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Targeting Mutant BRAF with Vemurafenib 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 who relapsed after or were refractory to purine analogues.

METHODS—We conducted in Italy and USA two phase-2 single-arm multicenter studies of vemurafenib (960 mg twice daily) given for a median of 16 and 18 weeks, respectively. Primary endpoints were complete remission rate and overall response rate. Patient enrollment was completed (n=28) in the Italian trial in April 2013 and is still open (n=26/36) in the American trial.

RESULTS—Drug-related adverse events were usually of grade 1-2, and those most frequently requiring dose reductions were rash and arthralgia/arthritis; secondary cutaneous tumors (treated with simple excision) developed in 6/50 patients. Overall response rates were 96% (25/26 evaluable Italian patients) and 100% (24/24 evaluable American patients), obtained after a median of 8 weeks and 12 weeks, respectively. Complete response rates were 34.6% (9/26) and 41.7% (10/24), respectively. In the Italian trial, after a median follow-up of 23 months, the median relapse-free and treatment-free survivals were respectively 19 and 25 months in complete responders, and 6 and 18 months in partial responders. In the American trial, 1-year progression-

free and overall survival were 73% and 91%, respectively. Frequent persistence of phospho-ERK+ bone marrow leukemic cells at the end of treatment suggests bypass MEK-ERK reactivation as a resistance mechanism.

CONCLUSIONS—A short oral course of vemurafenib proved safe and highly effective in relapsed/refractory hairy cell leukemia patients (Funded by AIRC, ERC, Roche/Genentech and others; EudractCT number: 2011-005487-13, ClinicalTrials.gov number NCT01711632).

Keywords

hairy cell leukemia; BRAF mutations; BRAF inhibitors; vemurafenib

INTRODUCTION

Hairy cell leukemia is a chronic mature B-cell malignancy with unique clinicopathologic and biologic features¹⁻⁴. Purine analogues (cladribine; pentostatin) induce durable complete remissions in ~80% of HCL cases^{5,6}. However, 30-50% of patients relapse and respond progressively less well to purine analogues^{7,8}, which can also cause cumulative myelotoxicity and immune-suppression. Thus, new therapeutic approaches are needed.

Tiacci et al. discovered the V600E mutation of BRAF, a kinase commonly mutated in solid tumors⁹, as the key genetic lesion of hairy cell leukemia^{10,11}, and Chung et al. reported that this mutation is acquired in the hematopoietic stem cell compartment¹². Tiacci et al. showed that, as in other BRAF-mutated neoplasms¹³, the V600E mutation in hairy cell leukemia constitutively activates the MAPK pathway^{14,15}. The same group demonstrated *in vitro*¹⁶ that BRAF inhibitors reverse the unique molecular signature² and morphology of patients' hairy cells, and potently induce their apoptosis, thus establishing BRAF-V600E as an attractive therapeutic target in hairy cell leukemia. Moreover, anti-leukemic activity of BRAF inhibitors has been anecdotally reported in these patients^{17,18}.

We conducted two phase-2 multicenter clinical trials, in Italy and USA, to define the efficacy and safety of an oral BRAF inhibitor, vemurafenib¹⁹, in patients with relapsed or refractory HCL. Patients in the Italian trial completed enrollment in April 2013 and have a median follow-up of almost 2 years from the end of treatment. The American trial has not yet completed patient enrollment but already met its primary endpoint (overall response rate). Here we report the results of both trials.

MATERIALS AND METHODS

Patients

In the Italian trial, 28 patients were enrolled (Table S1) meeting the following criteria: refractoriness to a purine analogue (no response or relapse within 1 year following treatment; n=6); early relapse after a purine analogue (between 1 and 2 years from the first course, or within 4 years from a second or later course, n=20); or whenever in case of significant bone marrow hypoplasia at relapse, n=1); or severe side effects from a previous purine analogue (n=1). Patients had to have Hb <11.0 g/dl and/or neutrophils <1,500/mm³ and/or platelets <100,000/mm³. The diagnosis was centrally confirmed by two authors (BF,

ET) through annexin-A1 immunostaining of marrow biopsy²⁰, BRAF-V600E detection by allele-specific PCR¹¹ and flow cytometry. Further details are provided in the study protocol available at NEJM.org.

In the American trial, 26 patients were enrolled (Table S2) meeting the following criteria: refractoriness to a purine analogue (as defined above); early relapse (between 1 and 2 years) after the first course of purine analogue; or ≥ 2 relapses after 2 years from a third or later course of purine analogue. Patients had to have neutrophils $\leq 1,000/\text{mm}^3$, Hb $\leq 10\text{g/dl}$, or platelets $\leq 100,000/\text{mm}^3$. BRAF-V600E was assessed with immunohistochemistry, PCR/Sanger sequencing and/or MSKCC IMPACT assay²¹ (the latter done in all patients) in a CLIA-certified laboratory. Further details are provided in the study protocol available at NEJM.org.

Study design

The Italian trial (B. Falini: principal investigator) is a phase-2, single-arm, multicenter study sponsored by the Institute of Hematology, University of Perugia, and designed by two academic investigators (BF and ET). Roche provided vemurafenib and an unconditional research grant. The planned enrollment of 28 patients was completed in 11 months (May 2012-April 2013) at 8 centers. Patients received oral vemurafenib 960 mg twice daily for a minimum of 8 weeks and, if not achieving complete response, a maximum of 16 weeks (Supplementary Figure 1). The first 3 patients received 20 weeks of vemurafenib. The primary endpoint was complete response. Secondary endpoints included time to response, relapse-free survival and safety. We also evaluated overall response, progression-free survival (PFS), treatment-free survival (TFS) and pharmacodynamic biomarkers.

