

# Utility of Quantitative Flow Cytometry Immunophenotypic Analysis of CD5 Expression in Small B-Cell Neoplasms

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• **Context.**—The value of assessing CD5 expression in the differential diagnosis of small B-cell neoplasms is well established. Assessment is usually done qualitatively.

**Objectives.**—To assess CD5 expression levels by quantitative flow cytometry immunophenotyping and to determine possible differences among various small B-cell neoplasms.

**Design.**—We performed 4-color flow cytometry analysis on specimens of peripheral blood and bone marrow aspirate and quantified CD5 expression in various small B-cell lymphomas and leukemias. We also assessed CD5 levels in peripheral blood samples of healthy blood donors.

**Results.**—Cases of chronic lymphocytic leukemia and mantle cell lymphoma had higher levels of CD5 compared with control B cells ( $P < .001$ ). Cases of marginal zone lymphoma and hairy cell leukemia had CD5 levels similar to control B cells ( $P = .35$  and  $P = .14$ , respectively), whereas cases of follicular lymphoma and lymphoplasma-

cytic lymphoma had significantly lower CD5 levels than control B cells ( $P < .001$  and  $P = .04$ , respectively). In B-cell neoplasms, a high level of CD5 expression was correlated with a homogeneous pattern of positive events, whereas lower CD5 levels were correlated with heterogeneous patterns of positive events.

**Conclusions.**—Using flow cytometric immunophenotypic analysis to quantify CD5 levels can aid in diagnosis. CD5 expression levels are higher in patients with chronic lymphocytic leukemia and mantle cell lymphoma, and expression is observed in a homogeneous pattern, as compared with other B-cell neoplasms that are either negative for CD5 or express CD5 at lower levels with a heterogeneous pattern. However, there is some overlap in CD5 expression levels between a subset of atypical chronic lymphocytic leukemia and marginal zone lymphoma cases.

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Patients with small B-cell neoplasms can present with predominantly blood or tissue involvement and indolent or aggressive disease, and those neoplasms show variable morphologic and immunophenotypic features. The most common small B-cell tumors include chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), mantle cell lymphoma, follicular lymphoma, and marginal zone lymphomas (MZLs) of extranodal, nodal, or splenic types. Accurate diagnosis of those tumors is essential for evaluating prognostic factors and for planning the types and timing of therapies. Flow cytometry immunophenotypic analysis is one of the cornerstones used in the diagnosis of

small B-cell neoplasms because it allows the simultaneous evaluation of numerous markers on a distinct cell population, and it has the additional advantage that this technology is widely available in clinical laboratories.<sup>1</sup>

As a part of the immunophenotypic workup of small B-cell neoplasms, assessment of CD5 expression is essential and is performed routinely by flow cytometry immunophenotyping.<sup>2,3</sup> CD5 is a 67-kDa glycoprotein, scavenger receptor, cysteine-rich, super family member that is expressed by T cells and has a role in cellular activation.<sup>4</sup> CD5 downregulates activated B-lymphocytes and protects against autoimmunity by inducing production of cytokines, such as IL-10.<sup>5</sup> CD5 is also expressed by B-cells, and levels of CD5 on normal B-cells are high during fetal life, are low in children and younger adults, and are high again in the elderly.<sup>4-6</sup> CD5<sup>+</sup> normal B cells are small lymphocytes with scant cytoplasm, round nuclei, and clumped chromatin. Immunophenotypically, normal CD5<sup>+</sup> B cells express CD23, immunoglobulin (Ig) M, and IgD.<sup>4</sup> In contrast with neoplastic CD5<sup>+</sup> B-lymphocytes, which are antigen-experienced B cells and commonly carry hypermutations of the variable region of the immunoglobulin heavy-chain genes, normal CD5<sup>+</sup> B-cells are not antigen experienced, and their variable region of the immunoglobulin heavy-chain genes are unmutated. CD5<sup>+</sup> B cells represent 15% to 25% of the B cells in peripheral blood as well as in secondary lymphoid organs in adults.<sup>4</sup>

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Expression of CD5 is almost universal in CLL/SLL and mantle cell lymphoma, and therefore, detection of CD5 is useful in the diagnosis of those diseases. However, CD5 can occasionally be expressed by other small B-cell neoplasms, such as MZL of various types and, less often, lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM), follicular lymphoma, and other less-frequent small B-cell neoplasms.<sup>7-11</sup> Because distinguishing the various types of small B-cell leukemias and lymphomas has clinical and therapeutic consequences, CD5 expression can complicate classification of those tumors in some instances.

