Recurrent $CDKN1B$ (p27) mutations in Hairy Cell Leukemia

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Key Points

1) Somatic CDKN1B (p27) mutations were identified in 16% (13/81) of hairy cell leukemia patients (HCL) and coexist with BRAFV600E mutations.

2) CDKN1B is the second most commonly mutated gene in HCL implicating altered cell cycle regulation and/or senescence in HCL.

Abstract

Hairy cell leukemia (HCL) is marked by near 100% mutational frequency of BRAFV600E mutations. Recurrent cooperating genetic events that may contribute to HCL pathogenesis or affect clinical course in HCL are currently not described. We therefore performed whole exome sequencing to explore the mutational landscape of purine analogue refractory HCL. In addition to the disease defining BRAFV600E mutations, we identified mutations in EZH2, ARID1A and recurrent inactivating mutations of the cell cycle inhibitor CDKN1B (p27). Targeted deep sequencing of CDKN1B in a larger cohort of HCL patients identify deleterious CDKN1B mutations in 16% of patients with HCL (n=13/81). In 11 of 13 patients the CDKN1B mutation was clonal, implying an early role of CDKN1B mutations in the pathogenesis of HCL. CDKN1B mutations were not found to impact clinical characteristics or outcome in this cohort. These data identify HCL as having the highest frequency of CDKN1B mutations amongst cancers and identify CDKN1B as the 2nd most commonly mutated gene in HCL. Moreover, given the known function of CDKN1B, these data suggest a novel role for alterations in regulation of cell cycle and senescence in HCL with CDKN1B mutations.


**Introduction**

Hairy-cell leukemia (HCL) is a rare, mature B-cell malignancy presenting with slowly progressing pancytopenia and splenomegaly. Classical HCL is successfully treated with chemotherapy, but eradication of minimal residual disease (MRD) is rarely achieved. Standard treatment fails in a minority of patients, with potentially fatal outcome.

Gain-of-function mutations of the BRAF serine/threonine protein kinase (BRAFV600E) have been identified in nearly all cases of classical HCL, and MAP kinase signaling is considered the key oncogenic pathway in HCL\(^2\). Chung *et al.* recently identified hematopoietic stem cells (HSCs) as the cell of origin of HCL by demonstrating that HSCs and subsequently cells along the hematopoietic hierarchy contain mutated BRAF.\(^3\) Currently, however, no other recurrently mutated genes are known to co-exist with BRAFV600E mutations in HCL. It is unclear if BRAFV600E mutations alone are sufficient to induce HCL. Moreover, it is also not known if additional mutations may be acquired in BRAFV600E-mutant HCL cells, resulting in acquired resistance to therapies commonly administered to patients with HCL such as purine analogs (PA). We therefore performed whole-exome sequencing (WES) in 3 HCL patients who were refractory to purine analogue (PA) treatment and received the BRAF inhibitor (BRAFi) vemurafenib followed by recurrence testing of novel mutations in a larger cohort of HCL patients.
Methods

Clinical samples were provided by SD, AH and TZ (n=10), DG and EM (n=50), TH (n=17), MD (n=1), JD (n=2) and XT (n=6) and studies were approved by local ethics committees. Exome-and targeted sequencing analysis was performed as previously described\textsuperscript{4,5}. For further details please see supplemental methods.
Results and Discussion

We first performed copy number variation (CNV) analysis based on WES data from these 3 initial patients. This revealed loss of chromosome 21 in all three patients (supplemental material). Although these findings were validated by Affymetrix Cytoscan HD Arrays, CNV analysis of 7 additional patients failed to reveal chromosome 21 abnormalities (supplemental table 1). Of note, 5/63 patients harbored deletions of 7q involving BRAF (7q34) and thereby causing loss of heterozygosity of the mutant BRAF-allele.

