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High prevalence of *MAP2K1* mutations in variant and IGHV4-34 expressing hairy-cell leukemia

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To understand the genetic mechanisms driving variant and IGHV4-34 expressing hairy-cell leukemia, we performed whole exome sequencing of ten patients including six with matched normal samples. Activating mutations in the *MAP2K1* gene (encoding MEK1) were observed in five of these ten patients and in 10 of 21 in a validation set (overall frequency 15 of 31) suggesting potential new therapeutic approaches for these patients.

Hairy-cell leukemia-variant (HCLv) is a chronic mature B-cell neoplasm with little available data regarding the genetic mechanisms driving pathogenesis. HCLv shares overlapping pathological features with classic hairy-cell leukemia (HCLc) but the two malignancies have distinct morphologies, immunophenotypes, molecular signatures, and clinical courses. Accordingly, they are classified separately by the latest World Health Organization guidelines. While purine analog therapy (i.e. cladribine or pentostatin) leads to a median survival of over 20 years and durable complete remissions in the vast majority of HCLc patients, it is largely ineffective in HCLv where only 50% of patients exhibit even a partial response.

It has recently been shown that 40% of HCLv samples and 10% of HCLc samples express the IGHV4-34 immunoglobulin variable heavy chain rearrangement (IGHV4-34+). Independent of the variant/classic diagnosis, IGHV4-34 expression is associated with higher disease burden at diagnosis, poor response to single agent cladribine, and shorter overall survival[1].

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AUTHOR CONTRIBUTIONS

JJW, EA, RJK, and PSM conceived the study and supervised analyses. JJW designed, created and performed the analyses. EA and RJK provided patient materials. LR extracted DNA and processed the patient samples. RLW performed the exome sequencing. MP conducted the Sanger sequencing and Taqman analysis. JKK designed and supervised the Taqman assay. SRD and ODA provided computational scripts. JJW, RJK, and PSM wrote the manuscript with contributions from all other authors.

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HCLc was recently shown to be almost universally driven by the oncogenic activating V600E mutation in the serine/threonine kinase *BRAF*[2]. This finding has been validated multiple times and targeted therapy for BRAF V600E has shown clinical benefit in refractory HCLc[3]. However, multiple reports have failed to identify any instances of *BRAF* V600 mutations in either HCLv or IGHV4-34+ HCLc[2,4–6].

We performed whole exome sequencing on ten HCL samples that were either variant (n=5), IGHV4-34 positive (n=3), or both (n=2) (Supplementary Table 1). Differentiating HCLv from splenic marginal zone lymphoma (SMZL) and similar entities can be difficult or sometimes even impossible, particularly without splenic pathology. However the presence of CD103 and absence of CD25 on nearly all of the HCLv cases supports this diagnosis, and the two CD103-negative cases were supported by splenic tissue analysis. Each tumor sample had a post-treatment blood sample to serve as a matched normal. Sequencing was performed on the Illumina HiSeq with paired-end 101 bp reads to an average depth of 46x (Supplementary Table 2). Analysis of sample purity showed that while all tumor samples had minimal normal contamination, four of the post-treatment blood samples still had high tumor content and were insufficient for identifying somatic variants (Supplementary Figure 1). These were therefore discarded, leaving six matched and four unmatched tumor samples (online Methods). Filtering the variant calls to identify candidate driver genes (online Methods) identified five recurrent targets: *MAP2K1*, *U2AF1*, *ARID1A*, *TP53*, and *TTN* (Table 1).

The most frequently mutated gene was *MAP2K1*, occurring in five of ten separate samples. We validated this finding in a set of 21 additional samples (Supplementary Table 1). Sanger sequencing of the affected exons (exons 2 and 3) revealed ten more mutation positive samples for an overall frequency of 48% (95% CI 30%-67%) (Supplementary Table 3). *MAP2K1* mutations occurred in all three subtypes of HCL examined (6 of 15 HCLv IGHV4-34-, 4 of 9 HCLv IGHV4-34+, and 5 of 7 HCLc IGHV4-34+ overall) with no significant differences in frequency ($p > 0.1$ for all pairwise comparisons by Fisher Exact Test). *MAP2K1* encodes the dual specificity kinase MEK1, which is a direct effector of BRAF and directly upstream of ERK1/2 in the MAPK pathway. The *BRAF* V600E mutation occurred in one of the initial ten whole exome discovery set samples as well as in two of the 21 validation set samples by TaqMan PCR and was mutually exclusive of *MAP2K1* mutations (Supplementary Table 3). *MAP2K1* mutations have been identified infrequently in the developmental disorder cardio-facio-cutaneous (CFC) syndrome, in several cancers including melanoma, ovarian carcinoma, colorectal carcinoma, and non-small cell lung cancer and have also recently emerged as a mode of resistance to BRAF *MAP2K1* V600E targeted therapy[7]. With the exception of one instance of SMZL[8], mutations have never been reported in any hematologic malignancy and never with an incidence approaching this frequency in any cancer.