The American trial (J. Park: principal investigator), a phase-2 single-arm multicenter study, enrolled patients consecutively from 1/2013-2/2015 at 6 sites. Patients received oral vemurafenib 960 mg twice daily, on a continuous schedule for 3 months. Patients with residual disease (by morphology or immunohistochemistry for CD20, PAX5, DBA44 and tartrate-resistant alkaline phosphatase) were allowed to receive up to 3 additional months of vemurafenib. The primary end point was overall response after 3 months of vemurafenib. Secondary end points were overall survival, progression-free survival, time-to-relapse and pharmacodynamic analyses.

In both trials, at disease relapse, defined by peripheral blood counts low enough to meet the respective initial eligibility criteria, retreatment with vemurafenib was allowed, for up to 12 weeks (Italian trial) and until disease progression or unacceptable toxicity (American trial).

Study assessments

Adverse events were graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) v4.03. Neoplastic cutaneous lesions were surgically excised but did not mandate dose modifications.

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

Marrow and peripheral blood samples were centralized at the Institute of Hematology, University of Perugia. The Hairy Cell Index (Supplementary Methods) in the marrow biopsy was calculated as previously described²².

In the American trial, marrow assessments were obtained after 1 and 3 months of vemurafenib and at end of treatment. For patients with splenomegaly at enrollment, abdominal CT was performed at baseline and at 3 months. All patients underwent dermatologic examination at baseline, after 1 month of vemurafenib, and every 3 months thereafter for at least 1 year after drug discontinuation.

In both trials, complete response required resolution of any cytopenias (neutrophils $\geq 1,500/\text{mm}^3$, platelets $\geq 100,000/\text{mm}^3$, Hb ≥ 1 g/dl), no evidence of hairy cells in the peripheral blood and marrow biopsy by non-immunologic stains, and no splenomegaly. Partial remission required: resolution of cytopenias; $\geq 50\%$ splenomegaly reduction; and $\geq 50\%$ reduction of leukemic infiltration in both the marrow and peripheral blood. In both trials, relapse after complete or partial remission was defined as re-appearance of cytopenia(s) on at least two consecutive occasions (the first of which counted as relapse date).

Pharmacodynamic analyses

Changes in leukemia cell morphology during vemurafenib exposure and bone marrow ERK phosphorylation at the end of treatment (in a blinded fashion with respect to patients' outcome post-treatment) were assessed as described^{14,16}. Leukemia disease burden was assessed by serial flow cytometric analysis of peripheral blood or marrow mononuclear cells^{5,23}. DNA from peripheral blood mononuclear cells was used to quantify BRAF-V600E allele burden using droplet digital PCR. Soluble IL2RA and IL1R-type II were measured in serum²⁴. Targeted genomic analysis was performed in peripheral blood mononuclear cells as described²⁵. Further details are provided in Supplementary Methods.

Statistical analysis

A Simon's minimax two-stage design²⁷ was adopted in both trials. Full details are provided in Supplementary Methods.

RESULTS

Patients

The characteristics of all patients (n=54) are shown in Table 1 and Tables S1-S2. Six patients with disease refractory to a purine analogue were in the Italian study and 1 in the American one, and had undergone a median of 2.5 and 1 prior therapies before vemurafenib, respectively. Patients relapsed early and/or repeatedly after purine analogues (21 and 25, respectively) had received a median of 4 and 3 prior therapies, respectively. Fifty-four percent (15/28) of the Italian patients were refractory to the immediate prior therapy as were 41% (9/22) (41%) of the American patients.

In the Italian trial, 26/28 patients completed the planned treatment (median weeks of vemurafenib: 16, range 8-20). Two patients went off-study after ≤ 1 week due to drug-

unrelated acute myocardial infarction and to consent withdrawal after grade-3 drug-related reversible pancreatitis.

In the American trial, 24/26 patients had a median of 4.5 (range 3-6) months of treatment. One patient died from drug-unrelated progressive pneumonia after 23 days on treatment, and another one withdrew consent after 1 month due to grade-3 drug-related reversible photosensitivity.

Adverse events

Table 2 shows drug-related adverse events of grade ≥ 2 observed in ≥ 1 patient in each trial. Tables S3-S4 shows all adverse events of any grade (including those not considered drug-related in the Italian study).

In both trials, common vemurafenib-related adverse events, mostly of grade 1-2 and all reversible, included skin toxicities (especially rash and photosensitivity), arthralgias/arthritis, pyrexia and bilirubin increase. Other relatively frequent drug-related adverse events (recorded in one and/or the other study) were skin papillomas, alopecia, palmar/plantar hyperkeratosis/dysesthesia, fatigue, and increase of serum lipase (including 2 patients with grade-3 pancreatitis), transaminases, alkaline phosphatase or fibrinogen.

In the Italian study, cutaneous basal cell carcinomas developed in 2 patients (one with a previous history of basal cell carcinoma) and a cutaneous superficial melanoma occurred in one patient. In the American study, cutaneous squamous cell carcinomas developed in 3 patients (all with a previous history of squamous cell carcinoma), and a cutaneous basal cell carcinoma developed in 1 patient. All these tumors were managed by simple excision.

A slight transient Hb decrease was noted in 10 Italian patients within the first 6 weeks of treatment, and neutropenia in 1 American patient.

In the Italian trial, the planned vemurafenib dose (twice daily 960 mg) was reduced for ≥ 3 weeks in 17 instances involving 15/26 (58%) patients to twice daily 720 mg (9 patients), 480 mg (3 patients) or 240 mg (3 patients); toxicities related to these events were mainly rash in 6 patients and increased creatinine or bilirubin, arthralgia, pancreatitis, and palmo-plantar dysesthesia, each in 1 patient.

In the American trial, 13 patients (50%) required dose reductions to twice daily 720 mg (2 patients), 480 mg (10 patients) or 240 mg (1 patient). Adverse events leading to dose modifications were arthralgia (5 patients), rash (5 patients), photosensitivity (1 patient), neutropenia (1 patient), and AST/ALT elevations (1 patient).

Response rate

The overall response rate was 96% (25/26 evaluable Italian patients) and 100% (24/24 evaluable American patients) in the two trials. The unusual presenting features of the only non-responding patient are described in Supplementary Results.

