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Progression of RAS-Mutant Leukemia during RAF Inhibitor Treatment

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SUMMARY

Vemurafenib, a selective RAF inhibitor, extends survival among patients with BRAF V600E–mutant melanoma. Vemurafenib inhibits ERK signaling in BRAF V600E–mutant cells but activates ERK signaling in BRAF wild-type cells. This paradoxical activation of ERK signaling is the mechanistic basis for the development of RAS-mutant squamous-cell skin cancers in patients treated with RAF inhibitors. We report the accelerated growth of a previously unsuspected RAS-mutant leukemia in a patient with melanoma who was receiving vemurafenib. Exposure to vemurafenib induced hyperactivation of ERK signaling and proliferation of the leukemic cell population, an effect that was reversed on drug withdrawal.

Approximately 50% of patients with metastatic melanoma harbor a somatic V600E mutation — or, less frequently, a V600K mutation — in the BRAF kinase.^{1–4} These activating mutations drive increased ERK signaling, promoting the proliferation and survival of melanoma cells.⁵ Selective RAF inhibitors, such as vemurafenib and dabrafenib, inhibit ERK signaling and arrest growth in tumors with BRAF V600E or BRAF V600K mutations.^{3,6–8} Treatment with vemurafenib or dabrafenib induces tumor regression in more than half of patients with BRAF V600E–mutant metastatic melanoma. Both drugs also improve the rate of progression-free survival, as compared with dacarbazine.^{7,9–11} Vemurafenib has also been shown to improve overall survival.¹⁰

However, in tumors and normal cells with wild-type RAF, these inhibitors stimulate ERK signaling in a RAS-dependent manner.^{8,12–16} This paradoxical activation is thought to explain why this class of drugs induces cutaneous neoplasia, such as keratoacanthomas and squamous-cell carcinomas.¹⁷ These drugs may also increase the incidence of primary melanomas.¹⁸ Molecular analysis of cutaneous squamous lesions indicates that many harbor activating mutations in RAS.^{19,20} To our knowledge, however, no published reports have documented induction of noncutaneous neoplasia.

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We present the case of a patient in whom there was development of clinically evident NRAS-mutated chronic myelomonocytic leukemia shortly after the initiation of vemurafenib therapy for metastatic BRAF-mutant melanoma.

CASE REPORT

A 76-year-old man with stage IV (T3aNxM1b) BRAF V600K-mutant melanoma presented for evaluation. Six years earlier he had received a diagnosis of stage IIA melanoma on his neck that had been widely excised. One month before presentation, a subcarinal mass developed that compressed his left main-stem bronchus and caused dyspnea. Bronchoscopic débridement and biopsy of the mass confirmed the diagnosis of metastatic melanoma, and he was subsequently referred to our institution. At that time, his white-cell count was elevated, at 18,100 cells per cubic millimeter (reference range, 4000 to 11,000), with an increase in the absolute monocyte count to 3000 cells per cubic millimeter (reference range, 0 to 1300) (Fig. 1A). He was initially treated with intravenous ipilimumab (3 mg per kilogram of body weight every 3 weeks, for a total of 4 doses). Progression of the tumor was documented at 12 weeks and 18 weeks from the date of initial treatment.

An analysis of genomic DNA from the biopsy specimen of the tumor revealed a BRAF V600K mutation. No RAS mutation was detected. The patient began taking 960 mg of vemurafenib twice a day. At the time vemurafenib was initiated, his white-cell count was 25,600 per cubic millimeter; the leukocytosis was attributed to a resolving postobstructive pneumonia. After 11 days, the patient noted a dramatic improvement in his breathing, although he also reported new, profound fatigue. On evaluation, it was noted that his white-cell count had become markedly elevated (80,900 per cubic millimeter), and there were also increases in the absolute number of monocytes (27,600 per cubic millimeter) and neutrophils (35,900 per cubic millimeter) (Fig. 1A). Vemurafenib was stopped, although on physical examination it was determined that a previously palpable subcutaneous tumor had nearly resolved.

A review of the peripheral-blood smear confirmed the monocytosis. A bone marrow-biopsy specimen and aspirate were obtained. On examination, the samples revealed increased numbers of monocytes and neutrophils, a left shift with dysplastic myeloid precursors, and 6% blasts (Fig. 1B). Immunophenotyping of bone marrow aspirate and peripheral blood with the use of flow cytometry identified a monocyte population characterized by expression of CD14, CD13, CD33, and HLA-DR, with aberrant expression of CD56 and CD2 (Fig. 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org). Together, the clinical and hematopathological findings were diagnostic of chronic myelomonocytic leukemia. Karyotypic analysis and fluorescence in situ hybridization revealed no cytogenetic abnormalities.

