

ORIGINAL ARTICLE

Targeting Mutant BRAF in Relapsed or Refractory Hairy-Cell Leukemia

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ABSTRACT

BACKGROUND

BRAF V600E is the genetic lesion underlying hairy-cell leukemia. We assessed the safety and activity of the oral *BRAF* inhibitor vemurafenib in patients with hairy-cell leukemia that had relapsed after treatment with a purine analogue or who had disease that was refractory to purine analogues.

METHODS

We conducted two phase 2, single-group, multicenter studies of vemurafenib (at a dose of 960 mg twice daily) — one in Italy and one in the United States. The therapy was administered for a median of 16 weeks in the Italian study and 18 weeks in the U.S. study. Primary end points were the complete response rate (in the Italian trial) and the overall response rate (in the U.S. trial). Enrollment was completed (28 patients) in the Italian trial in April 2013 and is still open (26 of 36 planned patients) in the U.S. trial.

RESULTS

The overall response rates were 96% (25 of 26 patients who could be evaluated) after a median of 8 weeks in the Italian study and 100% (24 of 24) after a median of 12 weeks in the U.S. study. The rates of complete response were 35% (9 of 26 patients) and 42% (10 of 24) in the two trials, respectively. In the Italian trial, after a median follow-up of 23 months, the median relapse-free survival was 19 months among patients with a complete response and 6 months among those with a partial response; the median treatment-free survival was 25 months and 18 months, respectively. In the U.S. trial, at 1 year, the progression-free survival rate was 73% and the overall survival rate was 91%. Drug-related adverse events were usually of grade 1 or 2, and the events most frequently leading to dose reductions were rash and arthralgia or arthritis. Secondary cutaneous tumors (treated with simple excision) developed in 7 of 50 patients. The frequent persistence of phosphorylated ERK-positive leukemic cells in bone marrow at the end of treatment suggests bypass reactivation of MEK and ERK as a resistance mechanism.

CONCLUSIONS

A short oral course of vemurafenib was highly effective in patients with relapsed or refractory hairy-cell leukemia. (Funded by the Associazione Italiana per la Ricerca sul Cancro and others; EudraCT number, 2011-005487-13; ClinicalTrials.gov number NCT01711632.)

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HAIRY-CELL LEUKEMIA IS A CHRONIC mature B-cell cancer with unique clinicopathologic and biologic features.¹⁻⁴ Purine analogues (cladribine and pentostatin) induce durable complete responses in approximately 80% of patients with this cancer.^{5,6} However, in 30 to 50% of patients, the disease relapses and there is a progressively worse response to purine analogues,^{7,8} which can also cause cumulative myelotoxic effects and immune suppression. Thus, new therapeutic approaches are needed.

Tiacci and colleagues found that the V600E mutation of BRAF, a kinase commonly mutated in solid tumors,⁹ was the key genetic lesion of hairy-cell leukemia,^{10,11} and Chung et al. found that this mutation is acquired in the hematopoietic stem-cell compartment.¹² Tiacci et al. found that, as in other BRAF-mutated neoplasms,¹³ the V600E mutation in hairy-cell leukemia constitutively activates the mitogen-activated protein kinase (MAPK) pathway.^{14,15} The same group found that BRAF inhibitors *in vitro*¹⁶ reverse the unique molecular signature² and morphologic characteristics of hairy cells and potentially induce their apoptosis, thus establishing BRAF V600E as an attractive therapeutic target in hairy-cell leukemia. Moreover, the antileukemic activity of BRAF inhibitors has been reported anecdotally in patients with this cancer.^{17,18}

We conducted two phase 2, multicenter clinical trials — in Italy and the United States — to investigate the efficacy and safety of an oral BRAF inhibitor, vemurafenib,¹⁹ in patients with relapsed or refractory hairy-cell leukemia. The enrollment of patients in the Italian trial was completed in April 2013, and the median follow-up is almost 2 years after the end of treatment. The enrollment of the patients in the U.S. trial has not yet been completed, but the primary end point (overall response rate) has already been met. Here we report the results of these two trials.

METHODS

PATIENTS

In the Italian trial, 28 patients were enrolled (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). All the patients met one of the following criteria: disease that was refractory to a purine analogue, defined as no response or a relapse within 1 year after treatment (6 patients); early relapse after treatment with a purine analogue, defined as re-

lapse that occurred between 1 and 2 years after the first course or within 4 years after a second or later course (20 patients) or in any case (regardless of timing) in which there was considerable bone marrow hypoplasia at the time of relapse (1 patient); or severe side effects from a previous purine analogue (1 patient). Eligible patients also had to have a hemoglobin level of less than 11.0 g per deciliter, a neutrophil count of less than 1500 per cubic millimeter, or a platelet count of less than 100,000 per cubic millimeter. The diagnosis was confirmed centrally by two of the authors with the use of annexin A1 immunostaining of a bone marrow–biopsy specimen,²⁰ detection of BRAF V600E by an allele-specific polymerase-chain-reaction (PCR) assay,¹¹ and flow cytometry. Further details are provided in the study protocol, available at NEJM.org.

In the U.S. trial, 26 patients have been enrolled (Table S2 in the Supplementary Appendix). All the patients met one of the following criteria: disease that was refractory to a purine analogue (as defined above; 1 patient); early relapse (between 1 and 2 years) after the first course of a purine analogue (1 patient); or two or more relapses that occurred more than 2 years after a third or later course of a purine analogue (24 patients). Eligible patients had to have a hemoglobin level of 10.0 g per deciliter or less, a neutrophil count of 1000 per cubic millimeter or less, or a platelet count of 100,000 per cubic millimeter or less. The presence of BRAF V600E was assessed by means of immunohistochemical testing, PCR assay or Sanger sequencing, or the Memorial Sloan Kettering Cancer Center IMPACT assay²¹ (the IMPACT assay was performed in all the patients) in a Clinical Laboratory Improvement Amendments–certified laboratory. Further details are provided in the study protocol.

STUDY DESIGN

The Italian trial was a phase 2, single-group, multicenter study sponsored by the Institute of Hematology, University of Perugia, and designed by the first and penultimate authors. Roche provided an unconditional research grant and vemurafenib at no cost. Otherwise, Roche had no role in the study. The planned enrollment of 28 patients was completed in 11 months (May 20, 2012, through April 23, 2013) at eight centers. Patients received oral vemurafenib at a dose of 960 mg twice daily for a minimum of 8 weeks and, if they did not have a complete response,

for a maximum of 16 weeks (Fig. S1 in the Supplementary Appendix). The first 3 patients received vemurafenib for 20 weeks. The primary end point was the rate of complete response. Secondary end points included the time to response, relapse-free survival, and safety. We also evaluated the overall response rate, progression-free survival, treatment-free survival (i.e., from the last vemurafenib dose until the initiation of further antileukemic treatment), and pharmacodynamic biomarkers.

