Immunohistochemistry for \( \text{BRAF} \) V600E in the Differential Diagnosis of Hairy Cell Leukemia vs Other Splenic B-Cell Lymphomas

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Key Words: BRAF; Immunohistochemistry; Hairy cell leukemia

ABSTRACT

Objectives: Recent reports have used immunohistochemistry (IHC) with a mutation-specific antibody to detect the \( \text{BRAF} \) V600E mutation, which is found in nearly all cases of hairy cell leukemia (HCL). To date, however, only a small number of non-HCL, splenic B-cell lymphomas have been examined by IHC.

Methods: We analyzed 121 cases, including 26 HCLs, 52 non-HCL splenic lymphomas, 22 chronic lymphocytic leukemias/small lymphocytic lymphomas (CLLs/SLLs), and 21 plasma cell neoplasms (PCNs) for \( \text{BRAF} \) V600E expression by IHC. Molecular testing for \( \text{BRAF} \) V600E was performed in a subset of cases, using allele-specific polymerase chain reaction and/or Sanger sequencing.

Results: Twenty-six (100%) of 26 HCL cases were positive by IHC vs one (1%) of 95 non-HCL cases. Positive staining was identified in one (2%) of 44 splenic marginal zone lymphomas (SMZLs), while each of 22 CLLs/SLLs, 21 PCNs, six unclassifiable splenic lymphomas, and two HCL variants were negative. IHC and molecular results were concordant in all cases examined (21 HCLs and 21 non-HCLs, including the \( \text{BRAF} + \) SMZLs).

Conclusions: The detection of \( \text{BRAF} \) V600E by IHC is useful in the distinction of HCLs from other splenic-based lymphomas, although the identification of at least rare SMZLs containing this abnormality illustrates the continuing need for a multiparameter approach to diagnosis.

Hairy cell leukemia (HCL) is a small B-cell neoplasm that presents with splenomegaly, bone marrow involvement, and peripheral blood cytopenias.1-3 Distinguishing HCL from other B-cell or plasma cell neoplasms that have bone marrow involvement, including splenic marginal zone lymphoma (SMZL) and HCL variant (HCLv), is critical for patient management because HCL is responsive to therapy with purine analogues, such as cladribine, while SMZL, HCLv, and other B-cell lymphomas are not.4-7 The diagnosis of HCL is typically based on the presence of appropriate morphologic features together with the demonstration of CD11c, CD25, and CD103 coexpression, along with CD123, by flow cytometry.2,8,9 Obtaining diagnostic flow cytometric results may sometimes be difficult, however, because the peripheral blood characteristically contains few malignant B cells, and bone marrow aspirate samples are often hemodilute due to marked reticulin fibrosis, a characteristic feature of HCL in the marrow. Formalin-fixed, paraffin-embedded (FFPE) trephine core biopsy specimens therefore usually contain the most representative tumor tissue sample for analysis. A variety of immunohistochemical stains have been used to address this differential diagnosis from trephine biopsy specimens, including cyclin D1, CD123, DBA.44, tartrate-resistant acid phosphatase, and annexin A1.10-18 Among these, annexin A1 has been reported to be the most sensitive and specific marker, but because T cells and granulocytes are also positive, interpreting this stain in bone marrow biopsy specimens is frequently challenging, especially when only small numbers of tumor cells are present.16 There therefore still remains a need for a better diagnostic marker for the workup of HCL from FFPE material.
Recently, molecular studies have shown the \textit{BRAF} c.1799T>A p.V600E mutation to be a highly sensitive and specific marker for HCL.\cite{19,22} This mutation has been reported in nearly all cases of HCL studied and has only rarely been described in non-HCL lymphoproliferative disorders. A variety of molecular methods have been used to evaluate for \textit{BRAF} mutations, including allele-specific polymerase chain reaction (PCR), high-resolution melt curve analysis, and Sanger sequencing, but these methods are costly and time-consuming, are not available in all laboratories, and often cannot be applied to decalcified FFPE core biopsy material.\cite{19-27} Recent studies have shown that immunohistochemistry (IHC) using the VE1 antibody, specific for \textit{BRAF} V600E, correlates with molecular findings in HCL and may represent a useful diagnostic approach for FFPE material.\cite{28-30} To date, however, only a small number of non-HCL, splenic B-cell lymphomas have been studied using \textit{BRAF} V600E IHC. In this study, we explore the utility of \textit{BRAF} V600E IHC in the differential diagnosis of HCL in a series of 121 cases, including 52 non-HCL splenic B-cell lymphomas.

