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In search of genetic factors predisposing to familial hairy cell leukemia (HCL): exome-sequencing of four multiplex HCL pedigrees

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Compliance with ethical standards

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To the Editor:

Hairy cell leukemia (HCL) is a rare chronic B-cell lymphoproliferative disorder named for its characteristic hair-like cytoplasmic projections from the malignant cells. HCL is classified as an indolent lymphoproliferative neoplasm, representing ~2% of all leukemias with ~1240 new cases diagnosed annually in the US; median age-at-onset is 55 years [1]. It affects males more than females (4:1), and whites more than African-Americans [1]. Although familial and sporadic HCL exhibit similar clinical features, no characteristic germline genetic variation has been found. Familial HCL is rare with fewer than 20 families reported in the literature. Thirteen of the 15 reported pedigrees had two affected individuals; the remaining two pedigrees harbored three, including the family reported here [2–5]. Investigators have speculated that HCL may be an HLA-linked disorder but, in aggregate, the data are inconclusive [3–5]. The discovery that a somatic *BRAF* mutation (V600E) was nearly universal in HCL (but absent in other B-cell neoplasms) provided major insight into disease biology, identifying a critical therapeutic target [6], but no germline genetic susceptibility variants have been identified. In this study we applied high-throughput sequencing technology to four multiplex HCL pedigrees, seeking to identify shared germline variants conferring HCL susceptibility. In addition, we used CRISPR/Cas9-based genome editing to introduce *CASP9* p.H237P, one of the variants shared by all four affected members of the largest pedigree, into a model cell line, followed by measurements of cellular caspase-9 activity and apoptotic response.

Exome-sequencing (ES) of HCL Families A, B, C, and D

ES of individuals in Family A (Fig. 1; four HCL patients and one unaffected) and Families B, C, and D (Fig. 1; two HCL patients in each; no unaffected individuals were available for sequencing) identified 3, 30, 23, and 21 deleterious variants that segregated in these families, respectively (Supplementary Tables S1A–D). None of the deleterious variants was observed twice. Among all segregating variants, including nondeleterious ones, we observed a single variant in *ECT2L* (p.A722T) in Families B and C (Supplementary Table S1E). On the gene level, there was a limited overlap in two-way comparisons (*NOTCH3*, *ORC1*, *TSHZ3*, *KCNT1*, *DYSF*, and *SHQ1*); however, all but one variant (*DYSF*, p.R285W) in these genes were classified as nondeleterious (Supplementary Table S1E).

Ontology and pathway analyses of genes with deleterious variants in four HCL pedigrees

To gain insight into whether genes with deleterious variants segregating within each family belonged to similar ontological categories or cellular pathways, we compared each of the four gene lists to pre-defined gene-sets in Gene Ontology Biological Process, Kyoto Encyclopedia of Genes and Genomes, Reactome and WikiPathways databases and

identified functional categories shared by the pedigrees' gene lists (Supplementary Table S2). Neutrophil-mediated immunity (*ATP8B4*, *PNP*, *SLC44A2*, and *RAB37*) and signaling by G-protein coupled receptor (*MC3R*, *FZD6*, *NMUR2*, *KSR2*, *RGS9*, and *GPR35*) were the most frequently shared ontological categories and pathways (3/4 pedigrees). Other pathways (shared by 2/4 families) included MAPK and RAS signaling (*IGF2*, *KSR2*, and *CACNA1A*), apoptosis (*CASP9*, *IGF2*), PI3K-Akt Signaling (*CASP9*, *IGF2*), response to DNA damage (*CASP9*, *INTS3*), and signaling by EGFR/ERBB4/FGFR/PDGF/SCF-KIT (*CASP9*, *KSR2*) (Supplementary Table S2).