Two weeks after the vemurafenib was discontinued, the patient's white-cell and monocyte counts had decreased, to 26,900 per cubic millimeter and 6400 per cubic millimeter, respectively. In an attempt to attenuate the rise in the white-cell count with vemurafenib, treatment was resumed at a lower dose (720 mg twice daily). However, within days, the patient's white-cell count again began to rise (Fig. 1A). Hydroxyurea (500 mg daily) was added to the vemurafenib treatment, but there was no significant reduction in the white-cell count. Vemurafenib was withheld once the white-cell count reached 69,000 per cubic millimeter. After 2 weeks, the white-cell count had fallen to 28,600 per cubic millimeter.

Computed tomographic scans obtained 8 weeks and 16 weeks after the patient started taking vemurafenib showed that there had been a partial response to treatment, with a 43% decrease in disease burden at 16 weeks, including a reduction in the mediastinal lymphadenopathy and resolution of a hepatic metastasis (Fig. 1C). Also noted at this interval

was the development of splenomegaly, which was consistent with progressive chronic myelomonocytic leukemia. At present, the patient is being treated with intermittent dosing of vemurafenib, guided by changes in white-cell counts.

RESULTS

Given the expansion and subsequent contraction of the peripheral monocyte population coincident with vemurafenib treatment, we considered the possibility that the patient had a preexisting chronic myelomonocytic leukemia driven by an activating mutation upstream of RAF kinase. We analyzed the patient's leukemia cells for mutations in NRAS and KRAS and in other alleles known to be associated with chronic myelomonocytic leukemia. Sanger sequencing of specimens derived from nucleated cells in whole bone marrow and from peripheral-blood mononuclear cells (PBMCs) obtained before vemurafenib therapy revealed an NRAS G12R mutation in genomic DNA (data not shown). Genotyping of hematopoietic-cell subsets from the patient's bone marrow on which fluorescence-activated cell sorting analysis was performed revealed the NRAS G12R mutation in the monocyte (CD14+), erythroid (CD71+), and megakaryocyte (CD41a+) lineage subsets but not in the cells of lymphoid (CD3+) lineage (Table 1). Comparison of the results of genotyping biopsy specimens of the melanoma and bone marrow revealed that the BRAF V600K mutation was unique to the melanoma, whereas the NRAS G12R mutation was unique to the transformed cells of myeloid lineage. The patient's lymphocyte population was wild type for both BRAF and NRAS.

We hypothesized that vemurafenib was causing the hyperactivation of ERK and stimulating the growth of preexisting NRAS-mutated chronic myelomonocytic leukemia cells, which resulted in preferential expansion of this subpopulation during treatment. To test this hypothesis, PBMCs were obtained from the patient 10 days after vemurafenib was withdrawn and again 5 days after it was reinitiated at a dose of 720 mg twice daily. Levels of phosphorylated ERK (pERK) and total ERK (tERK) in specific PBMC subpopulations were measured by staining for pERK or tERK, along with markers for the relevant populations; analysis of pERK was performed with the use of flow cytometry (see Fig. 2 in the Supplementary Appendix for details). We evaluated both the NRAS-mutant leukemic monocyte population (CD14+, CD56+, HLA-DR^{high}) and the wild-type T lymphocytes (CD3+) for levels of pERK and tERK at each time point ex vivo. Monocytes in the PBMC sample obtained while the patient was being treated with vemurafenib had an elevated pERK:tERK ratio as compared with the sample obtained while the patient was not taking the drug (1.25 vs. 0.98, $P = 0.02$), which is consistent with increased activation of ERK during treatment (Fig. 2A). Under the same conditions, vemurafenib-induced activation of ERK was not observed in NRAS wild-type lymphocytes. Furthermore, comparison of the pERK:tERK ratio between NRAS-mutant monocytes and wild-type T lymphocytes revealed a higher baseline level of ERK activation in the NRAS-mutant monocytes in the absence of vemurafenib (0.98 vs. 0.40, $P < 0.001$), which is consistent with a higher level of constitutive ERK activation in the NRAS-mutant leukemic cells.