The U.S. trial is an ongoing phase 2, single-group, multicenter study that was designed by the second and last authors. The role of Roche is the same as that in the Italian trial. The patients were enrolled consecutively from January 2013 through February 2015 at six sites. Patients received oral vemurafenib at a dose of 960 mg twice daily, on a continuous schedule for 12 weeks. Patients with residual disease (as assessed by means of morphologic or immunohistochemical testing for CD20, PAX5, DBA44, and tartrate-resistant alkaline phosphatase) were allowed to receive vemurafenib for up to 12 additional weeks. The primary end point was the overall response rate after 12 weeks of treatment with vemurafenib. Secondary end points were overall survival, progression-free survival, time to relapse, and pharmacodynamic outcomes.

In the two trials, treatment with vemurafenib was allowed at the time of disease relapse, which was defined as peripheral-blood counts that were low enough to meet the respective initial eligibility criteria. Retreatment was permitted for up to 12 weeks (in the Italian trial) or until disease progression or occurrence of an unacceptable level of toxic effects (in the U.S. trial).

STUDY ASSESSMENTS

Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.03. Neoplastic cutaneous lesions were surgically excised, but their presence did not mandate dose modifications.

In the Italian trial, assessments of response (blood counts, spleen size, bone marrow biopsy, and aspirate) were conducted monthly during treatment, together with a dermatologic examination, and every 3 months during follow-up (except for the bone marrow evaluation, which was performed every 6 months). Bone marrow

and peripheral-blood samples were assessed centrally at the Institute of Hematology, University of Perugia. The calculation of the Hairy Cell Index (the percentage of hematopoietic cellularity multiplied by the percentage of leukemic cells, as assessed on the basis on CD20 immunostaining, and divided by 10,000) in the bone marrow–biopsy specimen has been described previously.²²

In the U.S. trial, bone marrow assessments were performed after 1 and 3 months of vemurafenib treatment and at the end of treatment. For patients who had splenomegaly at enrollment, computed tomography of the abdomen was performed at baseline and at 3 months. All the patients underwent dermatologic examination at baseline, after 1 month of vemurafenib treatment, and every 3 months thereafter until at least 1 year after discontinuation of the drug.

In the two trials, the assessment of a complete response required the resolution of any cytopenias (as defined by the eligibility criteria in each of the two studies), the absence of evidence of hairy cells in the peripheral blood and in bone marrow–biopsy specimens as assessed by means of nonimmunologic stains, and the absence of splenomegaly. The assessment of a partial response required the resolution of cytopenias, a reduction in splenomegaly of at least 50%, and a reduction of at least 50% in leukemic-cell infiltration in both the bone marrow and peripheral blood. In the two trials, relapse after a complete or partial response was defined as the reappearance of cytopenia on at least two consecutive occasions (the first of which was considered to be the date of relapse).

PHARMACODYNAMIC ANALYSES

Changes in the morphologic features of the leukemia cells during vemurafenib exposure and bone marrow ERK phosphorylation at the end of treatment (as assessed by two investigators in a blinded manner) were evaluated as described previously.^{14,16} The leukemia disease burden was assessed by means of serial flow cytometric analysis of peripheral-blood mononuclear cells (PBMCs) or bone marrow mononuclear cells.^{5,23} DNA from PBMCs was used to quantify the BRAF V600E allele burden with the use of a droplet digital PCR assay. Soluble interleukin-2–receptor alpha (IL2RA) and interleukin-1 receptor type II were measured in serum.²⁴ Targeted genomic analysis was performed in PBMCs as described

Table 1. Demographic and Clinical Characteristics of the Patients at Baseline.

Characteristic	Italian Trial (N=28)	U.S. Trial (N=26)
Age — yr		
Median	57	62
Range	27–84	44–80
No. of previous therapies		
Median	3	3
Range	1–12	1–8
Disease refractory to immediate prior therapy — no./total no. (%)		
Yes	15/27 (56)	9/22 (41)
No	12/27 (44)	13/22 (59)
Prior splenectomy — no. (%)		
Yes	8 (29)	5 (19)
No	20 (71)	21 (81)
Hairy-cell involvement in bone marrow — %*		
Median	82.5	80
Range	4–95	25–100
Absolute neutrophil count — $\times 10^{-3}/\text{mm}^3$		
Median	0.8	0.8
Range	0.2–5.7	0.2–4.8
Hemoglobin — g/dl		
Median	12.2	10.5
Range	8.2–17.0	8.2–14.4
Platelet count — $\times 10^{-3}/\text{mm}^3$		
Median	70	78
Range	6–306	26–238

* The percentage of hairy-cell involvement in bone marrow was determined with the use of immunohistochemical testing.

previously.²⁵ Further details are provided in the Methods section in the Supplementary Appendix.

STATISTICAL ANALYSIS

A Simon's minimax two-stage design²⁶ was adopted in the two trials. In the Italian study, we calculated a sample size that would be sufficient to accept the alternative hypothesis (complete response rate, $\geq 30\%$) and reject the null hypothesis (complete response rate, $\leq 10\%$) in an intention-to-treat analysis, at an alpha level of 0.05 and a beta level of 0.2. Enrollment was closed in April 2013, when the prespecified number of patients (28 patients) had been enrolled.

In the U.S. study, the primary end point was the overall response rate at 12 weeks. A Simon's minimax two-stage design was used to differen-

tiate between a promising response rate of 40% and an unpromising rate of 20%. In the initial stage of the study, we determined that a total of 19 patients had to be enrolled; if 4 or more patients had a response, the study would continue enrolling, with a target final sample of 36 patients. The study would be considered a success if at least 11 of the 36 patients had a response. Type I and type II errors were both 0.10. Because of promising results (24 patients with a response), this report includes data on the first 26 patients enrolled.

In the two trials, survival was estimated with the use of the Kaplan–Meier method. In the Italian study, differences in the rate of survival were assessed by means of a two-sided log-rank test. In the U.S. study, the time to relapse was evaluated with the use of cumulative incidence functions that treated death in the absence of relapse as a competing risk.

The change in hairy-cell leukemia cells in peripheral blood and bone marrow and the ratio of BRAF V600E to BRAF wild-type DNA concentration at 12 weeks were evaluated with the use of a paired Student's t-test. Full details of the statistical analysis are provided in the Supplementary Appendix.

RESULTS

PATIENTS

The characteristics of all 54 patients are shown in Table 1 and in Tables S1 and S2 in the Supplementary Appendix. Six patients in the Italian study and 1 in the U.S. study had disease that was refractory to a purine analogue; these patients had undergone a median of 2.5 prior therapies and 1 prior therapy, respectively, before vemurafenib treatment. Patients who had an early or repeated relapse after treatment with a purine analogue (20 patients in the Italian study and 25 patients in the U.S. study) had received a median of 4 prior therapies and 3 prior therapies, respectively. A total of 54% of the patients in the Italian study (15 of 28 patients) and 41% of those in the U.S. study (9 of 22) had disease that was refractory to the immediate prior therapy.