The intensity of antigen expression, as well as its presence or absence, can be helpful in differential diagnosis. For example, surface immunoglobulin and CD20 expression are typically dim in CLL/SLL but are brighter in other small B-cell neoplasms and especially bright in MZL and hairy cell leukemia. Typically, those markers are assessed in a qualitative fashion, as is the case for CD5 in routine practice.

However, qualitative assessment of individual markers by visual observation of flow cytometry data can be highly variable because of factors such as instrumentation, fluorochromes, and gating strategies. One approach to overcome that variability is to quantify antigen expression more rigorously by assessing the number of molecules expressed per cell, and that approach has been used, for example, to quantify CD20 expression levels in small B-cell neoplasms.<sup>12,13</sup>

We hypothesized that small B-cell neoplasms also express varying levels of CD5 and that quantification of CD5 expression levels might be helpful in differential diagnosis. Therefore, we quantified CD5 expression levels in various types of small B-cell leukemia and lymphoma, as well as in peripheral blood B cells of healthy controls.

## MATERIALS AND METHODS

### Study Group

The study group included 58 bone marrow aspirate (89%) and 7 peripheral blood specimens (11%) obtained from patients with untreated typical CLL (n = 23; 35%), so-called atypical CLL (n = 8; 12%), mantle cell lymphoma (n = 12; 18%), MZL (n = 7; 11%), follicular lymphoma (n = 8; 12%), LPL/WM (n = 4; 6%), and hairy cell leukemia (n = 3; 5%). Although most cases of lymphoma were diagnosed on lymph nodes, for analysis purposes, we selected consecutive cases for which residual peripheral blood or bone marrow aspirate specimens were available. We considered peripheral blood and bone marrow specimens were uniform for comparison purposes; therefore, we excluded from analysis specimens derived from soft tissues, lymph nodes, or the spleen. All cases were analyzed in the Clinical Flow Cytometry Laboratory of the Department of Hematopathology at the University of Texas MD Anderson Cancer Center. The diagnosis of each tumor type was based on the criteria of the World Health Organization Classification.<sup>14</sup> Therefore, final diagnoses of those tumors incorporated laboratory data, morphologic findings, immunohistochemical results, extended flow cytometry immunophenotype, and the results of conventional karyotyping and fluorescence in situ hybridization.

In addition to the tumor group, we also assessed a control group composed of peripheral blood samples from 15 healthy donors, whose complete blood cell counts were within reference range. We refer to this group as *control* or *normal B cells*.

### Matutes Scoring System

The updated Matutes scoring system was used to define cases of CLL as *typical* or *atypical*.<sup>13,15</sup> The system has 5 criteria, each equal to 1 point, based on the immunophenotype. The criteria are (1) dim

intensity of surface immunoglobulin light chain, (2) negative or dim intensity of CD79b or CD22, (3) presence of CD5, (4) presence of CD23, and (5) absence of FMC7. For a marker to be considered *positive*, expression must be found on 30% or more of the monotypic lymphocytes, as compared with the isotype control. A score of 4 to 5 supported typical CLL and a score of 3 or less supported atypical CLL/SLL.

### Flow Cytometry Immunophenotype

Samples were collected in ethylenediaminetetraacetic acid anticoagulant tubes, stored at room temperature, and processed within 24 hours of collection. Whole-blood staining was performed using 4-color flow cytometry with a panel of 3 tubes. Each panel included a combination of directly conjugated monoclonal antibodies with fluorescein isothiocyanate/phycoerythrin (PE)/peridinin-chlorophyll protein/allophycocyanin. The combinations of antibodies were as follows: tube 1, CD19/CD5-labeled 1:1/CD45/CD20; tube 2,  $\kappa$ /CD5 1:1/CD45/CD19; and tube 3,  $\lambda$ /CD5 1:1/CD45/CD19. All antibodies were from BD Biosciences (San Diego, California).

