

## ORIGINAL ARTICLE

# BRAF Mutations in Hairy-Cell Leukemia

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## ABSTRACT

**BACKGROUND**

Hairy-cell leukemia (HCL) is a well-defined clinicopathological entity whose underlying genetic lesion is still obscure.

**METHODS**

We searched for HCL-associated mutations by performing massively parallel sequencing of the whole exome of leukemic and matched normal cells purified from the peripheral blood of an index patient with HCL. Findings were validated by Sanger sequencing in 47 additional patients with HCL.

**RESULTS**

Whole-exome sequencing identified five missense somatic clonal mutations that were confirmed on Sanger sequencing, including a heterozygous mutation in *BRAF* that results in the BRAF V600E variant protein. Since BRAF V600E is oncogenic in other tumors, further analyses were focused on this genetic lesion. The same *BRAF* mutation was noted in all the other 47 patients with HCL who were evaluated by means of Sanger sequencing. None of the 195 patients with other peripheral B-cell lymphomas or leukemias who were evaluated carried the BRAF V600E variant, including 38 patients with splenic marginal-zone lymphomas or unclassifiable splenic lymphomas or leukemias. In immunohistologic and Western blot studies, HCL cells expressed phosphorylated MEK and ERK (the downstream targets of the BRAF kinase), indicating a constitutive activation of the RAF–MEK–ERK mitogen-activated protein kinase pathway in HCL. In vitro incubation of BRAF-mutated primary leukemic hairy cells from 5 patients with PLX-4720, a specific inhibitor of active BRAF, led to a marked decrease in phosphorylated ERK and MEK.

**CONCLUSIONS**

The BRAF V600E mutation was present in all patients with HCL who were evaluated. This finding may have implications for the pathogenesis, diagnosis, and targeted therapy of HCL. (Funded by Associazione Italiana per la Ricerca sul Cancro and others.)

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**H**AIRY-CELL LEUKEMIA (HCL) IS A DISTINCT disease entity that is characterized by an indolent course, marked splenomegaly, progressive pancytopenia in many cases, and rare circulating tumor cells, usually with no lymphadenopathy.<sup>1</sup> The bone marrow, spleen, and liver are characteristically infiltrated by leukemic B cells that have abundant cytoplasm with hairy-looking projections and unique immunophenotypic features.<sup>2,3</sup> Currently, the treatment of HCL is based on highly effective purine nucleoside analogues.<sup>4</sup>

In spite of the remarkable progress in the diagnosis and treatment of HCL during the past 50 years, the underlying genetic alterations that cause the disease remain obscure.<sup>3</sup> Major obstacles to molecular characterization of HCL have been the scarcity of tumor cells available for analysis (since many patients have pancytopenia), the very low proliferative index of leukemic cells and the inability to grow them in immunodeficient mice, and the absence of human cell lines of authentic HCL origin.

No recurrent chromosomal translocations have been identified in HCL.<sup>1</sup> Gene-expression profiling studies revealed a unique molecular signature that in part accounts for the distinctive features of HCL cells, such as their morphologic appearance, adhesion properties, selective homing to extranodal sites, and marrow fibrosis.<sup>5</sup> However, these studies did not pinpoint any recurrent genetic alteration. Similarly, high-density genomewide single-nucleotide polymorphism (SNP) genotyping showed a remarkably balanced genomic profile in HCL.<sup>6</sup>

A powerful approach to a better understanding of the genetic basis of cancer is offered by genomewide massively parallel sequencing of tumor and normal cells from the same patient, a study that is aimed at identifying novel acquired alterations.<sup>7,8</sup> We performed in-solution exome capture followed by massively parallel sequencing to investigate the DNA of purified leukemic and paired normal cells from an index patient with HCL. Our goal was to identify potentially recurrent somatic mutations in protein-coding genes that might shed light on the biologic characteristics of HCL and offer new approaches to diagnosis and treatment.

## METHODS

### TUMOR AND NORMAL SAMPLES

We based our criteria for a diagnosis of HCL on the 2008 World Health Organization classification.<sup>1</sup> All 48 patients who were evaluated had the typical

clinical presentation and morphologic features of HCL and were positive for annexin A1<sup>2</sup> on immunohistochemical analysis, had coexpression of CD11c, CD25, and CD103 on flow cytometry, or both.

Using peripheral-blood samples from the index patient, we performed whole-exome sequencing on purified (by >90%) CD19-positive leukemic cells obtained at the time of disease onset and on purified (by >98%) CD19-negative mononuclear cells obtained after the patient had undergone chemotherapy. (Details of the procedures are available in the Supplementary Appendix, available with the full text of this article at NEJM.org.) The index patient provided written informed consent for genetic analysis under a protocol approved by the ethics committee at the University of Perugia. All other patients provided written or oral informed consent for the analysis of their sample material.

### WHOLE-EXOME SEQUENCING AND BIOINFORMATIC ANALYSES

The preparation of shotgun libraries from the leukemic and nonleukemic genomic DNA obtained from the index patient, followed by in-solution exome capture, was performed with the use of a commercial platform (Agilent) covering 38 Mb of coding exons (approximately 1.22% of the genome). After massively parallel sequencing with the Genome Analyzer IIX (Illumina), candidate somatic mutations were identified according to a bioinformatic pipeline (Fig. 1, and the Supplementary Appendix).