In the Italian trial, 26 of 28 patients completed the planned treatment (median duration of treatment, 16 weeks; range, 8 to 20). A total of 2 patients discontinued the study treatment at or before 1 week, owing to acute myocardial infarction (in 1 patient), which was considered by

the investigator to be unrelated to the study drug, and to withdrawal of consent after a drug-related event of grade 3 reversible pancreatitis (in 1).

In the U.S. trial, 24 of 26 patients received the study treatment for a median of 18 weeks (range, 12 to 24). One patient died from progressive pneumonia (which was considered by the investigator to be unrelated to the study drug) after 23 days of treatment, and another patient withdrew consent after 4 weeks owing to a drug-related event of grade 3 reversible photosensitivity.

RESPONSE RATE

The overall response rates were 96% (25 of 26 patients who could be evaluated) in the Italian study and 100% (24 of 24) in the U.S. study. The unusual presenting features of the one patient who did not have a response are described in the Results section in the Supplementary Appendix.

The rates of complete response were similar in the two trials, as were the rates of partial response. In the Italian study, after a median of 8 weeks, 35% of the patients (9 of 26) had a complete response and 62% (16 of 26) had a partial response (Table S1 in the Supplementary Appendix). Among the patients with a complete response, 1 had primary refractory disease, and among those with a partial response, 5 had primary refractory disease. A total of 4 patients with a complete response and 10 with a partial response had disease that was resistant to the last prior treatment.

The median time to recovery of the platelet count ($\geq 100,000$ per cubic millimeter) was 2 weeks, the median time to recovery of the neutrophil count (≥ 1500 per cubic millimeter) was 4 weeks, and the median time to recovery of the hemoglobin level (≥ 11.0 g per deciliter) was 8 weeks. Reversion of symptomatic splenomegaly and clearance of leukemia cells from the blood (as assessed by means of cytomorphologic testing) usually occurred within 2 weeks after the start of treatment. A substantial decrease in leukemic-cell infiltration into bone marrow was observed at the earliest time point evaluated (4 weeks) (data not shown). However, in all the patients with a complete response, immunohistochemical testing showed minimal residual disease ($\leq 10\%$ of leukemic cells) at the end of treatment (Fig. 1A and 1B).

In the U.S. trial, after 12 weeks of vemurafenib treatment, 42% of the patients (10 of 24 patients) had a complete response and 58%

(14 of 24) had a partial response (Table S2 in the Supplementary Appendix). Most patients had recovery of the neutrophil count (>1000 per cubic millimeter), the hemoglobin level (>10.0 g per deciliter), and the platelet count ($>100,000$ per cubic millimeter) by 28 days (Fig. 2A). Among the 10 patients who received vemurafenib at a twice-daily dose of 480 mg for more than 4 weeks, rates of complete response (40%) and partial response (60%) were similar to those observed among patients who received the standard dose.

In unplanned exploratory analyses in the two studies, treatment duration did not appear to influence the type of response (see the Supplementary Appendix). In addition, the rate of complete response was not associated with status with regard to prior splenectomy, burden of bone marrow disease, refractoriness of the disease to the last treatment, or number of prior therapies (Tables S1 and S2 in the Supplementary Appendix, and data not shown).

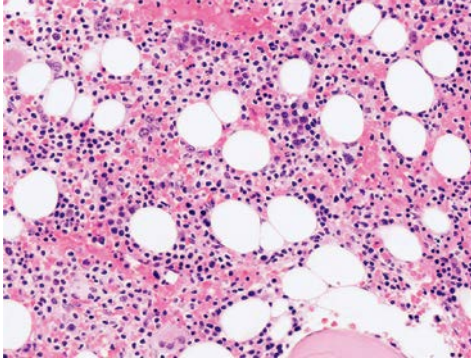
RESPONSE DURATION

In the Italian study, the median follow-up of the 25 patients who had a response was 23 months (range, 7 to 28) after the last vemurafenib dose. The median relapse-free survival was 9 months; the relapse-free survival was significantly longer among patients who had a complete response than among those who had a partial response (19 months vs. 6 months; hazard ratio for relapse, 0.26; 95% confidence interval [CI], 0.10 to 0.68; $P=0.006$) (Fig. 1C). The median treatment-free survival was 21.5 months in all 26 patients who could be evaluated, and (with the limitation of small numbers) it did not differ significantly between the group of patients who had a complete response and the group of those who had a partial response (25 months and 18 months, respectively; $P=0.21$) (Fig. 1C).

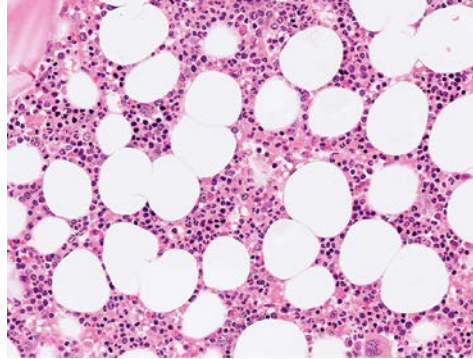
Of the 20 patients who had a relapse (5 patients after a complete response and 15 after a partial response), 7 did not require therapy at a median of 15 months (range, 4 to 18) after relapse, because their cytopenias were stably mild (median hemoglobin level, 14.2 g per deciliter [range, 12.4 to 17.5]; median neutrophil count, 1122 per cubic millimeter [range, 938 to 1724]; median platelet count, 86,000 per cubic millimeter [range, 63,000 to 269,000]). Conversely, in 13 of the 20 patients who had a relapse, cytopenia worsened, necessitating antileukemic treatment