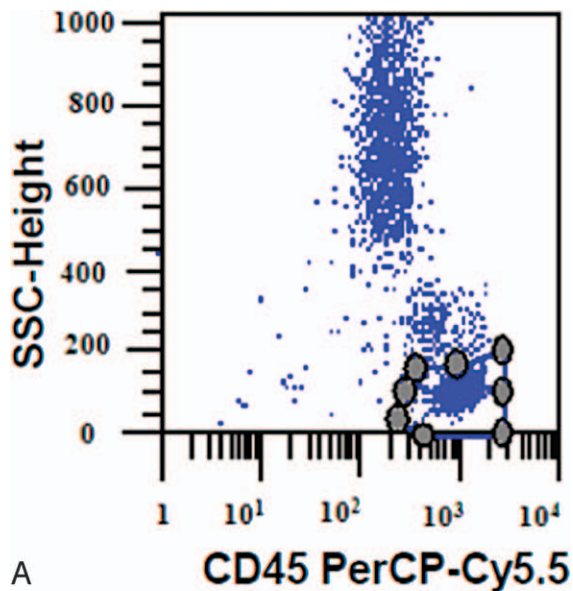
For quantitative assessment of CD5 expression levels, the CD5 clone L17F12 antibody was custom-conjugated with PE fluorochrome in a 1:1 ratio (BD Biosciences). QuantiBRITE PE beads, a lyophilized bead pellet conjugated with 4 levels of PE, were used for the conversion of position on the fluorescence 2 axis into the number of PE molecules bound per cell (BD Biosciences). The levels used for conversion were at 474, 5359, 23 843, and 62 336 molecules.

Cell suspensions admixed with fluorochrome-labeled antibodies contained 1 million cells in 1-mL Falcon tubes. Tubes were incubated for 10 minutes at 4°C, and red blood cell lysis was performed using the BD Biosciences Pharm Lyse buffer. Washing of tubes was done with 1× phosphate-buffered saline and 0.1 % sodium azide, and the pellet was resuspended in 1% paraformaldehyde. For  $\kappa$  and  $\lambda$  tubes, red blood cell lysis was done first to remove serum immunoglobulin, followed by staining with the antibodies. Other steps involved in the protocol remained the same.

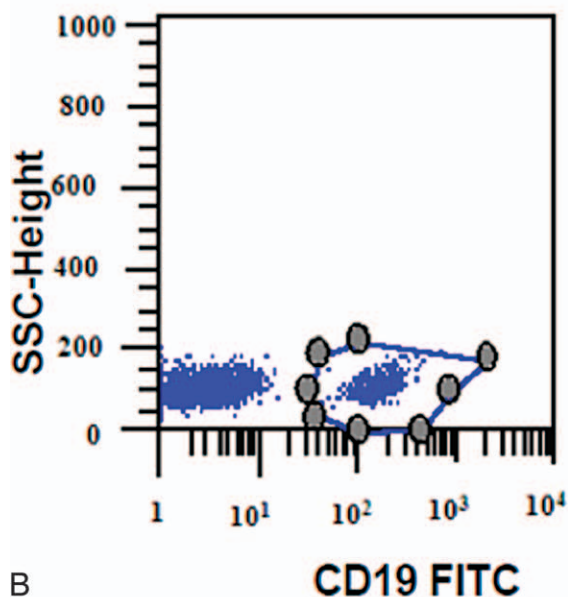
A BD Biosciences FACSCalibur cytometer was used for data acquisition. Calibration of the flow cytometer was achieved by using BD Biosciences CaliBRITE beads with BD Biosciences FACSComp software, and its daily optimization and compensation were performed with normal peripheral blood stained with CD3, CD4, CD8, and CD19 with BD Biosciences CellQuest Pro. The fluorescence 2 axis was converted into the number of antibodies per cell by using QuantiBrite beads. The instrument and compensation settings used to acquire QuantiBrite beads were also used to acquire the patient samples. Live gating was set on lymphocytes by using forward scatter height and side scatter height, and 20 000 events were acquired. Data analyses were performed using QuantiCalc (Verity Software House, Topsham, Maine). Gated CD45<sup>+</sup>/CD19<sup>+</sup> monotypic cells were used for the estimation of anti-CD5 antibodies bound per cell (ABC) and compared with CD45<sup>+</sup>/CD19<sup>+</sup> cells on the control samples. Tubes with anti-immunoglobulin  $\kappa$  and  $\lambda$  light chains were used to confirm the presence of a monotypic B-cell population.

The gating strategy for CD45<sup>+</sup>/CD19<sup>+</sup> monotypic B cells used to determine CD5 ABC is shown in Figure 1, A and B. The generated values of CD5 ABC were used to calculate mean, standard deviation, and ranges for each subtype of B-cell neoplasm. To determine whether patterns of expression were significantly different between controls and lymphoproliferative neoplasms, we used an unpaired *t* test with the 2-tailed option and 99% confidence interval.

