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Original Articles

Flow Cytometric Evaluation of CD38 Expression Assists in Distinguishing Follicular Hyperplasia from Follicular Lymphoma

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The distinction of follicular lymphoma (FL) from reactive follicular hyperplasia (FH) can be a diagnostic challenge in flow cytometry. In this study, the median fluorescent intensity (MFI) of CD38 as assessed by flow cytometry on B and T cell subpopulations in 102 lymph nodes specimens with histopathologically confirmed FL was compared with 55 cases of FH. The MFI of CD38 was highly significantly reduced in the neoplastic B cells in FL when compared with the reactive germinal center B cells in FH ($P < 1.0E-16$). The MFI of CD38 did not differ between the non-neoplastic B-cells in FL and nongerminal center B-cells in FH ($P = 0.14$) or between T-cells and non-neoplastic B-cells in FL ($P = 0.63$). A marginal increase in the MFI of CD38 was seen for T cells in FL compared with FH ($P = 0.04$). An increased difference in the MFI of CD38 was identified for T-cells compared with nongerminal center B-cells in FH ($P = 0.005$). No difference in CD38 expression was seen between Grades 1, 2, or 3 FL. The study also confirmed increased expression of CD10 ($P < 1.0E-9$), decreased CD19 ($P < 1.0E-22$), and CD20 ($P < 1.0E-16$) in FL in comparison with FH, as has been previously reported. This study identified decreased CD38 as a common finding in FL in comparison with FH and provides an additional tool to help differentiate FL from FH by flow cytometry. © 2009 Clinical Cytometry Society

Key terms: flow cytometry; CD38; follicular hyperplasia; follicular lymphoma

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The distinction of follicular lymphoma (FL) from reactive follicular hyperplasia (FH) can be a diagnostic challenge in flow cytometry. FL and FH share many immunophenotypic characteristics including an expansion of a CD10 positive germinal center–derived mature B-cell population. Surface light chain restriction is an immunophenotypic feature commonly used to differentiate FL from reactive FH. However, the presence of light chain restriction on a germinal center population cannot be used alone to make a diagnosis of FL. For example, prominent light chain restricted B-cell populations have been described in association with histologically reactive lymphoid proliferations (1). In addition, surface immunoglobulin can be variably decreased to absent on both FL (2) and in germinal center cells in FH, limiting the use of surface light chain restriction as a marker of clonality in this setting (3).

Therefore, the presence of additional immunophenotypic abnormalities such as the abnormal gain or loss in intensity of an antigen, or aberrant antigen expression is an important adjunct in the identification of lymphoma by flow cytometry. A relative reduction in the intensity of CD19 expression has been reported in cases of FL (4,5). However, reduced CD19 expression is not present in all cases of FL, and the assessment of intensity for this antigen can be difficult in cases with low-level involvement by FL. Other previously reported immunophenotypic differences seen in FL include a relative increase in expression of CD10 and HLA-DO, and reduced expression of CD20 and CD44 (4,6–8). However, these latter antigenic alterations are typically more subtle, and therefore often difficult to apply routinely in clinical practice.

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Aberrant Bcl-2 overexpression by germinal center B-cells in cases of FL has also been successfully used to differentiate between FL and FH (9,10). However, this analysis requires an additional fixation and permeabilization step that may be difficult with small samples, and can delay reporting of results. Additionally, Bcl-2 is not consistently expressed by all FLs (11). Because the pattern of antigenic abnormalities can vary from case to case, it is useful to have several potential immunophenotypic alterations to use in making a distinction between FL and FH.

The CD38 molecule is a transmembrane glycoprotein with a broad distribution among hematopoietic cells, being expressed by bone marrow progenitors, germinal center B cells, and plasma cells (12,13). In reviewing routine clinical cases, we had observed that the reactive germinal center population in FH coexpresses CD38 at an increased level when compared with the neoplastic germinal center population in FL, suggesting it might be useful in helping to differentiate the two entities.

In this study, we compare the median fluorescent intensity (MFI) of CD38 in reactive germinal center B-cells in cases of FH and the neoplastic germinal center B-cells in cases of FL in an effort to determine if the level of CD38 expression by flow cytometry can provide an additional helpful marker to differentiate these two conditions.

