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BRAF in the cross-hairs

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Abstract

Introduction: Hairy cell leukemia (HCL) is a rare, chronic B-cell lymphoproliferative disorder characterized by distinctive morphologic features and an indolent clinical course. The discovery of a recurrent activating mutation in *BRAF* (BRAF V600E) as a disease-defining genetic event in HCL has substantial diagnostic and therapeutic implications.

Areas covered: Herein the authors review the role of BRAF V600E and RAF-MEK-ERK signaling in the pathogenesis of HCL, anecdotal clinical reports of BRAF inhibitor monotherapy in management of relapsed or refractory HCL, larger phase 2 trials investigating efficacy of BRAF inhibitor therapy for HCL, adverse effects commonly associated with BRAF inhibitor therapy, including cutaneous toxicity, and mechanisms of therapeutic resistance.

Expert opinion: Ongoing and planned studies will help to optimize the use of BRAF inhibitor therapy for HCL by determining efficacy of BRAF inhibition in combination with other antigen targeted or molecularly targeted therapies, and more broadly, to determine how hematologists can best utilize and sequence emerging diagnostic and therapeutic modalities in the care of patients with newly diagnosed and relapsed or refractory HCL.

Keywords

hairy cell leukemia; targeted therapy; vemurafenib; BRAF; lymphoproliferative disorders

1. Introduction

Hairy cell leukemia (HCL) is a rare, chronic B-cell lymphoproliferative disorder first described by Bouroncle and colleagues in 1958 and subsequently characterized further by numerous other groups.[1–3] Only approximately 600–800 individuals are diagnosed each year with HCL in the U.S., with men more commonly affected than women (4:1 sex ratio) and Caucasians more frequently affected than non-whites.[4] The clinical course of HCL is generally indolent and characterized by splenomegaly, pancytopenia, and progressive bone marrow infiltration. While some individuals present with asymptomatic pancytopenia, others

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present with fatigue or infection in the setting of marrow failure.[5] In contrast to many other B-cell lymphoproliferative disorders, lymphadenopathy is uncommon at presentation.[6]

Purine analog therapies are an established, effective therapeutic modality for first-line treatment of HCL. Several studies have demonstrated complete response (CR) rates exceeding 90% among patients with HCL treated with a single 7-day continuous intravenous (IV) infusion of cladribine 0.1 mg/kg/day, with median duration of response exceeding 5 years in those achieving CR.[7,8] Alternative schedules of cladribine administration, including given as 0.14 mg/kg/day as a 2-hour IV infusion or subcutaneous injection, appear to have comparable efficacy.[9,10] Administration of pentostatin every 2 weeks until best response is associated with objective response rates >90% with similarly impressive response durability observed.[11,12] However, a subgroup of patients experiences suboptimal response durations, and serial therapy with purine analogs often yields responses of decreasing duration. Mechanisms of cladribine resistance include, among others, decreased intracellular levels of deoxycytidine kinase (dCK), which phosphorylates the prodrug cladribine to an active metabolite.[13] HCL bearing an unmutated immunoglobulin heavy chain variable region (IgHV) may exhibit greater B-cell receptor-mediated signaling and genomic instability, further contributing to defective induction of apoptosis and resistance to purine analogs.[14,15] Patients with measurable residual disease (MRD, also known as minimal residual disease) following purine analog therapy exhibit higher rates of relapse. Moreover, purine analog therapy is associated with myelosuppression and other toxicities, including possibly increased risk of secondary malignancies.[7] As such, there has been considerable interest in strategies to deepen response while limiting toxicities of conventional cytotoxic therapy. CD20-targeted monoclonal antibodies, tumor targeted immunotoxins, and oral molecularly targeted therapies demonstrate considerable promise in the treatment of HCL. As discussed subsequently, the discovery of a recurrent activating mutation in *BRAF* in patients with HCL has led to laboratory studies demonstrating the significance of this alteration in the pathogenesis of HCL and has been successfully translated into several clinical trials illustrating the utility of BRAF-targeted therapy for HCL.

2. *BRAF* and molecular pathogenesis of HCL

2.1. Introduction to the MAPK pathway.

The serine/threonine protein kinase BRAF is a key component of the RAS-RAF-MAPK signal transduction pathway (Figure 1). Broadly, signaling through the MAPK cascade leads to phosphorylation and activation of transcription factors in the nucleus, thereby regulating key cellular processes including proliferation, differentiation, and apoptosis.[16] Binding of a ligand to transmembrane receptor tyrosine kinase (RTK) leads to downstream activation of RAS proteins (GTPases; small G protein), which can activate downstream RAF protein kinases including BRAF, which in turn phosphorylate a further protein kinase (MEK family) with the capacity to phosphorylate both tyrosine and serine/threonine residues. MEK proteins can then activate an ERK (MAPK) protein, which in turn can translocate to the nucleus and serve as a kinase regulating transcription factors. Alterations at various stages of

these signaling pathways can serve to promote uncontrolled cell proliferation and inhibit apoptosis, thereby contributing to tumorigenesis.

2.2. *BRAF* mutations in human cancers.