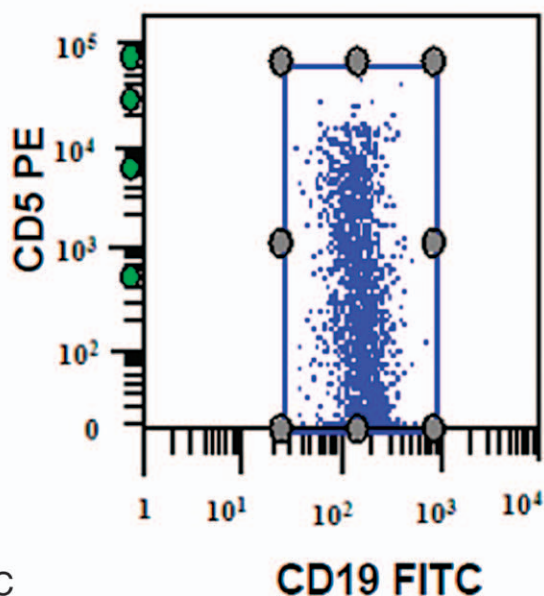
A negative control threshold for CD5 expression was established using 6 normal peripheral blood samples, gating on NK cells (CD5<sup>-</sup>CD19<sup>-</sup> lymphocytes). The calculated median CD5 ABC values ranged from 31 to 75, mean, 51; median, 41; and SD, 20; so B-cell populations with values greater than 75 were scored as positive.



A



B



C

Patterns of CD5 expression in B cells were classified as homogeneous if a cluster of cells spanned up to 1.25 log on the CD5 ABC scale; if the cluster showed a greater spread, the pattern was classified as heterogeneous.

### Cytogenetic Studies

Conventional cytogenetic analysis was performed on G-banded metaphase cells prepared from bone marrow aspirate specimens of CLL using standard procedures. Twenty metaphases were assessed, and the results were described using the International System for Human Cytogenetic Nomenclature.<sup>16</sup> A 5-locus fluorescence in situ hybridization panel (Vysis/Abbott, Des Plaines, Illinois) was also used to aid in detecting common cytogenetic abnormalities associated with CLL. Interphase cells were analyzed from bone marrow cultures using a panel of probes designed to detect deletion 13q14.3, deletion 13q34, trisomy 12, deletion of *TP53* at 17p13, and deletion of *ATM* at 11q22.3.

### Statistical Analysis

Statistical analysis was done by GraphPad Prism Software, Inc. (La Jolla, California) and unpaired two tailed t-test was used for statistical comparisons between groups. A *P* value of less than .05 was considered statistically significant.