MATERIALS AND METHODS

We retrospectively identified all cases of mature B-cell neoplasms with CD10 coexpression characterized by flow cytometry in our laboratory evaluated for lymphoma between July 2004 and January 2007. Of these cases, 102 cases were selected for retrospective flow cytometric analysis based on availability of histologic confirmation of FL from review of concurrent pathology reports. Cases with reported diagnoses compatible with an alternative diagnosis such as diffuse large B-cell lymphoma or Burkitt lymphoma were excluded from analysis. In addition, 55 cases with immunophenotypic features characteristic of polyclonal FH by flow cytometry were identified for retrospective flow cytometric review within the same time period. This study was conducted with the approval of the University of Washington Human Subjects Review Committee (02-3505-E 01).

Flow Cytometry

In all cases, the fresh tissue was collected in RPMI supplemented with 10% fetal calf serum. In preparation for flow cytometric analysis, the specimens were finely chopped and strained using 40 μm cell strainers [Becton Dickson (BD), San Jose, CA] during processing. The resulting cells were washed once in a solution composed of phosphate-buffered saline/0.3% bovine serum albumin/0.1% sodium azide, resuspended in 100 μL of RPMI/10% fetal calf serum, incubated with the antibodies for 15 min at 37°C, in the dark, exposed to 1.5 ml NH_4Cl containing 0.25% ultrapure formaldehyde (Polysciences, Warrington, PA) for 15 min at room temperature, and washed once in phosphate-buffered saline/bovine serum albumin/azide.

The following single eight-color antibody-fluorochrome combination was used for routine flow cytometric analysis of B-cells: kappa fluorescein isothiocyanate (FITC), lambda phycoerythrin (PE), CD19 phycoerythrin-Texas Red (PE-TR), CD20 phycoerythrin-cyanine 7 (PE-Cy7), CD45 Pacific Blue (PB), CD38 Alexa 594 (A594), CD10 allophycocyanin (APC), and CD5 allophycocyanin-cyanine 7 (APC-Cy7). The amount of each antibody used was based on titration experiments to optimize the signal-to-noise ratio. The flow cytometric data were analyzed using Macintosh G3 or G4 computers (Apple, Cupertino, CA) using software developed in our laboratory (B.L.W).

In all cases, flow cytometry was performed on a modified 4-laser, 10 color Becton Dickson LSRII flow cytometer using the following laser-fluorochrome combinations: (1) 407 nm violet laser, PB; (2) 488-nm blue laser, FITC, PE, PE-TR, PE-Cy5, PE-Cy7; (3) 594-nm yellow laser, A594; and (4) 635-nm red laser, APC, APC-Cy7. Daily instrument quality control was performed using UltraRainbow single peak and eight-peak beads (Spherotech) to allow consistent determination of fluorescence intensity over the course of the study.

In each case, events with very low forward scatter (largely representing degenerating cells) were excluded from analysis by forward and side-scatter gating. CD19 versus Side-scatter gating was used to identify the B-cells, and T-cells were identified by expression of CD5 without CD19 expression. The abnormal CD10 positive B-cell population and the non-neoplastic B-cell population were identified by gating on the population of interest. A multiparametric approach was used to define the abnormal and normal B-cell populations by gating on the unique antibody combinations that most separated the abnormal B-cells from the normal B-cells in each case. For example, in cases with dim CD19 expression, the abnormal population could be identified by its expression of CD20 despite the dim CD19 and the reduced CD19 expression allowed for separation between normal and neoplastic populations, in combination with other antigens. At no time was CD38 used to identify the population of interest. The CD20 versus CD10 plot was routinely used in cases of FH to define the normal and the follicular center populations. In each case, the mean and MFI of CD10, CD19, CD20, CD38, and CD45 was directly provided by the analysis software for each of the populations.

Statistical Analysis

Comparisons of the MFI of CD10, CD19, CD20, CD38, and CD45 between FL and FH were performed using the unpaired *t*-test (two tailed *P*-value) using Microsoft Excel software (Redmond, WA). The level of significance was set at the 0.05 level.