Complete and partial response rates were also similar in both trials. The Italian study showed 34.6% CRs (9/26 patients) and 61.4% PRs (16/26), after a median of 8 weeks (Table

S1). Complete and partial responders included 1 and 5 primary refractory ones, respectively. Notably, 4 complete and 10 partial responders were resistant to the last prior treatment. Median time for recovery of platelets ($\geq 100,000/\text{mm}^3$), neutrophils ($\geq 1,500/\text{mm}^3$) and Hb ($\geq 1 \text{ g/dl}$) was 2, 4 and 8 weeks, respectively. Reversion of symptomatic splenomegaly and clearance of leukemia cells from the blood (by cytomorphology) usually occurred within 2 weeks. A significant decrease in marrow leukemic infiltration was observed at the earliest time point evaluated (4 weeks) (not shown). However, in all complete responders, immunohistochemistry showed minimal residual disease ($\leq 10\%$) at the end of treatment (Figure 1A-B).

In the American trial, after 3 months of vemurafenib 10/24 (41.7%) patients achieved complete and 14/24 (58.3%) a partial response (Table S2). Most patients achieved recovery of neutrophils ($> 1,000/\text{mm}^3$), Hb ($> 10\text{g/dl}$), and platelets ($> 100,000/\text{mm}^3$) by 28 days (Figure 2A). In patients receiving twice daily vemurafenib 480 mg for > 1 month ($n=10$), similar complete and partial response rates were observed (6/10 and 4/10, respectively).

In unplanned exploratory analyses of both studies, treatment duration did not appear to influence response depth (Supplementary Results), and complete rate was not influenced by prior splenectomy, marrow disease burden, refractoriness to the last treatment or number of prior therapies (Tables S1-S2 and not shown).

Response duration

In the Italian study, the median follow-up for the 25 responding patients was 23 months (range 7-28) from the last vemurafenib dose. Median relapse-free survival was 9 months, being significantly longer in complete than in partial responders (19 and 6 months, respectively; p -value 0.006; HR 0.26, 95% CI 0.10-0.68; Figure 1C). Median treatment-free survival was 21.5 months in all 26 evaluable patients, and (with the limitation of small numbers) did not differ between complete and partial responders (25 and 18 months, respectively; p -value 0.21; Figure 1C). Indeed, of the 20 relapsed patients (5 after complete and 15 after partial responses), 7 did not require therapy at a median of 15 (range 4-18) months following relapse, as their cytopenia(s) are stably mild (median Hb 14.2 g/dl, range 12.4-17.5 g/dl; median neutrophils $1122/\text{mm}^3$, range $938\text{-}1724/\text{mm}^3$; median platelets: $86,000/\text{mm}^3$, range $63,000\text{-}269,000/\text{mm}^3$). Conversely, in 13/20 relapsed patients cytopenia(s) worsened requiring an anti-leukemic treatment at a median of 5 (range 0-16) months after relapse. Five of 25 patients (4 complete 1 partial responder) have not relapsed at the last follow-up (23-25 months post-treatment). Three of 4 complete responders showed no morphologic evidence of hairy cell leukemia in their last marrow biopsy at 13, 19 and 24 months, respectively, whereas the other patient lost the histologic complete response status at 12 months but maintained normal blood counts at the last follow-up (24 months). In unplanned exploratory analyses, relapse-free and treatment-free survival did not differ between patients receiving ($n=18$) or not receiving ($n=7$) additional treatment with vemurafenib after achievement of their best response, or between patients requiring ($n=14$) or not requiring ($n=11$) a dose reduction (not shown). However, as compared to the 18 non-splenectomized patients, the 7 splenectomized patients had shorter relapse-free survival (median of 11 and 6 months, respectively; HR 3.5, 95% CI 1.04-12.1; p -value 0.04) and

treatment-free survival (median of 25 and 11 months, respectively; HR 6.6, 95% CI 1.6-28; p-value 0.010).

In the American trial, median follow-up from the first vemurafenib dose among survivors was 11.7 months (range, 1.3 – 25.4). At one year, progression-free survival was 73% (95% CI: 55-97) and overall survival was 91% (95% CI: 79-99) (Figure 2B). Disease progression developed in 7 (3 complete and 4 partial responders) of 24 patients (29%) (Table S2). At one year following response, the cumulative incidence of relapse was 27% (95% CI: 7-51).

In both trials, vemurafenib retreatment at relapse produced some responses, detailed in Supplementary Results.

Pharmacodynamic analyses and resistance mechanisms

Hairy cell leukemia cells exposed *in vitro* to vemurafenib downregulate CD25 and lose their surface projections¹⁶. Consistently, blood and/or marrow flow cytometry documented loss of surface CD25 in a large fraction of leukemia cells at one or more time points during therapy in 15/19 (79%) evaluable Italian patients (including one already reported¹⁶) (Figure 3A), suggesting that measurement of surface or soluble CD25 may underestimate the residual HCL burden during vemurafenib treatment. Interestingly, in one patient with marked lymphocytosis, leukemic cells showed a clear smoothing of the cell membrane 2 and 3 days after starting vemurafenib (Figure 3B). Moreover, in 13/26 Italian patients a marrow biopsy taken the day after therapy completion was evaluable for phospho-ERK/PAX5 double immunostaining. In 6/13 patients (all partial responders), residual hairy cells (PAX5+) still expressing phospho-ERK were observed despite prolonged (16-20 weeks) exposure to vemurafenib (Figure 3C, left). In 7/13 patients (2 complete and 5 partial responders after 12-20 weeks of treatment), residual leukemic cells did not detectably express phospho-ERK (Figure 3C, right). Median progression-free survival in patients with or without residual phospho-ERK+ leukemic cells was 7.75 months (range 5-13) and 13 months (range 7.5-24), respectively (p-value 0.004; HR 10.3, 95% CI 2.1-51). Interestingly, residual disease measured by the hairy cell index was higher in patients with persistent versus undetectable phospho-ERK expression in leukemic cells (average hairy cell index: 0.139 versus 0.054; p-value 0.026, two-sided Mann-Whitney test). Thus, persistent ERK phosphorylation in residual hairy cells at the end of treatment may indicate a greater residual disease burden and a shorter progression-free survival, suggesting MEK/ERK reactivation is a resistance mechanism to vemurafenib.