## RESULTS

### Matutes Scoring System

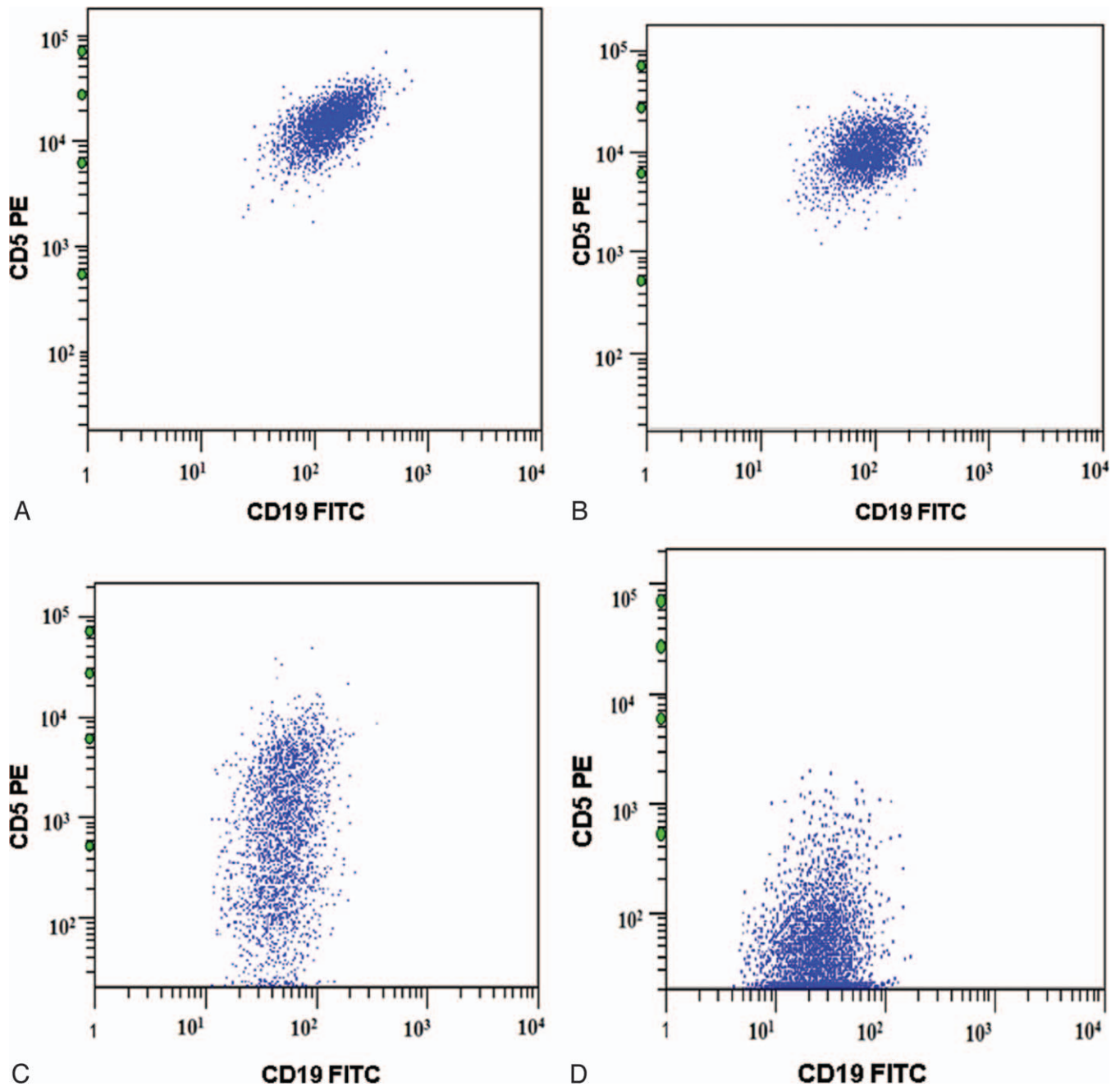
We used the updated Matutes scoring system<sup>15</sup> for the CLL subset, and 23 cases were classified as typical CLL and 8 cases as atypical CLL.

### Patterns of CD5 Expression in B cells

We observed 3 patterns of CD5 expression in B cells, with a threshold of 75 ABC to determine the pattern of CD5 expression for each case. In one pattern, events were seen on dot plots as a distinct, tight cluster of events greater than 75 ABC. For this study, we designated this occurrence a *homogeneous expression pattern* (or homogeneous positive). In the second pattern, events showed variable CD5 expression, partially greater than and partially less than the threshold of 75 ABC, and we designated this occurrence a *heterogeneous expression pattern* (or heterogeneous positive). A third pattern showed a tight cluster of events was entirely less than 75 ABC and was designated *homogeneous negative*. Representative patterns are shown in Figures 1 and 2.

All CD19<sup>+</sup> B cells from normal controls showed a heterogeneous pattern of CD5 expression (Figure 1, A through C). Most cases of CLL (21 of 23; 91%) (Figure 2, A), atypical CLL (7 of 8; 88%), and mantle cell lymphoma (11 of 12; 92%) (Figure 2, B) showed a homogeneous positive pattern. One case of atypical CLL showed a heterogeneous pattern. Among cases of MZL, 3 of 7 (43%) showed a heterogeneous pattern (Figure 2, C), 2 (29%) showed a homogeneous negative pattern, and 2 (29%) showed a homogeneous positive pattern. In contrast, 7 of 8 cases

←  
**Figure 1.** A and B, Gating strategy to determine the CD5 values of antibodies bound per cell in CD45<sup>+</sup>/CD19<sup>+</sup> B cells. C, This specimen is peripheral blood from a healthy donor, and the CD19<sup>+</sup> cells show a variable level of CD5 expression ranging from negative to positive. This is designated as a heterogeneous pattern of CD5 expression. Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin; SSC, side scatter.



