Brief report

Simple genetic diagnosis of hairy cell leukemia by sensitive detection of the *BRAF*-V600E mutation

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Hairy cell leukemia (HCL) is a distinct clinicopathologic entity that responds well to purine analogs but is sometimes difficult to differentiate from HCL-like disorders (eg, splenic marginal zone lymphoma and HCL variant). We recently identified the *BRAF*-V600E mutation as the disease-defining genetic event in HCL. In this study, we describe a new, simple, and inexpensive test for genetics-based diagnosis of HCL in whole-blood samples that detects *BRAF*-V600E through a sensitive allele-specific PCR qualitative assay followed by agarose-gel electrophoresis. This approach detected *BRAF*-V600E in all 123 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 after therapy, pointing to its pivotal role in HCL pathogenesis and maintenance of the leukemic clone. Conversely, 115 non-HCL chronic B-cell neoplasms, including 79 HCL-like disorders, were invariably negative for *BRAF*-V600E. This molecular assay is a powerful tool for improving the diagnostic accuracy in HCL. (*Blood*. 2012;119(1):192-195)

Introduction

Hairy cell leukemia (HCL) is a distinct entity usually characterized by splenomegaly (without lymphadenopathy), pancytopenia, and infiltration of BM, 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 morphologic and immunophenotypic criteria[1-4] that usually allow its distinction from HCL-like disorders of the 2008 World Health Organization classification, that is, splenic marginal zone lymphoma (SMZL) and splenic lymphoma/leukemia unclassifiable (SLLU; including HCL variant [HCL-v]).[5] A correct diagnosis is critical because 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] which 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 BM biopsies with low percentages of HCL cells. Moreover, immunocytochemistry for annexin-A1 is not readily applicable to routine hematologic samples, such as peripheral blood or diluted BM aspirate (because of 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,[9] a laborious procedure not amenable to a routine diagnostic setting.

Aims of this study were: (1) 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 (2) to assess the diagnostic accuracy of this test in a large cohort of HCL and HCL-like disorders.

Methods

Tumor samples

We studied 117 HCL patients: 96 before therapy and 21 after therapy, all with detectable disease (at least 0.1% leukemic cells). We also investigated 16 HCL patients after therapy in complete flow cytometric remission ($<$ 0.1% leukemic cells), 112 patients with other B-cell neoplasms (61 SMZL; 18 SLLU, including 11 HCL-v; 31 chronic lymphocytic leukemias [CLL]; 5 unclassifiable CD5-negative mature B-cell neoplasms), and 9 healthy blood donors (see supplemental Methods for details, available on the *Blood* Web site; see the Supplemental Materials link at the top of the

