Immunohistochemical Detection of Hairy Cell Leukemia in Paraffin Sections

The Role of Pax5 and CD103 Double Staining to Improve Specificity and Sensitivity

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Context.—In hematopathology practice, abnormal expression of CD103 on B cells is detected by flow cytometry in hairy cell leukemia (HCL) and, in combination with other phenotypic and morphologic findings, provides diagnostic specificity and sensitivity. Immunostaining on paraffin sections makes it possible to perform immunophenotyping in situ. However, normal bone marrow contains CD103-positive cells, which are not B cells, making it difficult to be certain about low-level involvement by HCL.

Objective.—To develop dual immunostaining for confirmation that CD103 is expressed in B cells, which may be highly desirable in assessing low-level involvement by HCL.

Design.—We developed a dual immunostaining approach using a B-cell marker, Pax5, expressed in the nucleus, in combination with a membrane marker, CD103.

Results.—We analyzed 6 HCLs, 7 marginal zone lymphomas, 12 lymphoplasmacytic lymphomas, 7 follicular lymphomas, 5 chronic lymphocytic leukemias, 5 mantle cell lymphomas, 1 multiple myeloma (lymphocytic variant), and 3 bone marrows not involved by any B-cell neoplasm. Our dual staining approach confirmed that only the neoplastic cells of HCL were positive for both CD103 and Pax5.

Conclusions.—This dual-staining method is particularly helpful in cases with low-level involvement by HCL and can be used for determining minimal residual disease after treatment.

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CD103 is an integrin αE chain that upon heterodimerization with the integrin β7 constitutes the receptor for E-cadherin. This interaction plays a role in various cellular interactions within the immune system.1 CD103 is expressed on intraepithelial T cells, some subsets of memory T cells (CD8 positive), and dendritic cells.1,2 In hematopathology practice, abnormal expression of CD103 on B cells is observed in hairy cell leukemia (HCL) and, in combination with other phenotypic and morphologic findings, provides diagnostic specificity and sensitivity. Until recently, the phenotypic workup for HCL predominantly relied on flow cytometry, which allows targeted analysis of B-cell subpopulations (gating on CD19/CD20–positive B cells and allowing separation of B cells based on intensity of CD20 expression and on side/forward scatter properties). However, when materials for flow cytometry are not submitted, evaluation could present a challenge. The development of a CD103 antibody for immunostaining on paraffin sections made it possible to perform phenotyping in situ.3 Morgan and colleagues4 showed high-quality CD103 immunostaining in bone marrows involved by HCL, including extensive and low-level infiltration. However, normal bone marrow contains CD103-positive cells, which may make it difficult to be certain about low-level involvement by HCL. Therefore, confirmation that CD103 is expressed in B cells may be highly desirable in such cases. In this regard, we developed a dual immunohistochemical stain based on CD103 and a nuclear B-cell marker, Pax5, to specifically identify the B cells of HCL that coexpress Pax5 and CD103.

MATERIALS AND METHODS

Sample Selection

Paraffin blocks including representative bone marrow biopsies, clots, or lymph node biopsies were obtained from the AmeriPath Northeast files (Shelton, Connecticut). The research protocol was approved by the Western Institutional Review Board. Classification of lymphomas based on morphologic and immunophenotypic features was conducted in accordance with the 2008 World Health Organization Classification of Tumors of Haematopoietic and Lymphoid Tissues.4 Diagnosis was confirmed by slide review, flow cytometry, and clinical information. Cases included 6 HCLs, 7 marginal zone lymphomas, 12 lymphoplasmacytic lymphomas, 7 follicular lymphomas, 5 chronic lymphocytic leukemias, 5 mantle cell lymphomas, 1 multiple myeloma (lymphocytic variant), and 3 bone marrows not involved by any B-cell neoplasm.