**Figure 2.** A, This dot plot of a peripheral blood specimen from a patient with chronic lymphocytic leukemia displays CD19<sup>+</sup> cells with a high expression level of CD5 and a compact distribution, which was designated a homogeneous positive pattern. B, This dot plot of a case of mantle cell lymphoma displays a homogeneous positive pattern of CD5 expression. C, This case of marginal zone lymphoma displays a heterogeneous pattern of CD5 expression. D, This case of follicular lymphoma displays dim reactivity with CD19, and a dim/negative pattern of CD5 expression. (The y-axis logarithmic numbers refer to antibodies bound per cell.) Abbreviations: FITC, fluorescein isothiocyanate; PE, phycoerythrin.

(88%) of follicular lymphoma showed a homogeneous negative pattern (Figure 2, D), and 1 of 8 (13%) showed a homogeneous positive pattern. One of 4 LPL/WM cases (25%) and 1 of 3 hairy cell leukemia cases (33%) showed a heterogeneous positive pattern, whereas the remaining cases showed a homogeneous negative pattern (Table 1).

Specific scores for 8 cases of atypical CLL/SLL are shown in Table 2. All cases were CD5<sup>+</sup> and CD23<sup>+</sup>, yielding a score of 1 point for each of the fulfilled criteria. All cases were FMC7<sup>+</sup>, as well as moderately positive for CD22 or CD79b,

assigning a score of 0 for each of these criteria, as detailed in Table 2.

#### CD5 ABC Values in B Cells of Normal Controls and Small B-Cell Neoplasms

CD5 expression levels in typical and atypical CLL cases were significantly higher than were expression levels in normal control B cells ( $P < .001$ ) (Figure 3; Table 1). Similarly, CD5 expression levels in mantle cell lymphoma cases were significantly higher compared with B-cells of

**Table 1. Quantitative Median Levels of CD5 Antibodies Bound per Cell and Patterns of CD5 Expression in B Cells From Healthy Controls and Small B-Cell Neoplasms**

Diagnosis	Pattern of Expression			Mean	SD	Range	P Value <sup>a</sup>
	Homogeneous Negative, No. (%)	Heterogeneous, No. (%)	Homogeneous Positive, No. (%)				
Normal, n = 15		15 (100)		315	142	145–568	
CLL/SLL, n = 23		2 (9)	21 (91)	8877	5708	169–21 017	<.001
Atypical CLL/SLL, n = 8		1 (12)	7 (88)	4475	4141	396–11 536	<.001
MCL, n = 12		1 (8)	11 (92)	5371	3331	126–10254	<.001
MZL, n = 7	2 (29)	3 (43)	2 (29)	234	253	19–770	.34
HCL, n = 3	2 (66)	1 (33)		178	139	96–338	.14
LPL, n = 4	3 (75)	1 (25)		140	139	34–344	.04
FL, n = 8	7 (88)		1 (12)	46	17	29–82	<.001

Abbreviations: CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma.

<sup>a</sup> P values reflect comparison with B cells from healthy controls.

normal controls ( $P < .001$ ) but were not different from CLL. In contrast, CD5 expression levels in other small B-cell neoplasms were lower. The cases of MZL had CD5 expression levels similar to normal control B-cells ( $P = .35$ ) (Figure 3), and those levels were significantly lower than CD5 expression levels in CLL, both typical and atypical ( $P < .001$ ). All 3 cases of hairy cell leukemia had CD5 expression levels similar to the control B cells ( $P = .14$ ). CD5 expression levels in follicular lymphoma and LPL/WM were significantly lower than levels of normal control B cells ( $P < .001$  and .41).

Conventional cytogenetic analysis was carried out in 20 of 23 cases (87%) of typical CLL (17 [85%] were diploid, 1 [5%] was hypodiploid, 1 [5%] was hyperdiploid, and 1 [5%] showed del[11q]). Seven of 8 cases (88%) of atypical CLL had conventional cytogenetic analysis (3 [43%] were diploid, 1 [14%] showed trisomy 12, 1 [14%] showed -Y, 1 [14%] showed +Y, and 1 [14%] was hyperdiploid). Fluorescence in situ hybridization analysis was carried out in 22 of 23 cases (96%) of typical CLL (9 [41%] showed a normal pattern, 6 [27%] showed del13q14.3/D13S319, 5 [23%] showed trisomy 12, 3 [14%] showed del11q22.3/ATM, 1 [5%] showed del17q13.1/TP53, 1 [5%] showed del13q34/LAMP1; and 3 of these cases [14%] had 2 abnormalities each). There was no difference in CD5 expression levels between cases with and without trisomy 12 among cases of CLL ( $P = .35$ ).