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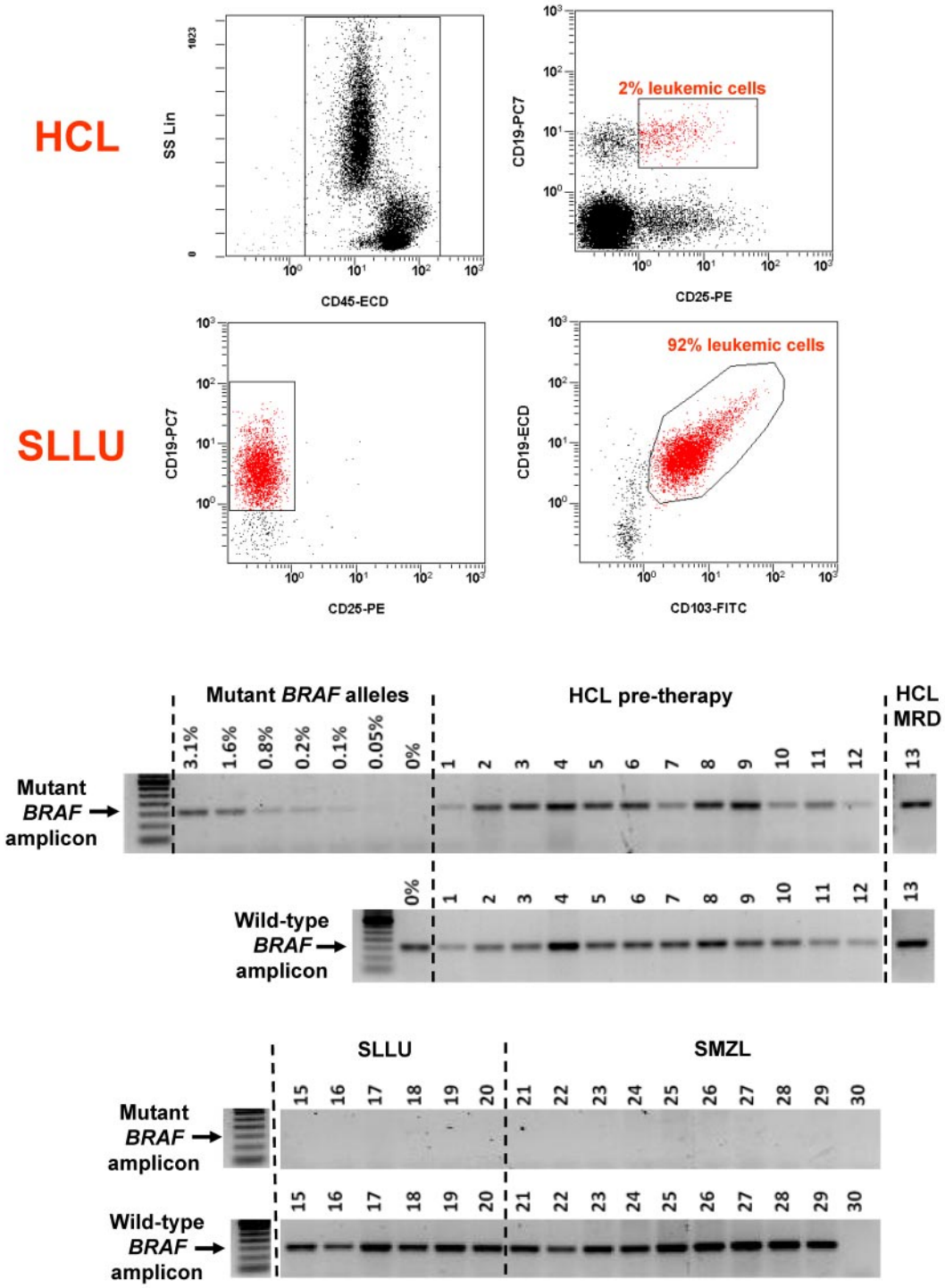

Figure 1. Flow cytometry and AS-PCR assays in HCL and HCL-like disorders. (Top panel) Flow cytometry dot plots of a whole-blood sample subjected to RBC lysis from a representative HCL patient (top graphs) and of purified peripheral blood leukemic cells from a representative patient with HCL-v (bottom graphs). HCL cells ($CD19^+/CD25^+$ red events in the top right panel) represent 2% of all nucleated cells ($CD45^+$ black events in the top left panel). HCL-v cells ($CD19^+/CD25^-$ and $CD19^+/CD103^+$ red events in the bottom left and right panels, respectively) represent 92% of all cells. (Bottom panel) Conventional agarose-gel electrophoresis of samples from 13 HCL patients (top panels; 12 pretreatment, 1 with residual disease after treatment) and 16 HCL-like patients (bottom gels; 6 SLLU, 10 SMZL), after AS-PCR for the mutant allele (1st and 3rd gel from the top) and for the wild-type allele (2nd and 4th gel from the top). In the top-most gels, serial dilutions of mutated and wild-type alleles (from 3.1% to 0% muted alleles) 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 *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 (ie, strong wild-type band) and negative for *BRAF*-V600E (ie, mutant band not visible). To facilitate the visualization of the results, the gel lane of HCL case 13 was repositioned in the 2 top gels and the gel lane of the 50-bp DNA ladder was repositioned in the 2 bottom gels.

online article). Samples from 23 HCL patients and 38 non-HCL patients were previously reported.[9] Diagnosis of HCL and non-HCL tumors conformed to the WHO 2008 classification.[1,5] 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.

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

Sample type	Leukemic cells	No. of cases	Mutated cases, no. (%)
Hairy cell leukemia			
Blood, pretreatment	1%-90%	81	81 (100)
Blood, MRD	0.1%-13%		10 (100)
Blood, complete remission	Not detectable	16	0 (0)
BM biopsies	30%-80%	14	14 (100)
Purified leukemic cells	≥ 90%	17	17 (100)
Splenic marginal zone lymphoma	2%-97%	61	0 (0)
Splenic lymphoma/leukemia unclassifiable	15%-97%	18*	0 (0)
Chronic lymphocytic leukemia	18%-98%	31	0 (0)
CD5-negative mature B-cell neoplasm unclassifiable	15%-97%	5	0 (0)
Healthy blood donors	Not present	9	0 (0)

AS-PCR indicates allele-specific PCR; HCL, hairy cell leukemia; and MRD, minimal residual disease.