Activating mutations in *BRAF* play a central role in the pathogenesis of melanoma. The Sanger Institute first reported missense mutations in *BRAF* within exon 15 of the kinase domain, leading to substitution of valine by glutamic acid and the activated BRAF V600E variant (previously identified as V599E due to a sequencing error) in a high proportion of melanoma cell lines and primary tumors.[17] To clarify nomenclature used in this manuscript, *BRAF* refers to the *BRAF* gene and BRAF refers to the protein product; the term *BRAF*V600E mutation, when used, refers to the mutant gene, exhibiting a thymidine to adenosine transversion at nucleotide 1799 (c.1799T>A), ultimately resulting in the BRAF V600E mutant protein. Functional consequences of this mutation include increased BRAF kinase activity and ERK1/2 phosphorylation. *BRAF* mutations appear to represent one of several progression events in the development of melanoma; such mutations are rarely observed in the setting of less invasive melanoma.[18] Oral kinase inhibitors selective for BRAF, such as vemurafenib and dabrafenib, have demonstrated considerable efficacy as single agents in patients with advanced melanoma bearing *BRAF* mutations.[19,20] However, activation of other receptor tyrosine kinase signaling pathways or reactivation of signaling via the MAPK pathway (e.g. via *NRAS* mutations) can provide compensatory survival signaling in the presence of BRAF inhibition.[21] NRAS activation in this setting can in turn bypass BRAF and reactivate MEK1/2 and ERK1/2 downstream. BRAF V600E mutant cancers are sensitized to ATP-competitive RAF inhibitors such as vemurafenib, which effectively block signaling through BRAF V600E monomers.[22] However, binding of a RAF inhibitor to a component of a RAF homodimer (e.g. CRAF-CRAF) or heterodimer (BRAF-CRAF) inhibits one component of the dimer but results in transactivation of the other and induction of downstream MEK-ERK signaling; RAF dimerization and drug binding to the ATP-binding site of one component of the dimer are dependent on RAS. [23,24] BRAF inhibitors can thus cause paradoxical downstream activation of the MAPK pathway in wild type BRAF cells, particularly in the presence of RAS activation, as well as in cells bearing BRAF V600E mutant protein if RAF dimerization is promoted due to aberrant splicing or increased RAS activation.[22] Combination of BRAF and MEK inhibitor therapy appears to enhance response, in part by addressing this potential mechanism of resistance.[25–27] Combination therapy additionally is associated with lower rates of proliferative skin lesions and acneiform rash compared with BRAF inhibitor monotherapy, possibly related to attenuation of MAPK pathway activation.

2.3. *BRAF* mutations in HCL.

Several years following the first observation of recurrent *BRAF* mutations in malignant melanoma, Tiacci et al. first reported heterozygous mutations in *BRAF* resulting in the BRAF V600E variant protein were remarkably specific to HCL among B-cell lymphoproliferative disorders.[28] Whole exome sequencing of paired leukemic and normal control DNA from an index patient identified a set of candidate somatic mutations, including BRAF V600E. Subsequent screening of 47 additional patients with HCL by PCR and direct Sanger sequencing confirmed the *BRAF*V600E variant in all patients, most commonly

occurring in a heterozygous fashion. Sequencing of *BRAF* exon 15 in 195 patients with other peripheral B-cell malignancies, including patients with HCL variant (HCLv), demonstrated no evidence of *BRAF* mutation. Phosphorylated (activated) ERK was observed in bone marrow specimens, consistent with downstream signaling from activated BRAF. Additionally, incubation of a BRAF inhibitor with primary HCL cells *in vitro* led to marked decrease in phosphorylated MEK/ERK, suggesting the potential benefit of BRAF inhibition in treatment of HCL. Shortly thereafter, other groups independently confirmed the specificity of the *BRAF*V600E variant for HCL among other lymphoid malignancies (including HCLv), using high-resolution melting analysis-based PCR[29] and later using more-sensitive allele-specific PCR methods (qualitative allele-specific PCR[30] and mRNA-based reverse transcription real-time quantitative PCR assays[31]). Alternative *BRAF* mutations in exon 11 have been observed in two patients with *BRAF*V600E-negative HCL. [32] In contrast to other BRAF V600E mutant cancers in which homozygous mutations in *BRAF* are frequently observed,[33] patients with HCL exhibit heterozygous or hemizygous (i.e. bearing only one copy of *BRAF*, with this copy containing the mutation) *BRAF* mutations.[28, 34] Investigators from the National Cancer Institute (NCI) additionally reported *BRAF* mutation status in 69 patients, 53 with HCL and 16 with HCLv; all 16 patients with HCLv and 11/53 patients with HCLc had wild-type *BRAF*. A subgroup of 13 patients exhibited the IgHV4–34 immunoglobulin rearrangement (HCL, n=5; HCLv, n=8), all with wild-type *BRAF*. [35] Of note, IgHV4–34 sequences were each more than 98% homologous to germline (unmutated).[35] The NCI group subsequently reported a high prevalence of activating mutations in the *MAP2K1* gene (encoding MEK1) among patients with HCLv (*MAP2K1* mutations in 10/24 patients) and IgHV4–34⁺ HCL (*MAP2K1* mutations in 5/7 patients) using whole exome or Sanger sequencing; such mutations appeared mutually exclusive of *BRAF*V600E.[36] However, other groups have observed *MAP2K1* mutations at lower frequency, with 1/14 HCLv patients with a detectable *MAP2K1* mutation by Sanger sequencing in one report[37] and 2/12 HCLv and *BRAF* V600E-negative HCL patients by targeted next-generation sequencing in another report.[38] These findings suggest multiple potential mechanisms of MAPK activation in classical HCL and a subset of patients with HCLv and the critical role of RAF-MEK-ERK signaling in both malignancies.