## COMMENT

Flow cytometry immunophenotypic analysis is essential for the diagnosis of small B-cell lymphomas and leukemias. In addition to establishing a surrogate indication for clonality by assessment of immunoglobulin light chains, expression of various antigens is characteristic of certain types of B-cell lymphoma. CD5 is particularly helpful to assess because this antigen is typically expressed in CLL/SLL and mantle cell lymphoma. However, rarely CD5 is expressed in other small cell neoplasms, and that can cause problems in the differential diagnosis. We hypothesized that small B-cell neoplasms express varying levels of CD5 and that quantification of CD5 expression levels might be helpful in differential diagnosis. Our results show that quantitative analysis of CD5 expression levels is indeed helpful. CD5 expression levels are typically high in CLL/SLL and mantle cell lymphoma, and the pattern of expression is distinctive. In contrast, CD5 expression levels are significantly lower in other types of small B-cell neoplasms as well as in control B cells from healthy donors, and the pattern of expression is usually heterogeneous.

There were some examples of overlap in mean CD5 ABC between CLL/SLL and MZL. In particular, the lower range of expression of some cases of CLL/SLL cases, particularly atypical CLL, overlapped with the upper range of some cases of MZL cases. There were only 2 cases of MZL with moderately high CD5 expression levels and a homogeneous positive pattern that could cause problems in the differential diagnosis. Therefore, CD5 expression levels would be theoretically helpful in 56 of 58 cases (97%) assessed in this study. In addition, the levels of CD5 expression in follicular lymphoma and LPL/WM were significantly lower than expression in control B cells. These low levels of CD5 expression could be helpful as an adjunct to distinguishing follicular lymphoma or LPL/WM from background, normal B cells in the assessment of minimal residual disease after therapy.

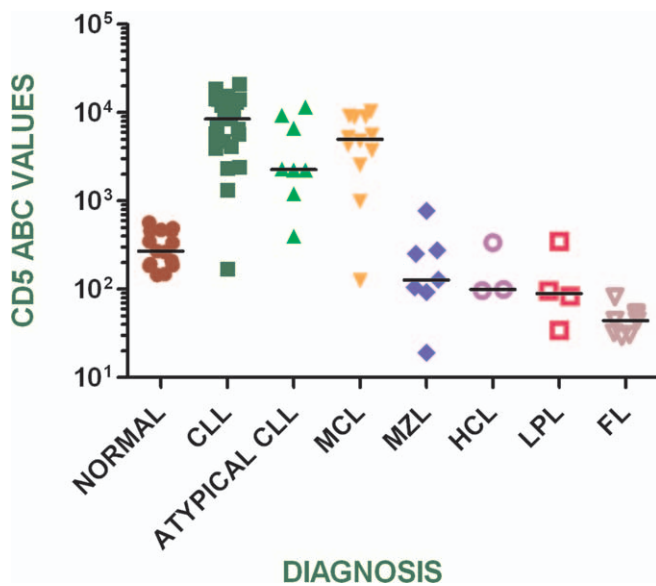
Others have used a quantitative approach to assess various markers in B-cell leukemias and lymphomas. Perhaps the most common antigen quantified has been CD20 in patients with CLL/SLL; however, CD5 quantification was also used by D'Arena et al<sup>17</sup> in a series of small B-cell neoplasms. D'Arena et al<sup>17</sup> demonstrated that the highest levels of CD5 were seen in CLL/SLL, mantle cell lymphoma, and MZL; however, the frequency of cases with high CD5 levels was variable: 61 of 61 (100%) in CLL/SLL, 8 of 10 (80%) in mantle cell lymphoma, and 1 of 6 (17%) in

**Table 2. Matutes Score for the 8 Cases of Atypical Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma<sup>a</sup>**

Case No.	Dim slg	CD5 <sup>+</sup>	CD23 <sup>+</sup>	FMC7 <sup>+</sup>	Weak or Negative CD22 or 79b	Total Score
1	0	1	1	0	0	2
2	1	1	1	0	0	3
3	1	1	1	0	0	3
4	1	1	1	0	0	3
5	0	1	1	0	0	2
6	0	1	1	0	0	2
7	1	1	1	0	0	3
8	0	1	1	0	0	2

Abbreviation: slg, surface immunoglobulin.

