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Letter
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CCNF (Cyclin F) as a Candidate Gene for Familial Hodgkin Lymphoma: Additional Evidence for the Importance of Mitotic Checkpoint Defects in Tumorigenesis

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Hodgkin lymphoma (HL) is a relatively infrequent neoplasm (2.6 cases/100,000 individuals; https://www.lls.org/facts-and-statistics)[1] of germinal center B cells, the etiology of which has a significant genetic component. Despite this, few studies have investigated genetic predisposition to familial HL (fHL); these have typically included very limited numbers of families and have not provided functional evidence supporting the role of the fHL-linked variants identified. Here, we performed whole exome sequencing (WES) on 22 HL patients from 11 families, followed by candidate gene prioritization and functional testing of variants in the *CCNF* (Cyclin F) gene, identified in 2 different families. Intriguingly, *CCNF* plays a role in DNA integrity and mitotic checkpoints, adding it to a growing list of genes in this category, potentially implicated in fHL.

HL is characterized by a paucity of malignant pathognomonic giant multinucleated Reed-Sternberg cells and large, mononuclear Hodgkin cells (HRS) cells; the bulk of the neoplasm is composed of nonmalignant inflammatory and stromal cells. While their rarity makes the study of HRS cell-intrinsic abnormalities a challenge, features including DNA aneuploidy,[2] nuclear DNA fragmentation, cell-cycle arrest,[3] altered mitotic checkpoint control,[4] deregulated expression of cell-cycle regulators,[5] and the formation of multinucleated cells[4,6] are well-documented.

Both twin and case-control studies have shown that the etiopathogenesis of HL has a significant genetic component.[7,8] While typically sporadic, familial clustering is found in approximately 4.5% of HL, allowing for Next Generation Sequencing-based investigations for potential drivers of disease.[9–15] Functional effects of fHL-associated variants (by expression in isogenic cell lines) have however been demonstrated by only 2 such studies: in the *KLHDC8B* gene (a promoter-disrupting chromosomal translocation in 1 family and a recurrent 5'UTR variant in 3 others)[9] and the *POT1* gene (2 different missense variants, in 2 families).[13] In both, the fHL-linked variants caused loss of function, leading to defective cytokinesis in the case of *KLHDC8B*, and increased telomere length and fragility in the case of *POT1*. A third study showed a decrease in microRNAs with tumor-suppressive functions, in blood samples from a family with compound-heterozygous variants (1 nonsense and 1 missense) in *DICER1*.[12] We set out to assess for variants in these genes and others, in a large series: 22 patients with HL, from 11 families (Figure 1).

This study was approved by the ethics committee of the Faculty of Medicine, UCLouvain (2014/03JUI/302 and 2018/06NOV/411). WES was performed on genomic (blood) DNA from all available samples (22 affected individuals from 11 families) at Integragen (France) and Macrogen (South Korea), using SureSelect capture kits (Agilent) on Illumina sequencers (Suppl. Table S1). Raw data were aligned to the reference human genome (GRCh38). Average coverage depth obtained (after duplicate read removal) was 107× (range: 65×–187×). Variant calling was performed using GATK 4.2 Haplotype Caller (following Broad Institute best practices) (Suppl. Materials and Methods). Variant call (.vcf) files were imported into Highlander 17.18 (https://sites.uclouvain.be/highlander/): the in-house bioinformatics framework of the Genomics and Bioinformatics platform of UCLouvain, consisting of a pipeline for variant visualization, annotation, and filtering, integrated with a local database currently containing 3153 (50–100× average coverage depth) germline WES and 1065 (200–400× average coverage depth) somatic WES, categorized by pathology.