### VALIDATION AND SCREENING OF CANDIDATE MUTATIONS

We used polymerase-chain-reaction (PCR) amplification and direct DNA sequencing of the same samples that were subjected to whole-exome sequencing to verify candidate nonsynonymous somatic variants (i.e., present in the tumor but not in its paired normal DNA) (Tables S1 and S2 in the Supplementary Appendix). Because the sensitivity of Sanger sequencing allows for the detection of heterozygous mutations only when they are present in a major clonal population, sequence variants that were reported in less than 25% of the reads were not included in this validation phase.

PCR and direct DNA Sanger sequencing of *BRAF* exon 15 in the 47 additional patients with HCL and in 195 patients with other peripheral B-cell lymphomas or leukemias were performed with the use of the primers e15F-5'-TACCTAACTCTTCATAA-TGCTTGC-3' and e15R-5'-GTAACCTCAGCAGCATC-

TCAGGG-3'. Cycling conditions (after the initial denaturation step at 94°C for 2 minutes) were a temperature of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 20 seconds for 40 cycles, followed by a final elongation at 72°C for 10 minutes. For 48 diffuse large B-cell lymphomas, primers and cycling conditions are reported in Tables S1 and S2 in the Supplementary Appendix. All 242 samples that we investigated had at least 30% neoplastic cells.

#### IMMUNOHISTOLOGIC AND WESTERN BLOTTING STUDIES

Details regarding the immunohistologic and Western blotting studies for phosphorylated extracellular signal-regulated kinase (phospho-ERK) and phosphorylated mitogen-activated protein-ERK kinase (phospho-MEK) in HCL samples (under basal conditions and after *in vitro* incubation with active BRAF inhibitors) are provided in the Supplementary Appendix.