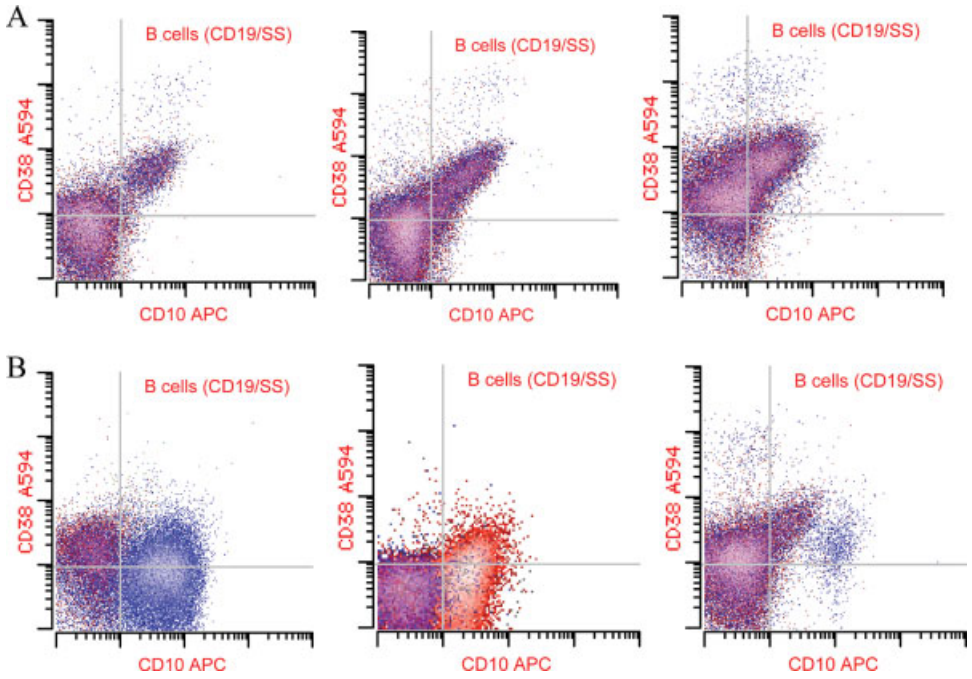

FIG. 1. Flow cytometric analysis of CD38 in follicular hyperplasia and follicular lymphoma. **A**: The CD10 positive germinal center B-cells express increased CD38 when compared with the nongerminal center B-cells in the same sample. **B**: The level of CD38 in cases of follicular lymphoma exhibits a relatively decreased level of CD38. Blue color corresponds to kappa expressing cells and red color corresponds to lambda expressing cells. Note both kappa-restricted neoplastic population with low CD38 and polyclonal reactive germinal center population with a relative increase in expression of CD38 are present in the last panel. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

RESULTS

The reactive germinal center B-cells in cases of FH expressed a characteristic increase in CD38 when compared with the nongerminal center (CD10 negative) B-cells within the same sample. In contrast, the neoplastic cells in cases of FL show a relatively reduced level of CD38 (Fig. 1) when compared with the reactive germinal center B cells in cases of FH ($P < 1.0E\text{-}16$) (Fig. 2).

The MFI of CD38 did not vary significantly between several bystander populations, serving as internal controls. Specifically, the MFI of CD38 did not differ significantly between the non-neoplastic B-cells in cases of FL and nongerminal center B-cells in FH ($P = 0.14$) or between T-cells and non-neoplastic B-cells in cases of FL ($P = 0.63$). A marginally increased significant difference in the MFI of CD38 was seen for T cells in FL compared