A Bone Marrow–Biopsy Specimen with Hematoxylin and Eosin Staining

Before Treatment

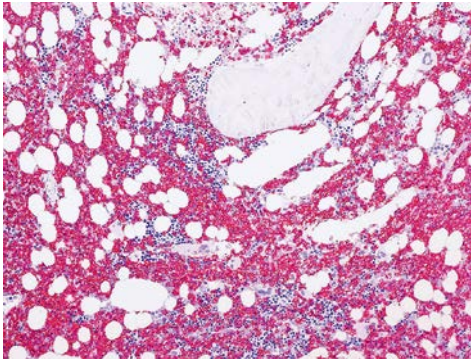


After Treatment

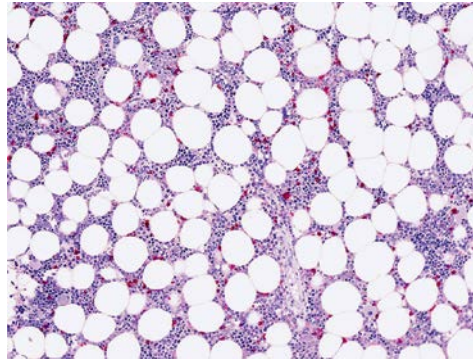


B Bone Marrow–Biopsy Specimen with CD20 Immunostaining

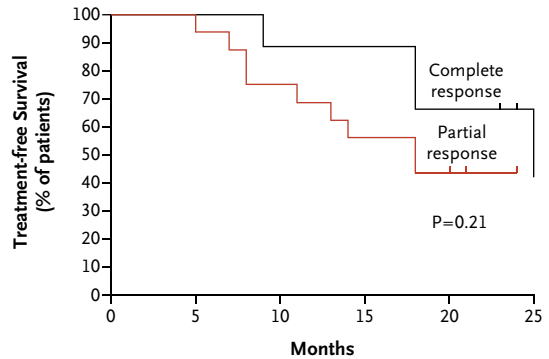
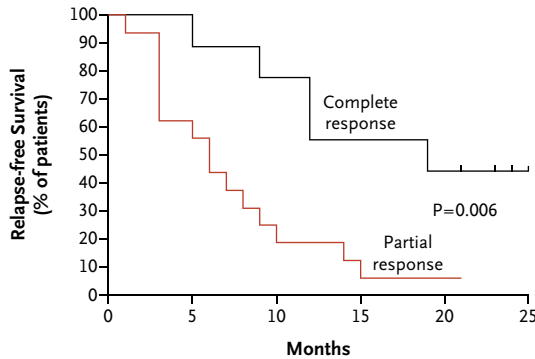
Before Treatment



After Treatment



C Relapse-free Survival and Treatment-free Survival

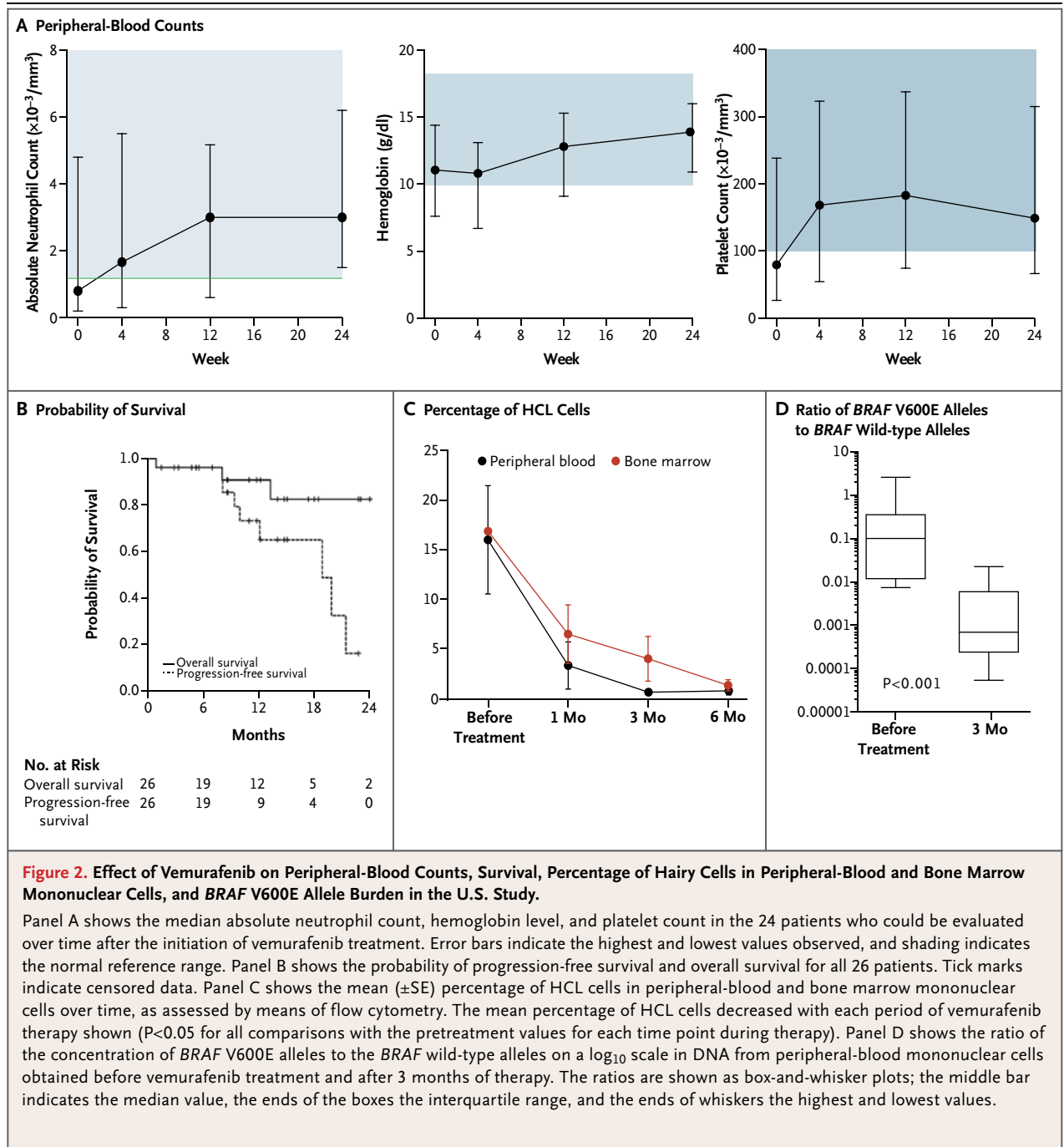


No. at Risk

	0	5	10	15	20	25
Complete response	9	8	7	6	4	4
Partial response	16	9	3	1	1	1

Figure 1. Extent and Duration of Response in the 25 Patients Who Had a Response to Vemurafenib in the Italian Trial.

Panel A shows hematoxylin and eosin staining of a bone marrow–biopsy specimen from a patient. The image shows infiltration by hairy-cell leukemia (HCL) cells with wide, clear cytoplasm that is recognizable by morphologic testing at baseline (left side) but not after 8 weeks of vemurafenib treatment (right side). Panel B shows CD20 immunostaining of the same bone marrow–biopsy specimen. The image shows approximately 75% leukemic-cell infiltration at baseline (left side) and only a few ($\leq 10\%$) residual scattered hairy cells after 8 weeks of vemurafenib treatment (right side). Panel C shows the rate of relapse-free survival (left side) and rate of treatment-free survival (right side) among patients who had a complete response or a partial response. The tick marks indicate censored data.



at a median of 5 months (range, 0 to 16) after relapse.