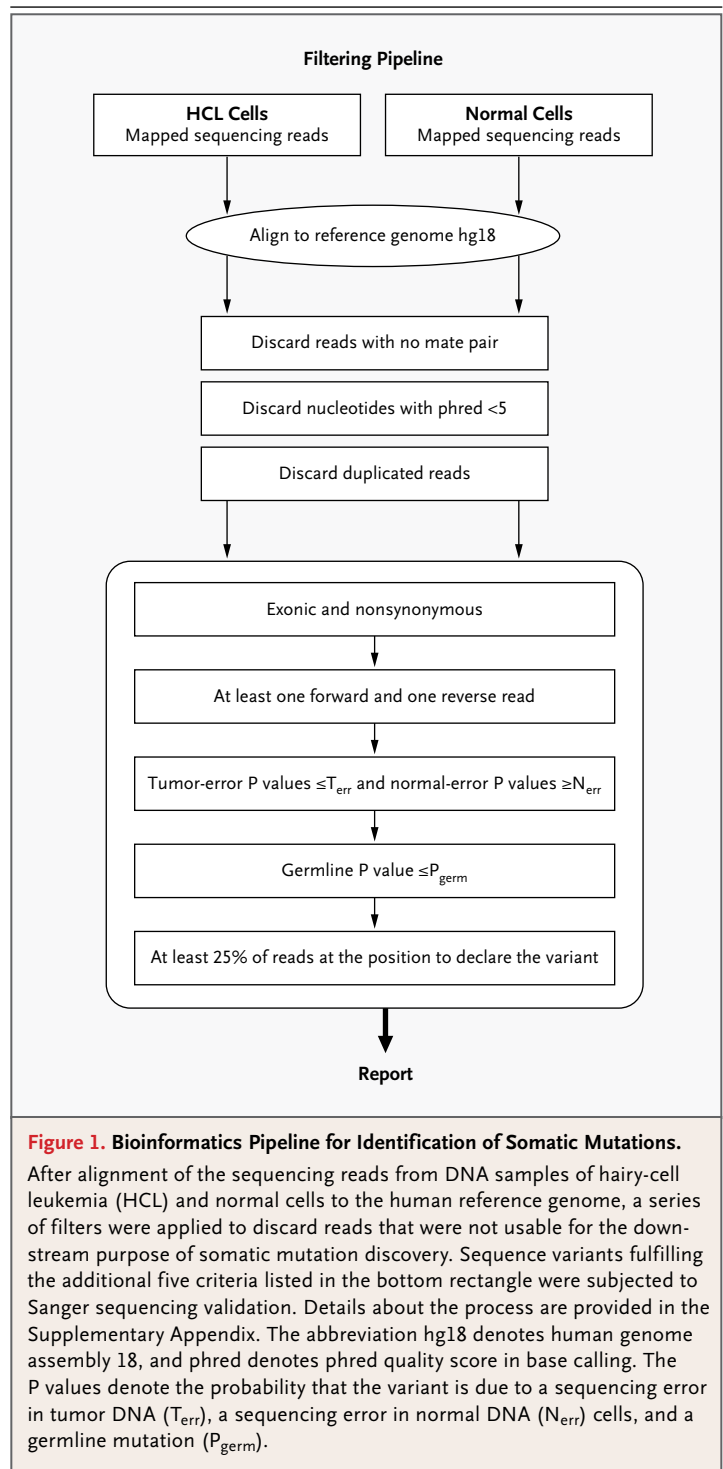
## RESULTS

#### CASE REPORT

A 47-year-old man presented with fever and pneumonia in March 2009. The hemoglobin level was 11.5 g per deciliter, the white-cell count was 2700 per cubic millimeter (with 49% circulating leukemic hairy cells), and the platelet count was 70,000 per cubic millimeter. Splenomegaly was present. A trephine biopsy sample of bone marrow showed typical HCL histologic features.<sup>1</sup> Immunohistochemical analysis revealed leukemic cells that were positive for CD20, DBA44, CD68, and annexin A1, and the expression of CD20, CD11c, CD25, and CD103 was documented on flow cytometry. The patient was started on therapy with pentostatin (deoxycoformycin), which resulted in complete hematologic remission after 5 months of therapy.

#### IDENTIFICATION OF CANDIDATE SOMATIC MUTATIONS

Whole-exome sequencing of genomic DNA from the patient's purified leukemic and nonleukemic cells produced approximately 42.5 million and 42.8 million reads, respectively, of 108 nucleotides (Table S3 in the Supplementary Appendix). After removal of low-quality and duplicate reads, the mean coverage depth of the exome was 71 (for tumor cells) and 70 (for normal cells) (median, 52 and 51, respectively), with 99% of the target exome covered by at least 1 read and 86% by at



least 10 reads. Using the statistical algorithm for variant identification (SAVI) (Supplementary Appendix), we identified five unique nonsynonymous variants that were present specifically in the tumor DNA (27 to 49% of the reads) and targeted BRAF, CSMD3, SLC5A1, CNTN6, and OR8J1 (Tables S4 and S5

in the Supplementary Appendix). All five variants were validated as somatic in origin after Sanger resequencing of paired tumor and normal DNA samples from the same patient. All mutations were heterozygous and introduced amino acid substitutions in the encoded protein (Table S5 in the Supplementary Appendix). The extremely low false discovery rate (nearly 0%) for the SAVI algorithm did not come at the expense of low sensitivity (i.e., the proportion of true variants identified). Indeed, sensitivity, as estimated from the number of known germline polymorphisms that are present in the SNP database (dbSNP) build 130 and that were detected according to the algorithm, was similar to estimates of sensitivity in previous studies<sup>9,10</sup> (Table S6 in the Supplementary Appendix).

#### **BRAF MUTATIONS IN HCL SAMPLES**

Whereas little is known about the biologic role of four of the five affected genes (Table S7 in the Supplementary Appendix), *BRAF* is the most frequently mutated gene that encodes a protein kinase in human cancers.<sup>11</sup> The amino acid substitution of glutamic acid for valine at position 600 of the *BRAF* protein (V600E) that was identified in our patient is a mutational hotspot in melanomas and papillary thyroid cancers, and its oncogenic activity in these cancers has been extensively documented.<sup>12,13</sup> Moreover, dysregulation of the mitogen-activated protein kinase (MAPK) pathways, of which *BRAF* is a component, was previously implicated in the pathogenesis of HCL.<sup>14</sup>

Therefore, we screened 47 additional samples from patients with HCL for this mutation by PCR and direct DNA Sanger sequencing of *BRAF* exon 15. The features of these patients are shown in Table 1. Strikingly, a T→A transversion occurring at position 1860 of the *BRAF* messenger RNA RefSeq NM\_004333.4 and resulting in the V600E variant was found in samples from all 47 patients with HCL in addition to the sample from the index patient. The mutation was somatic in origin, since it was absent in all 10 of the patients in whom matched nonleukemic DNA was evaluated.

In 30 of the 48 patients with the V600E variant (including the index patient), the high purity of leukemic cells (>90%) allowed for the analysis of the zygosity of the mutation without substantial interference of wild-type alleles contributed by contaminating nonleukemic cells. In 26 of these 30 patients, the mutation appeared as a double

peak (Fig. 2A), indicating a heterozygous lesion occurring in all cells of the leukemic clone. In the remaining 4 patients, only the mutant peak was observed (Fig. 2B), pointing to a homozygous or hemizygous clonal event.

In 17 of the 48 patients with HCL, the height of the mutated peak in the chromatogram relative to the proportion of leukemic cells in the analyzed samples (from 30 to 74%) was consistent with the clonal nature of the mutation. In the remaining patient with HCL (Patient 7 in Table 1), the fresh peripheral-blood sample that was initially analyzed and found to be wild type for *BRAF* turned out retrospectively to contain a proportion of leukemic cells that was well below the detection threshold of direct Sanger sequencing for a heterozygous clonal mutation (about 30% in our study). Because this patient was in remission, with less than 0.1% leukemic cells circulating in the peripheral blood, the only sample with considerable leukemic infiltration that we could investigate was an archival formalin-fixed, paraffin-embedded bone marrow biopsy specimen. Although the quantity and quality of genomic DNA extracted from this sample were very poor, we could perform Sanger sequencing on the cloned faint PCR product, and 4 of 26 sequenced clones were mutated. Thus, the *BRAF* mutation was detected in samples from all 48 patients with HCL that we analyzed and was clonal in at least 47 of them.

