone and paracortex. The germinal center in contrast showed lack of reactivity with the antibody in the lymphocytes, whereas the follicular dendritic reticulum cells and histiocytes reacted positively. **B,** high-power view of the same tonsil, showing the positive mantle zone lymphocytes in complete contrast to the germinal center lymphocytes. The follicular dendritic reticulum cells reacted positively with the antibody.

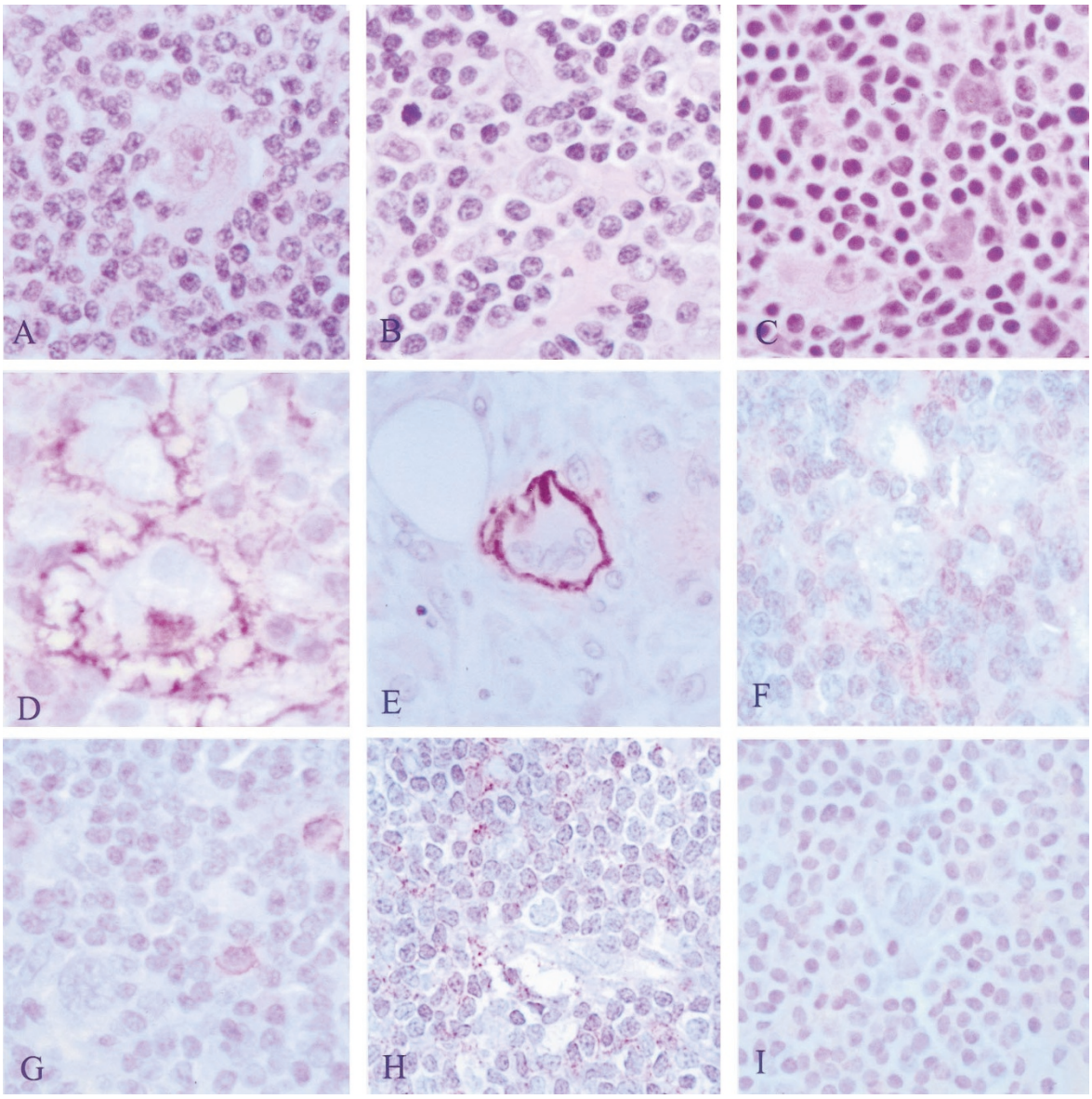

FIGURE 2. **A**, H&E section of lymphocyte-rich classical Hodgkin lymphoma, showing a mononuclear Reed-Sternberg cell variant in the center of the section. The background is composed of a population of small lymphocytes. **B**, H&E section of nodular sclerosing Hodgkin lymphoma, showing a Hodgkin cell in the center of the section. The background shows bands of fibrosis, occasional histiocytes, and plasma cells. **C,** H&E section of lymphocyte predominant Hodgkin lymphoma showing L&H cells and homogeneous background of small lymphocytes and a histiocyte. **D,** immunoperoxidase stain for CD44 in lymphocyte-rich classical Hodgkin lymphoma, showing both membranous and Golgi staining. There is variable reactivity in the background lymphocytes for CD44. **E**, immunoperoxidase stain for CD44 in nodular sclerosing Hodgkin lymphoma, showing only membranous reactivity of the multinucleated RS cell, with only focal reactivity in the background lymphocytes. **F**, immunoperoxidase stain for CD44 in lymphocyte predominant Hodgkin lymphoma, showing lack of reactivity with CD44 in L&H cell, with variable reactivity in the background lymphocytes. **G**, immunoperoxidase stain for CD44V6 in a representative case of lymphocyte-rich classical Hodgkin lymphoma, showing lack of reactivity in the RS variant. The background shows reactivity in only rare