A total of 5 of 25 patients (4 patients with a complete response and 1 with a partial response) had not had a relapse as of the last follow-up assessment (23 to 25 months after the end of treatment). Of the 4 patients with a com-

plete response, 3 had no morphologic evidence of hairy-cell leukemia in their last bone marrow–biopsy specimen at 13, 19, and 24 months, respectively, whereas the fourth patient did not have a complete response on histologic testing at the last follow-up (24 months).

In unplanned exploratory analyses, relapse-free survival and treatment-free survival did not differ significantly between the group of 18 patients who received additional treatment after having their best response and the group of 7 patients who did not receive additional treatment, or between the group of 14 patients who required a dose reduction and the group of 11 who did not (data not shown). However, the 7 patients who underwent splenectomy had a shorter relapse-free survival than did those who did not undergo splenectomy (median, 6 months vs. 11 months; hazard ratio for relapse, 3.55; 95% CI, 1.04 to 12.06; $P=0.04$) and shorter treatment-free survival (median, 11 months vs. 25 months; hazard ratio, 6.61; 95% CI, 1.56 to 28.00; $P=0.01$).

In the U.S. trial, among survivors at the time of data cutoff, the median follow-up from the first vemurafenib dose was 11.7 months (range, 1.3 to 25.4). At 1 year, the rate of progression-free survival was 73% (95% CI, 55 to 97) and the rate of overall survival was 91% (95% CI, 79 to 99) (Fig. 2B). Disease progression occurred in 7 of 24 patients (29%), including 3 patients who had had a complete response and 4 who had had a partial response (Table S2 in the Supplementary Appendix). At 1 year after response, the cumulative incidence of relapse was 27% (95% CI, 7 to 51). In both trials, vemurafenib retreatment at the time of relapse produced some responses (see the Supplementary Appendix).

ADVERSE EVENTS

Table 2 lists the drug-related adverse events of grade 2 or higher that were observed in at least 1 patient in either trial. Table S3 in the Supplementary Appendix list all the adverse events of any grade in the Italian trial, including those that were not considered by the investigator to be related to the study drug. Table S4 in the Supplementary Appendix lists drug-related adverse events in the U.S. trial that occurred in more than 5% of the 26 patients, as well as all adverse events of secondary neoplasms.

In both trials, common vemurafenib-related adverse events, mostly of grade 1 or 2 and all reversible, included toxic events of the skin (especially rash and photosensitivity), arthralgias or arthritis, pyrexia, and an elevated bilirubin level. Other relatively frequent drug-related adverse events (recorded in one or both studies) were skin papillomas, alopecia, palmar or plantar hyper-

keratosis or dysesthesia, and fatigue, as well as an increase in the serum lipase level (which in 2 patients was associated with grade 3 pancreatitis) and elevated levels of aminotransferases, alkaline phosphatase, or fibrinogen.

In the Italian study, cutaneous basal-cell carcinomas developed in two patients (including one patient who had a history of basal-cell carcinoma) and a cutaneous superficial melanoma developed in one patient, all during treatment with vemurafenib. In the U.S. study, cutaneous squamous-cell carcinomas developed in three patients (all of whom had a history of squamous-cell carcinoma) and a cutaneous basal-cell carcinoma developed in one patient. All these tumors were managed by simple excision.

A slight transient decrease in the hemoglobin level was noted in 10 patients in the Italian study within the first 6 weeks of treatment. Neutropenia was noted in 1 patient in the U.S. study.

In the Italian trial, the planned dose of vemurafenib (960 mg administered twice daily) was reduced for at least 3 weeks in 17 instances, involving 15 of 26 patients (58%), to 720 mg twice daily (9 patients), 480 mg twice daily (3 patients), or 240 mg twice daily (3 patients). Toxic effects that led to these dose reductions were mainly rash (in 6 patients) and increased creatinine or bilirubin level, arthralgia, pancreatitis, and palmar and plantar dysesthesia (in 1 patient each).

In the U.S. trial, 13 patients (50%) required reductions in the dose to 720 mg twice daily (2 patients), 480 mg twice daily (10 patients), or 240 mg twice daily (1 patient). Adverse events leading to dose modifications were arthralgia (in 5 patients), rash (in 5), photosensitivity (in 1), neutropenia (in 1), and elevations of the aspartate or alanine aminotransferase levels (in 1).

PHARMACODYNAMIC ANALYSES AND RESISTANCE MECHANISMS

Hairy-cell leukemia cells that are exposed to vemurafenib in vitro down-regulate CD25 and lose their surface projections.¹⁶ Flow cytometry in blood and bone marrow consistently showed a loss of surface CD25 in a large fraction of leukemia cells at one or more time points during therapy in 15 of 19 patients (79%) who could be evaluated in the Italian study (including 1 patient for whom data have already been reported¹⁶) (Fig. 3A). This finding suggests that the measurement of surface or soluble CD25 may underestimate the residual burden of hairy-cell leuke-

Table 2. Drug-Related Adverse Events of Grade 2 or Higher.*

Event	Italian Trial (N=28)			U.S. Trial (N=26)		
	Grade 2	Grade 3	Total	Grade 2	Grade 3	Total
	<i>number of patients (percent)</i>					
Arthralgia or arthritis	10 (36)	2 (7)	12 (43)	8 (31)	0	8 (31)
Rash or erythema	11 (39)	2 (7)	13 (46)	11 (42)	5 (19)	16 (62)
Cutaneous basal-cell carcinoma	2 (7)†	0	2 (7)	1 (4)	0	1 (4)
Cutaneous superficial melanoma	0	1 (4)	1 (4)	0	0	0
Cutaneous squamous-cell carcinoma	0	0	0	3 (12)‡	0	3 (12)
Skin papilloma	2 (7)	0	2 (7)	0	0	0
Photosensitivity reaction	2 (7)	0	2 (7)	0	2 (8)	2 (8)
Hyperkeratosis	3 (11)	0	3 (11)	0	0	0
Panniculitis	2 (7)	0	2 (7)	0	0	0
Pancreatitis	1 (4)§	2 (7)	3 (11)	0	0	0
Increased pancreatic enzymes	1 (4)	1 (4)	2 (7)	0	0	0
Hyperbilirubinemia	2 (7)	1 (4)	3 (11)	3 (12)	0	3 (12)
Any increased aminotransferase	1 (4)	0	1 (4)	3 (12)	1 (4)	4 (15)
Increased alkaline phosphatase	0	0	0	0	2 (8)	2 (8)
Pain in the hands or feet	2 (7)	0	2 (7)	2 (8)	0	2 (8)
Headache	1 (4)	0	1 (4)	0	0	0
Increased blood creatinine	2 (7)	0	2 (7)	1 (4)	0	1 (4)
Seborrheic keratosis	2 (7)	0	2 (7)	0	0	0
Asthenia	1 (4)	0	1 (4)	0	2 (8)	2 (8)
Musculoskeletal pain	1 (4)	0	1 (4)	0	0	0
Abdominal pain	1 (4)	0	1 (4)	0	0	0
Pyrexia	0	0	0	0	1 (4)	1 (4)
Nausea	0	0	0	1 (4)	0	1 (4)
Alopecia	0	0	0	1 (4)	0	1 (4)

* The adverse events listed here occurred in at least one patient in either trial. No grade 4 events were reported in either study.