#### **BRAF MUTATION IN OTHER B-CELL LYMPHOMAS OR LEUKEMIAS**

Given the high frequency of the *BRAF* V600E variant in patients with HCL, we next investigated whether the same variant was also present in other peripheral B-cell lymphomas or leukemias. Strikingly, none of the samples we investigated from 195 patients with these other disorders carried the mutation (Table S8 in the Supplementary Appendix). Samples from patients with wild-type *BRAF* also included some B-cell tumors in which clinical and morphologic features might have simulated those in HCL but that had a different prognosis and clinical management, such as 22 samples of splenic marginal-zone lymphoma (also frequently referred to as splenic lymphoma with villous lymphocytes) and 16 samples of unclassifiable splenic B-cell lymphoma or leukemia (Fig. 2C and 2D). The latter category includes HCL variant and splenic red-pulp small B-cell lymphoma, according to the 2008 World Health Organization classification.<sup>1</sup>

### EXPRESSION OF PHOSPHORYLATED MEK AND ERK

The V600E mutation results in the constitutive activation of BRAF kinase activity. Therefore, we assessed the phosphorylation status of MEK (the immediate downstream kinase target of BRAF) and ERK (the kinase phosphorylated by active MEK), using antibodies that specifically recognize phosphorylated MEK and ERK.

Because phosphoepitopes can be denatured by the decalcification process, we performed immunohistologic studies in five patients with BRAF-mutated HCL for whom optimally fixed and decalcified, paraffin-embedded bone marrow biopsy samples were available. In all cases, double immunohistochemical or immunofluorescence staining for a B-cell marker (PAX5 or CD20) and phospho-ERK showed phosphorylated ERK in HCL cells, which was abolished by preincubation of the anti-phospho-ERK antibody with its blocking phosphopeptide (Fig. 3A, and Fig. 1 in the Supplementary Appendix). Phospho-MEK could not be investigated because of the unreliable staining of the anti-phospho-MEK antibody in paraffin sections. In two of the five patients with BRAF-mutated HCL who were positive for phospho-ERK on immunohistochemical analysis, sufficient numbers of purified HCL cells were available for Western blot studies. In the two samples, the analysis showed phosphorylation of MEK and its substrate ERK (data not shown). These results are in keeping with findings in patients with HCL reported by Kamiguti et al.<sup>14</sup>

In vitro incubation of primary leukemic hairy cells from five additional patients with the specific active BRAF inhibitor PLX-4720 led to a marked decrease in phosphorylated MEK and ERK at low drug concentrations ( $\leq 1 \mu\text{M}$ ), whereas vehicle-treated cells retained MEK and ERK phosphorylation (Fig. 3B).

### DISCUSSION

By whole-exome sequencing of a DNA sample from a patient with HCL, we identified the BRAF V600E mutation as a genetic alteration that is invariably associated with this disease. The BRAF V600E mutation qualifies as a disease-defining genetic event in HCL because of three findings in our study: it was present in 100% of patients who encompassed the whole spectrum of HCL patients, including those presenting with leukocytosis or without

splenomegaly and those evaluated after therapy; it was present in the entire tumor-cell clone in virtually all patients; and it was not found in patients with other peripheral B-cell lymphomas or leukemias. Together, these factors strongly implicate the BRAF V600E mutation in the pathogenesis of HCL. Notably, among B-cell neoplasms (in which nonkinase genes are usually involved through translocations, deletions, or point mutations), HCL is the only one in which the disease-defining genetic lesion is an activating point mutation of a kinase-encoding gene. The frequency of the BRAF V600E mutation among the patients with HCL in our study was much higher than the reported frequency among patients with other BRAF-mutated human neoplasms, including melanomas (approximately 50%),<sup>11,15</sup> papillary thyroid carcinomas (approximately 40%),<sup>16</sup> and Langerhans'-cell histiocytosis (57%),<sup>17</sup> as well as those with other solid tumors, which have been reported to have a much lower frequency of the variant.<sup>11,18,19</sup>

A member of the serine-threonine kinase RAF family, the BRAF protein is part of the RAS-RAF-MAPK signaling pathway, which plays a major role in regulating cell survival, proliferation, and differentiation.<sup>20</sup> BRAF mutations (with V600E being the most common in human tumors) constitutively activate the MEK-ERK pathway, leading to enhanced cell proliferation, survival, and ultimately, neoplastic transformation.<sup>12,13,21</sup> In our study, all patients with HCL who carried BRAF mutations had the V600E phosphomimetic substitution, which occurs within the BRAF-activation segment and markedly enhances its kinase activity in a constitutive manner.<sup>22</sup>