lymphocytes. **H**, immunoperoxidase stain for CD44V6 in a representative case of nodular sclerosing Hodgkin lymphoma, showing lack of reactivity in the lacunar cell in the middle. The background shows variable reactivity on the background lymphocytes. **I**, immunoperoxidase stain for CD44V6 in a representative case of lymphocyte predominant Hodgkin lymphoma, showing lack of reactivity in the L&H cell in the center.

recurrent lesion did not contain residual neoplastic cells in the additional sections submitted for study. In the recurrent IF case, only the recurrent lesion was available for our evaluation, and showed variable positivity with CD44V6 in the neoplastic cells. The original material, which represented the initial presentation of disease 3 years earlier, was not available. The pattern of CD44 reactivity in the recurrent LP case was similar to that seen in the original diagnostic material.

DISCUSSION

Although the main objective of the current study was to evaluate the expression of CD44H in different subtypes of Hodgkin lymphoma, the relationship of this expression to their normal lymphoid counterparts is worth discussing. Many different techniques have been used to provide evidence to support a probable derivation of RS cells from either B-lymphocytes (24, 25), T lymphocytes (32, 33),

TABLE 2. The Reactivity of Different Subtypes of Hodgkin Lymphoma for CD44 and CD44V6 by Immunohistochemistry

Classification	CD44 Positive	CD44V6 Positive
LP (6)	0 (0%)	1 (17%)[a]
IF (3)	3 (100%)UP	1 (33%)[a]
LRCH (4)	1 (25%) UP	0 (0%)
	3 (75%) VP	
MC (4)	4 (100%) VP	0 (0%)
NS (25)	20 (80%) UP	1 (5%)*
	5 (20%) VP	

LP, lymphocyte predominent; IF, interfollicular; LRCHD, lymphocyte-rich classic Hodgkin; MC, mixed cellularity; NS, nodular sclerosing; UP, uniformly positive; VP, variably positive.