We next evaluated the effects of a RAF inhibitor, a MEK inhibitor, or both on ERK signaling and cell proliferation in NRAS-mutant leukemic cells in vitro (Fig. 2B). Unfractionated nucleated bone marrow cells obtained from the patient when he had not received vemurafenib for 3 days were cultured in methylcellulose-containing growth factors (stem-cell factor, interleukin-3, and interleukin-6) in the presence of the RAF inhibitor PLX4720, the MEK inhibitor PD325901, both inhibitors, or neither inhibitor. Cells cultured in the presence of PLX4720 showed a dose-dependent increase in the number of colony-forming units (CFUs) that was similar in magnitude to the increase seen with granulocyte-macrophage colony-stimulating factor (GM-CSF) (at a concentration of 10 ng per milliliter),

which was used as a positive control. When PD325901 was added, the stimulation induced by PLX4720 was reversed, and the number of colonies formed was similar to the number formed in untreated cells. When cells were treated with PD325901 alone, the outgrowth of colonies relative to untreated cells was markedly diminished. In all culture conditions tested, more than 90% of the CFUs were granulocyte–macrophage progenitor CFUs (Fig. 3 in the Supplementary Appendix). The NRAS G12R mutation was present in 100% of the colonies cultured with PLX4720 as compared with 84% of the colonies cultured in the absence of drug and 82% of those cultured with PD325901 alone (Fig. 4 in the Supplementary Appendix), indicating that the RAF inhibitor preferentially promoted the outgrowth of CFUs harboring the NRAS G12R mutation. A similar pattern was seen in fractionated colonies seeded with CD34+ bone marrow cells (data not shown).

Last, we evaluated the effects of the RAF inhibitor, the MEK inhibitor, or both on ERK activation in circulating leukemic cells. PBMCs obtained when the patient was not receiving vemurafenib were activated *ex vivo* with GM-CSF in the presence or absence of the RAF or MEK inhibitor. In the leukemic cells, ERK activation was enhanced in the presence of the RAF inhibitor, as indicated by an increase in the pERK:tERK ratio from 2.07 to 2.76 ($P = 0.002$) (Fig. 2C). In cells treated with the MEK inhibitor alone, ERK activation was inhibited, as compared with untreated cells (1.44 vs. 2.07, $P = 0.001$). Most notably, the addition of PD325901 to cells treated with PLX4720 diminished ERK hyperactivation, lowering the pERK:tERK ratio from 2.76 to 1.72 ($P = 0.001$). The lower pERK levels in the cells treated with PD325901 alone as compared with the cells treated with both drugs suggests that ERK hyper-activation is attenuated, but not abrogated, by the addition of PD325901. This observation indicates that *in vitro*, at the concentrations used in this case, ERK activation that is induced by the RAF inhibitor in this RAS-mutant leukemia can be suppressed by the addition of a MEK inhibitor.

DISCUSSION

We describe a case of accelerated progression of a RAS-mutant hematologic cancer in a patient treated with a selective inhibitor of RAF. On treatment with vemurafenib, we observed dose-dependent, reversible activation of ERK in the NRAS-mutated leukemic clones. At the same time, treatment with the RAF inhibitor caused regression of the patient's BRAF V600K–mutant melanoma. Our data are consistent with the preexistence of a clinically undiagnosed NRAS-mutant leukemic clone, which was specifically induced to proliferate on treatment with vemurafenib. Clinically, this leukemia is characteristic of chronic myelomonocytic leukemia. Mutations in NRAS or KRAS have previously been reported in as few as 11% and as many as 57% of patients with chronic myelomonocytic leukemia.^{21,22} The NRAS mutation was detected in the monocyte, erythroid, and megakaryocyte lineages but not in the lymphocyte lineage.