Activation of the MAPK pathway in HCL was previously described by other authors, who found that ERK was constitutively activated<sup>14</sup> and provided a survival signal for the leukemic hairy cells.<sup>23</sup> This signal was hypothesized to rescue HCL cells from the potentially proapoptotic effect of p38-JNK activation (triggered by the interaction of leukemic cells with the vitronectin-positive cells in the splenic red pulp).<sup>14,24</sup> ERK activation in HCL was reported to depend on MEK activation by a route, other than RAF activation, that is not yet known.<sup>14</sup> Our findings implicate mutated BRAF as the most likely trigger for constitutive MEK and ERK activation in HCL. This hypothesis is supported by our immunohistochemical and Western blot analyses for phospho-MEK and phospho-ERK, which showed that in the presence of BRAF muta-

tions, these downstream targets are activated in HCL cells and are dephosphorylated when treated with a specific active BRAF inhibitor. On the basis of our findings, it should be feasible to develop murine models of HCL by activating the RAS-RAF-MAPK signaling pathway in specific B-cell subpopulations. Further studies are required to

clarify whether mutations in other genes may cooperate with BRAF in the pathogenesis of HCL.

The BRAF V600E mutation may account for some immunophenotypic features of HCL — for example, the low-to-moderate expression of cyclin D1 (which is independent of CCND1 rearrangements or amplifications)<sup>25,26</sup> and the absence of

**Table 1. Clinical and Phenotypical Features of 48 Patients with Hairy-Cell Leukemia (HCL).\***

Patient No.	BRAF V600E Mutation	Sex	Age at Diagnosis yr	White-Cell Count at Diagnosis per mm <sup>3</sup>	Splenomegaly at Diagnosis	Immunophenotype of Leukemic B Cells†				Previous Therapy
						ANXA1	CD25	CD11c	CD103	
1‡	Yes	M	62	2,470	No	Positive	Positive	NA	Positive	Interferon
2	Yes	M	47	9,400	Yes	NA	Positive	Positive	Positive	None
3	Yes	F	41	3,760	Yes	Positive	NA	Positive	Positive	Rituximab
4	Yes	M	62	6,710	Yes	Positive	Positive	Positive	Positive	None
5	Yes	M	56	6,240	Yes	NA	Positive	Positive	Positive	None
6	Yes	F	61	8,819	Yes	NA	Positive	Positive	Positive	None
7	Yes	M	39	2,980	Yes	Positive	NA	NA	NA	Splenectomy, interferon, pentostatin, 2-CDA, rituximab, 2-CDA
8§	Yes	M	46	2,700	Yes	NA	Positive	Positive	Positive	None
9	Yes	M	60	5,470	Yes	NA	Positive	Positive	Positive	Pentostatin, 2-CDA, splenectomy
10	Yes	M	33	4,120	Yes	NA	Positive	Positive	Positive	None
11‡	Yes¶	M	46	11,390	Yes	NA	Positive	Positive	Positive	Pentostatin, 2-CDA, splenectomy, interferon, FCR, CHOP, 2-CDA
12	Yes	F	68	5,300	No	Positive	NA	NA	NA	None
13	Yes	M	70	9,350	No	NA	Positive	Positive	Positive	None
14	Yes	M	48	2,100	No	Positive	Positive	Positive	NA	None
15	Yes	M	75	3,720	No	Positive	NA	Positive	NA	None
16	Yes¶	M	39	4,800	Yes	Positive	Positive	Positive	Positive	Pentostatin, 2-CDA, rituximab, splenectomy
17‡	Yes	M	61	2,500	Yes	Positive	NA	NA	NA	None
18‡	Yes	M	63	1,300	Yes	Positive	NA	NA	NA	None
19	Yes¶	F	60	7,800	Yes	NA	Positive	Positive	Positive	None
20	Yes	F	47	8,010	Yes	NA	Positive	Positive	Positive	None
21	Yes	M	55	2,330	Yes	NA	Positive	Positive	Positive	None
22	Yes	M	63	3,680	Yes	NA	Positive	Positive	Positive	None
23	Yes	M	33	9,900	No	Positive	Positive	Positive	Positive	None
24	Yes	F	68	2,900	Yes	Positive	NA	Positive	Positive	None
25	Yes	M	59	4,880	Yes	Positive	NA	Positive	Positive**	None

Table 1. (Continued.)