While HCL bears the immunophenotypic profile of a mature B-cell and clonal rearrangements of immunoglobulin heavy and light chain genes, *BRAF* mutations arising in a more primitive hematopoietic cell population may serve as a leukemia-initiating event. Chung et al. identified *BRAF*V600E mutations in purified hematopoietic stem cells (HSCs) and pro-B cell populations, in addition to within the HCL population, in 14 patients with HCL. Subsequent transplantation of 3×10^3 HSCs from the bone marrow of a patient with untreated HCL into irradiated immunocompromised mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice, NSG mice) was sufficient to generate a circulating population of hematopoietic cells with HCL immunophenotype, with persistence of detectable *BRAF* V600E mutation within bone marrow hematopoietic cells in the transplanted mice.[39] In a conditional knockin murine model, the authors further demonstrated expression of the mutant allele in HSCs (using an *Mx1-Cre* system) resulted in development of anemia, thrombocytopenia, splenomegaly, and increased circulating CD25 (soluble IL-2 receptor)—

all characteristics of HCL[40]—with recapitulation of this phenotype on transplantation into lethally-irradiated mice (although no hairy cells were seen, and the *Mx1-Cre BrafV600E* mouse is not intended to model human HCL).[39] These data are consistent with one prior report using the *Mx1-Cre* conditional knockin murine model in which somatic expression of *BrafV600E* led to development of nonlymphoid leukemia of histiocytic type, splenomegaly, and bone marrow failure.[41] Chung et al. further noted when expression of the mutant allele was restricted to B-lineage cells (under the control of a *CD19-Cre* system), mice did not develop this lethal hematopoietic disorder and exhibited no overtly abnormal phenotype aside from increased MAPK signaling in B-cells and increased self-renewal of B-cell precursors. Lymphoid-primed multi-potent progenitors (LMPPs) bearing *BRAFV600E* additionally exhibited aberrant self-renewal but impaired myeloid differentiation. Vemurafenib treatment led to spleen size reduction and restoration of more normal myelopoiesis/erythropoiesis in mice transplanted with HSCs bearing the mutant allele. The discovery of *BRAF*-mutated HCL HSCs with self-renewal capacity provides further insight into the pathogenesis of HCL and additional support for investigating BRAF inhibition as a therapeutic modality for HCL.

2.4. Preclinical data: inhibiting BRAF in HCL.

Tiacci and colleagues subsequently characterized the effects of vemurafenib on primary leukemic samples from patients with HCL and other B-cell malignancies.[42] They observed reliable and dose-dependent decreases in MEK/ERK phosphorylation specific to HCL bearing *BRAFV600E*. They noted corresponding decrease in transcriptional output from the BRAF-MEK-ERK pathway as evaluated by gene set enrichment analysis (mRNA levels reflecting expression of 48 genes induced by BRAF-mutated melanoma and colorectal carcinoma). BRAF-inhibitor treated HCL cells, but not malignant B-cells of other histology (including HCLv), experienced regression of hair-like projections and became smaller and smoother. This group further detected substantial downregulation of other genes typically upregulated in patients with HCL, including CD25, and more broadly, noted silencing of the transcriptional profile uniquely exhibited by HCL, further supporting the central role of *BRAF* mutations in the molecular pathogenesis of HCL. Additionally, whereas vemurafenib treatment led to upregulation of pro-apoptotic activators in HCL cells, co-culture with bone marrow stromal cells led to increases in ERK phosphorylation and blunting of apoptosis; co-treatment with dabrafenib and the MEK inhibitor trametinib appeared to restore ERK dephosphorylation and promote apoptosis *in vitro* in two patients, suggesting that suppressing persistent signaling through the MAPK pathway might help to overcome resistance to BRAF inhibitor monotherapy in the context of the bone marrow microenvironment.

3. Early experience and anecdotal reports of BRAF inhibitor therapy in the treatment of HCL

Following the initial reports of recurrent *BRAF* mutations in patients with HCL, Dietrich et al. reported the first anecdotal clinical use of the oral BRAF inhibitor vemurafenib in a patient with HCL. In brief, a 51 year-old man with HCL exhibited persistent multilineage cytopenias, extensive bone marrow infiltration, and massive splenomegaly despite prior

therapy with cladribine, two courses of pentostatin, and combination therapy of cladribine and rituximab. He began vemurafenib 240 mg twice daily, with reduction of splenomegaly within days, correction of cytopenias within weeks, and overall achievement of complete response, with only low-level MRD detectable within the bone marrow. The vemurafenib dose was slowly escalated. His course was notable for development of erythema nodosum and arthralgias, with the latter occurring at higher doses of vemurafenib; treatment was discontinued after 56 days of treatment.[43,44] A follow-up report from the same group on this patient noted immunohistochemical (IHC) studies performed on bone marrow samples during treatment suggested abrogation of ERK phosphorylation in HCL cells. At 6 months post-treatment, the patient remained in ongoing CR without detectable MRD.[44]

Others quickly reported responses to vemurafenib observed in isolated patients with relapsed or refractory HCL, including at doses of 240 mg twice daily[45,46] or 960 mg twice daily, [47] with relapse noted 2 months post-vemurafenib in one case[45] and 7 months post-vemurafenib in another.[46,48] In the latter case, response was quickly observed upon retreatment with vemurafenib 240 mg twice daily and maintained even with further reduction to 240 mg daily.[48] Other case reports have described the use of vemurafenib in the treatment of HCL in unique clinical situations, including response in a patient with primary refractory HCL and concomitant aspergillus pneumonia requiring mechanical ventilation, providing anecdotal evidence vemurafenib might have clinical application in the setting of active infection, when prompt neutrophil recovery and avoiding myelosuppressive therapy might be particularly desired.[49] Another report described response to vemurafenib in a patient with refractory leptomeningeal involvement by HCL.[50] This experience parallels responses to vemurafenib observed in patients with metastatic melanoma bearing BRAF V600E mutations with brain metastases.[51] While cladribine crosses the blood brain barrier, given the suboptimal responses to cladribine observed in the few reported cases of CNS involvement by HCL, vemurafenib might be an early therapeutic consideration. Additionally, a report from The Ohio State University described the use of another kinase inhibitor selective for mutant BRAF, dabrafenib 150 mg twice daily, as co-treatment for relapsed HCL and melanoma both bearing the *BRAF*V600E mutation on a clinical trial. Ongoing CR of both malignancies observed; in contrast to other reports in which patients discontinued BRAF inhibitor therapy for HCL within several months, this patient continued dabrafenib for >18 28-day cycles.[52] In the rare patient with HCL and a synchronous or metachronous solid malignancy, genomic profiling may identify occasional opportunities for simultaneous targeting of malignancies with BRAF or combined BRAF/MEK inhibition.