In the American trial, pre-vemurafenib the mean percentage of leukemic cells in blood and marrow mononuclear cells by flow cytometry was 16.0% and 16.8%, respectively. This decreased to 3.4% and 6.9%, respectively, after 1 month of treatment (p-value <0.05 - Figure 2C) and to 0.74% and 4.2%, respectively, by 3 months. Bone marrow hairy cells decreased even further after 6 months to a mean of 1.3% (Figure 2C). Likewise, digital PCR revealed a >100-fold decrease of BRAF-V600E allele burden in peripheral blood mononuclear cells after 3 months of vemurafenib ($p=0.0001$ compared to pretreatment; Figure 2D). Accordingly, phosphorylated ERK1/2 rapidly decreased in marrow biopsies after 1 month of vemurafenib (Figure S3A). Finally, in all patients serum levels of soluble IL2RA and IL1R type II, well established hairy cell leukemia biomarkers^{27,28}, markedly declined, relative to

the baseline, by a mean of 30% and 27%, respectively, within 24 hours of vemurafenib and by $\geq 5\%$ after 3 months (Figure S3B-C).

In one American patient refractory to vemurafenib retreatment, targeted sequencing of 300 genes (Table S3) identified 2 separate activating subclonal *KRAS* mutations (along with a new *RUNX1* mutation) at relapse after the initial vemurafenib treatment, in addition to the recurrence of the BRAF-V600E mutation. *KRAS* mutations were not seen before vemurafenib or at remission (day 120) despite high *KRAS* sequencing coverage (range: 496X-1165X) (Figure 4B; Table S5). Because in BRAF-V600E mutant malignancies activating *RAS* mutations represent a known mechanism of vemurafenib resistance through MEK/ERK rephosphorylation²⁹, the *KRAS* mutations newly acquired in this patient at relapse, although subclonal, likely contributed to the subsequent insensitivity to vemurafenib (Figure 4C).

DISCUSSION

In both trials, an oral course of vemurafenib (960 mg twice daily for 16-18 months) was rapidly and highly effective (96%-100% response rate) with low-grade toxicity in hairy cell leukemia patients who were not only heavily pre-treated but often (41%-53% of cases) refractory to the last prior therapy. The median time to response was 8 weeks in the Italian trial and 12 weeks in the American trial. Further treatment did not appear to improve response depth or duration. Retreatment with vemurafenib at relapse was effective, although to a lesser extent in Italian patients relapsing after partial response versus after complete response.

Unlike vemurafenib dosing of indefinite duration as in metastatic melanoma³⁰⁻³², we limited initial vemurafenib treatment to a few months (even if this perhaps reduced response depth and/or duration) due to the concern of vemurafenib-induced secondary tumors³³⁻³⁵. Although the latter mostly affect the skin and are of low malignant potential, their development may be less acceptable in an indolent leukemia (however refractory or multiply relapsed) compared to metastatic melanoma.

The response rate, depth and duration in hairy cell leukemia was higher than in metastatic melanoma³⁰⁻³², possibly due to the less complex hairy cell leukemia genome landscape (dominated by BRAF-V600E¹⁰) and the much lower proliferative index ($\leq 1\%$) of hairy cell leukemia versus metastatic melanoma³⁶⁻³⁷. However, in both trials marrow residual hairy cells with their associated BRAF-V600E allele burden were consistently present at the end of treatment, even after complete response. In about half of evaluable Italian patients, these cells exhibited persistent phospho-ERK expression despite prolonged ongoing exposure to vemurafenib, which appeared to correlate with a higher residual leukemic burden and shorter progression-free survival. This suggests that, in at least some patients, leukemic cells develop alternative mechanisms for reactivating MEK/ERK to bypass BRAF blockade. *In vitro* studies from the Italian group suggest that the marrow microenvironment might oppose vemurafenib-induced ERK dephosphorylation and apoptosis¹⁶. Intriguingly, it was proposed³⁸ that, passing through the vitronectin-rich splenic red pulp, hairy cells receive vitronectin-mediated pro-apoptotic signals that are transduced by p38/JNK activation but are

overcome by concomitant anti-apoptotic signals from constitutive MEK-ERK activation. We noted that prior splenectomy appeared to associate with shorter response duration. Thus, lack of hairy cell recirculation through the spleen resulting in their continuous homing to the protective marrow microenvironment may favor the persistence of ERK phosphorylation in leukemic cells at the end of treatment, a feature also apparently associated with a shorter response duration. Furthermore, one patient in the American trial acquired at relapse two activating *KRAS* mutations likely contributing to disease progression on vemurafenib retreatment.

Vemurafenib-related adverse events were manageable and similar to those observed in melanoma (e.g., rash, arthralgias and secondary cutaneous tumors). Importantly, lack of significant myelotoxicity makes vemurafenib an ideal salvage treatment for multiply relapsed hairy cell leukemia patients with pancytopenia and scarce marrow reserve due to previous chemotherapies.

Lower vemurafenib doses (e.g., 240 mg twice daily) from the beginning of treatment proved effective in anecdotal relapsed/refractory hairy cell leukemia patients¹⁷. In our studies, the starting dose, 960 mg twice daily, was reduced only if toxicity occurred, and mostly to 720 mg (in Italian patients) or 480 mg (in American patients) twice daily, followed sometimes by re-escalation. Prospective clinical trials are needed to formally evaluate toxicity, response rate and survival with lower vemurafenib doses and/or longer treatment durations. Clinical trials are also warranted to evaluate the other clinical BRAF inhibitor, dabrafenib, which *in vitro* studies from the Italian group¹⁶ and single case reports^{18,39} suggest to be effective in refractory/relapsed HCL.