The proliferative effect of the RAF inhibitor on the NRAS-mutant leukemia *in vivo* was recapitulated *in vitro* and was correlated with enhanced ERK signaling. As predicted, ERK signaling was attenuated on cotreatment with a MEK inhibitor. MEK inhibitors have recently been shown to prolong the survival of patients with BRAF V600E–mutated melanoma²³ and can prolong progression-free survival when combined with a RAF inhibitor.²⁴ We speculate that the addition of a MEK inhibitor to this patient's treatment regimen would suppress the vemurafenib-induced leukemic proliferation *in vivo*. However, MEK inhibitors have not been approved by the Food and Drug Administration, and we were unable to obtain a MEK inhibitor to test this hypothesis clinically. The patient is currently receiving maintenance therapy with vemurafenib, with interruptions in administration when the white-cell count approaches 100,000 per cubic millimeter. Our findings are consistent

with a prior report of RAS-dependent paradoxical ERK activation in BCR-ABL T315I-mutant chronic myeloid leukemic cells exposed to imatinib, nilotinib, or dasatinib in vitro.²⁵

This case report shows that paradoxical ERK activation by RAF inhibitors is not restricted to proliferations such as squamous-cell carcinomas and keratoacanthomas. It is clear that paradoxical activation can be seen in other premalignant lesions in which there are RAS mutations or other genetic changes that result in upstream activation of this pathway. Since premalignant and malignant solid tumors are also frequently driven by RAS mutations or activation of upstream receptor tyrosine kinases, we anticipate that additional cases of neoplasia stimulated by RAF inhibitors will be encountered as the use of the drugs vemurafenib and dabrafenib increases. Specifically, our experience with this patient suggests that a rapid rise in the white-cell count in any patient treated with a RAF inhibitor should be investigated promptly and the drug withheld until the cause of the rise in white cells is clarified. In light of these findings, the administration of RAF inhibitors as adjuvants will require careful monitoring; they should be used only in the context of a clinical trial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The Clinical Course of a Patient with BRAF V600K–Mutant Melanoma and NRAS G12R–Mutant Chronic Myelomonocytic Leukemia on Treatment with Vemurafenib

The white-cell count and absolute monocyte count (Panel A) were monitored over the course of treatment. Solid triangles indicate doses of ipilimumab and the open triangle represents the time at which the bone marrow–biopsy specimen and the peripheral-blood smear were obtained. Shaded areas represent the periods of treatment with vemurafenib (Vem), administered at doses of either 960 mg or 720 mg twice daily. Myeloid dysplasia is seen in the peripheral-blood smear and the bone marrow aspirate (Panel B, arrows). The insets in Panel B show the biopsy specimen and aspirate at higher magnification. Computed tomographic (CT) scans show the response of the metastatic melanoma after treatment with

vemurafenib at 8 and 16 weeks (Panel C). The red circles denote the location of melanoma metastases in the subcarinal lymph nodes (top row) and in the liver (bottom row).

Figure 2. RAS-Mutant Leukemia Cells, Hyperactivated in the Presence of Selective RAF Inhibitors

Peripheral-blood mononuclear cells (PBMCs) were collected from the patient when he was (+) and was not (-) receiving treatment with vemurafenib (720 mg twice daily). ERK signaling was assessed by measuring levels of phosphorylated ERK (pERK) with flow cytometry. The ratio of the median fluorescence intensity (MFI) of staining for pERK was compared with the MFI of staining for total ERK (tERK) in the aberrant monocyte population (CD14+, CD56+, HLA-DR^{high}) and the lymphocyte population (CD3+) in the presence or absence of vemurafenib (Panel A). Total bone marrow mononuclear cells were plated in methylcellulose in the presence or absence of PLX4720 (2 μM or 20 μM),

PD325901 (200 nM), or granulocyte–macrophage colony-stimulating factor (GM-CSF) (10 ng per milliliter). After 7 days, the total number of colony-forming units (CFUs) was enumerated (Panel B). PBMCs were treated for 4 hours with PLX4720 (2 μ M or 20 μ M) or PD325901 (100 nM) in the presence of GM-CSF (10 ng per milliliter). The ratio of the MFI of pERK to the MFI of tERK in the monocyte population was determined with the use of flow cytometry (Panel C). The graphs depict the mean of the experimental triplicates (Panel A), duplicates (Panel B), or quadruplicates (Panel C), and the T bars represent the standard error of the mean. In Panels A and C, the P values were calculated with the use of a paired t-test.

Table 1

Detection of BRAF V600K or NRAS G12R Mutations According to Tissue Type.

Tissue	BRAF V600K Mutation	NRAS G12R Mutation
Melanoma	Detected	Not detected
Monocyte (CD14+)	Not detected	Detected
Megakaryocyte (CD41a+)	Not detected	Detected
Erythroid (CD71+) lineage	Not detected	Detected
Lymphocyte (CD3+)	Not detected	Not detected