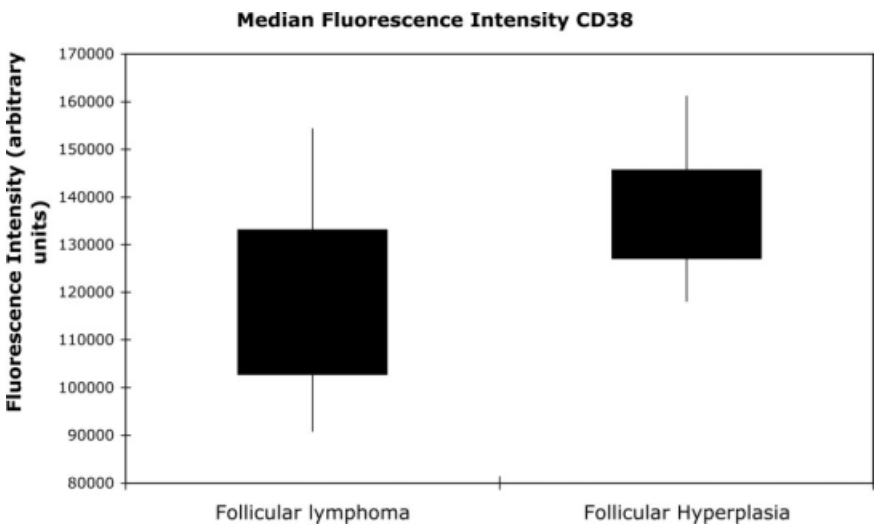

FIG. 2. CD38 expression in follicular lymphoma and follicular hyperplasia analyzed by flow cytometry. The median fluorescent intensity of CD38 is significantly decreased in follicular lymphoma when compared with follicular hyperplasia ($P < 1.0E\text{-}16$). The black box represents one standard deviation about the mean, and the whiskers represent the range of values.

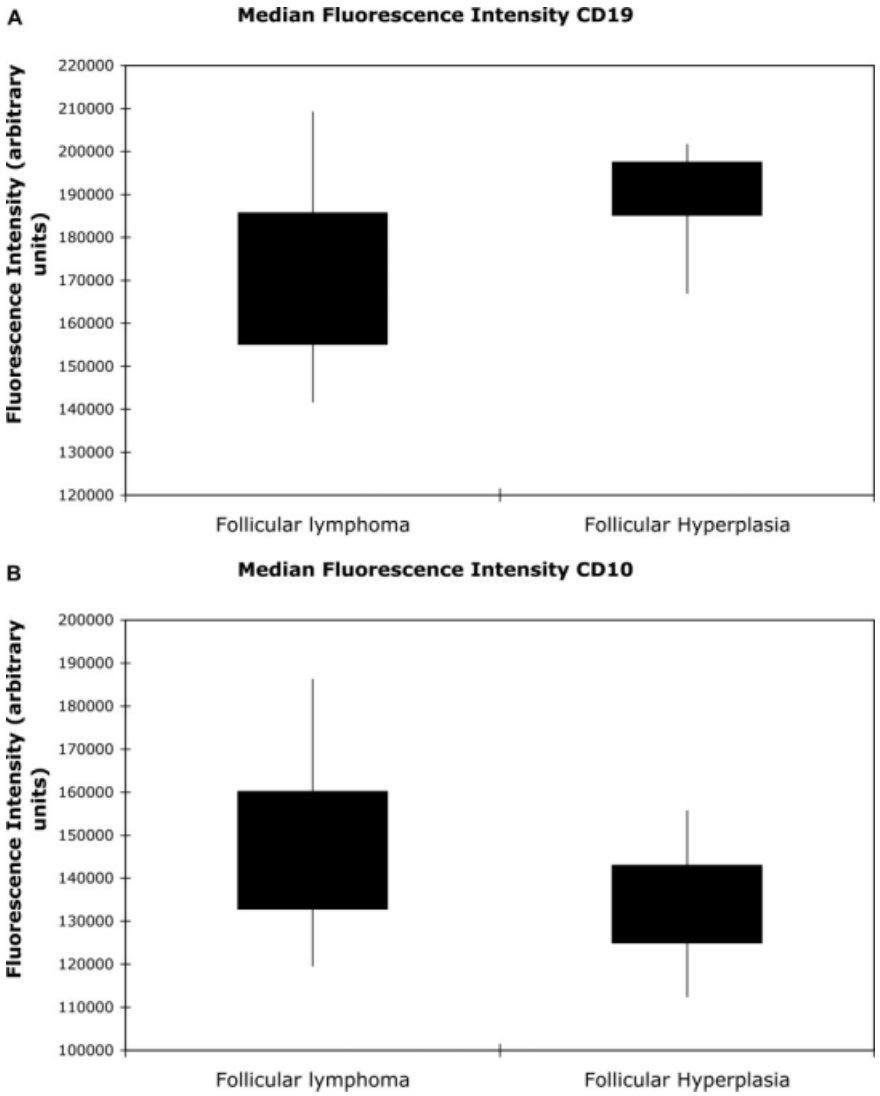

FIG. 3. CD19 and CD10 expression in follicular lymphoma and follicular hyperplasia analyzed by flow cytometry. **A**: The median fluorescent intensity of CD19 is significantly decreased in follicular lymphoma when compared with follicular hyperplasia ($P < 1.0E-22$). **B**: The median fluorescent intensity of CD10 is significantly increased in follicular lymphoma when compared with follicular hyperplasia ($P < 1.0E-9$). The black box represents one standard deviation about the mean, and the whiskers represent the range of values.

with FH ($P = 0.04$). An increased significant difference in the MFI of CD38 was identified for T-cells compared with nongerminal center B-cells in FH ($P = 0.005$).