Further “real world” evidence in support of vemurafenib’s efficacy in HCL comes from a report of 21 patients with HCL treated across different centers in Europe, five of whom had been previously reported in the literature (Table 1).[53] Of note, interpretation of this series is limited as a retrospective collection of single patients treated with variable vemurafenib dosing regimens and durations, without uniform indications for treatment, procedures for dose adjustment or discontinuation, or methods and timing of response assessment. Patients were heavily pre-treated (median of 3 prior therapies, range 0–12) and most received initial therapy with vemurafenib 240 mg twice daily (n=17), with a minority of patients treated at higher doses or later receiving an escalated dose. Median duration of therapy was 90 days (range, 56–266). All patients achieved hematologic improvement, with objective response

observed in 20 of 21, with the non-responder's course characterized by development of acute myeloid leukemia (AML) during vemurafenib therapy; 6 of 15 patients undergoing reassessment by bone marrow biopsy achieved CR.[53] Median relapse-free survival (RFS) was 14 months, with achievement of CR associated with greater time to retreatment or death (event-free survival, EFS). Of note, the definition of relapse in the series of patients treated off-trial included deterioration of peripheral blood counts below remission thresholds, including absolute neutrophil count (ANC) $<1,000/\mu\text{L}$, hemoglobin $<10\text{g/dL}$, or platelets $<100,000/\mu\text{L}$. An American phase 2 trial detailed in the subsequent section used similar criteria, while the Italian phase 2 trial used hematologic parameters of ANC $<1,500/\mu\text{L}$, hemoglobin $<11\text{g/dL}$, and platelets $<100,000/\mu\text{L}$; these differences limit comparison between the reports.[53,54] Six patients in the European series of patients treated off-trial were retreated with vemurafenib and attained response (of whom 4 continued low-dose vemurafenib continuously).[53] These data further suggest vemurafenib doses well below those commonly used in treatment of advanced melanoma may be sufficient to achieve clinically significant BRAF inhibition and response in most patients with HCL, and may be hypothesis-generating. However, a prospective clinical trial would be required to evaluate rates and durability of response to standard (960 mg twice daily) vs. lower doses of vemurafenib in patients with HCL.

4. Clinical trials investigating BRAF inhibitors in the treatment of HCL

Following these early reports, multicenter trials evaluated the safety and efficacy of vemurafenib in patients with R/R HCL more systematically. Two similar phase 2 multicenter trials, conducted in Italy and in the United States, were conducted and presented in a combined report; of note, the Italian trial began prior to the American trial, enrolled more rapidly, and had more extended follow-up available (Table 1).[54] The Italian trial enrolled 28 patients, largely refractory to purine analogs (no response or relapse <1 year, $n=6$), or with early relapse (1–2 years following first course or <4 years following a second or later course). Patients received vemurafenib 960 mg twice daily for a minimum of 8 weeks, followed by response assessment, with therapy extended for up to 8 additional weeks if CR was not achieved (20 weeks total for the first three patients). The American trial enrolled 26 patients with R/R HCL, largely with two or more relapses >2 years following a third or later course of purine analog therapy ($n=24$). Similar to the Italian trial, patients received vemurafenib 960 mg PO daily continuously for 12 weeks, with an additional 12 weeks of therapy permitted for patients with residual evidence of morphologic disease thereafter. Retreatment with vemurafenib for relapse was permitted in both trials. Approximately half of patients in both trials required vemurafenib dose reduction to 240–720 mg twice daily, largely due to cutaneous toxicities (as described below) and arthralgias. Two patients in the Italian trial discontinued therapy within one week of initiation due to myocardial infarction ($n=1$, thought unrelated to vemurafenib) or grade 3 reversible drug-related pancreatitis ($n=1$), and two patients in the U.S. trial discontinued therapy prematurely (death due to pneumonia thought unrelated to vemurafenib, $n=1$; grade 3 reversible photosensitivity, $n=1$). All evaluable patients exhibited evidence of hematologic improvement, and nearly all (25 of 26 in the Italian study, and 24 of 24 in the American study) achieved objective response. Rates of CR were similar in the Italian (35%) and U.S. (42%) trials. The single patient not

exhibiting objective response to vemurafenib had bulky splenomegaly and lymphadenopathy prior to treatment, on addition to pancytopenia; he experienced regression of splenomegaly and nodal masses, improvement in neutropenia, and resolution of anemia with therapy, but with reduction in spleen size short of criteria for PR. Regression of splenomegaly, clearance of circulating HCL cells, and recovery of neutrophil and platelet counts was commonly observed within 4 weeks of treatment in both studies. Response rates did not appear different in the American trial among 10 patients treated with vemurafenib 480 mg (vs. 960 mg) twice daily for >4 weeks, although the study was not designed or powered to assess for differences in response rate between these groups. Median relapse-free survival (RFS) among responders was 9 months in the Italian study at median follow-up of 23 months (range, 7–28), with follow-up measured from the last dose of vemurafenib; as noted in the prior section, milder cytopenias were considered evidence of relapse in the Italian study than in the off-trial series from Europe[53], and 7/20 relapsing patients in the Italian study did not require further therapy at the time of the report.[54] Cumulative incidence of relapse was 27% at 1 year after response in the American trial, with median follow-up of 11.7 months (range, 1.3–25.4), with follow-up measured from the first dose of vemurafenib. One-year progression-free survival (PFS) was 73% in the American trial. Of note, in both trials, relapsing patients requiring further antileukemic therapy were permitted retreatment with vemurafenib, with 8/8 evaluable patients achieving at least a minor response on retreatment in the Italian trial and 4/6 evaluable patients achieving PR on retreatment in the U.S. trial, with one patient experiencing rapid progression upon retreatment as discussed below.