[a]Cases of Hodgkin lymphoma with subsequent recurrence.

and dendritic cells (34–36). The neoplastic cells in lymphocyte predominant Hodgkin lymphoma originate from a B-lymphocyte, a suggestion confirmed by single cell polymerase chain reaction (PCR) studies (37, 38). Germinal center B cells have been proposed as the normal counterpart of L&H variants, as supported by the presence of somatic hypermutation of the immunoglobulin genes and BCL-6 expression (37, 39). Although the origin of the neoplastic cells in CHL has recently also been generally agreed upon (with few exceptions) to be a B-lymphocyte of germinal center derivation, significant differences exist between the neoplastic cells in LP and CHL. The presence of CD20 on the neoplastic cells in CHL has been documented by various studies. The percentage of positive cases varied from study to study and the range reported in the literature is from 11 to 58% in paraffin sections (17, 18) and up to 80% in frozen sections (18). In addition, absence of CD45 expression in cases of CHL (19) contrasts with the positivity of both antigens (90% in case of CD20 and 70% in case of CD45) in paraffin sections in LP cases (17, 19). These immunophenotypic variations suggest differences in the maturational states of the neoplastic cells in the two entities. Also the variable reports confirming and denying the presence of B-cell clonality in RS cells, in addition to the absence of Ig messenger RNA and consequently the protein product in CHL (40–44), clearly speak to the biological differences between LP and CHL. In our study, the variable reactivity of RS cells with CD44 in all subtypes of CHL and its lack of reactivity in LP provide further evidence of the biological difference between these two entities. Kanzler *et al.* (45) have shown crippling somatic mutations in RS cells, suggesting a germinal center B-cell origin, although there was no evidence of ongoing mutations in the cells analyzed. If we are to agree with Kanzler *et al.* that RS cells are of germinal center origin, then the variably bright expression of CD44 is abnormal, considering the documented lack or weak expression of this antigen by normal germinal center B cells (10).

The most significant finding of the current study is the potential diagnostic utility of CD44 in the classification of Hodgkin lymphoma. Like CD30 and CD15, CD44 did react with the neoplastic cells in CHL of nodular sclerosis (NS), mixed cellularity (MC), and lymphocyte rich (LRCH) subtypes. Our series did not include any cases of the lymphocyte-depleted subtype, reflecting the rarity of this entity. However, the antibody did not show positivity in any of the cases of the lymphocyte predominant subtype examined, irrespective of whether they represented a primary diagnosis or recurrence. This suggests that CD44 might be useful to distinguish CHL from LP. These findings are consistent with the study by Beham-Schmid *et al.* (26) on NS cases in which expression of CD44 was demonstrated on the lacunar cells in NS in all but two cases examined. However, their study did not evaluate other subtypes of Hodgkin lymphoma. Perhaps the major utility of our finding is in distinguishing LRCH from LP because these two entities can have similar morphologies. In the present study, all six LP cases lacked reactivity with CD44, whereas the four cases of LRCH reacted positively. The addition of CD44 to the panel of Hodgkin lymphoma immunohistochemical markers may be useful in separating these two entities with the presence of CD44 positivity, favoring the latter diagnosis. However, evaluating CD44 staining is challenging because the antigen is also expressed by mature background T and B cells, dendritic cells, and histiocytes. In addition, the staining in some cases was quite variable in intensity from cell to cell, and the cases varied as to the number of positive neoplastic cells. However, in most of the cases, the majority of the neoplastic cells showed reactivity. This variability in staining is parallel to what Picker *et al.* (13) showed in their study of non-Hodgkin lymphoma. In the latter study, Picker and colleagues did not find any correlation between the stage of the disease and the expression of CD44H. However, in the study by Drillenburg *et al.* (46), the authors found a correlation between the expression of CD44H and CD44V6 and tumor dissemination. Interestingly, these authors also found a correlation between the expression of CD44H and disease-related death in patients with localized nodal disease. In our study, the neoplastic cells of CHL cases reacted with CD44H irrespective of the disease stage at presentation or the Recurrence State. This further highlights the difference between Hodgkin and non-Hodgkin lymphomas. Similarly, no correlation was found between CD44 expression and the stage of the disease in LP cases.