The mutations we identified in *MAP2K1* gene map to the negative regulatory region, the catalytic core (but not the active site), and the intervening linker[7] (Figure 1). All but one of the mutations are substitutions, most of which have been identified before either in patient samples from other types of cancer or by *in vitro* screens and have been shown to strongly increase basal enzymatic activity and cell proliferation[9–12]. The only non-substitution

mutation is a forty-eight nucleotide in-frame deletion (amino acids 42 through 57) that almost entirely removes the auto-inhibitory helix-A[13]. A highly similar deletion (residues 44 through 51) has been shown to increase basal activity of the enzyme by 60-fold[14]. These findings are of profound importance since MEK inhibitors have been under intense development as a mechanism to suppress mitogenic signaling and as a means of complementing clinical benefit of mutant BRAF inhibitors[15]. Current MEK inhibitors function by an allosteric mechanism dependent on the same N-terminal auto-inhibitory domains where these mutations cluster. The most common mutation in our cohort, C121S, as well as I103N and Q56P have previously been shown to confer resistance to current MEK inhibitors[9,10] while the K57N mutation remains sensitive[11]. Thus, while the current repertoire of MEK inhibitors are not expected to be clinically beneficial for all patients, MEK1 and its effector ERK remain very attractive targets for therapy in this leukemia. The uniquely high incidence of *MAP2K1* mutations may also be of diagnostic utility for this disease. To validate the association of *MAP2K1* mutations with these forms of HCL we also investigated 20 cases of IGHV4-34-negative HCLc (Supplementary Table 1). Seventeen of these samples were *BRAF* V600E positive and one sample had a *MAP2K1* F53L mutation (Supplementary Table 3), supporting previous findings that *BRAF* V600E is largely specific to IGHV4-34 negative HCLc. The robust segregation of mutations in *BRAF* and *MAP2K1* with different forms of HCL demonstrates that the mutations are not redundant in this context, possibly due to quantitative effects on pathway activation, qualitative effects on feedback pathways, or other mechanisms.

Results from our whole exome sequencing revealed four additional candidate driver genes each mutated in two of ten patients: *TP53, U2AF1, ARID1A* and *TTN*. We did not pursue *TTN* because it encodes the longest protein in the genome with a well-described muscle-specific function and the mutations are therefore likely to be passengers. *TP53* is the most commonly mutated gene across all cancers and somatic mutation and loss of heterozygosity at this locus have previously been associated specifically with HCLv[16]. U2AF1 is a component of the splicing machinery that recognizes and binds to the 3’ splice site. The hotspot S34F mutation was found in two of the ten discovery set samples and has been observed before in several other hematopoietic malignancies[17] and in a small fraction of lung adenocarcinomas[18]. Amongst hematopoietic malignancies *U2AF1* mutations have previously been restricted to myeloid disorders however the splicing machinery component *SF3B1* is mutated in roughly 10% of chronic lymphocytic leukemias, another mature B-cell malignancy. We performed Sanger sequencing of this hotspot in our validation set and identified one additional case (Supplementary Table 3). While this could reflect the true frequency, it should also be noted that in the two initial S34F positive samples the variant allele frequency was low (17% and 22%), possibly due to being sub-clonal, and could be below the level of detection for Sanger sequencing. Two of the initial ten samples contained truncating mutations in the tumor suppressor *ARID1A*, a core component of the ATP-dependent chromatin remodeling complex SWI/SNF. Loss of function mutations in *ARID1A* have been identified in many different cancers including several B cell malignancies[19]. In conclusion, we find a remarkably high frequency of *MAP2K1* mutations driving variant and IGHV4-34 expressing hairy cell leukemia. The development of inhibitors towards these

mutated forms of MEK1 or its presumed target ERK could transform the treatment of patients with this disease.

Online Methods

Sample Collection

DNA was extracted from peripheral blood of patients being treated on or screened for HCL protocols at the National Institutes of Health, approved by the Investigational Review Board of the National Cancer Institute. Informed consent was obtained from all research participants under study NCT01087333. The diagnosis of HCLc and HCLv and molecular characterizations of IGHV rearrangements were performed as previously described[20]. DNA Samples were extracted using a Precision System Science automated robot (PSS USA).

