SIMPLE GENETIC DIAGNOSIS OF HAIRY CELL LEUKEMIA
BY SENSITIVE DETECTION OF THE BRAF-V600E MUTATION

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Running title: Detection of BRAF V600E mutation in HCL

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Hairy cell leukemia (HCL) is a distinct clinico-pathological entity that responds well to purine analogs but is sometimes difficult to differentiate from HCL-like disorders (e.g., splenic marginal zone lymphoma and HCL-variant). We recently identified the BRAF-V600E mutation as the disease-defining genetic event in HCL. Hereby, we describe a new, simple and inexpensive test for a genetics-based diagnosis of HCL in whole-blood samples, that detects BRAF-V600E through a sensitive allele-specific polymerase-chain-reaction qualitative assay followed by agarose-gel electrophoresis. This approach detected BRAF-V600E in all 117 leukemic HCL samples investigated containing as few as 0.1% leukemic cells. BRAF-V600E was detected at different time points during the disease course, even post-therapy, pointing to its pivotal role in HCL pathogenesis and maintenance of the leukemic clone. Conversely, 112 non-HCL chronic B-cell neoplasms, including 76 HCL-like disorders, were invariably negative for BRAF-V600E. This molecular assay is a powerful tool for improving the diagnostic accuracy in HCL.
Introduction

Hairy cell leukemia (HCL) is a distinct entity usually characterized by splenomegaly (without lymphadenopathy), pancytopenia, and infiltration of bone marrow, spleen and liver by leukemic B cells with “hairy” appearance. In contrast to other chronic B-cell leukemias, HCL cells circulate at low percentages in the blood\(^1\), and exhibit distinct functional features and gene expression profile\(^2,3\).

HCL diagnosis relies on morphological and immunophenotypic criteria\(^4\) that usually allow its distinction from HCL-like disorders of the 2008-World Health Organization (WHO) classification, i.e. splenic marginal zone lymphoma (SMZL) and splenic lymphoma/leukemia unclassifiable (SLLU, including HCL-variant - HCL-v)\(^5\). A correct diagnosis is critical since purine analogs (pentostatin and cladribine) are highly effective only in HCL\(^4\).

The most problematic cases can be diagnosed using Annexin-A1 immunostaining\(^6\)-\(^8\), that we previously reported to be highly sensitive and specific for HCL among B-cell lymphomas\(^6\). However, because Annexin-A1 is also expressed by myeloid and T cells\(^6\), this immunohistochemical staining may be difficult to interpret in bone marrow biopsies with low percentages of HCL cells. Moreover, immunocytochemistry for Annexin-A1 is not readily applicable to routine hematological samples, such as peripheral blood or diluted bone marrow aspirate (due to dry tap), that are also usually poor in HCL cells and rich in neutrophils and T cells.

An ideal solution would be a sensitive and specific test for a genetics-based diagnosis of HCL. We recently identified \(BRAF\)-V600E as the HCL-defining genetic lesion (present in all HCL cases, absent in other B-cell neoplasms)\(^9\) by Sanger sequencing of \(BRAF\) exon-15. However, this technique required \(\geq 30\%\) leukemic cells for reliably detecting a clonal heterozygous mutation. Thus, the rare HCL cells typically present in the
blood of most patients had to be purified through cell sorting, a laborious procedure not amenable to a routine diagnostic setting.

Aims of this study were: i) to develop a sensitive, easy and inexpensive test for the routine clinical diagnosis of HCL in blood samples, based on BRAF-V600E detection by allele-specific PCR (AS-PCR) followed by conventional agarose-gel electrophoresis; and ii) to assess the diagnostic accuracy of this test in a large cohort of HCL and HCL-like disorders.
Materials and Methods

Tumor samples

We studied 117 HCL patients: 96 pre-therapy and 21 post-therapy, all with detectable disease. We also investigated 14 HCL patients post-therapy in complete flow-cytometric remission (<0.1% leukemic cells), 112 patients with other B-cell neoplasms (60 SMZL; 16 SLLU, including 11 HCL-v; 31 chronic lymphocytic leukemias-CLL; 5 unclassifiable CD5-negative mature B-cell neoplasms) and 9 healthy blood donors. See Supplementary Material for details. Samples from 23 HCL patients and 38 non-HCL patients were previously reported9. Diagnosis of HCL and non-HCL tumors conformed to the WHO-2008 classification1,5. Patients gave verbal or written consent for the analysis of their sample material in accordance with the Declaration of Helsinki, and the study was approved by the local ethics committee at Perugia University.