Depth of response observed in the American and Italian trials was quantified by several means. Mean percentage of HCL cells among bone marrow mononuclear cells decreased from 16.8% pre-treatment to 6.9% and 1.3% after 4 and 24 weeks of vemurafenib, respectively.[54] Additionally, digital PCR studies, to quantify the concentration of molecules with mutated *BRAF*V600E (c.1799T>A) DNA, noted >100-fold reduction in the median ratio of *BRAF*V600E to wild-type alleles following 12 weeks of vemurafenib therapy, though the mutant alleles remained detectable in all patients. While soluble CD25 (IL-2 receptor alpha chain) is an established biomarker of HCL burden[55] and levels declined considerably with vemurafenib treatment in the U.S. trial, surface CD25 is downregulated by vemurafenib exposure *in vitro*[42] and this phenomenon was observed in HCL cells of 15 of 19 evaluable patients in the Italian trial, suggesting CD25 levels may underestimate levels of residual HCL in this setting.[54] Additionally, 6 of 13 patients in the Italian trial undergoing bone marrow biopsy immediately post-vemurafenib demonstrated residual HCL with expression of phosphorylated ERK. In a *post-hoc* exploratory analysis, patients with residual phospho-ERK exhibited shorter progression free survival compared with those having undetectable phospho-ERK.[54] Residual phospho-ERK expression in the course of vemurafenib therapy likely reflects persistent signaling through the MAPK pathway and may serve as a biomarker of therapeutic resistance.

5. Toxicities of BRAF inhibitors in the treatment of HCL

Cutaneous toxicities are observed in the vast majority of patients with advanced *BRAF* V600E-mutant melanoma treated with single-agent vemurafenib.[56] While pruritic maculopapular rash, hyperkeratosis, and hand-foot syndrome are common and generally

manageable with topical corticosteroids, exfoliants, oral antihistamines and other supportive measures, and while photosensitivity is typically manageable with sun protection strategies, approximately 20–25% of patients develop keratoacanthoma or cutaneous SCC, generally managed with surgical excision or destructive therapy. Of some note, *HRAS* mutations are frequently observed in cutaneous SCC lesions arising on vemurafenib therapy.[57] As described in section 2.2, vemurafenib may paradoxically increase signaling through the MAPK pathway in this setting, particularly in the presence of co-existing activating mutations in *RAS*. Progression of chronic myelomonocytic leukemia (CMML) bearing an activating mutation in *NRAS* has also been observed in a patient with *BRAF* mutant melanoma treated with vemurafenib, providing further clinical evidence BRAF inhibitor monotherapy can accelerate RAS-driven malignancies.[58]

Early reports and individual case reports further provided initial insight into toxicities of vemurafenib in patients with HCL, including development of benign seborrheic keratoses, [45] generalized acneiform rash,[50] moderate arthralgias,[47] and weight loss.[48] The larger phase 2 trials permitted more uniform assessment of the nature, frequency, and severity of vemurafenib toxicities in patients with HCL more homogeneously treated and prospectively followed than in the prior anecdotal reports. Grade 2–3 rash was observed in 46% and 62% of participants in the Italian and American trials, respectively.[54] Among the 54 patients treated across the Italian and U.S. studies, the incidence of skin cancer was <20%, with few cases of SCC (n=3), basal cell carcinoma (BCC, n=3), melanoma (n=1) arising during vemurafenib therapy. A few cases of keratoacanthoma (n=3) were observed in the European series of 21 largely low-dose vemurafenib recipients, though only a single case of SCC was observed.[53] Grade 2–3 arthralgias were observed in 43% and 31% of patients in the Italian and American trials, respectively, and led to dose reduction of vemurafenib in 5 patients in the American study; largely manageable arthralgias have been observed even with low dose vemurafenib.[53] Reversible mild transaminitis has been observed even in the setting of lower doses of vemurafenib.[53] Transaminitis and hyperbilirubinemia, generally grade 1–2 and reversible, has been observed in a minority of patients in larger series.[53,54] In the aforementioned “real world” series from Europe, one patient receiving vemurafenib for HCL developed pure erythroid leukemia (AML-M6b) associated with an activating mutation in *PI3K* (E545K) with evidence of phospho-ERK by IHC in the AML population. [53] Whether a causal relationship exists between vemurafenib exposure and development or progression of AML remains uncertain; this patient had received 5 prior lines of chemotherapy, which could predispose to myeloid neoplasia, and lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) levels were elevated prior to vemurafenib therapy and later tracked with AML activity. While regression of peripheral blasts and decrease in LDH/ALT were observed following vemurafenib discontinuation, overt erythroid leukemia became evident 1.8 months thereafter. The significance of phospho-ERK by IHC in the AML population remains unclear and implicating paradoxical activation of ERK during vemurafenib therapy in the pathogenesis of this case of AML remains speculative.

The extent to which several of the toxicities of vemurafenib likely mediated by RAS activation might be attenuated by concurrent MEK inhibition, as they appear to be in patients with advanced melanoma, remains uncertain in patients with HCL.

