Brief Report

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LYMPHOID NEOPLASIA

Genomic analysis of hairy cell leukemia identifies novel recurrent genetic alterations

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Key Points

- *KMT2C* mutations occur in 15% and 25% of patients with cHCL and vHCL, respectively, along with *CCND3* and *U2AF1* mutations each in 13% of vHCLs.
- *NF1*, *NF2*, *N/KRAS*, and *IRS1* alterations contribute to clinical resistance to vemurafenib treatment in patients with cHCL.

Classical hairy cell leukemia (cHCL) is characterized by a near 100% frequency of the *BRAF*V600E mutation, whereas ~30% of variant HCLs (vHCLs) have *MAP2K1* mutations. However, recurrent genetic alterations cooperating with *BRAF*V600E or *MAP2K1* mutations in HCL, as well as those in *MAP2K1* wild-type vHCL, are not well defined. We therefore performed deep targeted mutational and copy number analysis of cHCL (n = 53) and vHCL (n = 8). The most common genetic alteration in cHCL apart from *BRAF*V600E was heterozygous loss of chromosome 7q, the minimally deleted region of which targeted wild-type *BRAF*, subdividing cHCL into those hemizygous versus heterozygous for the *BRAF*V600E mutation. In addition to *CDKN1B* mutations in cHCL, recurrent inactivating mutations in *KMT2C* (*MLL3*) were identified in 15% and 25% of cHCLs and vHCLs, respectively. Moreover, 13% of vHCLs harbored predicted activating mutations in *CCND3*. A change-of-function mutation in the splicing factor *U2AF1* was also present in 13% of vHCLs. Genomic analysis of de novo vemurafenib-resistant cHCL identified a novel gain-of-function mutation in *IRS1* and losses of *NF1* and *NF2*, each of which contributed to resistance. These data provide further insight into the genetic bases of cHCL and vHCL and mechanisms of RAF inhibitor resistance encountered clinically. (*Blood*. 2017;130(14):1644-1648)

Introduction

Hairy cell leukemia (HCL) comprises the clonal hematologic malignancies of classical (cHCL) and variant (vHCL). Although cHCL and vHCL share expression of CD11c and CD103, only cHCL expresses CD25, CD123, CD200, and annexin A1. Furthermore, cHCL and vHCL differ in therapeutic response and prognosis, with vHCL responding poorly to purine analogs (PAs), with a median survival less than half that of cHCL.[1-5] In addition, the mutations present in each HCL subtype are distinct, with *BRAF*V600E mutations in ~100% of cHCLs,[6,7] whereas ~30% of vHCLs harbor activating mutations in *MAP2K1*,[8,9] encoding the MEK1 kinase just downstream of BRAF. These data have furthered our understanding of and therapeutic approaches for HCL; however, studies of diverse cancers marked by the *BRAF*V600E mutation suggest that additional alterations are frequently required for tumor initiation and/or progression in *BRAF*V600E-mutant cells.[9-13] To this end, we recently detected coexistence of *CDKN1B* and *BRAF*V600E mutations in 16% of cHCLs.[14] Currently, however, additional examples of recurrent genomic alterations that coexist with *BRAF*V600E or *MAP2K1* mutations in HCL are not known, nor have recurrent mutations in MEK1 wild-type vHCL been identified. We therefore performed deep sequencing and copy number (CN) analysis to gain additional insights into the pathogenesis and mechanisms of therapeutic resistance in HCL.

Methods

Diagnostic bone marrow or peripheral blood mononuclear cells (MNCs) were obtained from 53 patients with cHCL (22 treatment naïve, 4 relapsed/refractory