Immunohistochemistry

Immunohistochemical testing was performed on paraffin sections using rabbit antibody to CD103 (clone EPR4166, Abcam, Cambridge, Massachusetts) and mouse monoclonal antibody to Pax5 and CD103.
Pax5 (clone 1EW, Leica Microsystems, Buffalo Grove, Illinois). The CD103 antibody was used at 1:500 dilution with incubation for 15 min; the Pax5 antibody was a premixed solution provided by the manufacturer, with incubation for 20 minutes. After the deparaffinization step, slides were washed and treated with a heat-induced epitope retrieval using EDTA, pH 8.0, for 20 minutes in the Leica Bond Automation immunohistochemistry system. Slides were washed again and incubated with CD103 or Pax5 antibody. Next, slides were washed and treated with Bond Polymer Refine Detection steps, including a second horseradish peroxidase-conjugated antibody and diaminobenzidine detection solution (Leica). Slides were then washed and incubated with CD103 or Pax5 antibody. After that, slides were washed and treated with Bond Polymer Refine Red Detection steps, including a second antibody conjugated with alk-phos (Leica). Slides were then counterstained with hematoxylin, dehydrated, and mounted. The primary antibody step defined the color of CD103 and Pax5 detection. If CD103 antibody was applied first and Pax5 second, then CD103 stain was brown and Pax5 was red. If Pax5 antibody was applied first and CD103 second, then CD103 was visualized as red and Pax5 as brown.

**RESULTS**

While implementing CD103 immunostaining in our laboratory, we noted that some bone marrows not involved by HCL contained variable numbers of CD103-positive cells, with a single cell or cluster distribution pattern (Figure 1). As seen in Figure 1, A (CD20), some areas of bone marrow contained only a few B cells in a single-cell distribution pattern. However, CD103 immunostaining (Figure 1, B) identified significantly more positive cells as single cells and in clusters. This finding indicated that making a definitive interpretation about low-level HCL infiltration may be difficult and cause erroneous interpretation.

Therefore, we undertook the development of a simultaneous (double) immunostain for Pax5 (nuclear protein expressed in both normal and neoplastic B cells) and CD103. As seen in Figure 1, C, simultaneous Pax5 immunostaining separates scattered B cells from CD103-positive cells in this bone marrow. Furthermore, we also performed the procedure so that Pax5 staining generated a brown reaction product in the nucleus, whereas CD103 was detected as a red membrane stain with similar results (Figure 1, D). Based on our experience, both color combinations worked very well, with slightly better quality achieved using Pax5 detected by the red reaction product and CD103 as brown. Figure 1, D, illustrates the need for double staining, as small clusters of Pax5-positive B cells were localized close to CD103-positive cell clusters but did not coexpress the markers.

Implementation of double staining for Pax5/CD103 allows straightforward diagnosis of both low-level and extensive involvement by HCL. As shown in Figure 2, double staining allows resolution of single B cells expressing CD103 and separates normal CD103-negative B cells from CD103-positive...
positive B cells (hairy cells) in the clot section containing mostly peripheral blood elements (a small population of HCL was detected by flow cytometry). As noted above, both combinations (CD103 brown/Pax5 red, Figure 2, A, and CD103 red/Pax5 brown, Figure 2, B) worked very well, with slightly more defined membrane staining when CD103 was visualized by a brown reaction product. The large arrows in Figure 2, A and B, indicate single double-positive hairy cells. The small arrow in Figure 2, B, shows a CD103-positive non-B cell. Scattered Pax5-positive, CD103-negative B cells are seen in Figure 2, A and B. Figure 3 illustrates a case with extensive involvement by HCL. In such cases double staining would not be necessary, as it is not difficult to align areas of extensive B-cell infiltration on slides separately stained for a B-cell marker and CD103.