*Including 11 HCL variants.

Qualitative AS-PCR for *BRAF*-V600E

Briefly, the assay consists of 2 PCRs sharing the same reverse primer (5′-GTAACTCAGCAGCATCTCAGGG-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′-AGGTGATTTTGGTCTAGCTACAG**A**-3′ (mutated base in bold) and 5′-AGGTGATTTTGGTCTAGCTACAG**T**-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 supplemental Methods 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 (Figure 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 123 HCL patients (101 before treatment; 22 with residual or relapsing disease) and all tested positive (100% diagnostic sensitivity; Table 1, Figure 1), including 23 samples previously known to harbor *BRAF*-V600E by Sanger sequencing.[9] Notably, among the newly reported 94 HCL cases, 24 (17 before treatment, 7 after treatment) had only 0.1% to 5% leukemic cells and 11 were analyzed 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,[9] 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 after conventional therapy establishes the rationale for using active BRAF inhibitors[10] in this setting. Our test may also serve as a new tool (in addition to IHC, flow cytometry, and Ig gene rearrangement analysis[11,12]) to assess minimal residual disease (MRD) after therapy, although the clinical relevance of MRD in HCL remains unclear.[12]

We next evaluated the diagnostic specificity of our test by analyzing blood samples from 9 healthy donors and 16 HCL patients in complete flow cytometric remission (< 0.1% leukemic cells) after therapy, and all tested negative (Table 1, Figure 1). Specificity was also assessed in 115 patients with non-HCL chronic B-cell neoplasms. Because absence[9] or very rare occurrence[13-17] of *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 115 cases, 79 patients with SMZL and SLLU, of which 65 previously unreported.[9] Notably, all 112 cases tested negative (Table 1, Figure 1), showing a 100% diagnostic specificity of our assay and further confirming in a larger patient series the absence of *BRAF*-V600E in HCL-like disorders. Considering that 37 HCL-like cases had ≥ 40% neoplastic cells and that our test can detect 0.1% mutant alleles, these data also argue against the presence of small *BRAF*-V600E–mutated subclones (down to 0.5% of a whole leukemic population representing ≥ 40% of the sample) in HCL-like disorders. This finding further supports the concept that, among B-cell lymphomas and leukemias, *BRAF*-V600E is the genetic lesion defining HCL.[9] Although we have collectively analyzed 99 HCL-like disorders (SMZL and SLLU, the latter including 19 HCL-v) without finding *BRAF*-V600E in any of them (this article and Tiacci et al[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 BM. In this setting, it appears superior to annexin-A1 immunostaining, which may be difficult to interpret (because of annexin-A1 expression by myeloid and T cells) unless a technically demanding double staining for annexin-1 and a B-cell marker (eg, PAX5) is performed. Our gel-based AS-PCR is considerably more sensitive than a recently described high-resolution-melting analysis (HRMA)–based PCR, which was applied to fewer HCL samples (n = 48) containing more (≥ 10%) leukemic cells.[18] Notably, our test detected *BRAF*-V600E in all blood samples having < 10% HCL cells (37 of 88 samples; 27 before therapy; 10 after therapy) and does not require the expensive instrumentation needed for HRMA.

In conclusion, our sensitive, simple, and reliable assay confirms the constant presence of *BRAF*-V600E in HCL (as also reported in Boyd et al[18] and Arcaini et al[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

Contribution: 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 the writing of the manuscript; F.F., L.T., A.A., D.C., E.S., P.F.d.C., C.D.B, A.P., R.F., and G.I. provided patients' samples and clinicopathologic data, and commented on the manuscript; A.S. purified leukemic cells from patients' peripheral blood samples; and B.F. led the project, supervised the study, and wrote the manuscript.

Conflict-of-interest disclosure: E.T. and B.F. applied for a patent on the clinical use of BRAF mutants in HCL. The remaining authors declare no competing financial interests.

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