The MFI of several of other antigens evaluated showed significantly different levels of antigen expression between the neoplastic cells and the reactive follicular center B-cells. As reported in previous studies (4,5,7), the CD19 ($P < 1.0E-22$) and CD20 ($P < 1.0E-16$) MFI were significantly reduced in FL when compared with cases of FH. In addition, the MFI of CD10 ($P < 1.0E-9$) was significantly increased in FL compared with cases of FH (Figs. 3 and 4). Although the MFI of CD45 in cases of FL was slightly decreased when compared with that of the reactive FH cases, the difference was only marginally significant ($P = 0.04$).

Sixty-eight of the 102 cases of FL were given specific histopathologic grades on review of concurrent pathology reports with 8 classified as Grade 3, 22 Grade 2, and 38 Grade 1. The MFI of CD10 was marginally significantly increased between cases with Grades 1 and 3 FLs ($P < 0.05$), but not between Grades 1 and 2 or between Grades 2 and 3. No significant difference in MFI of CD38, CD19, CD20, or CD45 expression was detected between grades of FL. No significant differences were identified when Grade 1 and 2 FL cases (low grade) were combined and together compared with Grade 3 FL (intermediate grade).

The level of expression of CD19, CD20, CD10, and CD38 individually or in combination for each case of FL was compared with the range of expression seen in FH, and those cases of lymphoma having a level of expression of one or more of these antigens outside two standard deviations of the levels seen in FH were identified (see Table 1). No single antigen showed as high a percentage of cases outside of two standard deviations as all four antigens in combination, although CD19 was the most commonly abnormal at 83.3% of cases. Although CD38 alone was abnormal in only 66.7% of cases, its

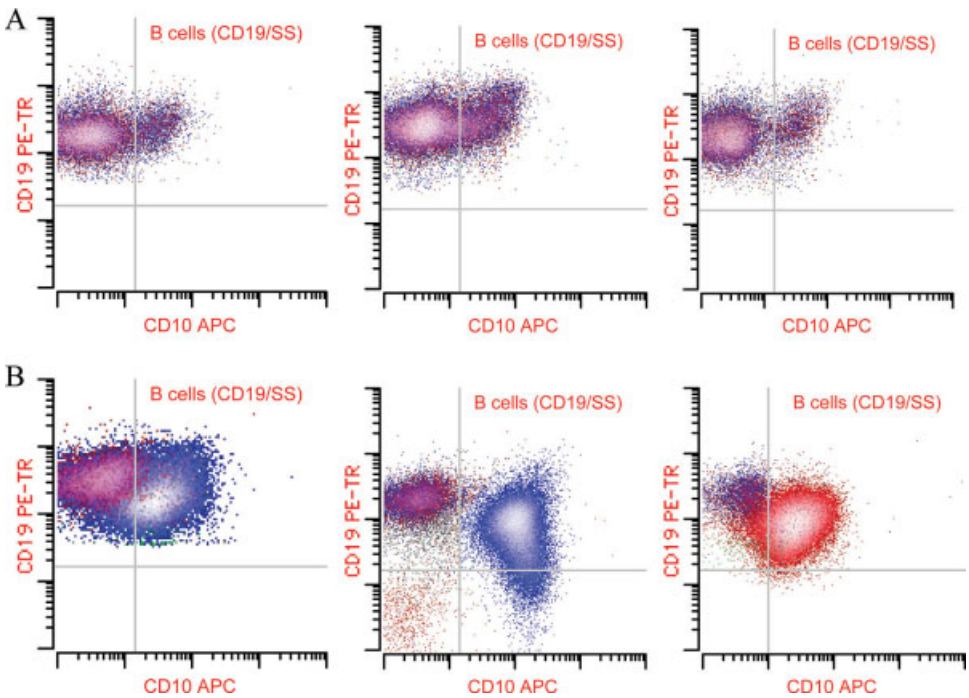

FIG. 4. Flow cytometric analysis of CD19 and CD10 in cases of follicular lymphoma and follicular hyperplasia. **A**: CD19 is not decreased in the CD10 positive germinal center population in cases of follicular hyperplasia. **B**: CD19 is variably decreased in cases of follicular lymphoma (CD10 positive population) when compared with the polyclonal non-neoplastic B-cells within the same sample. Blue color represents kappa surface light chain expressing cells and red color represents lambda surface light chain expressing cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

presence was required to reach the highest level of abnormality seen with any combination, i.e. 98.0%. One or more of the remaining three antigens in combination were abnormal in 93.1% of cases.