In addition to the BRAF-V600E mutation, WES identified 15 to 37 somatic mutations per patient (supplemental table 2). Each case had several mutations previously associated with cancer or mutations in genes annotated in the COSMIC database (Figure 1A). All mutations called by WES were visually inspected in the Integrated Genome Viewer (IGV). Among these mutations we identified a frame shift deletion of EZH2 (NM_004456: p.K406KfsX17), which has been described in myelodysplastic syndromes and a missense mutation of ARID1A (NM_139135: p.K1515Q), a gene that has previously been seen to be mutated in HCL.3,7

We were able to compare the mutational landscape before and after BRAFi treatment in a patient with aggressive relapse after vemurafenib. Six new mutations emerged with BRAFi treatment (supplemental Figure 1). No mutations directly or indirectly activating RAS signaling and thus explaining resistance to BRAFi were identified. A stop gain in KDM6A (NM_021140: p.Q333*) was identified at relapse. KDM6A encodes a histone demethylase that specifically demethylates histone H3, which has been shown to be recurrently mutated in bladder cancer and rarely in acute myeloid leukemia (AML) or head and neck cancer. Mutations in ARID1A, EZH2, and KDM6A suggest that the epigenetic regulation machinery is recurrently targeted in HCL.

Surprisingly, 2/3 patients with refractory HCL that underwent WES were found to harbor somatic, inactivating mutations of CDKN1B (Figure 1A, NM_004064: p.W60* and p.A167QfsX37). To determine whether CDKN1B mutations are recurrent in HCL, we developed a custom, targeted next-generation sequencing panel and sequenced CDKN1B (exon 1 and 2), BRAF (exon 15) and MAP2K1 (exon2 and 3) in 81 patients with HCL and five patients with HCL-variant diagnosed according to standard criteria. All 81 patients with classical HCL were found to harbor BRAFV600E mutations. One of five patients with HCLv had a MAP2K1 (NM_002755: p.K57T) mutation. We identified 17 mutations of CDKN1B...
including four splice site, 11 non-sense and two missense mutations affecting 13 out of 81 (16%) patients with classical HCL (Table 1, Figure 1B, supplemental table 2 and 3). All but one sample harbored at least one CDKN1B nonsense or splice site variant, except P0811 where a missense mutation was identified. Three patients had more than one mutation implying selective pressure to inactivate CDKN1B. Comparison of allele frequencies of BRAF- and CDKN1B mutations revealed that the majority (11/13, including treatment naive patients) of CDKN1B mutations had allele frequencies very similar to those of the BRAF mutant clone (supplemental Figure 2). This suggests that CDKN1B mutations are early lesions and contribute to HCL pathogenesis. CDKN1B mutations were not present in five BRAFV600E-negative HCLv patients.

CDKN1B (p27) is a critical element of cell cycle control and a known tumor suppressor\textsuperscript{12}. CDKN1B binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls cell cycle progression in G1 phase. Germ line mutations of CDKN1B cause a multiple endocrine neoplasia 1-like (MEN1) phenotype. Menin, the product of MEN1, enhances expression of CDKN1B and CDKN2C suggesting a functional link between MEN1 and CDKN1B\textsuperscript{13}. Recently, 8% (14/180) neuroendocrine tumors of the small intestine (NET) were reported to harbor frameshift CDKN1B deletions and 14% (7/50) NETs had hemizygous deletions encompassing CDKN1B.\textsuperscript{14} CDKN1B mutations occur at very low frequencies in other malignancies (<5%) and have not been shown to co-occur with BRAF V600E. Comparison of the frequency of CDKN1B mutations across different cancers revealed that HCL has the highest incidence of CDKN1B mutations across cancer (Figure 1C). CDKN1B alterations have not been reported in other B-lymphoid malignancies, including the Cancer Genome Atlas (TCGA) study of Diffuse Large B-cell Lymphoma\textsuperscript{15}. WES of 113 patients with CLL did not reveal variants for CDKN1B (unpublished data). In T-Prolymphocytic leukemia, only one CDKN1B mutation was identified but almost 50% harbor hemizygous deletions of CDKN1B\textsuperscript{16}.