† In one patient, two basal-cell carcinomas developed; the other patient had a history of basal-cell carcinoma.

‡ All three patients had a history of squamous-cell carcinoma.

§ The patient with grade 2 pancreatitis in the Italian trial had an elevated lipase or amylase level (or both) but did not have clinical symptoms or radiologic abnormalities.

mia during vemurafenib treatment. In 1 patient with marked lymphocytosis, leukemic cells showed a clear smoothing of the cell membrane 2 and 3 days after the initiation of vemurafenib (Fig. 3B).

In 13 of 26 patients in the Italian study, a bone marrow–biopsy specimen obtained the day after the completion of therapy could be evaluated for phosphorylated ERK and PAX5 double immunostaining. In 6 of the 13 patients (all of whom had a partial response), residual hairy cells (PAX5 positive) that were still expressing phosphorylated ERK were observed, despite prolonged exposure (16 to 20 weeks) to vemurafenib

(Fig. 3C). In 7 of the 13 patients (2 patients with a complete response and 5 with a partial response after 12 to 20 weeks of treatment), residual leukemic cells did not detectably express phosphorylated ERK (Fig. 3C). In a post hoc exploratory analysis, the median progression-free survival was 8 months (range, 5 to 13) in patients with residual phosphorylated ERK–positive leukemic cells and 13 months (range, 8 to 24) in patients without such cells (hazard ratio for disease progression, 10.33; 95% CI, 2.10 to 50.81; P = 0.004).

The residual disease, as measured by the Hairy Cell Index (ranging from 0 to 1, with

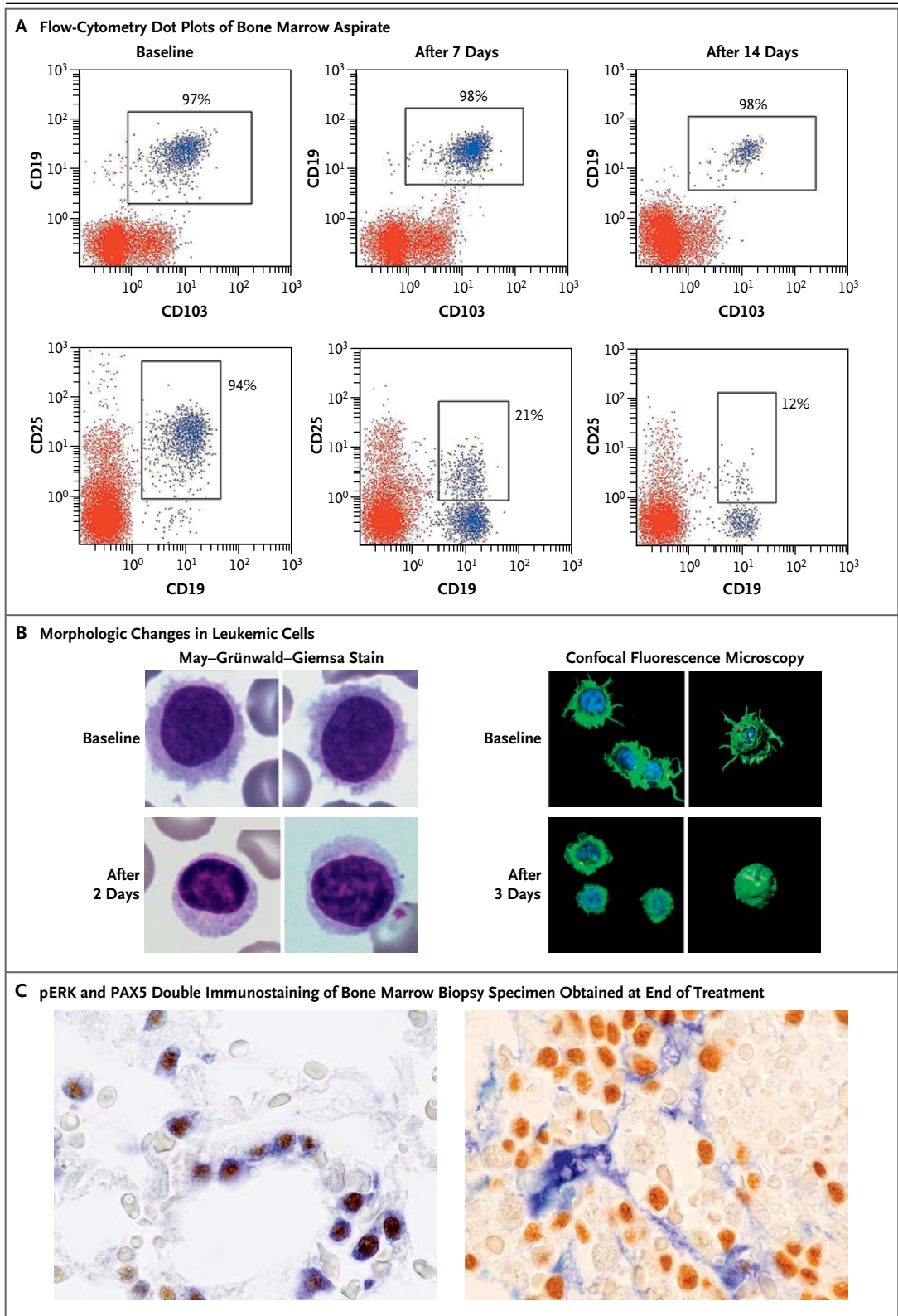


Figure 3 (facing page). Phenotypic and Molecular Changes of HCL Cells during Treatment with Vemurafenib in the Italian Trial.