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 HCL 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, as successfully exploited in metastatic melanoma⁴⁰.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Italian study

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American study

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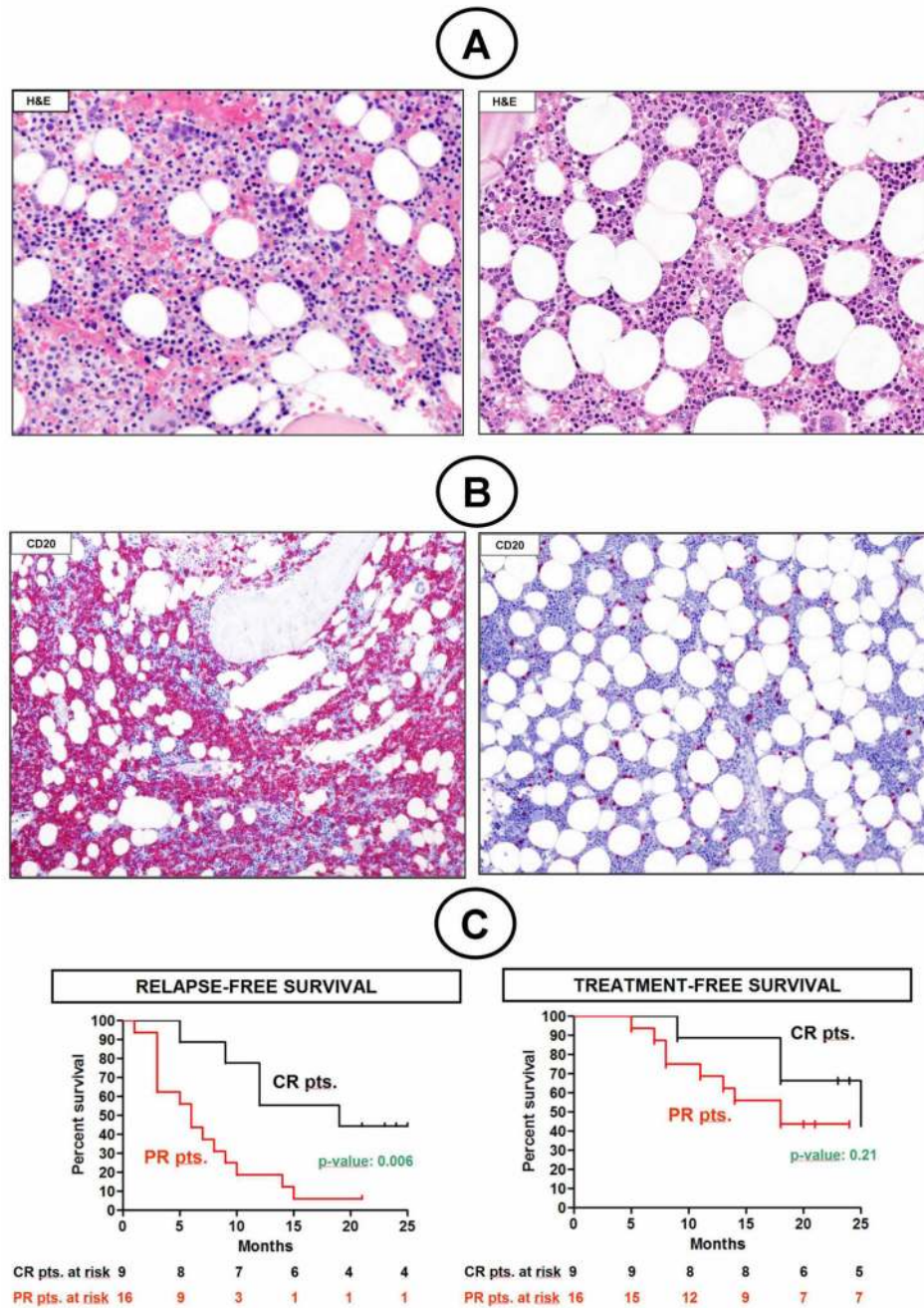


Figure 1. Response depth and duration in the 25 patients of the Italian trial responding to vemurafenib

(A) Hematoxylin-eosin staining of a BM biopsy from a patient showing infiltration by leukemic HCL cells with wide, clear cytoplasm recognizable by morphology at baseline (left panel), but not after 8 weeks of vemurafenib (right panel). (B) CD20 immunostaining of the same BM biopsy showing ~75% leukemic infiltration at baseline (left panel), and only few ($\leq 10\%$) residual scattered hairy cells after 8 weeks of vemurafenib (right panel). (C)

Relapse-free (left) and treatment-free survival (right) of patients obtaining a CR (black line) or PR (red line).