The correlation of CD44V6 with aggressive behavior in non-Hodgkin lymphoma, as documented previously (14, 15), prompted us to explore its relation to prognosis in Hodgkin lymphoma. Beham-Schmid *et al.* (26) addressed the prognostic utility of CD44 splice variants in the nodular sclerosis subtype only using

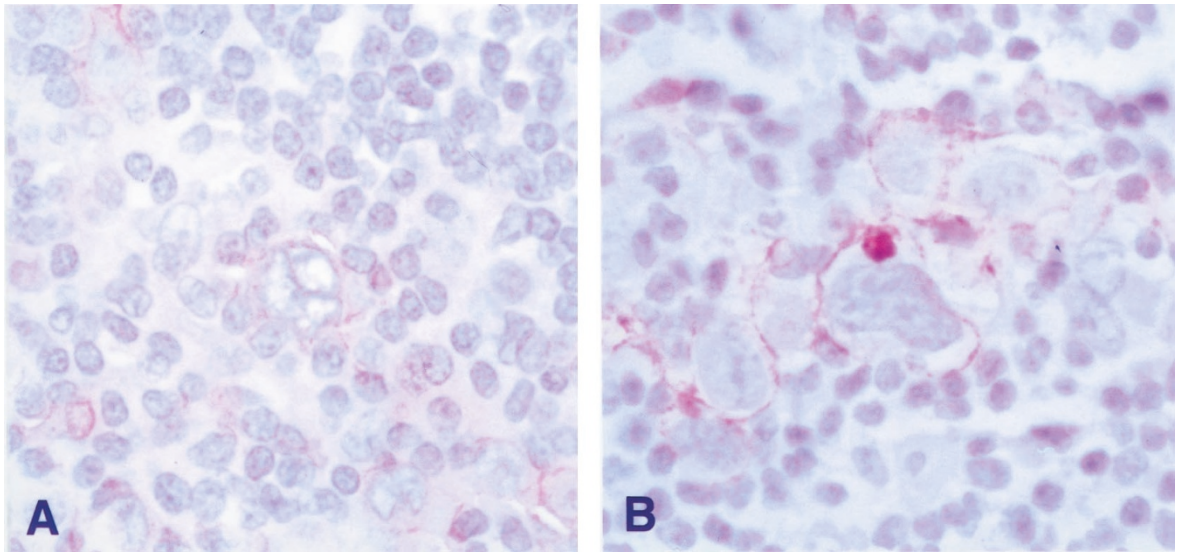

FIGURE 3. **A**, immunoperoxidase stain for CD44V6 in a recurrent case of lymphocyte predominant Hodgkin lymphoma, showing positive reactivity in the L&H cells. The background shows faint reactivity on the lymphocytes. **B**, immunoperoxidase stain for CD44V6 in a recurrent case of interfollicular Hodgkin lymphoma, showing positive reactivity in a Hodgkin cell. The background shows variable reactivity on the lymphocytes and in the histiocytes above and to the right of the neoplastic cell.

two parameters: relapse and bone marrow involvement. They did not find a significant correlation between the level of expression of CD44V6 and either of these parameters. Our series included three cases of different subtypes (LP, IF, and NS) that suffered subsequent relapse, and all of these cases showed positive reactivity with CD44V6 in a variable number of the neoplastic cells. Reactivity with CD44V6 was detected in one of the three cases (LP) in both the initial presentation and the recurrent material. The finding of reactivity with CD44V6 in the absence of CD44H for the recurrent LP case is rather unusual. In the study of Beham-Schmid *et al.* (26), no reactivity with the splice variants, including CD44V6, was encountered in cases that lacked reactivity for the standard or hematopoietic form of CD44 (CD44H). One explanation for this finding might be that the case under discussion represents LP, and this entity was not included in the latter study. Another explanation is that LP represents a neoplasm of B cells at a different stage of maturation from NS CHL. As for CD44V6 negative cases, the Recurrence State is unknown to us in a significant number of them, therefore we can not draw any conclusions as to the possibility of recurrence in the CD44V6 negative cases. Although the number of recurrent cases is quite small in the current study and precludes drawing a conclusion as to the significance of this antibody in determining the risk for recurrence in Hodgkin lymphoma, the suggestion of an association with relapse irrespective of subtype is worthy of further investigation.

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