Qualitative AS-PCR for \textit{BRAF-V600E}

Briefly, the assay consists of two PCR reactions sharing the same reverse primer (5'-GTAACCTCGACGCATCTCAGGG-3') but differing for the forward primer, which is complementary to either the wild-type (T) or the mutated (A) base causing the V600E replacement. Forward primers were: 5'-AGGTGATTTTGGTCTAGCTACAGA-3' (mutated base in bold) and 5'-AGGTGATTTTGGTCTAGCTACAGT-3' (wild-type base in bold). After mutant-AS-PCR, detection of a band on agarose-gel electrophoresis indicates the presence of the mutation. Conversely, absence of the band indicates lack of the mutation as long as the wild-type-AS-PCR (positive control) gives a readily visible product. See Supplementary Material for details.
Results and Discussion

We first assessed the analytical sensitivity of our AS-PCR in serial dilutions of DNA from a BRAF-V600E homozygous sample with DNA from a BRAF wild-type sample, and established the lower detection limit to be 0.1% of mutant alleles (Fig. 1), corresponding to 0.2% of diploid tumor cells harboring a clonal heterozygous BRAF-V600E mutation (or 0.1% if the mutation is homozygous).

We then analyzed samples from 117 HCL patients (96 pre-treatment; 21 with residual or relapsing disease) and all tested positive (100% diagnostic sensitivity) (Table 1, Fig. 1), including 23 samples previously known to harbor BRAF-V600E by Sanger sequencing. Notably, among the newly reported 94 HCL cases, 21 (15 pre-treatment, 6 post-treatment) had only 0.1%-5% leukemic cells and 11 were analyzed at different time-points after the onset of the disease (range 1-26 years).

These findings demonstrate the excellent analytical and diagnostic sensitivity of our test. They also confirm and extend our previous report that BRAF-V600E occurs and persists over the disease course in virtually all HCL cases, further supporting the view that BRAF-V600E represents the key pathogenetic event in HCL and therefore a new therapeutic target. Indeed, persistence of BRAF-V600E at partial remission or relapse following conventional therapy establishes the rationale for using active-BRAF inhibitors in this setting. Our test may also serve as a new tool (in addition to immunohistochemistry, flow cytometry and immunoglobulin gene rearrangement analysis) to assess minimal residual disease (MRD) following therapy, although the clinical relevance of MRD in HCL remains unclear.

We next evaluated the diagnostic specificity of our test by analyzing blood samples from 9 healthy donors and 14 HCL patients in complete flow-cytometric remission (<0.1%
leukemic cells) post-therapy, and all tested negative (Table 1, Fig. 1). Specificity was also assessed in 112 patients with non-HCL chronic B-cell neoplasms. Because absence\textsuperscript{9} or very rare occurrence\textsuperscript{13-17} of \textit{BRAF}-V600E has been already reported in several B-cell tumors, we focused on HCL-like disorders that, being rare, have been so far poorly investigated. Therefore, we included, among the 112 cases, 76 patients with SMZL and SLLU, of which 62 previously unreported\textsuperscript{9}. Notably, all 112 cases tested negative (Table 1, Fig. 1), showing a 100% diagnostic specificity of our assay and further confirming in a larger patient series the absence of \textit{BRAF}-V600E in HCL-like disorders. Considering that 34 HCL-like cases had \( \geq 40\% \) neoplastic cells and that our test can detect 0.1\% mutant alleles, these data also argue against the presence of small \textit{BRAF}-V600E-mutated subclones (down to 0.5\% of a whole leukemic population representing \( \geq 40\% \) of the sample) in HCL-like disorders. This finding further supports the concept that, among B-cell lymphomas and leukemias, \textit{BRAF}-V600E is the genetic lesion defining HCL\textsuperscript{9}. Although we have collectively analyzed 96 HCL-like disorders (SMZL and SLLU, the latter including 19 HCL-variant) without finding \textit{BRAF}-V600E in any of them (this paper and\textsuperscript{9}), we cannot exclude that this mutation may be rarely found in these and other B-cell neoplasms if a larger number of cases is investigated.

Our diagnostic test is especially useful for patients with a low tumor burden in the blood (as typically occurs in HCL) or bone marrow. In this setting, it appears superior to Annexin-A1 immunostaining, that may be difficult to interpret (due to Annexin-A1 expression by myeloid and T cells) unless a technically demanding double staining for Annexin-1 and a B-cell marker (e.g., PAX5) is performed. Our gel-based AS-PCR is considerably more sensitive than a recently described HRMA (High-Resolution-Melting Analysis)-based PCR, which was applied to fewer HCL samples (\( n=48 \)) containing more (\( \geq 10\% \)) leukemic cells\textsuperscript{18}. Notably, our test detected \textit{BRAF}-V600E in all blood samples
having <10% HCL cells (33/80 samples; 24 pre-therapy; 9 post-therapy) and does not require the expensive instrumentation needed for HRMA.