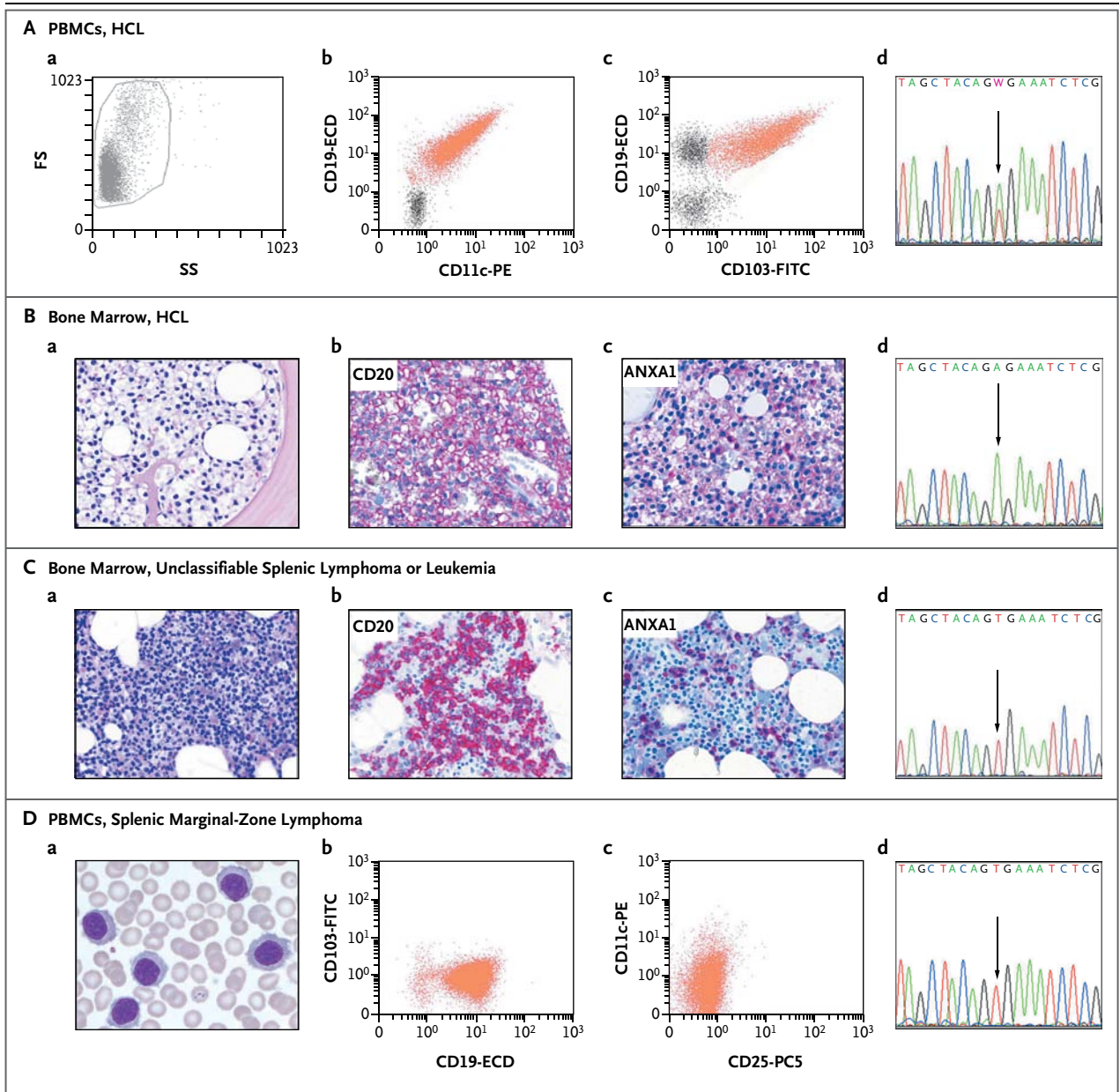
Patient No.	BRAF V600E Mutation	Sex	Age at Diagnosis yr	White-Cell Count at Diagnosis per mm <sup>3</sup>	Splénomegaly at Diagnosis	Immunophenotype of Leukemic B Cells†				Previous Therapy
						ANXA1	CD25	CD11c	CD103	
26‡	Yes	F	79	2,900	Yes	Positive	NA	NA	NA	None
27‡	Yes	M	57	1,300	Yes	Positive	NA	NA	NA	None
28‡	Yes	M	72	2,000	Yes	Positive	NA	NA	NA	None
29‡	Yes	M	74	9,300	Yes	Positive	NA	NA	NA	Splenectomy, interferon
30‡	Yes	M	71	7,400	Yes	Positive	NA	NA	NA	Splenectomy, interferon
31‡	Yes	M	52	1,300	Yes	Positive	NA	NA	NA	None
32	Yes	M	66	51,000	NA	NA	Positive	Positive	Positive	None
33	Yes	M	52	NA	NA	NA	Positive	Positive	Positive	None
34	Yes	M	80	24,600	NA	NA	Positive	Positive	Positive	2-CDA
35	Yes	F	77	34,700	Yes	NA	Positive	Positive	Positive	Interferon
36	Yes	M	49	12,100	NA	NA	Positive	Positive	Positive	None
37	Yes	M	51	37,900	NA	NA	Positive	Positive	Positive	None
38	Yes	M	35	23,500	Yes	NA	Positive	Positive	Positive††	None
39	Yes	M	45	7,800	NA	NA	Positive	Positive	Positive	None
40	Yes	F	49	18,900	NA	NA	Positive	Positive	Positive	None
41	Yes	M	44	2,610	Yes	NA	Positive	Positive	Positive	Interferon, pento- statin plus inter- feron, 2-CDA, splenectomy
42	Yes	M	56	7,500	Yes	NA	Positive	Positive	Positive	None
43	Yes	M	48	14,110	Yes	NA	Positive	Positive	Positive	None
44	Yes	M	41	4,170	Yes	Positive	NA	NA	NA	None
45	Yes	M	65	2,190	Yes	Positive	NA	NA	NA	None
46	Yes	M	56	36,070	Yes	NA	Positive	Positive	Positive	None
47	Yes¶	M	67	39,000	Yes	NA	Positive	Positive	Positive	Cyclophosphamide
48	Yes	M	56	12,420	No	NA	Positive	Positive	Positive	None