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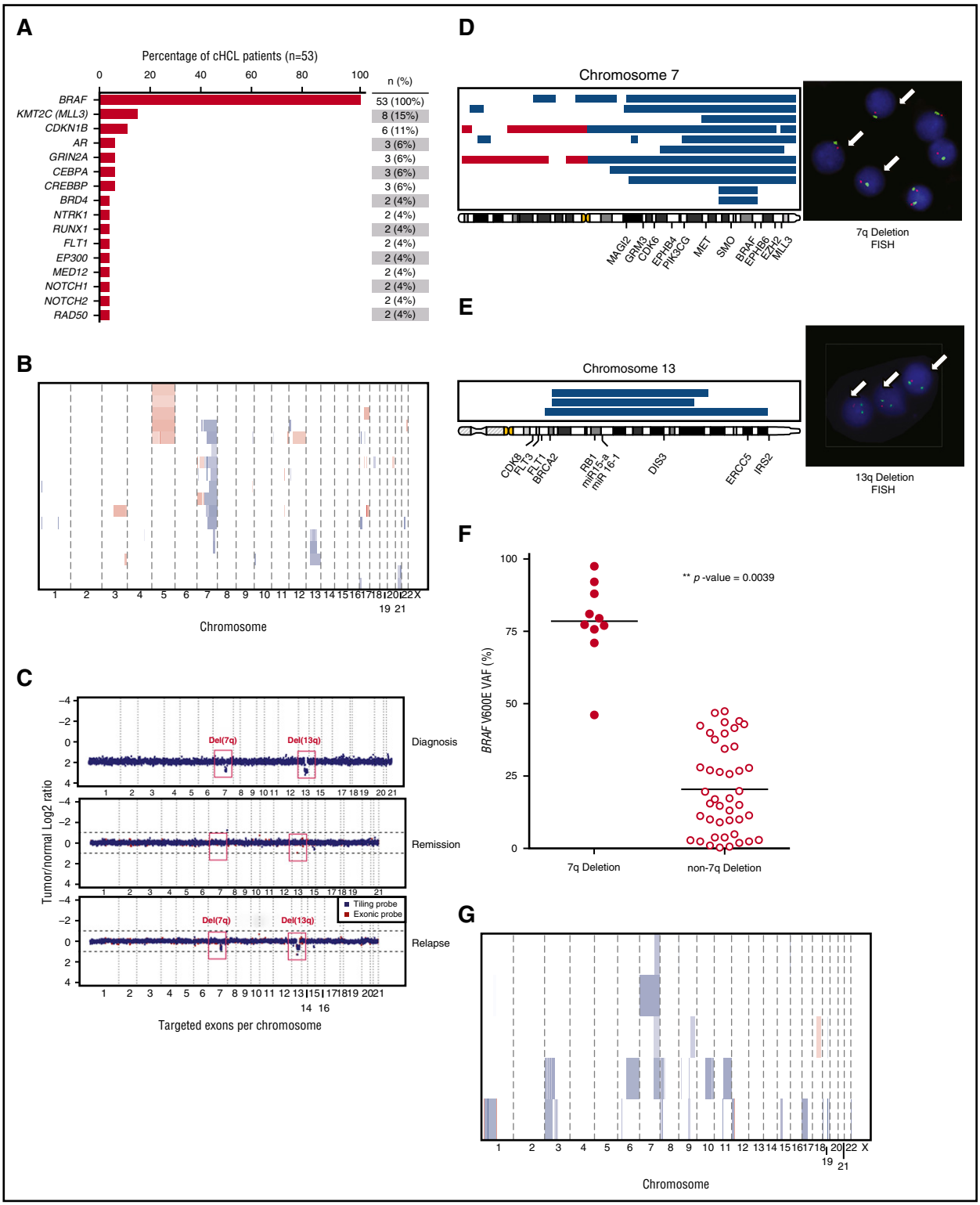

Figure 1. Genomic alterations in cHCL and vHCL. (A) Histogram of mutations in cHCL cohort (n = 53 patients) present in ≥2 patients. (B) CN analysis of the cHCL cases. Curated segmentation data for 53 cHCL samples. In the red-blue scale, white corresponds to a normal (diploid) CN log ratio, blue is a deletion, and red is a gain. (C) CN variation plots of peripheral blood MNCs from a single patient with cHCL at initiation, remission, and relapse from BRAF inhibitor treatment illustrating deletion of 7q and 13q [del(7q) and del(13q), respectively] regions at times of treatment initiation and relapse but not in disease remission. Genes mapped in the region of del(7q) with representative fluorescence in situ hybridization (FISH) with 7q deletion (white arrows; probes: red = 7q31; green = centromeric probe chromosome 7 [CEP7]) (D) and del(13q) with representative FISH with 13q14 deletion (white arrows; probes: red = 13q14; green = 13q34) (E). (F) *BRAF*V600E variant allele frequency (VAF) in patient cases with or without del(7q). (G) CN analysis of 8 cases of vHCL.