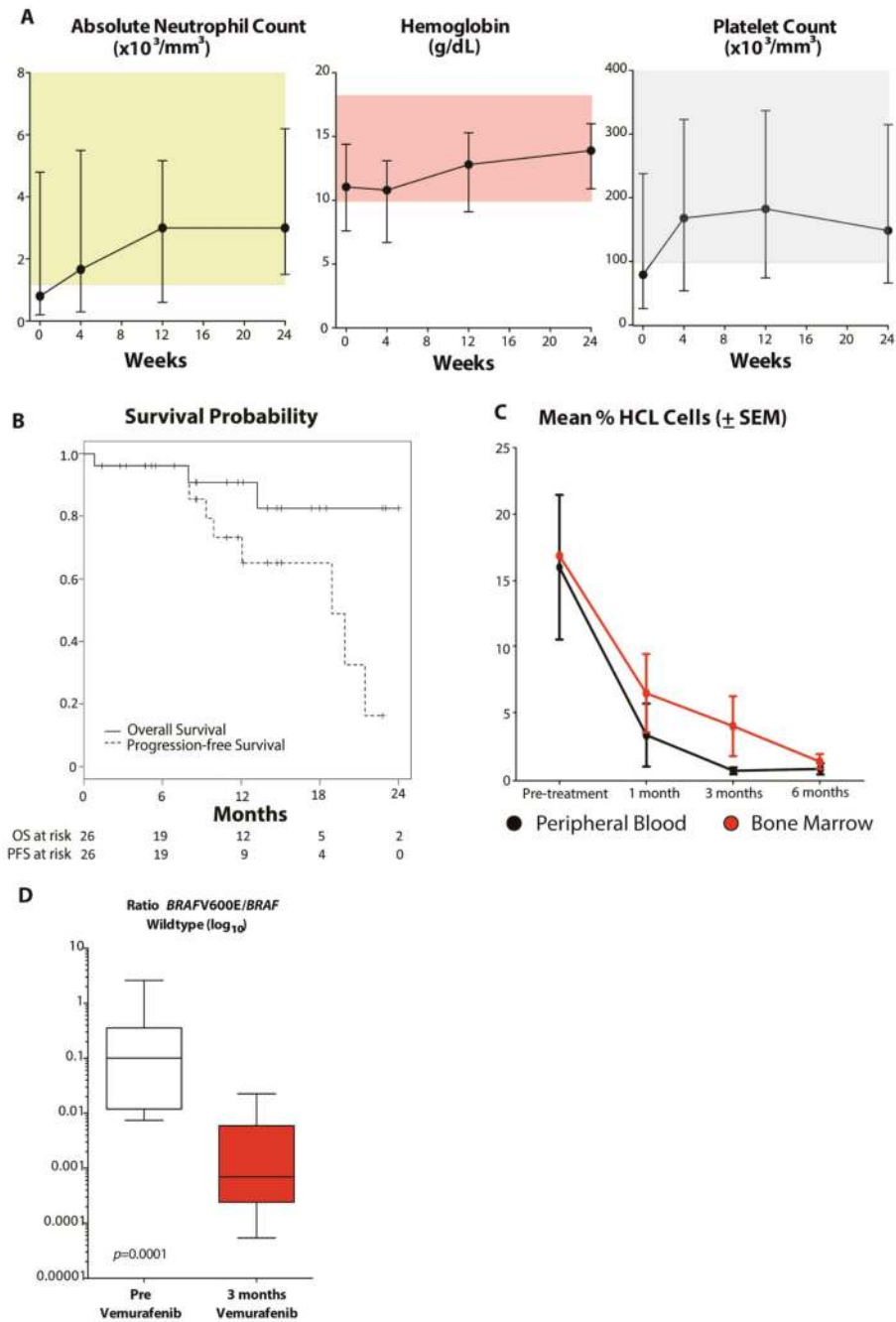


Figure 2. Effect of vemurafenib on peripheral blood counts, survival, flow cytometric enumeration of hairy cells, and *BRAFV600E* allele burden

(A) Median value of the absolute neutrophil count, hemoglobin and platelet cell counts of the 24 evaluable patients over time after initiating vemurafenib treatment. Error bars denote the upper and lower limits. (B) The probability of progression-free survival and overall survival for all 26 patients. Tick marks indicate censored data. (C) Mean percentage of HCL cells in peripheral blood and bone marrow mononuclear cells over time of patients from this study (\pm standard error of mean (SEM)). Mean percentage of HCL cells decreased with each

period of vemurafenib therapy shown ($p < 0.05$ comparing percentage of HCL cells for each time point on therapy relative to pretreatment values). (D) Ratio of the concentration of *BRAF*V600E:*BRAF*wildtype alleles (\log_{10}) in DNA from peripheral blood mononuclear cells pretreatment with vemurafenib and following 3 months of vemurafenib. The ratios are displayed as box-and-whisker plots with the median value as the middle bar, the ends of the boxes as upper and lower quartile values, and ends of whiskers as highest and lowest values.