In conclusion, our sensitive, simple and reliable assay confirms the constant presence of \textit{BRAF}-V600E in HCL (as also reported in \textsuperscript{18,19}) and its absence in HCL-like disorders, and adds to the already available armamentarium for improving the diagnostic accuracy in HCL and HCL-like disorders.

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**Authorship contributions and disclosure of conflicts of interest**

E.T. designed the study, decided the allele-specific PCR strategy, analyzed and interpreted the data, and wrote the manuscript. G.S. developed the allele-specific PCR assay and performed all related experiments, analyzed and interpreted the data, and contributed to write the manuscript. F.F., L.T., A.A., D.C., E.S., P.F.C., C.D.B, A.P., R.F.
and G.I. provided patients’ samples and clinico-pathological data, and commented on the manuscript. A.S. purified leukemic cells from patients’ peripheral blood samples. B.F. led the project, supervised the study and wrote the manuscript. B.F. and E.T. applied for a patent on the clinical use of BRAF mutants in HCL.
References


Table 1. Results of AS-PCR in HCL cases and other B-cell neoplasms

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Leukemic cells</th>
<th>Number of cases</th>
<th>Mutated cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hairy cell leukemia</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Blood, pre-treatment</td>
<td>1%-90%</td>
<td>76</td>
<td>76 (100%)</td>
</tr>
<tr>
<td>- Blood, MRD</td>
<td>0.1%-13%</td>
<td>10</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>- Blood, complete remission</td>
<td>Not detectable</td>
<td>14</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>- Bone marrow biopsies</td>
<td>30%-80%</td>
<td>14</td>
<td>14 (100%)</td>
</tr>
<tr>
<td>- Purified leukemic cells</td>
<td>≥90%</td>
<td>17</td>
<td>17 (100%)</td>
</tr>
<tr>
<td><strong>Splenic marginal zone lymphoma</strong></td>
<td></td>
<td>60</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Splenic lymphoma/leukemia unclassifiable</strong></td>
<td></td>
<td>16*</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Chronic lymphocytic leukemia</strong></td>
<td></td>
<td>31</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>CD5-negative mature B-cell neoplasm unclassifiable</strong></td>
<td></td>
<td>5</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Healthy blood donors</strong></td>
<td>Not present</td>
<td>9</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

MRD, minimal residual disease. *Including 11 HCL-variant.
FIGURE LEGEND

Figure 1. Flow cytometry and AS-PCR assays in HCL and HCL-like disorders

**Top)** Flow cytometry dot plots of a whole-blood sample subjected to red blood cell lysis from a representative HCL patient (upper panels) and of purified peripheral blood leukemic cells from a representative patient with HCL-v (lower panels). HCL cells (CD19+/CD25+ red events in the upper right panel) represent 2% of all nucleated cells (CD45+ black events in the upper left panel). HCL-v cells (CD19+/CD25- and CD19+/CD103+ red events in the lower left and lower right panels, respectively) represent 92% of all cells.

**Bottom)** Conventional agarose-gel electrophoresis of samples from 13 HCL patients (upper panels; 12 pre-treatment, 1 with MRD post-treatment) and 16 HCL-like patients (bottom panels; 6 SLLU, 10 SMZL), after AS-PCR for the mutant allele (1st and 3rd panel from the top) and for the wild-type allele (2nd and 4th panel from the top). In the upper panel, serial dilutions of mutated and wild-type alleles (from 3.1% to 0%) are also included to show the analytical sensitivity of the mutant-AS-PCR (≥0.1% mutated alleles). All HCL samples gave rise to a mutant \textit{BRAF}-V600E band as opposed to none of the HCL-like samples. One of the latter (SMZL case 30), which did not give rise to the wild-type band either, was not evaluable in this particular experiment (shown on purpose), but on repetition turned out to be evaluable (i.e., strong wild-type band) and negative for \textit{BRAF}-V600E (i.e., mutant band not visible). To facilitate the visualization of the results, the gel lane of HCL case 13 was repositioned in the two upper panels and the gel lane of the 50-bp DNA ladder was repositioned in the two lower panels.
FIGURE 1

HCL

SLLU

Mutant *BRAF* alleles

Mutant *BRAF* amplicon

Wild-type *BRAF* amplicon

HCL pre-therapy

HCL MRD

SLLU

SMZL
Simple genetic diagnosis of hairy cell leukemia by sensitive detection of the \textit{BRAF-V600E} mutation

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