Investigation of the *BRAF* V600E Mutation by Pyrosequencing in Lymphoproliferative Disorders

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**Key Words:** Pyrosequencing; Lymphoma; Hairy cell leukemia; *BRAF* mutations; PTLD

**Abstract**

The presence of the *BRAF* c.1799T>A V600E mutation was recently described in cases of hairy cell leukemia (HCL) but not in other common lymphomas. However, many uncommon subtypes of lymphoma have not been studied. We designed a *BRAF* pyrosequencing assay specific for the V600E mutation, which has a sensitivity of 5% and is applicable to paraffin-embedded tissue. DNA was sequenced in 9 cases of HCL; 6 cases of variant HCL; 10 cases each of nodal marginal zone lymphoma (NMZL), extranodal marginal zone lymphoma (ENMZL), posttransplantation lymphoproliferative disorder (PTLD), and large granular lymphocyte (LGL) proliferations; 11 cases of peripheral T-cell lymphoma (PTCL); and 12 cases of anaplastic large cell lymphoma (ALCL). All (100%) cases of HCL were positive for *BRAF* mutations. No mutations were identified in variant HCL, NMZL, ENMZL, PTLD, PTCL, ALCL, or LGL proliferations. Among lymphoproliferative disorders, *BRAF* mutations are restricted to HCL.

Hairy cell leukemia (HCL) is a distinctive mature B-cell neoplasm that primarily involves the bone marrow, spleen, and peripheral blood and has a relatively indolent course. The vast majority of patients manifest marked splenomegaly, progressive pancytopenia, and only a small number of circulating neoplastic cells. The leukemic cells have a distinctive morphologic appearance, including abundant cytoplasm with circumferential “hairy” projections and a characteristic immunophenotypic profile including coexpression of CD11c, CD25, CD103, and Annexin A1 (ANXA1). The latter antibody has been shown to be a highly specific marker for HCL and was included in the 2008 World Health Organization (WHO) classification as a valid marker to differentiate HCL from other B-cell lymphoproliferative disorders, especially hairy cell leukemia variant (HCLv) and splenic marginal zone lymphoma (SMZL).

Gene expression studies of HCL have shown a distinctive molecular signature that, at least in part, explains some of the morphologic and clinical findings associated with the disease. However, until recently, no chromosomal or molecular abnormality was specifically associated with HCL. This situation changed when Tiacci et al identified a heterozygous point mutation on codon 15 of the *BRAF* gene that results in the substitution of glutamic acid for valine at position 600 of the *BRAF* protein (*BRAF* c.1799T>A, p.V600E) in 48 cases of HCL. The *BRAF* V600E mutation was detected in that study using whole exome and Sanger sequencing of enriched, purified leukemic cells from peripheral blood samples. Other investigators have used allele-specific polymerase chain reaction (PCR), high-resolution melting curve analysis (HRMA), real-time PCR, pyrosequencing, and most recently a commercial
The objectives of the current study were to describe a specific BRAF V600E pyrosequencing protocol to confirm the presence of the BRAF V600E mutation in HCL and to investigate its presence in other uncommon subtypes of lymphoproliferative disorders that were not included in the original series.

Materials and Methods

Case Selection

Following approval for the study by the University of Nebraska Institutional Review Board, a natural language search of the pathology department files was conducted to identify cases of HCL. Cases of HCL with available pathological material including H&E-stained slides, immunostains, Wright-Giemsa–stained bone marrow and peripheral blood smears, pathology reports, clinical data, and the results of ancillary studies were retrieved. The originally assigned diagnoses were reviewed by 3 of the authors (J.A.L., P.A., T.C.G.), without knowledge of the BRAF mutation status, using the diagnostic criteria established in the 2008 WHO classification.1 Briefly, HCL cases had the classic morphologic features (oval to indented nucleus, abundant clear cytoplasm with “hairy” projections, diffuse infiltration pattern) and coexpressed CD11c, CD25, and CD103 on flow cytometry or were positive for ANXA1 on immunohistochemical staining, or both. HCLv was characterized by the presence of a B-cell lymphoproliferative disorder resembling HCL but showing 1 or more variant features defined in the WHO criteria including leukocytosis, absence of monocytopenia, prominent nucleoli, convoluted nuclei, or a variant immunophenotype, including negativity for CD25 and/or absence of ANXA1 expression. For cases in which only formalin-fixed paraffin-embedded (FFPE) tissue was available for molecular testing, the extent of tumor involvement was assessed independently by the reviewers, using a combination of the H&E-stained slides and immunostains for CD20 (L26, DAKO, Carpinteria, CA) and ANXA1 (MRQ-3, Cell Marque, Rocklin, CA). CD25 immunostaining was performed on 2 cases (4C9, Leica Microsystems, Buffalo Grove, IL) without flow cytometric results. Immunoperoxidase staining was performed according to standard laboratory procedures. For cases in which frozen cell suspensions were available for testing, the percentage of tumor involvement was obtained after reviewing the flow cytometry histograms. Only HCL and HCLv cases with available nondecalcified FFPE tissue or frozen cell suspensions containing at least 10% of atypical lymphoid cells were tested.