6. Understanding and overcoming therapeutic resistance to BRAF inhibitors for HCL

As noted previously, despite impressive rates of response to BRAF inhibitor therapy in patients with advanced melanoma bearing BRAF V600E mutations, most patients develop progression of disease while on vemurafenib monotherapy, with median progression free survival 5.3 months in one pivotal randomized trial.[20] In contrast, patients with HCL rarely experience disease progression during active therapy with vemurafenib, demonstrate response to lower doses than used in melanoma, exhibit longer progression-free survival after starting vemurafenib therapy than patients with melanoma despite typically fixed duration of therapy, and frequently retain sensitivity on re-treatment. Nonetheless, several mechanisms of resistance to BRAF inhibitor therapy may be common between malignancies, including reactivation of signaling through the MAPK pathway. As described in section 2.2, transactivation of a drug-unbound RAF protomer in the setting of BRAF inhibitor monotherapy may paradoxically induce MEK and ERK phosphorylation.[23] RAS activation in the setting of BRAF inhibition would further amplify this effect and potentiate downstream MEK1/2 and ERK1/2 activation. Residual ERK1/2 phosphorylation has been observed as a biomarker of resistance in persisting HCL populations among vemurafenib-treated patients,[48,53,54] with some evidence MEK inhibition may help to overcome this resistance.[42] One patient treated with vemurafenib for relapsed HCL in the U.S. trial developed progressive cytopenias and splenomegaly on retreatment, and genomic profiling identified two subclonal activating mutations in *KRAS*, in addition to *BRAFV600E*; the *KRAS* mutations had not been observed prior to vemurafenib therapy or at initial response to vemurafenib despite high sequencing coverage.[54] However, other genomic alterations outside the MAPK pathway may contribute to the pathogenesis of HCL and drive therapeutic resistance. A subset of patients with HCL exhibit somatic alterations in the tumor suppressor *CDKN1B* co-existent with *BRAFV600E* mutations.[59] Loss of *CDKN1B* function may result in loss of BRAF mutation-driven senescence in patients with HCL, as observed in other BRAF mutant cancers, and thereby constitute a cooperating genetic event. Additional alterations in *IRS1*, *NF1*, and *NF2* in a patient with *de novo* resistance to vemurafenib; gain-of-function mutations in *IRS1* may enhance transmission of signaling through insulin-like growth factor-1 receptors to the PI3K-AKT and MAPK pathways, thereby enhancing AKT and ERK activation, and loss of *NF1*, a known negative regulator of RAS proteins, may potentiate signaling through the MAPK pathway in the presence of BRAF inhibition.[34] Recurrent mutations in the histone methyltransferase *KMT2C* were additionally observed in a subset of patients with HCL bearing *BRAFV600E* mutations.[34] Further understanding of mechanisms leading to persistent signaling through the MAPK pathway, as well as parallel pathways, will continue to inform development of targeted therapies complementary to BRAF inhibition.

Recently, Caesar et al. reported on the first patient with HCL treated with a MEK inhibitor. In brief, a 74 year-old man with R/R HCL previously reported to have responded to vemurafenib [45] experienced progressive cytopenias and bone marrow infiltration by HCL following >3 years of largely continuous low-dose vemurafenib therapy. The addition of rituximab led to only minimal, transient improvement. He was found to have multiple new

activating mutations in *KRAS* and *MAP2K1*, in addition to *BRAF*V600E. Consistent, with other preclinical studies[42], ERK phosphorylation was abolished *ex vivo* in the patient's tumor cells by exposure to trametinib (a MEK inhibitor), but not vemurafenib. Treatment of the patient himself with the MEK inhibitor cobimetinib concurrent with vemurafenib led to suppression of ERK phosphophorylation, resolution of B symptoms, and recovery of cytopenias, with further hematologic improvement with escalation of the cobimetinib dose[60]. As has been observed with combination BRAF/MEK-targeted therapy in melanoma,[25–27] this approach has the potential to overcome MAPK pathway-dependent mechanisms of resistance as well as to reduce the risk of adverse events driven by persistent RAS signaling. Prospective clinical trials are needed, and are ongoing, to assess whether combination BRAF/MEK-targeted therapy (vs BRAF inhibitor monotherapy) is associated with reduced cutaneous toxicity and more durable responses in patients with HCL.

Preclinical studies have additionally shown that ERK inhibition has potent anti-tumor activity in BRAF-inhibitor resistant melanoma cell lines and murine xenograft models with activating mutations in BRAF[61,62]. BVD-523 (ulixertinib), a novel small-molecule ERK1/2 kinase inhibitor, has been shown to inhibit ERK1 and ERK2 kinases potently and selectively in a reversible, ATP-competitive fashion[63] and has demonstrated activity in patients with advanced solid tumors bearing mutations in BRAF, MEK, or NRAS.[64] Such an approach is of interest as a potential complementary therapy to BRAF or MEK inhibition to provide further downstream suppression of the MAPK pathway through ERK inhibition. While patients with HCLv and a small subgroup of patients with HCL (most notably IgHV4–34⁺ HCL) lack the *BRAF*V600E mutation, some of these patients exhibit activating mutations in *MAP2K1* (encoding MEK1), with prevalence varying by report.[36–38] Such patients may benefit from such approaches targeting the MAPK pathway downstream of BRAF.

Finally, approaches not directly targeting RAF-MEK-ERK signaling are of interest in overcoming resistance to BRAF inhibitor monotherapy. As discussed in the subsequent commentary, other approaches to eradicate MRD, such as CD20-targeted monoclonal antibodies, may further deepen responses to molecularly targeted therapies by eliminating small resistant clones.