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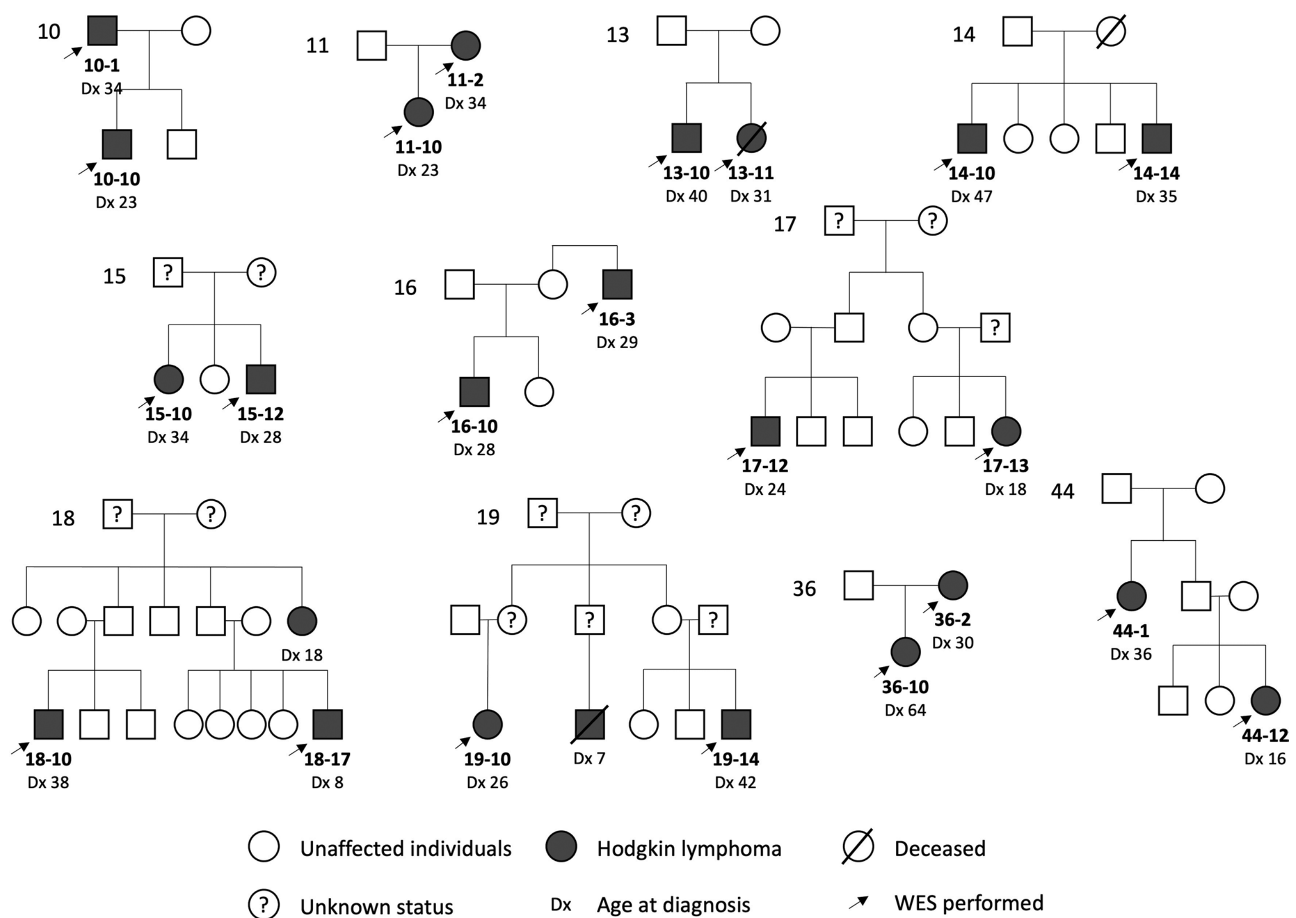

Figure 1. Eleven families with 2 or more Hodgkin Lymphoma-affected members each, analyzed by WES. WES = whole exome sequencing.

WES data were filtered to retain variants shared by the affected members within each family. The series being composed of DNA from pairs of closely related affected individuals (6 families with 2 affected first-degree relatives: Families 10, 11, 13, 14, 15, and 36; 3 families with 2 affected second-degree relatives: Families 16, 17, and 44; 2 families with 3 affected second-degree relatives, from 2 of whom DNA was available: Families 18 and 19) (Figure 1), this yielded a large number of shared (disease cosegregating) variants (66,359 ± 8824; mean ± SD) per family (Suppl. Table S2). In order to identify potential genetic drivers of fHL, we retained disease cosegregating variants that are: (1) absent-to-rare in the general population and (2) predicted to affect protein function (Suppl. Materials and Methods, Suppl. Table S3). A total of 813 variants in 802 genes, visually curated for alignment quality, satisfied these criteria across the 11 families (20–109 genes per family; median = 83 genes) (Suppl. Table S2). This included 41 variants in 41 cancer-associated genes (of 1126 genes currently included in the OncoKB Cancer Gene List; https://www.oncokb.org/cancerGenes, updated September 2023) (Suppl. Table S4), identified in 1 family each. No variants satisfying these criteria were identified in *KLHDC8B*, *POT1*, or *DICER1*. Because we did not obtain adequate WES coverage of the recurrent fHL-associated *KLHDC8B* variant position (GRCh38 chr3:49171662, c-158C>T),[9] we performed PCR followed by Sanger sequencing of a 352 base-pair amplicon containing it; the variant was absent in all samples in our series (data not shown). Pathway overrepresentation analyses performed on the 802 genes (Suppl. Materials and Methods) did not identify any significantly enriched pathways.