In addition, DNA from cases registered in the Nebraska Lymphoma Study group database, previously chosen for gene expression profiling because of the presence of 70% or more atypical lymphoid cells, were studied, including cases of NMZL, ENMZL, PTLD, PTCL, and ALCL.
Finally, cases of LGL proliferations with available frozen cell suspensions were identified in the department database. These cases have been previously studied with flow cytometry and shown to contain more than 10% atypical lymphoid cells.

DNA Extraction

Sections cut from FFPE blocks of nondecalcified tissue to obtain 1 cm² of tissue were placed in 2 mL of microcentrifuge tubes and deparaffinized using CitriSolv solution (Decon Labs, King of Prussia, PA) and 100% ethanol. The tissue was resuspended in 180 μL of buffer ATL (Qiagen, Valencia, CA) to which 20 μL of proteinase K (Qiagen) were added. The mixture was incubated overnight at 50°C followed by a final incubation at 90°C for 60 minutes to inactivate proteinase K. Genomic DNA extraction was performed on a QIAcube instrument (Qiagen) using the QIAamp DNA FFPE tissue kit (Qiagen) according to the manufacturer’s directions.

Frozen cell suspensions obtained at the time of tissue biopsy or flow cytometric immunophenotyping were retrieved from the –80°C freezer. The bone marrow aspirate specimens that were analyzed using flow cytometry had been previously separated with Ficoll-Hypaque gradient centrifugation according to standard laboratory procedures. For fresh or frozen tissues, genomic DNA was extracted using a phenol/chloroform solution following proteinase K digestion at 50°C for 1 hour. The DNA concentration was measured with spectrophotometry and adjusted to a final concentration of 0.005 μg/μL in ×1 Tris ethylenediaminetetraacetic acid.

PCR Amplification

Primers were chosen using the Pyrosequencing Assay by Design software (Qiagen). PCR amplification was performed on a 151–base pair region of exon 15 in the BRAF gene including codon 600 using forward (5'-TGATTTTTGGTCTAGCTACA-3') and reverse (5'-biotin CTAGTA-ACTCAGCAGCATCTCAGG-3') primers (Sigma, St Louis, MO). The reverse primer was biotinylated at the 5’ end to facilitate product isolation for the pyrosequencing reaction. Each PCR mix contained 0.01 μg of DNA, 0.3 μmol/L of each primer, 0.2 mmol/L of each dNTP (Invitrogen, Grand Island, NY), 3.0 mmol/L magnesium chloride (Qiagen), and 0.8 U of HotStar Taq polymerase (Qiagen) in ×1 PCR buffer (Qiagen) for a total volume of 25 μL. PCR was performed on a BIO-METRA thermocycler (Lab tecpo, Horsham, PA) according to the following protocol: initial denaturation at 95°C for 15 minutes, 45 cycles of 53°C for 20 seconds, 72°C for 20 seconds, and 95°C for 20 seconds, followed by a final extension at 72°C for 5 minutes.

Pyrosequencing

Single-stranded DNA templates for pyrosequencing were obtained with the assistance of the PyroMark Q24 Vacuum Prep Workstation (Qiagen) according to manufacturer’s instructions. Briefly, 10 μL of PCR product was immobilized on streptavidin-coated Sepharose high-performance beads (GE Healthcare, Piscataway, NJ) and processed to obtain single-stranded DNA. This DNA template was then incubated with 25 μL of 0.3 μmol/L sequencing primer (5'-TGATTTTTGGTCTAGCTACA-3') on the PyroMark Q24 heat-block at 80°C to 85°C for 2 minutes. Pyrosequencing was performed using the following dispensation order (CGAT-GAGTC) on a PyroMark Q24 system using software version 50-0416, revision 2.0.6, and PyroMark Gold Q96 reagent kit (Qiagen) following the manufacturer’s guidelines.