Conclusion:

Advances in BRAF inhibitor therapy for HCL constitute remarkably brisk translation of genomic findings from bench-to bedside. Within 12 months of the first report of recurrent BRAF V600E mutations in HCL, an anecdotal report was published regarding a patient with relapsed/refractory treated with a BRAF-selective kinase inhibitor (vemurafenib) who exhibited response, and a phase 2 trial in Italy had opened and accrued its first patient. Soon thereafter, another phase 2 trial had opened in the United States, and further anecdotal patients were treated with vemurafenib with varying schedules of dose and duration (generally lower than the standard 960 mg twice daily investigated in phase 2 trials). These phase 2 trials have since established the efficacy of vemurafenib given over a defined period in more homogeneously treated groups of patients, though only a minority of patients achieve CR following BRAF inhibitor monotherapy, and relapse typically follows discontinuation of

BRAF inhibitor therapy. Common toxicities of vemurafenib observed in patients with HCL parallel those commonly observed in melanoma and include rash, cutaneous SCC, keratoacanthoma, photosensitivity, mild elevations in liver biochemical tests, and arthralgias. Even when given at low doses, however, vemurafenib appears active in patients with HCL, though the efficacy and toxicity of lower vs standard dose vemurafenib has not been established by prospective clinical trials. Paradoxical activation of the MAPK pathway appears to represent both a key mechanism of resistance to BRAF inhibitor monotherapy as well as RAS-driven toxicities, such as cutaneous SCC. Further development of strategies to complement BRAF inhibitor therapy, by effecting downstream inhibition of the MAPK pathway or via mechanisms independent of this pathway, have the potential to enhance responses in patients with HCL.

Expert opinion:

With the initial reports in the early 1990s that a single cycle of cladribine could bring about durable CR in the majority of patients with newly-diagnosed or relapsed HCL, successful management of HCL with a brief course of therapy became a new standard.[65,66] Subsequent developments in monoclonal antibodies, antibody drug conjugates, and targeted therapies have further broadened the therapeutic landscape in HCL. Our understanding of the pathogenesis of HCL has been further refined by the discovery of recurrent BRAF V600E mutations, which appear to arise within an HSC population with capacity for self-renewal. This observation quickly lent itself to diagnostic applications, as a sensitive and specific molecular marker of HCL among lymphoproliferative disorders, as well as to rapid investigation of oral molecularly targeted therapy. Additional advances of note are recognition that MRD negativity in CR is associated with superior durability of response in patients with HCL treated with chemotherapy[67] and refinement of MRD assessment by multiparameter flow cytometry; droplet digital PCR designed to detect low-level *BRAF* V600E alleles may serve as an additional sensitive molecular assay for MRD assessment. [68] As such, we enter this new era in HCL therapy with considerably improved tools for monitoring and management. Yet, HCL remains incurable with currently available therapy and purine analog therapy remains a mainstay of frontline treatment.

Several ongoing and forthcoming clinical trials will characterize the safety and efficacy of several approaches designed to enhance responses to BRAF inhibitor monotherapy. An international trial of dabrafenib in combination with trametinib (combined BRAF/MEK inhibition) for patients with HCL and other rare BRAF V600E mutant cancers is ongoing (NCT02034110); the potential implications of this combination in overcoming resistance to vemurafenib monotherapy and attenuating risk of toxicities driven by paradoxical MAPK activation has been described previously herein. Starting with combination BRAF/MEK inhibitor therapy (vs BRAF inhibitor monotherapy) in patients with HCL is thus of interest, though prospective clinical trials will be needed to compare these approaches, as was done in patients with BRAF V600E mutant melanoma. These forthcoming studies will offer further insight into risks and principal mechanisms of resistance to combination BRAF/MEK blockade in patients with HCL. Additionally, enrollment recently begun on a multicenter phase 2 trial of vemurafenib in combination with the humanized anti-CD20 monoclonal antibody obinutuzumab in patients with newly diagnosed HCL bearing BRAF

V600E (NCT03410875). Patients in the latter trial receive obinutuzumab following an initial 28-day cycle of vemurafenib monotherapy. Tiacci and colleagues recently reported composite rates CR or CR with incomplete platelet recovery of 100% among 25 evaluable patients with relapsed or refractory HCL treated with the combination of vemurafenib and rituximab, with 14/23 evaluable patients achieving MRD negativity by allele-specific PCR with a limit of detection of 0.05% *BRAF*V600E alleles; PFS was 100% at median 14 months and 9/10 patients achieving MRD negativity who underwent follow-up bone marrow evaluation maintained MRD negativity.[69] Compared with rituximab, obinutuzumab exhibits greater antibody-dependent cellular cytotoxicity and cellular phagocytosis, and in patients with chronic lymphocytic leukemia (CLL, another B-cell lymphoproliferative disorder), obinutuzumab appears to exhibit greater antitumor activity.[70] As such, treatment of patients earlier in their disease course and utilizing a more potent antibody represents an attractive chemotherapy-free approach with the potential of attaining rapid and deep responses, though will require considerable follow-up to assess durability of response.

Of note, moxetumomab pasudotox, a CD22-targeted recombinant immunotoxin was recently approved by the U.S. Food and Drug Administration for treatment of relapsed or refractory HCL; 30% of patients with HCL who had received 2 prior therapies exhibited CR lasting 6 months in a pivotal phase 2 trial, though risks of capillary leak syndrome and hemolytic uremic syndrome, and high rates of immunogenicity remain concerns and may limit feasibility of combination therapy.[71] The oral Bruton's tyrosine kinase inhibitor ibrutinib, which has considerable efficacy in other B-cell malignancies including chronic lymphocytic leukemia (CLL), is under investigation in an ongoing phase 2 trial (NCT01841723) in patients with HCL unfit for or with relapse following purine analog therapy and in patients with HCLv at a dose of 420 mg daily (as used in CLL) or 840 mg daily. After a pre-specified number of responses were observed at the 420 mg dose level, investigators reported objective responses in 13/28 (46%; CR, n=4; PR, n=9) evaluable patients, with an additional 8/26 (29%) achieving stable disease and reported clinical benefit in symptoms or cytopenias. Grade 3 infection was observed in 25% of patients but therapy was generally acceptably tolerated on long-term administration and 20 patients remained on ibrutinib.[72] Further investigation of these approaches, including in patients with less advanced HCL and more extensive "real world" experience, will help to place them within the hierarchy of currently available therapies for HCL, including BRAF inhibitors.