Finally, we confirmed that the double-staining procedure is very specific in separating HCL from other B-lineage neoplasms. Marginal zone lymphoma may raise a differential diagnosis with HCL, particularly when splenomegaly is present. Figure 4, A, illustrates a case of splenic marginal zone lymphoma, which shows absence of CD103 on neoplastic B cells. The infiltrate was quite subtle, and separate CD103 and B-cell marker staining could have caused an erroneous conclusion that B cells were positive for CD103, as small clusters of B cells and CD103-positive cells were seen in close proximity. Another B-cell neoplasm, which may involve spleen, is lymphoplasmacytic lymphoma. As seen in Figure 4, B, neoplastic Pax5-positive cells in a representative case do not express CD103. Sometimes HCL is positive for CD10, which may raise a differential diagnosis with follicular lymphoma (particularly in the absence of flow cytometry). Figure 4, C, illustrates a typical case of follicular lymphoma involving the lymph node. The neoplastic cells are negative for CD103. An unusual diagnostic difficulty may occur in differentiating the lymphocytic variant of multiple myeloma (CD20 positive, cyclin D1 positive) from a subtype of HCL expressing cyclin D1. Figure 4, D, shows that this variant of multiple myeloma can be differentiated from HCL by lack of CD103 expression in the neoplastic cells. Overall, double-staining analysis was done in 3 bone marrows not involved by any B-cell neoplasm, 5 cases of chronic lymphocytic leukemia, 5 cases of mantle cell lymphoma, 7 cases of follicular lymphoma, 7 cases of marginal zone lymphoma, 12 cases of lymphoplasmacytic lymphoma, 1 case of multiple myeloma (lymphocytic variant), and 6 cases of HCL. Our findings show that expression of CD103 was seen only on the neoplastic B cells of HCL in the analyzed cases.

**DISCUSSION**

Data in the literature and our results indicate that CD103 is expressed normally by non-B cells. The cells normally expressing CD103 include dendritic cells and subsets of T cells. Although it was believed that these CD103-positive cells are very scant in bone marrow, our findings indicate that their numbers are variable. Some bone marrow biopsies may contain only a few of these cells, but others show distinct clusters of CD103-positive cells (Figure 1, B through D). A recent publication regarding CD103 immunostaining on paraffin sections noted that CD103 is expressed in the cytoplasm of some normal bone marrow cells, suggesting that this cytoplasmic pattern allows normal cells to be distinguished from hairy cells characterized by membrane CD103 expression. We observed that non-B cells in bone marrow biopsies tend to express CD103 in the cytoplasm. However, the staining pattern in some non-B cells includes membrane signal, and some hairy cells show cytoplasmic CD103 expression. This makes it difficult to be definitive in
differentiating non-B cells from possible hairy cells based exclusively on the CD103 staining pattern, especially when B cells are scant. As shown in Figure 1, if B-cell marker and CD103 staining were done separately, one could conclude that small clusters of CD103-positive B cells were present, leading to an erroneous diagnosis.

Apart from HCL, expression of CD103 is seen in HCL variant, a subset of splenic marginal zone lymphomas and rare cases morphologically classified as splenic red pulp lymphoma with villous lymphocytes, prolymphocytic leukemia, and diffuse large B-cell lymphoma. The positive result of molecular testing for BRAF V600E mutation provides a diagnosis of HCL when phenotypic findings raise a differential diagnosis of HCL versus other B-cell neoplasms. However, at a low level of involvement some HCLs show negative molecular results for the BRAF V600E mutation. The antibody for the BRAF V600E mutation-containing protein became available recently, and immunostaining for the mutated BRAF appears to be even more sensitive than molecular testing. However, this antibody (VE1) also reacts with a subset of cells in small lymphocytic leukemia and apparently produces a nonspecific reaction with some normal bone marrow cells, likely mast cells. Our approach using a simple double-staining technique for the B-cell nuclear transcription factor Pax5 and CD103 allows definitive separation of non-B cells from neoplastic B cells expressing CD103. This may be particularly useful in assessing specimens for low-level involvement by HCL. Also, double staining for Pax5 and CD103 may be very helpful in follow-up biopsies for HCL after treatment when residual low-level HCL persists and/or CD20 is negative because of anti-CD20 antibody administration.

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