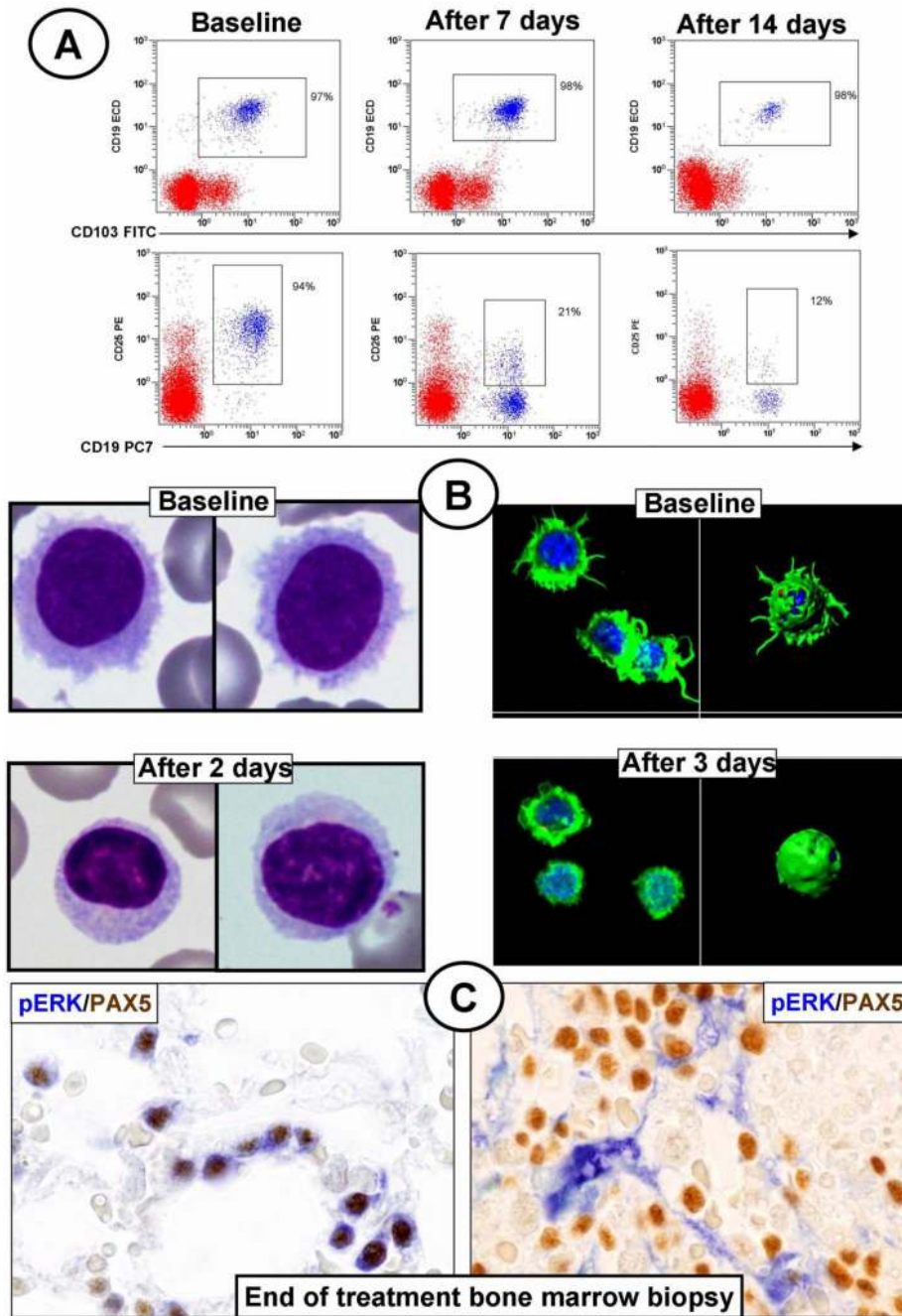


Figure 3. Phenotypic and molecular changes of HCL cells during treatment with vemurafenib in the Italian trial

(A) Flow cytometry dot plots of a patient's BM aspirate gated on CD45 cells and showing at baseline (left panels) CD25 expression by 94% of leukemic cells (CD19+/CD103+, upper row; CD19+/CD25+, bottom row). A progressively loss of CD25 expression (but not of CD19 or CD103 expression) is seen over 1 and 2 weeks of treatment with vemurafenib (down to 21% and 12%, respectively; middle and right panels, respectively). The remaining cells (red events) are normal CD45+ hematopoietic cells. (B) *Left panels*. May-Grünwald-

Giemsa staining of a patient's peripheral blood smear featuring leukemic cells rich in hairy projections at baseline (top), but not after 2 days of vemurafenib treatment (bottom). *Right panels.* Confocal fluorescence microscopy analysis of blood leukemic cells purified from the same patient shows prominent surface projections (stained in green by phalloidin) at baseline (top), but not after 3 days of vemurafenib treatment (bottom); these changes are clearly evident both in electronically magnified two-dimensional and three-dimensional reconstructed images of representative cells; (C) Double immunostaining of BM biopsy taken the day after the end of treatment and double stained for PAX5 (a B-cell marker; in brown) and phospho-ERK (pERK; in blue). In some responding patients (one exemplified in the left panel) persistence of pERK+ leukemic hairy cells is observed; conversely, in other patients (one exemplified in the right panel) residual hairy cells do not detectably express phospho-ERK, with stromal cells strongly positive for phospho-ERK as internal positive control.