Whole Exome Sequencing

DNA (1μg) was fragmented using a Covaris S2 Focused-ultrasonicator (Covaris, Woburn, MA) to a mean size of 300bp. Fragment ends were repaired and phosphorylated with T4 DNA Polymerase (NEB), Klenow-fragment (NEB) and T4 Polynucleotide Kinase (NEB). A 3'-A overhang was introduced with Klenow exo-minus (NEB) followed by ligation to Illumina paired-end adapters. Ligation products with a mean size of 240bp +/− 20% were isolated on a Caliper LabChip XT (PerkinElmer, Waltham, MA) followed by amplification using Illumina PCR primers InPE1.0, InPE2.0 and PCR primer indices. Pooled, indexed libraries were captured using the Agilent SureSelect Human All Exon, 50Mb Kit (Agilent, Santa Clara, CA) according to the manufacturer's protocol and sequenced on an Illumina HiSeq 2000 (Illumina, San Diego, CA)

Mutation and Sample Purity Analysis

After assessing sample purity (Supplementary Note), somatic mutation calls for individuals RB31, BL26, BL14, 10984, RG06, and RG01 were combined with non-reference variants in the tumor samples for individuals BL42, HH14, 10748, and 10821. These pooled variants were then filtered against public and in-house databases to remove common variants and possible artifacts. To identify candidate driver genes we further filtered for non-silent variants occurring at conserved positions. All such variants are listed in Supplementary Table 4. Finally, the count of such variants for each gene was used to identify recurrently targeted genes (i.e. in more than one sample), insisting that at least one such mutation be clearly somatic (i.e. in one of the six samples where this could be discerned). See Supplementary Note for more details.

Sanger Sequencing

Exons two and three of *MAP2K1* and exon 2 of *U2AF1* were sequenced in 21 additional HCL patients. Forward and reverse primers were tailed with M13 tags for downstream sequencing by M13 sequencing primers. DNA was sequenced using BigDye Terminator V3.1 sequencing kit (Applied Biosystems) and the 3730xl DNA Analyzer (Applied Biosystems). Results were analyzed using Variant Reporter V1.0 (Applied Biosystems). Supplementary Table 5 lists sequences for primers used in the sequencing assay.

TaqMan Real-Time PCR

The Custom Taqman SNP Genotyping Assay (ABI) for *BRAF* V600E was designed to detect *BRAF* c.1799T>A (Supplementary Table 5). Assays were run in duplicates with a final 1X concentration of probe mix and Taqman Universal PCR Master Mix (ABI) using 10-20 ng of template DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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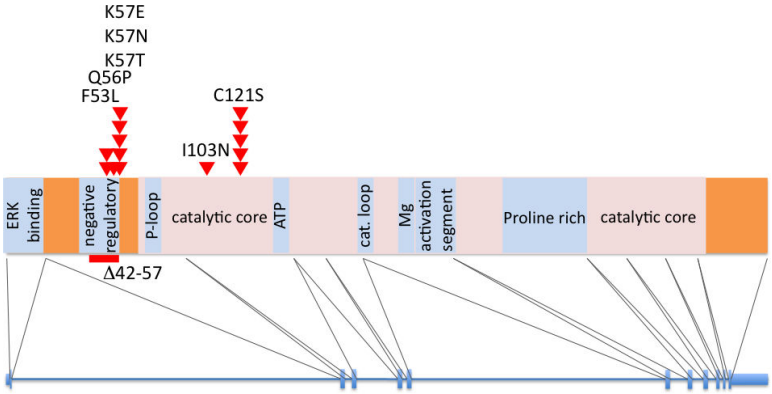

Figure 1. Distribution of mutations in *MAP2K1*

Schematic of the MEK1 protein and *MAP2K1* gene structure. Somatic mutations identified in variant and IGHV4-34 expressing hairy cell leukemia samples cluster in exons 2 and 3 encoding the N-terminal auto-regulatory domain. Triangles above the protein indicate substitutions and the bar below indicates the in-frame deletion.

Table 1

Recurrent mutated genes from whole exome sequencing

Reference sequences are NM_002755 (*MAP2K1*), NM_001025203 (*U2AF1*), NM_006015 (*ARID1A*), NM_001126114 (*TP53*), and NM_003319 (*TTN*).

Diagnosis	Patient	*MAP2K1*	*U2AF1*	*ARID1A*	*TP53*	*TTN*
HCLv IGHV4-34-	RB31	c.361T>A p.Cys121Ser				
	10821				c.782+2T>G	
	10984	c.159T>A p.Phe53Leu	c.101C>T p.Ser34Phe		c.711G>T p.Met237Ile; c.427G>A p.Val143Met	
	BL14	c.125_172del p.Leu42_Lys57del		c.4716C>G p.Tyr1572*; c.4709C>G p.Ser1570Cys		
	RG01					c.42055C>T p.Arg14019*
HCLv IGHV4-34+	RG06					
	BL26		c.101C>T p.Ser34Phe			
HCLc IGHV4-34+	10748			c.1690_1691insC p.Gln564fs		c.57477G>A p.Met19159Ile
	HH14	c.171G>T p.Lys57Asn				
	BL42	c.308T>A p.Ile103Asn				