We have determined the sensitivity of the assay to be 5% using the HT29 colonic cell line with mutant DNA diluted in wild-type DNA from samples of peripheral blood or placental tissue. Samples showing more than 5% incorporation of any nucleotide outside the wild-type sequence were considered positive for the presence of a mutation.

Results

Nine cases of HCL with available pathologic material containing more than 10% of neoplastic cells were available for mutation testing. Detailed clinical information and results of the immunophenotype are shown in Table 1. In 2 cases, flow cytometry immunophenotyping was not performed at the time of diagnosis and therefore the expression status of CD11c, CD25, or CD103 was unknown. However, in addition to the presence of classic clinical and morphologic features, the 2 cases expressed CD20, CD25, and ANXA1 on immunohistochemistry, confirming the diagnosis of HCL. A BRAF V600E mutation was identified in all cases of HCL by means of pyrosequencing (Figure 1). For heterozygous mutations, the expected percentage for a mutation is about 50% of the tumor cellularity. In our series, the average T>A mutation on HCL cases was 19.8%, which correlates with half of the average 47.3% of estimated neoplastic cells.

Six cases of variant HCL were identified (Table 1). Five cases did not express CD25, of which 2 also had leukocytosis and 2 others were negative for ANXA1 expression. The remaining case expressed CD25 but had leukocytosis, and the neoplastic cells were negative for ANXA1 expression. The BRAF V600E mutation was absent in all 6 cases of HCLv.

None of the cases of NMZL, ENMZL, PTLD, PTCL, ALCL, or LGL proliferations contained the BRAF V600E mutation (Table 2). Cases of PTLD were seen after solid organ transplantation (heart 2, kidney 3, liver 3, and lung 2) at a median interval of 28.5 months after transplantation (range, 6 months to 8 years) and were characterized by the presence of an Epstein-Barr virus–positive monomorphic cellular proliferation. The PTCL group included 2 cases of...
Table 1
Results of *BRAF* V600E Mutation Analysis in HCL and the Variant HCL Disorder

<table>
<thead>
<tr>
<th>Age, y/Sex</th>
<th>Organ</th>
<th>Tumor, %</th>
<th>WBC, 1,000 ×10⁹/L</th>
<th>CD11c</th>
<th>CD25</th>
<th>CD103</th>
<th>ANXA1</th>
<th>DNA Source</th>
<th><em>BRAF</em> V600E Mutation, No. (%)</th>
<th>Mutant, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hairy cell leukemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46/F</td>
<td>BM</td>
<td>40</td>
<td>7.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FFPE</td>
<td>Positive</td>
<td>19</td>
</tr>
<tr>
<td>62/F</td>
<td>BM</td>
<td>70</td>
<td>6.0</td>
<td>ND</td>
<td>+</td>
<td>*</td>
<td>ND</td>
<td>FFPE</td>
<td>Positive</td>
<td>6</td>
</tr>
<tr>
<td>93/F</td>
<td>BM</td>
<td>50</td>
<td>1.3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FFPE</td>
<td>Positive</td>
<td>34</td>
</tr>
<tr>
<td>77/F</td>
<td>BM</td>
<td>30</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FFPE</td>
<td>Positive</td>
<td>14</td>
</tr>
<tr>
<td>43/M</td>
<td>BM</td>
<td>60</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>FFPE</td>
<td>Positive</td>
<td>27</td>
</tr>
<tr>
<td>55/M</td>
<td>Bone</td>
<td>90</td>
<td>6.8</td>
<td>ND</td>
<td>+</td>
<td>*</td>
<td>ND</td>
<td>FFPE</td>
<td>Positive</td>
<td>39</td>
</tr>
<tr>
<td>45/M</td>
<td>BM</td>
<td>11</td>
<td>3.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NTA</td>
<td>Frozen</td>
<td>Positive</td>
<td>10</td>
</tr>
<tr>
<td>64/F</td>
<td>BM</td>
<td>38</td>
<td>5.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NTA</td>
<td>Frozen</td>
<td>Positive</td>
<td>19</td>
</tr>
<tr>
<td>56/M</td>
<td>BM</td>
<td>37</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NTA</td>
<td>Frozen</td>
<td>Positive</td>
<td>10</td>
</tr>
</tbody>
</table>