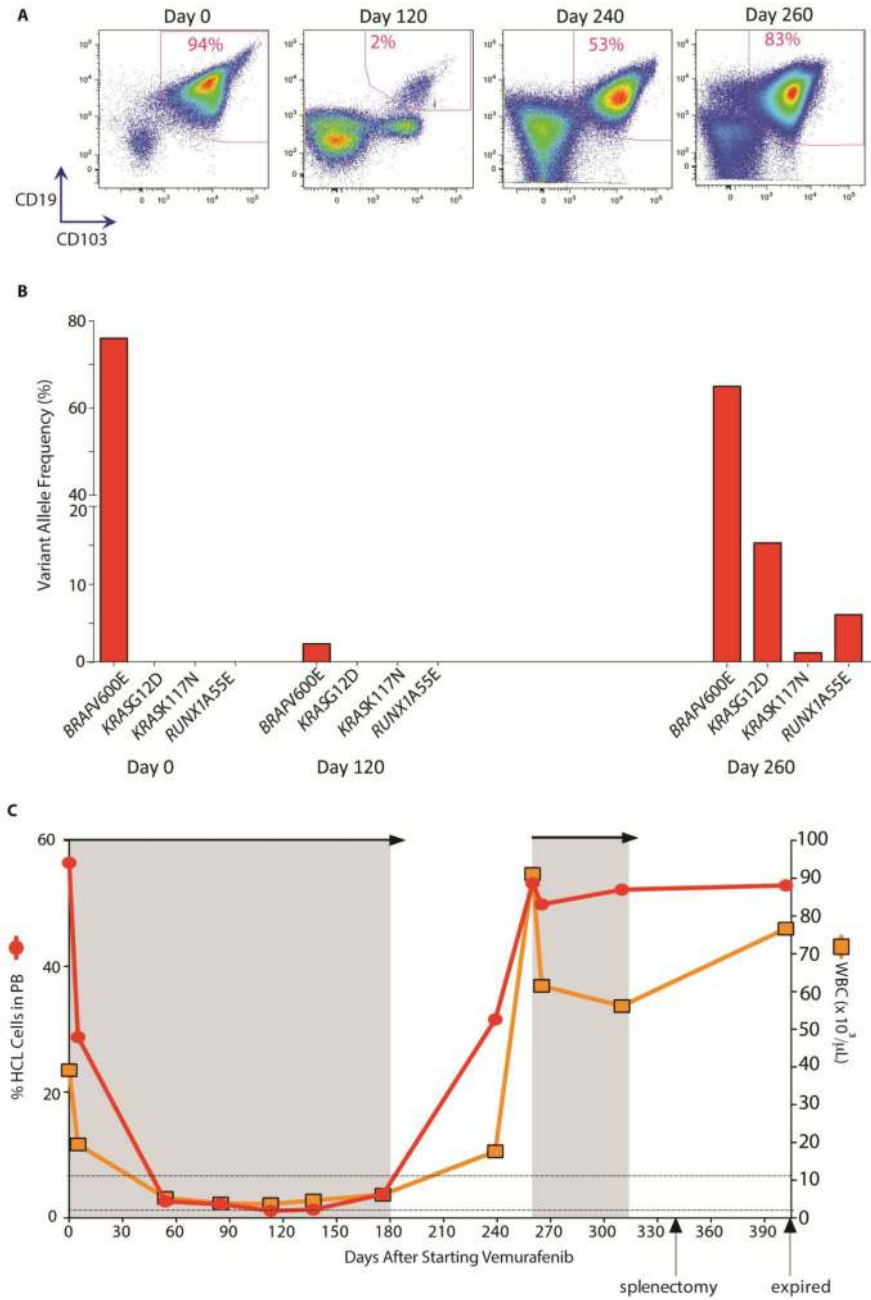


Figure 4. Serial genomic analysis reveals activating *KRAS* mutations associated with development of acquired vemurafenib resistance in *BRAFV600E*-mutant HCL
(A) Serial flow cytometric analysis for HCL cells in peripheral blood mononuclear cells throughout the initial course of therapy (days 0-180) and vemurafenib retreatment (days 260-320). Percentages in FACS plots denote percentage of HCL (CD19/CD103) cells amongst PB MNCs. **(B)** Somatic mutations identified in PB MNCs following 0, 120, and 260 days of vemurafenib administration. The variant allele frequency (%) is shown for mutations seen at each timepoint. **(C)** Graph of percentage of HCL cells in peripheral blood

(left axis) and total WBC count (right axis). Shaded areas represent periods of vemurafenib treatment and retreatment.

Table 1
Baseline demographic and clinical characteristics of the patients

Characteristic	Italian Trial (N=28)	American Trial (N=26)
Age		
Median – yr	57	62
Range – yr	27 - 84	44 - 80
Number of previous therapies		
Median	3	3
Range	1 - 12	1 - 8
Refractory to immediate prior therapy		9*
Yes	15	13*
No	12	
Prior splenectomy – % of patients		
Yes	28	19
No	69	81
Extent of HCL		
Median hairy cell involvement in bone marrow (range) [¶] - %	82.5 (40 - 95)	80 (25 – 100)
Median absolute neutrophil count (range), – ×10 ³ /mm ³	0.8 (0.2 - 5.7)	0.8 (0.2 – 4.8)
Median hemoglobin (range), – g/dl	12.2 (8.2 - 17)	10.5 (8.2 – 14.4)
Median platelet count (range), – ×10 ³ /mm ³	70 (6 - 306)	78 (26 – 238)

*The data on refractoriness to immediate prior therapy was available in 22 patients.

[¶]By immunohistochemistry.

Table 2
Drug-related adverse events of grade ≥ 2 *

Adverse Event	Italian trial				American Trial			
	No. of patients (%)				No. of patients (%)			
	Grade 2	Grade 3	Grade 4	Total	Grade 2	Grade 3	Grade 4	Total
Arthralgia/Arthritis	10 (36)	2 (7)	0	12 (43)	8 (31)	0	0	8 (31)
Rash/Erythema	11 (39)	2 (7)	0	13 (46)	11 (42)	5 (19)	0	16 (62)
Cutaneous basal cell carcinoma	2 ^o (4)	0	0	1 (4)	1 (4)	0	0	1 (4)
Cutaneous superficial melanoma	0	1 (4)	0	1 (4)	0	0	0	0
Cutaneous squamous cell carcinoma	0	0	0	0	3 (11) [¶]	0	0	3 (11)
Skin papillomas	2 (7)	0	0	2 (7)	0	0	0	0
Photosensitivity reaction	2 (7)	0	0	7 (11)	0	2 (8)	0	2 (8)
Hyperkeratosis	3 (11)	0	0	3 (11)	0	0	0	0
Panniculitis	2 (7)	0	0	2 (7)	0	0	0	0
Pancreatitis	1 [^] (4)	2 (7)	0	3 (11)	0	0	0	0
Pancreatic enzymes increased	1 (4)	1 (4)	0	2 (7)	0	0	0	0
Hyperbilirubinaemia	2 (7)	1 (4)	0	3 (11)	3 (11)	0	0	3 (11)
Transaminases increased	1 (4)	0	0	1 (4)	3 (11)	1 (4)	0	4 (15)
Alkaline phosphatase increased	0	0	0	0	0	2 (8)	0	2 (8)
Pain to the extremities	2 (7)	0	0	2 (7)	2 (8)	0	0	2 (8)
Headache	1 (4)	0	0	1 (4)	0	0	0	0
Blood creatinine increased	2 (7)	0	0	2 (7)	1	0	0	1 (4)
Seborrhoeic keratosis	2 (7)	0	0	2 (7)	0	0	0	0
Asthenia	1 (4)	0	0	1 (4)	0	2 (8)	0	2 (8)
Musculoskeletal pain	1 (4)	0	0	1 (4)	0	0	0	0
Abdominal pain	1 (4)	0	0	1 (4)	0	0	0	0
Pyrexia	0	0	0	0	0	1 (4)	0	1 (4)
Nausea	0	0	0	0	1 (4)	0	0	1 (4)
Alopecia	0	0	0	0	1 (4)	0	0	1 (4)

* Occurring in at least 1 patient.

^o In one patient two basal cell carcinomas developed; the other patient had a previous history of basal cell carcinoma.

[¶] All 3 patients had previous history of squamous cell carcinoma.

[^] Lipase and/or amylase elevation only (no clinical symptoms nor radiological abnormalities).