\* CHOP denotes cyclophosphamide, doxorubicin, vincristine, and prednisone; FCR fludarabine, cyclophosphamide, and rituximab; NA not available; and 2-CDA 2-chlorodeoxyadenosine (cladribine).  
 † ANXA1 antibody was assessed on immunohistochemical analysis, and CD25, CD11c, and CD103 were assessed on flow cytometry.  
 ‡ A sample from this patient was previously studied by means of gene-expression profiling.<sup>5</sup>  
 § Samples from this patient, the index patient, were subjected to whole-exome sequencing.  
 ¶ The BRAF V600E mutation was present in a homozygous or hemizygous fashion.  
 || CD103 antibody labeled 19% of HCL cells.  
 \*\* CD103 antibody labeled 22% of HCL cells.  
 †† CD103 antibody labeled 39% of HCL cells.

p27.<sup>27</sup> In melanoma cells, the presence of BRAF V600E leads to the activation of the MEK-ERK pathway with concomitant transcriptional constitutive expression of cyclin D1 and p27 down-regulation in an adhesion-independent manner.<sup>28-30</sup> Moreover, MEK-ERK pathway-induced activation

of an AP1-protein complex containing the jun D transcription factor (JUND)<sup>31</sup> has been implicated in the expression of the HCL marker CD11c.

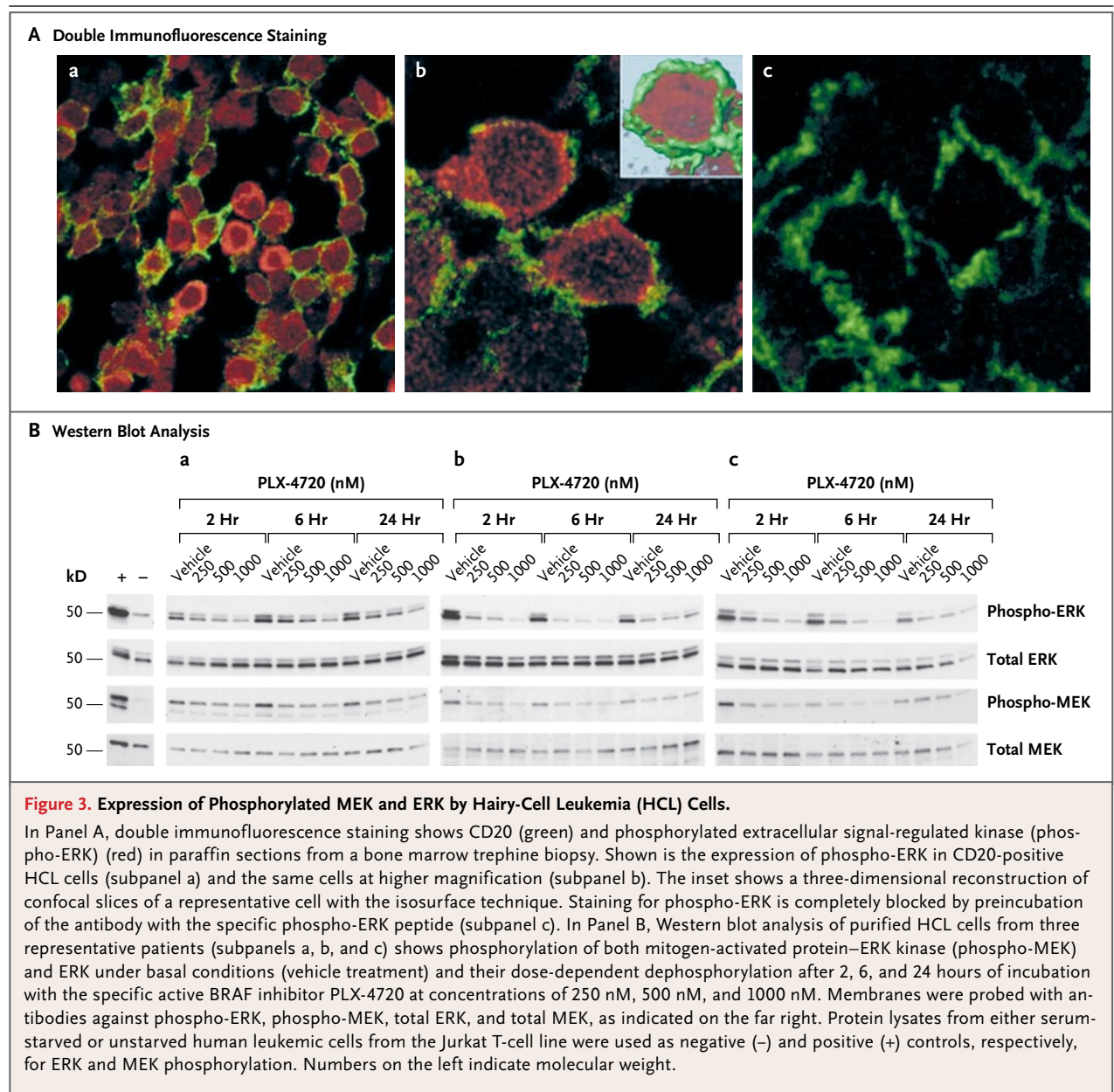
BRAF V600E was present in all 48 patients with HCL in our study. However, given the relatively small number of patients who were evalu-