Within the next few years, we will begin to see clinical results reflecting therapy of HCL with combinations of BRAF inhibitors and CD20-targeted monoclonal antibody therapy as well as combinations of BRAF and MEK inhibitors. It is challenging to supplant a therapy with morphologic CR rates consistently exceeding 75% and with responses frequently lasting >5 years, such as cladribine for HCL, and in the community setting we anticipate purine analog therapy, with or without a CD20-targeted monoclonal antibody, remaining standard in the next several years. However, if studies evaluating a fixed duration of therapy with such a combination demonstrate high-rates of MRD-negative CR and durability is confirmed, comparison of such an approach in frontline management of HCL with purine analog based therapy may be warranted. Such a combinatorial approach may be attractive in using multiple mechanisms to overcome therapeutic resistance in patients with HCL and thereby intensify depth of remission, while avoiding both the acute and possible-long term

toxicities of cytotoxic therapy as well as the expense and adverse effects associated with chronic administration of targeted therapy. Further advances in targeting MAPK-mediated signaling as well as other pathways relevant in resistance to vemurafenib in HCL will likely lead to investigation of novel combination approaches as well. Intermittent use of low-dose BRAF inhibitor therapy, alone or in combination with other targeted therapy, is also likely to become a more widely utilized strategy for chronic management of patients with refractory or multiply relapsed HCL.

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Article highlights:

- The discovery of recurrent somatic BRAF V600E mutations in the vast majority of patients with HCL has clear diagnostic and therapeutic implications.
- Monotherapy with the BRAF inhibitor vemurafenib is associated with high rates of clinical response, even at doses lower than commonly utilized in treatment of melanoma
- Vemurafenib therapy is associated with high rates of cutaneous adverse events, which are generally manageable with supportive measures and dose interruption/reduction, though squamous cell carcinomas arising during therapy typically require excision
- HCL can exploit multiple mechanisms of resistance to BRAF inhibitor monotherapy, including paradoxical activation of the MAPK pathway

Figure 1:

Simplified depiction of the RAS-RAF-MAPK signal transduction pathway. Binding of a ligand to a transmembrane receptor tyrosine kinase. Dimerization of receptor subunits leads to phosphorylation of cytoplasmic domains, leading to recruitment of adaptor proteins and guanine nucleotide exchange factors in turn leading to activation of RAS GTPase proteins, which activate RAF kinases (e.g. BRAF), in turn phosphorylating and activating MEK family kinases. MEK proteins can then phosphorylate ERK proteins, leading to ERK activation and translocation to the nucleus, where ERK serves to activate transcription factors, including c-myc, and regulate gene expression governing cell cycle entry and proliferation. PI3K is able to be activated directly via receptor tyrosine kinases following ligand binding and may additionally be activated by RAS proteins. Several commercially available and investigational inhibitors of BRAF, MEK1/2, and ERK1/2 are depicted as well.

Table 1:
Clinical responses in largest reported series investigating vemurafenib therapy for HCL

Yr=years, ORR=objective response rate; CR=complete response; PR=partial response; MR=minor response; RFS=relapse free survival; PFS=progression free survival; OS=overall survival

Reference	N	Median Age, Yr (Range)	Initial Vemurafenib Dosing	Vemurafenib Duration	Clinical Responses in Evaluable Patients	Response Durability €	Retreatment
[54]; Italian phase 2 trial	28	57 (27–84)	960 mg PO twice daily†	8–16 weeks *	ORR: 96% (25 of 26) CR: 35% (9 of 26) PR: 62% (16 of 26)	Median RFS 9 months (19 months for those achieving CR)	10 pts (CR, n=2; PR, n=2; MR, n=4; not evaluable, n=2)
[54]; U.S. phase 2 study	26	62 (44–80)	960 mg PO twice daily ‡	12 weeks ‡	ORR: 100% (24 of 24) CR: 42% (10 of 24) PR: 58% (14 of 24)	1-year PFS 73% 1-year OS 91%	6 pts (PR, n=4; PD, n=1; not evaluable, n=1)
[53]; Patients treated off-study at several European centers	21	64 (45–89)	240 mg PO twice daily (n=17); 240 mg PO once daily (n=1); 480 mg PO daily (n=2); 960 mg PO	90 days (median); 56–266 days (range)	ORR: 95% (20 of 21) CR: 40% (6 of 15 evaluable patients) PR: 60% (9 of 15 evaluable patients)	Median RFS 14 months 1-year OS 88%	6 pts (PR, n=2; blood count recovery without confirmed PR, n=4)
			twice daily (n=2)		patients)		

Footnotes:

* 3 patients received vemurafenib for 20 weeks;

‡ Dose reductions permitted for intolerance;

‡ Therapy for an additional 12 weeks permitted for patients with residual disease after treatment;

€ The Italian phase 2 study defined relapse in the setting of milder cytopenias than the U.S. phase 2 study or the European off-trial series, including absolute neutrophil count (ANC) <1,500/μL, hemoglobin <11g/dL, or platelets <100,000/μL, compared with hematologic parameters of ANC <1,000/μL, hemoglobin <10g/dL, and platelets <100,000/μL in the other series.