Hairy cell leukemia variant

<table>
<thead>
<tr>
<th>Age, y/Sex</th>
<th>Organ</th>
<th>Tumor, %</th>
<th>WBC, 1,000 ×10⁹/L</th>
<th>CD11c</th>
<th>CD25</th>
<th>CD103</th>
<th>ANXA1</th>
<th>DNA Source</th>
<th><em>BRAF</em> V600E Mutation, No. (%)</th>
<th>Mutant, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>70/M</td>
<td>PB</td>
<td>51</td>
<td>7.3</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>Frozen</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>77/F</td>
<td>PB</td>
<td>80</td>
<td>NA</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>NTA</td>
<td>Frozen</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>72/F</td>
<td>BM</td>
<td>20</td>
<td>56.4</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>NTA</td>
<td>FFPE</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>83/F</td>
<td>BM</td>
<td>30</td>
<td>9.5</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>FFPE</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>69/M</td>
<td>BM</td>
<td>25</td>
<td>32.0</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>NTA</td>
<td>FFPE</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>59/F</td>
<td>Spleen</td>
<td>80</td>
<td>49.9</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>FFPE</td>
<td>Negative</td>
<td>0</td>
</tr>
</tbody>
</table>

ANXA1, Annexin A1; BM, bone marrow; PB, peripheral blood; FFPE, formalin-fixed paraffin-embedded; HCL, hairy cell leukemia; NA, not available; ND, not done; NTA, no FFPE tissue available.

* An immunohistochemical stain for CD25 was performed on FFPE in these cases; in all others, CD11c, CD25, and CD103 expression was assessed with flow cytometry.

Figure 1
Detection of *BRAF* V600E mutation in lymphoproliferative disorders with A and T peaks at codon 600 highlighted. The nucleotide dispensation is indicated at the bottom of the pyrogram. Dispensations at position 2 (G), 5 (G), 8 (T), and 9 (C) are used as controls to determine the peak size (scale factor) of a single nucleotide incorporation. A, Pyrogram of a nodal marginal zone lymphoma showing a wild-type *BRAF* sequence. B, Pyrogram of a case of hairy cell leukemia showing a heterozygous *BRAF*c.1799T>A mutation (34% mutant sequence).

Table 2
Results of *BRAF* V600E Mutation Analysis in Other Lymphoproliferative Disorders

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Cases</th>
<th>Median Age, y</th>
<th>DNA Source</th>
<th><em>BRAF</em> V600E Mutation, No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nodal marginal zone lymphoma</td>
<td>10</td>
<td>68</td>
<td>Frozen</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Extranodal marginal zone lymphoma</td>
<td>10</td>
<td>62</td>
<td>Frozen</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Posttransplantation lymphoproliferative disorder, monomorphic type</td>
<td>10</td>
<td>20</td>
<td>Frozen</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PTCL</td>
<td>11</td>
<td>76</td>
<td>Frozen</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ALC</td>
<td>12</td>
<td>32</td>
<td>Frozen</td>
<td>0 (0)</td>
</tr>
<tr>
<td>T/NK-LGL proliferations</td>
<td>10</td>
<td>64</td>
<td>Frozen</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

ALCL, anaplastic large cell lymphoma; PTCL, peripheral T-cell lymphoma; LGL, large granular lymphocyte; NK, natural killer.
angioimmunoblastic T-cell lymphoma and 9 cases of peripheral T-cell lymphoma, not otherwise specified. Among the ALCL cases, 9 showed anaplastic lymphoma kinase (ALK) expression, whereas the remaining 3 cases were ALK negative. Immunophenotyping of the LGL proliferations demonstrated a natural killer cell phenotype in 4 cases, while the remaining 6 cases showed a T-cell phenotype.

The total turnaround time of the BRAF pyrosequencing assay was approximately 4 hours, including 1 hour and 45 minutes of hands-on time, 2 hours for the PCR amplification on a thermocycler, and 22 minutes on the pyrosequencer.

**Discussion**

We have described a pyrosequencing assay with new amplification primers specific for detecting BRAF V600E mutation in lymphoproliferative disorders. The assay has a rapid turnaround time and a sensitivity of about 5% of mutant alleles and works on paraffin-embedded nondecalcified material.