Genotyping of *CASP9* p.H237P in 129 lymphoproliferative disorder (LPD) pedigrees and general lymphoid cancer population (560 cases vs. 555 controls)

Based on biological plausibility, the *CASP9* p.H237P variant that was observed in all four affected individuals in the largest pedigree was selected for further evaluation. We genotyped this variant in 129 families affected with various LPD (Supplementary Table S3A). Two families harbored this variant. In one pedigree (Family E) *CASP9* p.H237P segregated in two siblings, one with CLL and one with NHL (Supplementary Fig. S1). In contrast, in pedigree F *CASP9* p.H237P only one of two members with CLL carried *CASP9* p.H237P, as did two unaffected siblings (Supplementary Fig. S2).

To ascertain the frequency of *CASP9* p.H237P in the general lymphoid cancer population and controls, we performed TaqMan genotyping of the H237 position; the variant was present in one case (a diffuse large cell lymphoma) among 560 versus one of 555 controls (Supplementary Table S3B) ($p = \text{NS}$).

IonTorrent/Ampliseq targeted sequencing of genomic regions including *CASP3/8/9* in sporadic HCL

Targeted sequencing of *CASP3*, *CASP8*, and *CASP9* was performed in germline DNA of 55 sporadic HCL samples (Supplementary Table S3C). Eleven missense variants were observed in *CASP8* and *CASP9* in the blood DNA of sporadic HCL patients (Supplementary Table S4). Variant p.H237P was not detected in these cases. Most observed variants ($N = 7$) were common ($>1\%$); only p.R192C in *CASP9* (observed once in our cohort) was both rare (ExAC = 0.04%) and predicted to be deleterious.

Protein structural modeling of caspase-9 p.H237P and p.R192C

The role of histidine (H237) in the caspase mechanism is hypothesized to function as a proton-abstracting base from the catalytic cysteine, thereby enhancing the nucleophilicity of C287 (Supplementary Fig. S3) [7–9]. Structural analysis of R192 revealed that, unlike H237, it is a solvent-exposed residue and it is not a part of the caspase-9 active site (Supplementary Fig. S4). To evaluate the functional impact of these two variants, we used sequence-based software PROVEAN and a sequence/structure-based approach PolyPhen-2 to model the substitution of H237 and R192 with all 19 standard amino acids (Supplementary Table S5).

Both impact analysis approaches estimated that H237 is highly conserved and does not tolerate any substitutions. Variant impact analysis for R192 predicted that this substitution was probably damaging or deleterious (Supplementary Table S5). However, given that R192 is not part of the active site and is a solvent-exposed residue, the likelihood of p.R192C affecting caspase-9 function is moderate.

Functional validation of caspase-9 p.H237P and p. R192C in HCT116 cells

CRISPR editing of HCT116 cells resulted in homozygous, but no heterozygous, *CASP9* p.H237P, and *CASP9* p.R192C clones. We observed significantly decreased (p value = $1.3\text{E}-05$) caspase-9 (Fig. 2a) and caspases-3 and -7 enzyme activity (p value = $1.0\text{E}-05$) in HCT116 cells harboring homozygous *CASP9* p.H237P (Fig. 2b). Thus, this observation suggests that the homozygous *CASP9* p.H237P variant completely inactivates this key caspase and impedes apoptosis. We measured caspase-9, -3, and -7 activities in *CASP9* p.R192C HCT116 cells (Fig. 2c, d), and found it to be an activating variant: caspase-9 and caspase-3/7 activities were ~30–50% higher in CRISPR-edited cells compared with unedited HCT116 (p values = $5.0\text{E}-07$ and $3.0\text{E}-05$, respectively).

Discussion

ES of four multiplex HCL families in this study did not identify common variants or genes among the pedigrees. However, ontology and pathway analyses uncovered common functional categories. One of them, neutrophil activation, degranulation, and neutrophil-mediated immunity categories were found perturbed in 3/4 pedigrees (Families B (*ATP8B4*), C (*PNP*) and D (*SLC44A2*; *RAB37*). Although in the innate immune response, neutrophils function primarily as anti-microbial agents, their role in tumor initiation, progression, and surveillance is increasingly recognized [10]. At the time of diagnosis about half of HCL patients present with neutropenia. In addition, HCL patients frequently exhibit decreased number and/or function of T-, B-, and NK-cells, resulting in an overall compromised immune surveillance [11]. Interestingly, one of the variants identified in this study, a pathogenic nonsense variant (p.R58*) in *PNP* (Purine Nucleoside Phosphorylase) that segregated in both HCL patients in pedigree C, was previously described in a patient with PNP-deficiency, a rare autosomal recessive disease, which clinically manifests as severe combined immunodeficiency [12].