\textbf{Materials and Methods}

\textbf{Case Selection}

FFPE tissue samples were identified from the case files of the Cleveland Clinic and University of Pittsburgh Medical Center. Twenty-six cases of HCL were identified, including five bone marrow core biopsy specimens, 11 bone marrow aspirate clot sections, nine spleens, and one mediastinal mass. Additional cases included 44 splenectomy samples involved by SMZL, 22 bone marrow core biopsy specimens involved by chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), 21 plasma cell neoplasms (PCNs) in bone marrow core biopsy specimens, six splenectomy samples involved by unclassifiable splenic lymphomas, and two splenectomy samples involved by HCLv.

\textbf{IHC}

IHC was performed using the mouse monoclonal anti-\textit{BRAF} V600E antibody (clone VE1; Spring Bioscience, Pleasanton, CA) diluted 1:175 with Ventana antibody diluent with casein and incubated for 16 minutes. Automated staining used XT OptiView DAB IHC v3 software on a Ventana BenchMark XT and detection with multimer-based OptiView DAB IHC with AMP (Ventana Medical Systems, Tucson, AZ). IHC staining was scored as positive with diffuse cytoplasmic staining of the tumor cells, while isolated nuclear staining seen in rare cases was interpreted as negative since this pattern was also seen in the case-specific negative control.

\begin{table}
\caption{Results of \textit{BRAF} V600E Immunohistochemistry (IHC)}
\begin{tabular}{ll}
\hline
\textbf{Diagnosis} & \textbf{No. (\%)} Positive for \textit{BRAF} V600E IHC \\
\hline
Hairy cell leukemia (n = 26) & 26 (100) \\
Hairy cell leukemia variant (n = 2) & 0 \\
Splenic marginal zone lymphoma (n = 44) & 1 (2) \\
Unclassifiable splenic lymphoma (n = 6) & 0 \\
Chronic lymphocytic leukemia (n = 22) & 0 \\
Plasma cell neoplasm (n = 21) & 0 \\
\hline
\end{tabular}
\end{table}

\textbf{Image II} Hairy cell leukemia (HCL). \textbf{A}, Bone marrow core biopsy specimen involved by HCL (H&E, x500). \textbf{B}, Strong and diffuse positive staining for \textit{BRAF} V600E by immunohistochemistry (x500).
**BRAF Mutational Analysis**

In a subset of cases, **BRAF** mutation status was analyzed by allele-specific PCR and/or Sanger sequencing. PCR (BRAF RGQ assay; Qiagen, Manchester, UK) was performed on the Rotor Gene Q (Qiagen) following the manufacturer’s instructions. Sanger sequencing of **BRAF** exon 15 was performed using the Applied Biosystems 3730 DNA analyzer (Life Technologies, Grand Island, NY) as previously described.

**Results**

Results of **BRAF** V600E IHC in 121 cases are summarized in Table 1. All HCL cases (26/26 [100%]) were positive for **BRAF** V600E with diffuse, strong cytoplasmic staining. Similar staining results were seen in all sample types, including spleens, decalcified bone marrow trephine biopsy specimens, and bone marrow clot sections.

Two cases of HCLv were negative for **BRAF** V600E IHC. All cases of CLL/SLL (n = 22), PCNs (n = 21), and unclassifiable splenic lymphoma (n = 6) were negative for **BRAF** V600E. One case of SMZL (1/44 [2%]) was positive for **BRAF** V600E by IHC, with strong cytoplasmic staining similar to that seen in HCL. This case, from a 78-year-old man who had massive splenomegaly (2,250 g), showed a dimorphic white pulp growth pattern on routine H&E sections and cytologic features typical of SMZL. Flow cytometric studies showed a monotypic B-cell population coexpressing CD5 and CD23, and immunohistochemical stains showed the neoplastic cells to be negative for cyclin D1 and LEF1. Considering all 121 cases, **BRAF** V600E IHC had a sensitivity of 100% and a specificity of 98.9% for HCL.

![Image 1](image1.png) Hairy cell leukemia variant. A, Peripheral blood lymphocytes are small to medium sized with nucleoli and cytoplasmic projects (Wright-Giemsa, ×600). B, Splenectomy specimen showing diffuse red pulp involvement (H&E, ×500). C, Negative staining for **BRAF** V600E by immunohistochemistry (×500).
**Image 3** Chronic lymphocytic leukemia (CLL). 
A, CLL involving bone marrow (H&E, ×500). 
B, Negative staining for BRAF V600E by immunohistochemistry (×500).