A significant discovery in HCL was the description of the BRAF c.1799T>A p.V600E mutation in 48 patients using whole exome and Sanger sequencing. In that study, the analysis was restricted to cases with at least 30% of neoplastic cells (15% mutant DNA), the reported threshold for detecting heterozygous mutations with Sanger sequencing. However, techniques that are more sensitive than Sanger sequencing may allow for the detection of less common mutations involving the analyzed gene segment, depending on the primers and dispensation order used. Recently, a pyrosequencing protocol was reported for HCL that used a commercial BRAF kit. However, neither the methodology nor the quantitative sensitivity was described in detail. Furthermore, the primer sequences used were not included in the report because they are proprietary information of the manufacturer. Other investigators have used a pyrosequencing approach to detect BRAF exon 15 mutation in HCL with results similar to ours.

This study demonstrated the presence of a BRAF V600E mutation in HCL and its absence in HCLv, supporting the observations made in previous reports. Our findings are in agreement with those of other investigators who have shown that B-cell lymphoproliferative disorders usually considered in the differential diagnosis of HCL, including SMZL, HCLv, and SLLU, were negative for the BRAF V600E mutation.

In 2009, Arons et al described an interesting subgroup of HCL with IGHV4-34 gene usage. More recently, these investigators demonstrated that all the cases of HCL expressing IGHV4-34 as well as the HCLv cases demonstrated a wild-type BRAF exon 15. Moreover, almost 50% of HCL cases showing a wild-type BRAF exon 15 demonstrated preferential use of the IGHV4-34 family. These authors previously showed that cases of otherwise classic HCL expressing IGHV4-34 had greater WBC counts, lower response rate, and shorter overall survival. Although information on IGH family usage is not available in other series, these findings suggest that a subset of otherwise typical HCL, a different molecular mechanism may be present.

To date, some subtypes of lymphoma have not been investigated for the presence of BRAF mutations. In the current study, we showed the absence of a BRAF V600E mutation in cases of NMZL, ENMZL, monomorphic PTLD, PTCL, ALCL, and LGL proliferations, findings that support the high specificity for the BRAF V600E mutation in the diagnosis of HCL. Rare examples have been seen of acute lymphoblastic leukemia, multiple myeloma, B-cell chronic lymphocytic leukemia/lymphoma, B-cell prolymphocytic leukemia, and other non-Hodgkin lymphomas reported to harbor the BRAF V600E mutation.

This mutation has also been demonstrated in approximately 57% of patients with Langerhans cell histiocytosis and is observed in numerous solid tumors. Therefore an appropriate B-cell infiltrate should be identified before concluding that HCL is present in the bone marrow. Although

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investigation of the BRAF exon 15 mutation status is likely to become an adjuvant test in the laboratory diagnosis of HCL, the results of the mutation screening should be interpreted in the context of other morphologic and immunophenotypic findings.

Characteristically HCL presents with a low number of circulating neoplastic cells and has poorly cellular bone marrow aspirates because of the marked reticulin fibrosis. FFPE tissue of an aspirate or clot section containing an adequate amount of neoplastic cells can be used to identify the BRAF mutation. However, corresponding core biopsy sections treated with some acid-decalcifying solutions cannot be used for molecular studies because of depurination of the DNA. Recently, a monoclonal BRAF V600E mutation–specific antibody (VE1) was developed and tested in cases of intracerebral metastases of solid tumors, primary papillary thyroid carcinomas, and primary central nervous system neoplasms, revealing a high correlation between immunohistochemistry staining and BRAF mutation status. The potential diagnostic role of this monoclonal antibody in the evaluation of bone marrow samples involved by lymphoproliferative disorders deserves further investigation. Until that information is available, several molecular techniques for detecting the BRAF V600E mutation are being used.

In summary, we have described herein a new pyrosequencing assay specific for investigating BRAF V600E mutations in lymphoproliferative disorders. The assay sensitivity of about 5% of mutant alleles coupled with a rapid turnaround time make it particularly useful in the clinical setting. Our data confirm that among lymphoproliferative disorders, BRAF V600E mutation is restricted to HCL. The adjunctive investigation of the BRAF mutation status may further aid in the differential diagnosis of B-cell lymphoproliferative disorders.

References