Panel A shows flow-cytometry dot plots of a patient's bone marrow aspirate that was gated on CD45 cells. At baseline, CD25 was expressed by 94% of leukemic cells (top row, CD19+ and CD103+; bottom row, CD19+ and CD25+). A progressive loss of CD25 expression (but not of CD19 or CD103 expression) is seen over treatment periods of 7 days and 14 days (decreasing to 21% at 7 days and 12% at 14 days). The remaining cells (red dots) are normal CD45+ hematopoietic cells. In Panel B, May-Grünwald-Giemsa staining of a patient's peripheral-blood smear (at left) shows leukemic cells rich in hairy projections at baseline (top) but not after 2 days of vemurafenib treatment (bottom). Confocal fluorescence microscopic analysis of blood leukemic cells purified from the same patient (at right) shows prominent surface projections (stained green by phalloidin) at baseline (top) but not after 3 days of vemurafenib treatment (bottom). These changes are evident in electronically magnified two-dimensional images (left column) and reconstructed three-dimensional fluorescence images (right column) of representative cells. Panel C shows double immunostaining of a bone marrow-biopsy specimen obtained the day after the end of treatment and double-stained for PAX5 (a B-cell marker; brown) and phosphorylated ERK (pERK; blue). In some patients with a response, the persistence of pERK-positive leukemic hairy cells was observed (left side), whereas in other patients (right side), residual hairy cells did not detectably express pERK, with stromal cells strongly positive for pERK as an internal positive control.

higher values indicating greater leukemic infiltration), was higher in patients with persistent phosphorylated ERK in leukemic cells than in those with undetectable expression of phosphorylated ERK in leukemic cells (mean Hairy Cell Index, 0.139 vs. 0.054; $P = 0.03$ by a two-sided Mann-Whitney test). Thus, persistent ERK phosphorylation in residual hairy cells at the end of treatment may indicate a greater burden of residual disease and shorter progression-free survival, which suggests that reactivation of MEK and ERK is a resistance mechanism to vemurafenib.

In the U.S. trial, before vemurafenib treatment, the mean percentage of leukemic cells in PBMCs and bone marrow mononuclear cells as assessed by means of flow cytometry was 16.0% and 16.8%, respectively. These values decreased to 3.4% and 6.9%, respectively, after 4 weeks of treatment (Fig. 2C) and to 0.7% and 4.2%, re-

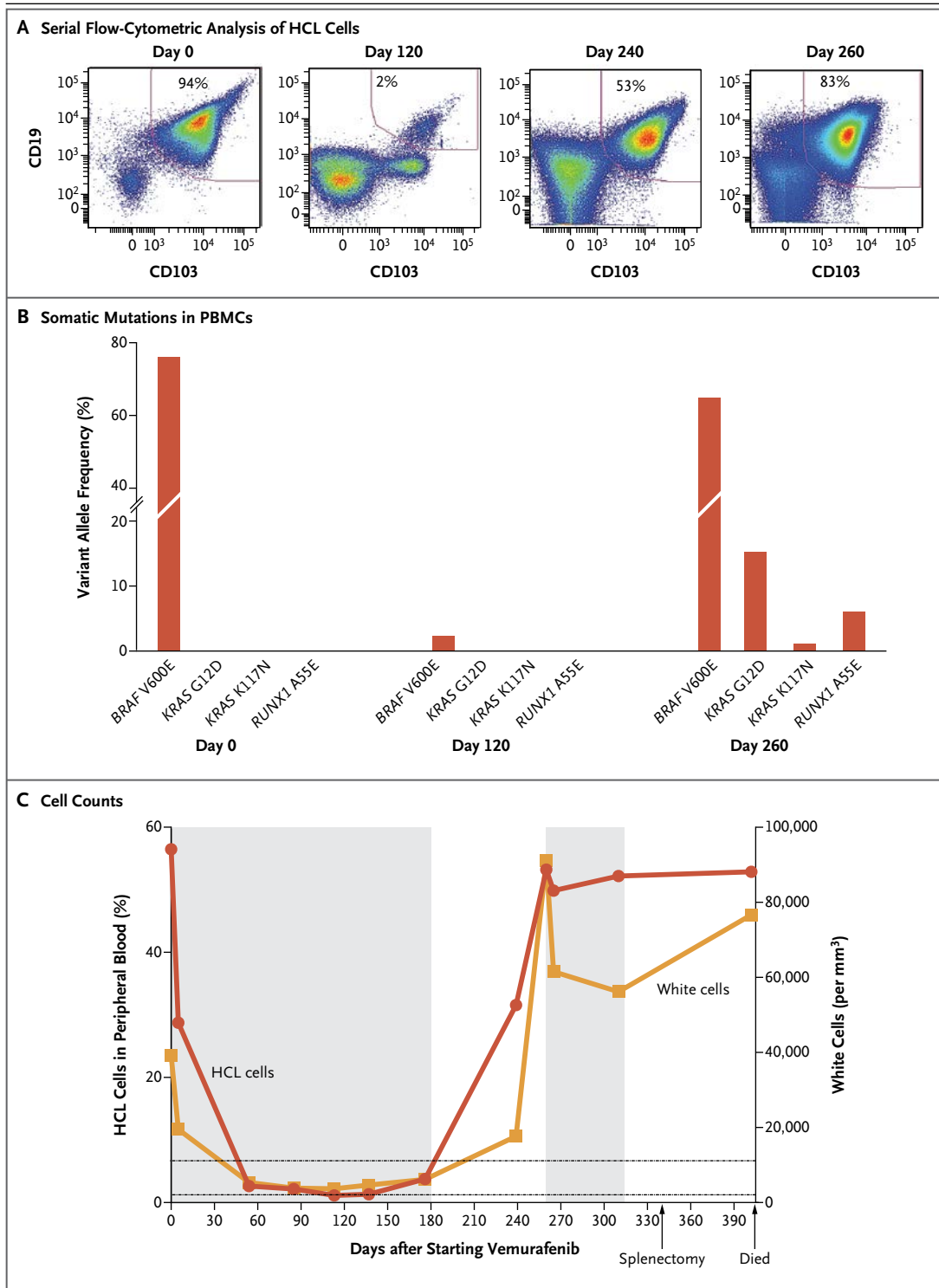
spectively, by 12 weeks ($P < 0.05$ for all comparisons). The percentage of hairy cells in bone marrow decreased even further after 24 weeks to a mean of 1.3% (Fig. 2C). Likewise, the droplet digital PCR assay, which was performed to quantify the concentration of molecules with mutated BRAF V600E DNA, revealed a decrease by a factor of more than 100 in the BRAF V600E allele burden in PBMCs after 12 weeks of vemurafenib therapy ($P < 0.001$ for the comparison with the pretreatment value) (Fig. 2D). Consistent with this finding, phosphorylated ERK1/2 rapidly decreased in bone marrow-biopsy specimens after 4 weeks of vemurafenib therapy (Fig. S3A in the Supplementary Appendix).

Finally, in all the patients, serum levels of soluble IL2RA and interleukin-1 receptor type II, which are well-established biomarkers of hairy-cell leukemia,^{27,28} declined markedly, as compared with baseline, by a mean of 30% and 27%, respectively, within 24 hours after the initiation of vemurafenib. After 3 months, the mean levels had declined by at least 95% (Fig. S3B and S3C in the Supplementary Appendix).