In the largest pedigree (Family A), we identified segregating variant p.H237P in *CASP9*, a gene known to be associated with multiple pathways, including apoptosis, response to DNA damage, PI3K-AKT, and TNF alpha signaling. Our functional evidence suggests that *CASP9* p.H237P, as a loss-of-function variant, impedes apoptosis but heterozygous p.H237P alone is likely insufficient to result in a clinical phenotype. Notably, in a study in which *CASP9* p.H237P was observed in two of seven patients with immunodeficiency and lymphoproliferation, the affected individuals also carried heterozygous variation in *TNFRSF13B* and *PRF1* [13]. The authors note that both *TNFRSF13B* and *PRF1* variants have been previously associated with immunodeficiencies in homozygous or compound heterozygous conditions and concluded that *CASP9* p.H237P may cooperate with other genetic factors in the disorders. There was only one recurrent, nondeleterious, segregating

variant observed in two of our families (*ECT2L* p.A722T; Families B and C) that may merit follow-up. Both recurrent somatic and germline variation in *ECT2L* has been observed in early T-cell precursor acute lymphoblastic leukemia [14]. However, samples from additional family members were not available to conduct additional genotyping on this variant in our pedigrees.

In summary, we observed multiple deleterious germline variants, segregating within each of the four multiplex HCL families. There was only a limited overlap between the pedigrees on a variant or gene level, however, we identified several functional pathways shared by the pedigrees. Functional follow-up of p.H237P in caspase-9 revealed that it is a loss-of-function mutation when in a homozygous state, but likely is phenotypically silent when heterozygous, and, thus, likely requires a second somatic hit and/or additional variation. We conclude that genetic predisposition to familial HCL could be mediated by multiple pedigree-specific variants/genes that belong to several common signaling pathways, or by yet unidentified noncoding genetic elements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Family A

Family B

Family C

Family D

Fig. 1. Pedigree diagrams of four multiplex HCL families.

Patients affected with HCL are indicated by black fill. When known, age(s) (in years) when a patient was diagnosed with HCL or other malignancies and the age at the latest follow-up or death are shown. Only siblings, parents, and children (>45 years old) of HCL-affected individuals are shown on the diagrams. Exome-sequenced individuals are indicated with asterisks. Abbreviations for cancers: ColCa colon, GastCa gastric, HNCa head and neck, ProstCa prostate, SoftSarc soft tissue sarcoma.

Fig. 2. Caspase-9 and caspase-3/7 activities in staurosporine-treated CRISPR-unedited HCT116 cells and in HCT116 cells carrying caspase-9 p.H237P and p.R192C substitutions. Caspase activity was measured in genetically unedited HCT116 cells (WT) and in CRISPR/Cas9-edited HCT116 cells carrying a homozygous *CASP9* p.H237P substitution (H237P). A luminescence-based assay was used to measure (a) Caspase-9 activity, and (b) Caspase-3/7 activity. Caspase activity was measured in genetically unedited HCT116 cells (WT) or in CRISPR/Cas9-edited HCT116 cells carrying a homozygous *CASP9* p.R192C substitution (R192C). A luminescence-based assay was used to measure (c) Caspase-9 activity, and (d) Caspase-3/7 activity. RLU relative luminescence units, D 0.5% media containing DMSO as a vehicle, S 1 μ M staurosporine, 16 and 24 h incubation time in hours. T-tests were performed for staurosporine-treated WT and CRISPR-edited cells for 16 and 24 h time points. * p value < 1.0E-03; ** p value < 1.0E-04.