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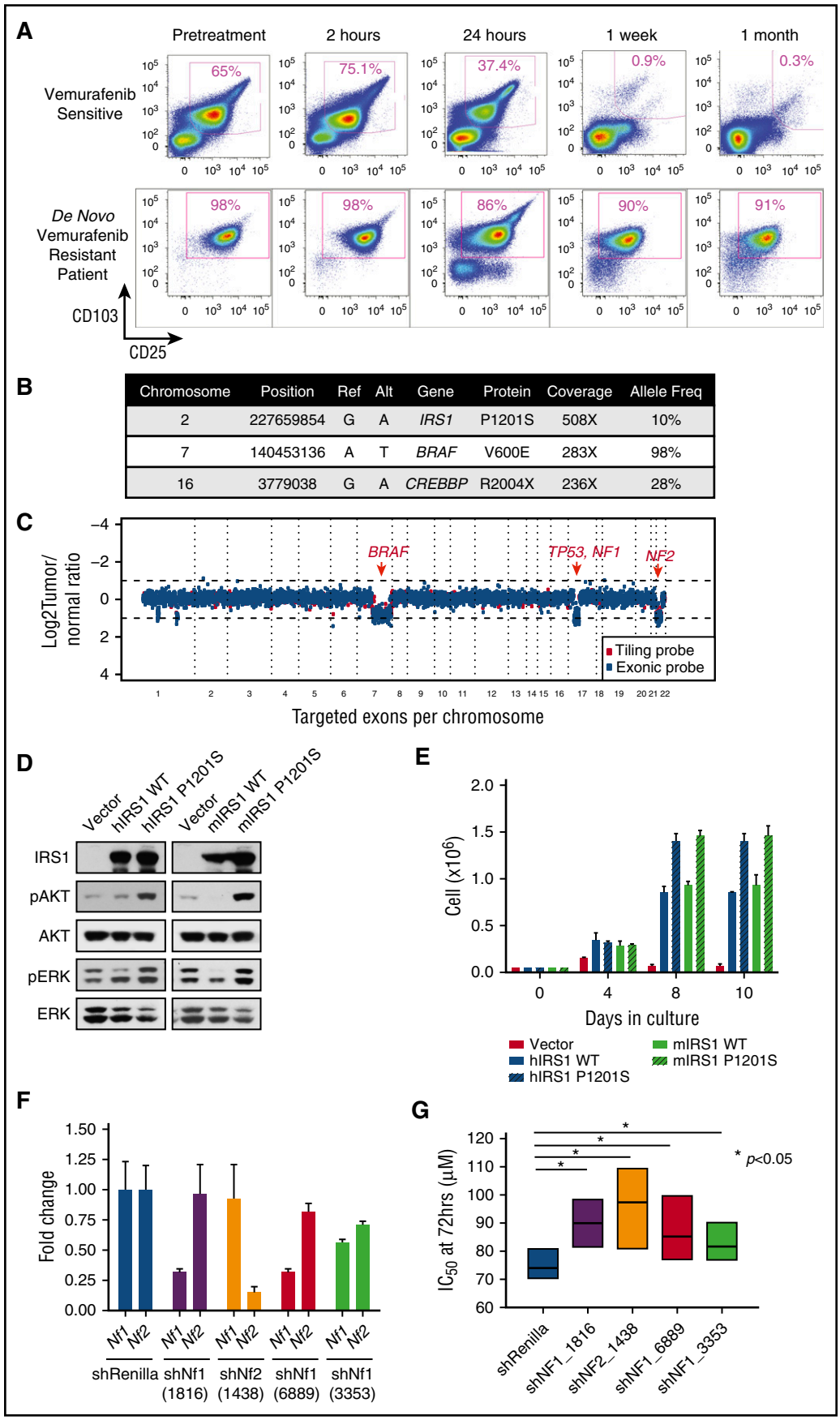

Figure 2.

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and PA refractory, and 27 relapsed/refractory) and 8 with vHCL (all relapsed/refractory) from Memorial Sloan-Kettering Cancer Center, University Hospital Heidelberg, and the Munich Leukemia Laboratory. Twenty-six of these patient cases were previously sequenced for *CDKN1B* alone.[14] Treatment groups were defined as treatment naïve (those patients receiving no prior treatment), relapsed/refractory (patients with HCL who relapsed after therapy using a PA ≥1 year but ≤2 years after the first course or ≤4 years after a second or later course of a PA), or relapsed/refractory and PA refractory (patients with no response to PAs or any relapse ≤1 year after the initiation of PAs).[15] Patient samples were collected after obtaining written informed consent. The use of human materials was approved by the institutional review boards of each institution in accordance with the Declaration of Helsinki.

Analysis of MNCs, fluorescence-activated cell sorter–purified HCL cells, and, for some cases, granulocytes (11 cHCL and 3 vHCL patient cases) was performed using the MSK-IMPACT targeted next-generation sequencing assay, which sequences all coding regions of 585 genes recurrently mutated in leukemias, lymphomas, and solid tumors (supplemental Table 1, available on the *Blood* Web site). Sequencing and analysis were performed as previously described.[16-18] The FACETS algorithm was used to estimate tumor purity, ploidy, and allele-specific CN from sequencing data of tumor-normal pairs as previously described.[18,19] Additional experimental details are described in the supplemental Methods.