<sup>a</sup> Criteria for score as indicated at the top of each column: 1 point if the criterion is fulfilled; 0 points if the criterion is not fulfilled (according to Moreau et al; *Am J Clin Pathol* 1997; 108: 378-382).



**Figure 3.** Comparison of quantitative expression of CD5 between B cells of normal controls and small B-cell neoplasms. Abbreviations: ABC, antibodies bound per cell; CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; HCL, hairy cell leukemia; LPL, lymphoplasmacytic lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma.

MZL cases. Our experience differs somewhat in that almost all cases of mantle cell lymphoma expressed CD5, and 29% of cases of MZL had a homogeneous positive pattern of CD5. Although the explanation for these differences between our results and the earlier study is not readily apparent, the levels of calibration were different for ABC. D’Arena and colleagues used sets of beads carrying 1400, 14 000, 36 600, and 182 000 molecules, compared with the beads in this study, with 474, 5359, 23 843, and 62 336 molecules. Other factors that may explain the differences between studies include the staining protocol, the antibody clone, and the fixation methods.<sup>18</sup>

Compared with earlier studies, we analyzed a wider spectrum of small B-cell neoplasms for CD5 expression levels, including CLL with typical and atypical features, mantle cell lymphoma, MZL, follicular lymphoma, LPL/WM, and hairy cell leukemia. We also showed distinctive patterns of CD5 expression in various lymphoproliferative disorders. The CLL/SLL cases demonstrated high expression levels of CD5 in a homogeneous pattern in virtually all cases in our study. This was also true for cases of mantle cell lymphoma. In contrast, MZLs had significantly lower CD5 expression levels and more often had a heterogeneous pattern of CD5 expression. Other small B-cell neoplasms assessed in this study rarely expressed CD5 and only at very low levels. There were differences between our results and the study by D’Arena et al.<sup>17</sup> In the earlier study, the authors showed that CD5 levels were much higher in CLL and mantle cell lymphoma cases, although only in about 20% of cases. D’Arena et al.<sup>17</sup> also reported that levels of CD5 expression in follicular lymphoma were higher than that of normal controls, which is the opposite of what we observed. The cause of these discrepancies between our study and D’Arena et al.<sup>17</sup> are not readily apparent, but gating strategies may have played a role.

Since the studies of D’Arena et al,<sup>17</sup> published in 2000, few studies have focused on quantitative analysis by flow cytometry immunophenotyping. However, antigen intensity is commonly estimated qualitatively in routine practice (ie, bright, moderate, or dim). Although valuable, qualitative assessment has limitations. Historically, routine assessment often uses percentages and compares expression with the distribution of an isotype. Typically, 20% greater than the isotype boundary is used as a cutoff to designate a marker as positive or negative, and that is clearly arbitrary. Therefore, small changes in the isotype gating may lead to artifactual results with false-positives or false-negatives. Another inherent problem with qualitative assessment of antigen expression is that fluorochromes used to label antibodies carry different intensities; for example, fluorescein isothiocyanate is inherently dim, whereas PE is inherently bright, and dim fluorochromes more often show a suboptimally low signal to noise ratio. Relatively dim expression could be missed with dimmer fluorochromes, creating a possible false-negative result.

Many of the shortcomings of qualitative antigen assessment can be overcome by the use of quantitative flow cytometry, which assigns an absolute value to the different fluorescence levels and, with standardization, may achieve similar values between different instruments and different laboratories. Therefore, we think quantitative assessment of antigen expression is going to achieve a place in the workup of small B-cell neoplasms as well as other diseases. There are recent examples to support this idea. In patients with CLL/SLL, quantitative analysis of CD38 and CD20 has been used to predict prognosis.<sup>12,19</sup> Similarly, quantification of CD64 expression by neutrophils has been used to assess inflammatory processes, and CD38 quantification by CD8<sup>+</sup> T cells is used as a prognostic marker in acquired immunodeficiency syndrome.

However, we also acknowledge that there are multiple technical considerations in the adoption of a quantitative flow cytometry assay, which include the methodology used to quantitate, the use of fixative or phosphate-buffered saline, variations in the Quantibrite lot numbers, the use of clones and their valency (Fab versus immunoglobulin), the saturation of the antibody, and the 1:1 conjugation. Some of these issues will likely be overcome as quantitative assessments achieve wider use.

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