**Figure 2. BRAF V600E Mutation in Hairy-Cell Leukemia (HCL), as Compared with Other Peripheral B-Cell Neoplasms.**

In Panel A, flow cytometric analysis of a sample from a patient with HCL shows peripheral-blood mononuclear cells (PBMCs) partly with high forward scatter (FS) and side scatter (SS) (black events in the plot in subpanel a), expressing CD19 together with CD11c and CD103 (red events in subpanels b and c, respectively). Direct DNA Sanger sequencing of purified leukemic cells reveals a heterozygous T→A mutation (subpanel d, arrow). ECD denotes phycoerythrin–Texas Red, FITC fluorescein isothiocyanate, and PE phycoerythrin. In Panel B, paraffin sections from a bone marrow–biopsy specimen obtained from a second patient with HCL shows diffuse marrow infiltration by HCL cells (subpanel a, hematoxylin and eosin) that are positive for CD20 antibody (subpanel b) and ANXA1 antibody (subpanel c). Purified HCL cells harbor a homozygous or hemizygous T→A mutation (subpanel d, arrow). In Panel C, paraffin sections from a bone marrow–biopsy specimen obtained from a patient with unclassifiable splenic lymphoma or leukemia show marked marrow infiltration by leukemic cells (subpanel a, hematoxylin and eosin) that express CD20 (subpanel b) but not ANXA1 (subpanel c, with positive myeloid cells used as an internal control). Purified leukemic cells do not carry the T→A mutation (subpanel d, arrow). In Panel D, leukemic cells in a PBMC sample obtained from a patient with splenic marginal-zone lymphoma show the typical morphologic features with polar villi (subpanel a, May–Grünwald–Giemsa stain). On flow cytometry, leukemic cells express CD19 but not CD103 (red events in subpanel b). These cells only weakly express CD11c and are negative for CD25 (red events in subpanel c). The leukemic cells do not carry the T→A mutation (subpanel d, arrow). PC5 denotes phycoerythrin–cyanine 5.





ated, we cannot rule out the possibility that some patients with HCL may not carry the BRAF V600E mutation. It must also be stressed that all samples that were analyzed in this study contained at least 30% neoplastic cells, which was the threshold for detecting a heterozygous clonal mutation by direct Sanger sequencing in our study. The detection of BRAF V600E in HCL samples containing lower proportions of leukemic cells will require more sensitive molecular techniques. If antibodies against the BRAF V600E variant protein become available, immunohistochemical analysis may be an alternative approach.

Among the 242 cases of peripheral B-cell lymphomas that we studied, BRAF V600E was restricted to HCL. However, it is possible that B-cell lymphomas other than HCL may occasionally carry BRAF mutations in exon 15 and that we did not discover them because of the limited number of cases in some lymphoma subgroups (Table S8 in the Supplementary Appendix). BRAF mutations have been reported in rare cases of B-cell acute lymphoblastic leukemia (6 of 213)<sup>32-34</sup> and peripheral B-cell lymphoma (4 of 164),<sup>35</sup> but none of these mutations produced the BRAF V600E variant. More recently, BRAF mutations were identified

in 8 of 199 patients (4%) with multiple myeloma and encoded the BRAF V600E variant protein in 4 of them.<sup>36</sup>

Overall, our findings point to analysis of BRAF mutations as a potential new diagnostic tool to distinguish HCL from other B-cell lymphomas with similar clinical and morphologic features, such as the HCL variant and splenic marginal-zone lymphoma, none of which carried the BRAF mutation in our study. This distinction is clinically relevant, since HCL, but not HCL-like disorders, responds to therapy with interferon or purine analogues.<sup>4</sup> The absence of BRAF mutations in the HCL variant further supports the view that this entity is different from HCL and thus justifies its inclusion in the category of unclassifiable splenic B-cell lymphoma or leukemia in the 2008 World Health Organization classification.<sup>37</sup>

Finally, the BRAF V600E mutant is a potential therapeutic target in patients with HCL who do not have a response (or have a suboptimal response) to initial therapy with purine analogues, as well as in patients with repeated relapses or unacceptable toxic effects.<sup>4</sup> Notably, BRAF V600E inhibitors<sup>38-40</sup> have shown remarkable activity in patients with BRAF-mutated metastatic melanoma.<sup>41</sup> These results, along with our *in vitro* finding that a specific active BRAF inhibitor causes MEK and ERK dephosphorylation in primary HCL cells, warrant

the clinical testing of active BRAF inhibitors, either alone or in combination with compounds acting downstream of BRAF (e.g., MEK or ERK inhibitors), in patients with HCL.

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#### APPENDIX

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