DISCUSSION

CD38 is a multifunctional transmembrane glycoprotein that serves as both an antigen and as an ectoenzyme (12). Its expression is tightly regulated, appearing on bone marrow progenitors, activated germinal center B-cells, and terminally differentiated plasma cells (14). CD38 has also been attributed a role in signaling leading to cellular activation and proliferation (15). Despite these observations, the biological function of CD38 remains poorly understood.

In this study, we compared the expression of CD38 between FH and FL and identified a decreased expression of CD38 in FL, which was highly statistically significant ($P < 1.0E\text{-}16$). The biological significance of decreased CD38 expression in FL is not clear. However, FL characteristically demonstrates a reduced Ki-67 defined proliferative rate by immunohistochemistry when compared with that seen in FH (16). Given the proposed relationship between CD38 and cellular activation and proliferation, the reduced CD38 expression seen in cases of FL may reflect an overall reduced proliferative activity similar to that seen with Ki-67 staining by immunohistochemistry. An attempt to identify differences in CD38 expression between different grades of FL, generally being associated with some differences in proliferative rate (17), was unsuccessful, perhaps reflecting a high degree of overlap because of the subjective grading of this disorder.

Although most non-neoplastic populations did not show significant differences in the MFI of CD38, verifying a lack of a generalized bias in the assessment of CD38 expression, T-cells in cases of FH did exhibit a significantly increased expression of CD38 when compared with nongerminal center B-cells in FH ($P = 0.005$). The significance of this finding is not clear; however, CD38

Table 1
Percentage of Follicular Lymphoma Cases Whose Level of Expression of CD19, CD20, CD38, and/or CD10 Falls More Than Two Standard Deviations Outside that Seen in Follicular Hyperplasia

Antigen	% Outside 2 SD
CD38	66.7
CD19	83.3
CD20	78.4
CD10	42.2
CD38, CD19	91.2
CD38, CD20	91.2
CD38, CD10	86.3
CD19, CD20	87.3
CD19, CD10	90.2
CD20, CD10	89.2
CD38, CD19, CD20	94.1
CD38, CD19, CD10	96.1
CD38, CD20, CD10	98.0
CD19, CD20, CD10	93.1
CD38, CD19, CD20, CD10	98.0

has been associated with T-cell activation (12) and specialized helper T cells are reported to help orchestrate the stepwise development of B-cell immunity in localized environments such as germinal centers (18). Although this relative increase in CD38 on T cells in FH may well reflect their activation as part of the on-going immune response, further study is needed.

Abnormal expression of CD38 alone is present in roughly two-thirds of cases of FL in comparison with normal follicle center B cells. Consequently, CD38 expression alone is not sufficient to definitely establish immunophenotypic abnormality in all cases of FL. However, the use of CD38 in combination with CD19, CD20 and CD10 allows for the identification of nearly all cases of FL, and CD19, CD20, and CD10 alone or in combination do not allow for identification of as large a percentage of cases. This suggests the utility of inclusion of CD38 in reagent panels for the evaluation of follicle center B cell populations and the importance of multiparametric evaluations for diagnosis of neoplasia. These findings also suggest that the clear majority of cases of FL may be identified by simple surface immunophenotyping without the need to evaluate for cytoplasmic antigens such as Bcl-2, which is more laborious and not as suitable for initial screening evaluations. The assessment of surface light restriction as a surrogate for clonality is also commonly used for the identification of neoplastic B cell populations; however, reliance on changes in the expression of other antigens, as illustrated here, is likely a more definitive approach, as clonal expansions of follicle center B cells may be seen in some normal individuals (1). Our study demonstrates the utility of CD38 for the distinction between neoplastic populations in FL and reactive germinal center B-cell populations in FH by flow cytometry.

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