Results and discussion

We performed targeted sequencing of 585 genes recurrently mutated in hematologic and solid tumors across 53 patients with cHCL. *BRAF*V600E mutation was present in 100% of patients with cHCL (Figure 1A; supplemental Figures 1 and 2; supplemental Table 2), and the next most commonly mutated genes in cHCL were the histone methyltransferase *KMT2C* (*MLL3*) and *CDKN1B*, occurring in 15% (8 of 53) and 11% (6 of 53) of patients, respectively. *KMT2C* was affected by predicted loss-of-function mutations throughout the coding region (supplemental Figure 2B). Other recurrent mutations in cHCL affected genes involved in transcriptional regulation (*BRD4*, *CEBPA*, *CREBBP*, *RUNX1*, *EP300*, and *MED12*), Notch signaling (*NOTCH1* and *NOTCH2*), and DNA repair (*RAD50*; Figure 1A; supplemental Figure 1; supplemental Table 2).

The most recurrent CN alterations in cHCL were deletions of chromosomes 7q and 13q and gains of chromosome 5. Chromosome 7q and 13q deletions were confirmed by fluorescence in situ hybridization (Figure 1B-E; supplemental Table 3). These CN changes were evident in peripheral blood MNCs at disease initiation and relapse but not during remission, consistent with their somatic nature in HCL cells (Figure 1C). Although recurrent 7q deletions have previously been reported in cHCL,[2-4,14] additional genes in the minimally deleted region of 7q beyond *BRAF* included *SMO* (7q32) and *BRAF* (7q34) itself. As a result, 7q deletion resulted in loss of heterozygosity of the *BRAF*V600E allele (Figure 1D,F), as evidenced by the higher *BRAF*V600E variant allele frequency of 7q-deleted versus 7q-diploid cHCL (79% vs 22%, respectively; Wilcoxon matched-pairs signed rank test $P = .0039$). Recurrent 13q deletions in cHCL included the tumor suppressor *RB1* and the *miR-15a* and *miR-16-1* microRNA cluster at 13q14.3, which have been well studied in chronic lymphocytic leukemia[20] (Figure 1E).

Prior genomic analyses identified *MAP2K1* mutations in ~30% of patients with vHCL, as well as individuals with mutations in *CCND3*, *TP53*, *U2AF1*, and *ARID1A*.[8] Sequencing across 8 additional patients with vHCL identified change-of-function mutations in both *CCND3* and *U2AF1*, each occurring in 13% (1 of 8) of patients with vHCL, and mutations in *TP53* (38%; 3 of 8 patients; supplemental Figure 3A-B; supplemental Table 4). Of note, the reported *TP53* mutations occurred at well-known hotspots, and 1 *TP53* mutation (*TP53* P301*fs**44) was homozygous. The *CCND3* and *U2AF1* mutations were absent in cHCL, suggesting additional genetic differences between cHCL and vHCL. *CCND3* mutations (p.R271*fs*; p.D286*fs*) are predicted to lead to loss of the PEST domain and increased expression of CCND3[21,22] (supplemental Figure 3C). The *U2AF1* mutation identified occurred at a previously described hotspot region[23] and altered the RNA splicing preferences of the protein distinct from loss of function[24] (supplemental Figure 3D). These findings may have therapeutic relevance, because it is speculated that *CCND3* mutations confer sensitivity to CDK4/CDK6 inhibitors,[22] whereas those in *U2AF1* confer sensitivity to spliceosome inhibitors.[25] Other mutations affecting genes involved in transcriptional regulation (*CEBPA*, *CREBBP*, *DDX3X*, and *PBRM1*) and chromatin remodeling (*KMT2C* [*MLL3*], *KDM6A*, and *KDM5C*) were also identified (supplemental Figure 3A-B).