In one patient in the U.S. study who had disease that was refractory to vemurafenib retreatment, targeted sequencing of 300 genes (Table S6 in the Supplementary Appendix) identified two separate activating subclonal KRAS mutations (along with a new RUNX1 mutation) at the time of relapse after the initial vemurafenib treatment, in addition to the recurrence of the BRAF V600E mutation. KRAS mutations had not been seen before vemurafenib treatment or at remission (day 120) despite high KRAS sequencing coverage (range, 496× to 1165×) (Fig. 4B, and Table S5 in the Supplementary Appendix). Because in BRAF V600E-mutant cancers activating RAS mutations represent a known mechanism of vemurafenib resistance by means of the rephosphorylation of MEK and ERK,²⁹ the KRAS mutations that were newly acquired in this patient at relapse, although subclonal, probably contributed to the subsequent insensitivity to vemurafenib (Fig. 4C).

DISCUSSION

In these two trials involving patients with hairy-cell leukemia, an oral course of vemurafenib (at



a dose of 960 mg twice daily for 16 to 18 weeks) was rapidly and highly effective, with response rates of 96% in the Italian study and 100% in the U.S. study and with low-grade toxic effects in

patients who not only were heavily pretreated but often had disease that was refractory to their last therapy (56% of patients in the Italian study and 41% of those in the U.S. study). The median

Figure 4 (facing page). Activating *KRAS* Mutations Associated with the Development of Acquired Vemurafenib Resistance in *BRAF* V600E–Mutated HCL, as Assessed by Serial Genomic Analysis.

One patient in the U.S. study had disease that was refractory to vemurafenib retreatment and underwent targeted sequencing of 300 genes. Panel A shows the serial flow-cytometric analysis of HCL cells in peripheral-blood mononuclear cells (PBMCs) from this patient throughout the initial course of therapy (days 0 to 180) and vemurafenib retreatment (days 260 to 320). The sectioned-off areas in the fluorescence-activated cell-sorting plots indicate HCL cells (CD19 and CD103), with percentages of HCL cells among the PBMCs. Panel B shows the somatic mutations that were identified in PBMCs after 0, 120, and 260 days of vemurafenib treatment. The variant allele frequency is shown for mutations seen at each time point. Panel C shows the percentage of HCL cells in peripheral blood (left axis) and the total white-cell count (right axis) in this patient. Shaded areas represent the periods of vemurafenib treatment and retreatment. The dashed lines at the bottom of the graph indicate the upper and lower limits of a normal white-cell count.

time to response was 8 weeks in the Italian trial; the response rate was assessed at 12 weeks in the U.S. trial. A longer duration of treatment did not appear to improve the type or duration of the response. Retreatment with vemurafenib at the time of relapse was effective in the two trials, although to a lesser extent in the patients who had a relapse after a partial response than in those who had a relapse after a complete response in the Italian study.

Rather than administering vemurafenib for an indefinite duration, as is done in patients with metastatic melanoma,³⁰⁻³² we limited the initial vemurafenib treatment to a few months (even if this perhaps reduced the type or duration of response) owing to concern about vemurafenib-induced secondary tumors.³³⁻³⁵ Although such tumors affect primarily the skin and are of low malignant potential, their development may be less acceptable in patients with an indolent leukemia (however refractory or multiply relapsed) than in those with metastatic melanoma.

The rate, type, and duration of response that we observed among patients with hairy-cell leukemia were superior to those that have been observed in patients with metastatic melanoma,³⁰⁻³² possibly owing to the less complex genome landscape of hairy-cell leukemia (dominated by *BRAF* V600E¹⁰) and the much lower proliferative index ($\leq 1\%$ of proliferating cells) of hairy-cell

leukemia as compared with metastatic melanoma.^{36,37} However, in the U.S. trial, residual hairy cells in bone marrow with their associated *BRAF* V600E allele burden were consistently present at the end of treatment, even after a complete response. In approximately half the patients who could be evaluated in the Italian study, these cells showed persistent expression of phosphorylated ERK despite prolonged ongoing exposure to vemurafenib, which appeared to correlate with a higher burden of residual leukemia and shorter progression-free survival. This finding suggests that, in at least some patients, leukemic cells develop alternative mechanisms for reactivating MEK and ERK to bypass *BRAF* blockade.

In vitro studies conducted by the Italian group suggest that the bone marrow microenvironment might oppose vemurafenib-induced ERK dephosphorylation and apoptosis.¹⁶ It was proposed³⁸ that as hairy cells pass through the vitronectin-rich splenic red pulp, they receive vitronectin-mediated proapoptotic signals that are transduced by activation of p38 and Jun N-terminal kinase (JNK) but are overcome by concomitant antiapoptotic signals from the constitutive activation of MEK and ERK. We found that prior splenectomy appeared to be associated with a shorter duration of response. Thus, a lack of hairy-cell recirculation through the spleen, resulting in their continuous homing to the protective bone marrow microenvironment, may favor the persistence of ERK phosphorylation in leukemic cells at the end of treatment — a feature that is also apparently associated with a shorter duration of response. Furthermore, two activating *KRAS* mutations developed in one patient in the U.S. trial who had a relapse, which probably contributed to disease progression during vemurafenib retreatment.

Vemurafenib-related adverse events were manageable and were similar to those observed in patients with melanoma (e.g., rash, arthralgias, and secondary cutaneous tumors). The absence of clinically significant myelotoxic effects makes vemurafenib an ideal salvage treatment for patients with multiply relapsed hairy-cell leukemia who have pancytopenia and scarce bone marrow reserve owing to previous chemotherapies.

In anecdotal cases, lower doses of vemurafenib (e.g., 240 mg twice daily) from the beginning of treatment proved effective in patients with relapsed or refractory hairy-cell leukemia.¹⁷

In our studies, the starting dose of 960 mg twice daily was reduced only if an unacceptable level of toxic effects occurred; in most cases, the dose was reduced to a twice-daily dose of 720 mg (in the Italian study) or 480 mg (in the U.S. study), followed sometimes by reescalation. Prospective clinical trials are needed to formally evaluate toxicity, response rate, and survival with lower vemurafenib doses or longer treatment durations than those we used in these trials. Clinical trials are also warranted to evaluate the other clinical BRAF inhibitor, dabrafenib, which *in vitro* studies by the Italian group¹⁶ and single case reports^{18,39} have suggested to be effective in refractory or relapsed hairy-cell leukemia.

We used vemurafenib as single agent. However, BRAF inhibitors could be combined with anti-CD20 monoclonal antibodies (e.g., rituximab) to potentially eradicate BRAF inhibitor-resistant hairy-cell leukemia cells. The finding of MAPK-pathway reactivation as a likely mechanism of resistance to vemurafenib in some patients also establishes a rationale for combined BRAF and MEK blockade, which has been used successfully in patients with metastatic melanoma.⁴⁰

In conclusion, we found that vemurafenib is an active targeted drug for patients with relapsed or refractory hairy-cell leukemia.

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APPENDIX

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