**Image 4** Splenic marginal zone lymphoma (SMZL) with BRAF V600E mutation. 
A, Splenectomy specimen involved by SMZL showing marked white pulp expansion (H&E, ×40). 
B, White pulp nodules contain small cores of small lymphocytes with prominently expanded marginal zones (H&E, ×200). 
C, Positive staining for BRAF V600E (×200).
**Table 2**

Concordance of Molecular Results With Immunohistochemistry (IHC) Results for the BRAF V600E Antibody

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BRAF Positive by Molecular Studies, No.</th>
<th>BRAF Negative by Molecular Studies, No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF IHC positive (n = 22)</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>BRAF IHC negative (n = 20)</td>
<td>0</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 3**

Results of Prior Studies

<table>
<thead>
<tr>
<th>Study</th>
<th>SMZL</th>
<th>Unclassifiable Splenic Lymphoma</th>
<th>HCLv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andrilis et al 28</td>
<td>0/8</td>
<td>0/6</td>
<td>0/2</td>
</tr>
<tr>
<td>Akarca et al 23</td>
<td>0/3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Wang et al 20</td>
<td>0/6</td>
<td>NA</td>
<td>0/3</td>
</tr>
</tbody>
</table>

HCLv, hairy cell leukemia variant; IHC, immunohistochemistry; NA, not assessed; SMZL, splenic marginal zone lymphoma.

**Discussion**

**BRAF** mutational analysis using either allele-specific PCR or Sanger sequencing was performed in 21 cases of HCL and 21 cases of non-HCL, including six CLLs/SLLs, three PCNs, six SMZLs (including the one IHC-positive case), four unclassifiable splenic lymphomas, and two cases of HCLv. All HCL cases (21/21 [100%]) were positive for the **BRAF** mutation **Table 2**. Of the 21 non-HCL cases, the SMZL case with **BRAF** V600E by IHC was confirmed to contain a c.1799 T>A V600E mutation by Sanger sequencing, while the remaining 20 were negative for the mutation. Results of IHC therefore showed complete concordance with molecular methods.

In this study, each of 26 HCLs (100%) were positive for **BRAF** V600E by IHC vs one (1%) of 95 non-HCL cases. Results of IHC were completely concordant with molecular studies in all cases examined. Staining in all positive cases was strong and diffuse with minimal background, confirming the results of others using similar detection methods. Each of 22 CLLs/SLLs and 21 PCNs involving bone marrow, which are also in the differential diagnosis of HCL, were negative. Significant nonspecific staining was not seen, highlighting the applicability of **BRAF** V600E IHC in bone marrow samples with this protocol.

**BRAF** V600E staining was positive in one (2%) of 44 SMZLs and negative in each of six (0%) unclassifiable splenic lymphomas. The mutated SMZL case was somewhat unusual, in that flow cytometry identified a CD5-positive, CD23-positive phenotype suggestive of CLL. In light of the clinical presentation with massive splenomegaly, the morphologic features on routine H&E, and absence of LEF1 staining, however, this case was classified as SMZL rather than CLL/SLL. Most important, the markedly nodular growth pattern observed in this splenectomy specimen clearly did not represent an HCL. Only a limited number of SMZL and unclassifiable splenic lymphomas have been previously examined to date **Table 3**. This report therefore represents the largest series of non-HCL splenic lymphomas examined for **BRAF** V600E by IHC, as well as the first reported case of SMZL containing a **BRAF** V600E mutation. Recently, however, a case of unclassifiable splenic lymphoma containing **BRAF** V600E was described. Taken together, these findings illustrate that, although rare, non-HCL splenic-based lymphomas may show **BRAF** V600E by IHC and/or molecular methods.

HCLv, recognized as a provisional entity in the 2008 World Health Organization classification, also presents with splenomegaly and a diffuse and interstitial growth pattern in the bone marrow that resembles classic HCL. In the peripheral blood, HCLv frequently shows prominent nucleoli and a marked leukocytosis, which are rarely seen in classic HCL. Moreover, flow cytometric studies show HCLv to lack CD25 and, usually, CD123. When peripheral blood is available for morphology and flow cytometry, the distinction between these two diagnoses is relatively straightforward. Addressing this differential diagnosis from bone marrow trephine biopsy specimens, however, often remains challenging. HCLv has been shown to lack the **BRAF** V600E by molecular methods. In the current study, two cases of HCLv, each confirmed by both splenic and bone marrow morphology, lacked **BRAF** V600E expression. Similar results have also been reported by IHC.
in a small number of HCLv tested (Table 3). These findings confirm the utility of BRAF V600E IHC in addressing this differential diagnosis from core biopsy material.

In conclusion, this report highlights the utility of BRAF V600E IHC in the differential diagnosis of HCL and other splenic-based lymphomas, especially for cases in which the most representative tumor is found in FFPE tissue. This technique allows for rapid, readily interpreted, sensitive, and specific detection of this abnormality using methods available in most diagnostic laboratories and that was completely concordant with the molecular results. Because at least rare examples of BRAF-mutated non-HCL lymphomas do exist, however, results remain best interpreted in the context of morphology and an appropriate panel of other routine diagnostic markers.

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References