To test the clinical impact of CDKN1B mutations we correlated CDKN1B mutations with prior treatment and response. While 2/5 of the initial refractory patients had CDKN1B mutations, 2/8 pre-treated patients and 10/43 treatment naïve patients had CDKN1B mutations (supplemental table 4, 6; Fisher test p=0.73). An influence of CDKN1B mutation on response to standard treatment was not detected (supplemental table 5, 6; Fisher test p=1.00).
Across BRAF mutant cancers, upregulation of cell cycle inhibitors such as \textit{CDKN2A} (INK4/ARF), \textit{CDKN1A} and \textit{CDKN1B} leads to senescence and constitutes a tumor suppressor mechanism. In fact, these genes are recurrently inactivated by genetic mechanisms during pathogenesis of many tumors. For example, BRAF-induced senescence in premalignant naevi\cite{17} is circumvented by deletion or mutation of \textit{CDKN2A} in invasive melanoma [\textit{supplemental Figure 2A}]. Thus, the identification of recurrent inactivating mutations of \textit{CDKN1B} in a \textit{BRAFV600E}-mutant cancer suggests that \textit{CDKN1B} loss may serve as a mechanism to impair cell cycle control and/or circumvent oncogene induced senescence.

In addition to affecting cell cycle regulation and senescence, \textit{CDKN1B} is a known opponent of cyclin D1, a gene regulated in a \textit{BRAFV600E}-dependent manner and highly expressed in HCL.\cite{18} Thus, identification of inactivating mutations on \textit{CDKN1B} in HCL further indicate pathway convergence\cite{19}.

In summary, we demonstrate that \textit{CDKN1B} is inactivated in 16\% of patients with classical HCL and is the 2\textsuperscript{nd} most commonly mutated gene in HCL. These results implicate cell cycle deregulation in the pathogenesis of HCL and suggest that \textit{CDKN1B} serves as an important tumor suppressor in this disease.
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Author contributions:

SD designed research, performed research, analyzed data, and wrote the paper
JH performed research
SL performed research
BH analyzed data
DG provided clinical samples
SJ provided clinical samples
MD provided clinical samples
MO analyzed data
ME provided follow up of patients
XL performed research
MS performed research
BW analyzed data
XT provided clinical samples and follow up
JD provided clinical samples
MA analyzed data
CD provided clinical samples and follow up
CK wrote the paper
MG performed research
AJ performed research
SF wrote the paper
WH analyzed data, wrote paper
MM performed research
TH performed research and provided clinical samples
AH provided clinical samples
DR performed research
BB analyzed data
HG wrote the paper
EM provided clinical samples
OAW performed research, designed research, wrote the paper
TZ designed research, performed research, analyzed data, and wrote the paper

Conflict of interest:

The authors declare no conflict of interest.
Table 1. **CDKN1B** (p27) mutations in **BRAF**V600E-mutant (classical) hairy cell leukemia.

<table>
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<th>Sample Identifier</th>
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Literature:

Figure legends

Figure 1. Recurrent CDKN1B mutations in classical hairy cell leukemia (HCL).

A) Cancer consensus and COSMIC annotated gene mutations identified in HCL by whole exome sequencing. Patient 3 progressed after vemurafenib treatment and was sequenced before and after BRAFi (vemurafenib) treatment. Exome sequencing identified recurrent inactivating somatic mutations of CDKN1B (for complete list of somatic mutations see supplemental table 1b).

B) Gene regions of CDKN1B and distribution of mutations in HCL. Binding regions of important interaction partners are shown below the gene diagram. 17 CDKN1B mutations were identified in 13 patients. In cases where normal material was not available, for the remaining mutations with sufficient read coverage (mean= 1259 reads, supplemental table 1) the allele frequencies indicated somatic origin (supplemental figure 2).

C) Frequency of CDKN1B mutations across cancer entities. Mutation frequencies were from http://www.cbioportal.org/public-portal/. HCL shows the highest CDKN1B mutations frequency in cancer.
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