As with cHCL, chromosome 7q deletions were also present in vHCL (consistent with previous reports of 7q deletions in 20% of vHCLs[3,4]; (Figure 1G; supplemental Figure 3A; supplemental Table 5). In addition, we also identified recurrent 3p deletions in vHCL. This region includes a critical tumor suppressor locus encoding *VHL*, *SETD2*, *BAP1*, and *PBRM1* and is commonly deleted in renal and lung carcinomas.[26]

In addition to providing knowledge of disease biology and novel therapeutic targets, we also sought to understand if any of the mutations identified might affect response to therapy. For example, we previously described *KRAS* mutations in a patient with cHCL who developed vemurafenib resistance.[15] Interestingly, here we identified an activating mutation in *NRAS* in a treatment-naïve patient with cHCL (supplemental Figure 2A; supplemental Table 2). Moreover, we also detected mutations in the kinases *NTRK1* and *FLT1* in *BRAF*V600E-mutant cHCL, the functional impact of which is not known. In addition, 1 patient experienced complete de novo vemurafenib resistance when treated in the phase 2 clinical trial of vemurafenib for relapsed/refractory cHCL (Figure 2A). Genomic analysis of the pretreatment sample uncovered a clonal hemizygous *BRAF*V600E mutation, as well as heterozygous deletions of *BRAF*, *NF1*, *NF2*, and *TP53* and subclonal mutations in *CREBBP* and *IRS1* (Figure 2A-C). *IRS1* encodes a signaling adaptor protein that relays signals from IGF-1R to the MAPK and PI3K-AKT pathways. Stable expression of mutant *IRS1*P1201S activated PI3K-AKT signaling and phosphorylated ERK1/2, leading to cytokine-independent growth of Ba/F3 cells (Figure 2D-E). Both *NF1* and *NF2* encode tumor suppressors that have been experimentally implicated in RAF inhibitor resistance in epithelial cancer cells[27,28] but have not been described in the context of cHCL or clinical RAF inhibitor resistance in hematologic malignancies. Consistent with the heterozygous deletion of *NF1* and *NF2*, the de novo vemurafenib-resistant patient showed decreased expression of both *NF1* and *NF2*, whereas patients without *NF1* or *NF2* copy loss who were responsive to vemurafenib as part of the same clinical trial did

Figure 2. Gain-of-function mutation in *IRS1* and *NF1/2* loss result in de novo vemurafenib resistance in cHCL. (A) Serial flow cytometric analyses of cHCL cells ($CD103^+/CD25^+$) in the peripheral blood of a patient with cHCL before vemurafenib initiation and during the first month of treatment (top), as well as a different patient before vemurafenib initiation until time of death (bottom). Mutations (B) and CN alterations (C) detected in pretreatment cHCL sample from the vemurafenib-resistant patient. (D) Western blot analysis of human (hIRS1) and mouse IRS1 (mIRS1) complementary DNA constructs in wild-type (WT) or mutant forms on AKT and ERK phosphorylation related to empty vector. (E) Cell growth of IRS1-expressing Ba/F3 cells from (D) after interleukin-3 withdrawal. (F) Quantitative reverse transcription polymerase chain reaction of *Nf1* and *Nf2* expression after anti-*Nf1* or -*Nf2* short hairpin (shRNA) knockdown. The numbers below each shRNA indicate the shRNA oligonucleotide sequence (as shown in supplemental Methods). (G) IC50, 50% inhibitory concentration (IC_{50}) of BRAFV600E-expressing Ba/F3 cells to vemurafenib with or without knockdown of m*Nf1* or m*Nf2*.

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not demonstrate decreased expression of *NF1*/*NF2* (supplemental Figure 4A-B). To understand the functional role of Nf1 and Nf2 loss and the potential contribution to RAF inhibitor resistance, we performed short hairpin RNA–mediated downregulation of *Nf1* or *Nf2* in Ba/F3 cells stably expressing *BRAF*V600E. Silencing of either *Nf1* or *Nf2* alone (Figure 2F-G) or concomitant downregulation of *Nf1* and *Nf2* simultaneously (supplemental Figure 4C-D) conferred vemurafenib resistance in vitro.

Combined, these data identify numerous novel drivers of HCL. Although activating MAPK mutations are critical for both cHCL and vHCL, our data suggest additional shared cooperating alterations, as well as disease-specific alterations targeting *BRAF*, *KMT2C*, and *CDKN1B* in cHCL and *MAP2K1*, *CCND3*, *U2AF1*, *TP53*, and *KMT2C* in vHCL. Finally, these data nominate several novel potential therapeutic approaches for vHCL and identify diverse causes for RAF inhibitor resistance through activation of signaling pathways parallel to the MAPK pathway.

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Authorship

Contribution: B.H.D., J.T., H.W., J.M.B., S.S., E.K., Y.R.C., S.S.C., L.W., S.X.L., C.C.O., B.S.T., M.F.B., and O.A.-W. performed the experiments and analyzed data; B.G., S.D., J.M.B., J.H., T.W., C.C.O., R.T., T.H., M.S.T., J.H.P., T.Z., and O.A.-W. collected patient material and clinical data; and B.H.D., T.Z., and O.A.-W. wrote the manuscript.

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