We next focused on the (31/802) genes with rare, predicted pathogenic, disease cosegregating variants in more than 1 family (Suppl. Table S5). These 31 candidates were categorized by function, based on manual curation of public databases and peer-reviewed published literature (Suppl. Figure S1, Suppl. Table S6). The 2 largest categories grouped genes coding for: (1) large structural proteins participating in tissue/organ development and function (9 genes, in 10 families) and (2) highly polymorphic proteins with sensory and neural functions (6 genes, in 9 families). Ranking of all 31 gene candidates based on expression pattern (in B cells, immune cells) and function (in pathways and/or interactomes implicated in tumorigenesis) however suggested greater relevance of genes in the remaining categories: (3) DNA stability and mitotic checkpoints, (4) receptor trafficking and cell signaling, (5) cellular metabolism, (6) cellular and mitochondrial stress response, and (7) thrombotic and vascular response (Suppl. Figure S1, Suppl. Table S6).

Of particular interest to us were genes in category (3), which regulate mitotic progression and checkpoints, DNA stability, and response to DNA damage: relevant given the characteristic genomic and morphologic features of HRS cells. Moreover, *KLHDC8B*[9] and *POT1*,[13] the 2 genes with functionally tested variants previously published for fHL, fall into this category. Ten variants in 5 genes: *CCNF*, *CROCC*, *HJURP*, *MCM10*, and *USP45*, identified in 5 of 11 families (Suppl. Tables S5, S6), fall into this category. Amongst these, we prioritized *CCNF* for in vitro testing of 2 fHL-associated variants: c.1022A>G (p.Tyr341Cys) in Family 19 and c.1123C>T (p.Arg375Trp) in Family 13. Both are absent or extremely rare in the general population and are predicted to have a pathogenic effect on protein function by 14 and 15 in silico tools, respectively (Table 1). Not only do public gene expression databases (BioGPS, EMBL-EBI, and The Human Protein Atlas) show *CCNF* is highly expressed in B cells, but also somatic mutations in this gene have been previously

reported in endemic Burkitt lymphoma, associated with Epstein-Barr virus (EBV) infection.[16] Moreover, loss of CCNF function in cells has been shown to cause genomic and morphologic phenotypes reminiscent of HRS cells, as detailed below.

CCNF codes for the F-box protein Cyclin F (FBXO1), which associates with SKP1 (S-phase kinase-associated protein 1), CUL1 (Cullin1), and RBX1 (RING-box protein 1) to form the cell-cycle–regulated $SCF^{CyclinF}$ E3 ubiquitin ligase complex. CCNF, via a hydrophobic patch in its Cyclin-N (substrate-binding) domain, is responsible for binding specific proteins for ubiquitination and proteasomal degradation. Its targets (listed in Suppl. Table S7) are proteins that function at precise, limited points during mitosis. Loss of CCNF or its inability to bind targets leads to their inappropriate persistence, causing defects in DNA integrity, repair and replication,[17–21] failure of mitotic checkpoints that would otherwise block progression through the cell cycle,[22–24] and formation of micronuclei.[25]

The 2 heterozygous variants identified in our series (coding for p.Y341C and p.R375W, confirmed by PCR-Sanger sequencing on blood-DNA from the respective family members; data not shown) are both located in the Cyclin-N substrate-binding domain of CCNF (Figure 2A). Immunoprecipitation of

Table 1

***CCNF* Variants Identified in 2 Hodgkin Lymphoma Families**

Gene	Family	Chr	Pos	Codon (DNA)	AA (Protein)	Predicted Damaging by	MAF in Local Database	gnomAD_WES
CCNF	19	16	2445550	c.1022A>G	p.Tyr341Cys	14	0.000147[a]	Absent
	13		2448883	c.1123C>T	p.Arg375Trp	15	Absent	2/251426 (0.000007955)

Both variants are heterozygous. Chr, Pos = genomic position (GRCh38), codon change (DNA), and corresponding amino acid (AA) change (Protein); predicted damaging by = number of programs (out of 20) that predict the variant would affect protein function.

[a]c.1022A>G (p.Tyr341Cys) was identified (germline, heterozygous) in 1 (breast cancer) sample in the local WES database.

AA = amino acid; gnomAD_WES = allele number and frequency in gnomAD v2.1.1 (125,748 WES); MAF = minor allele frequency; WES = whole exome sequencing.

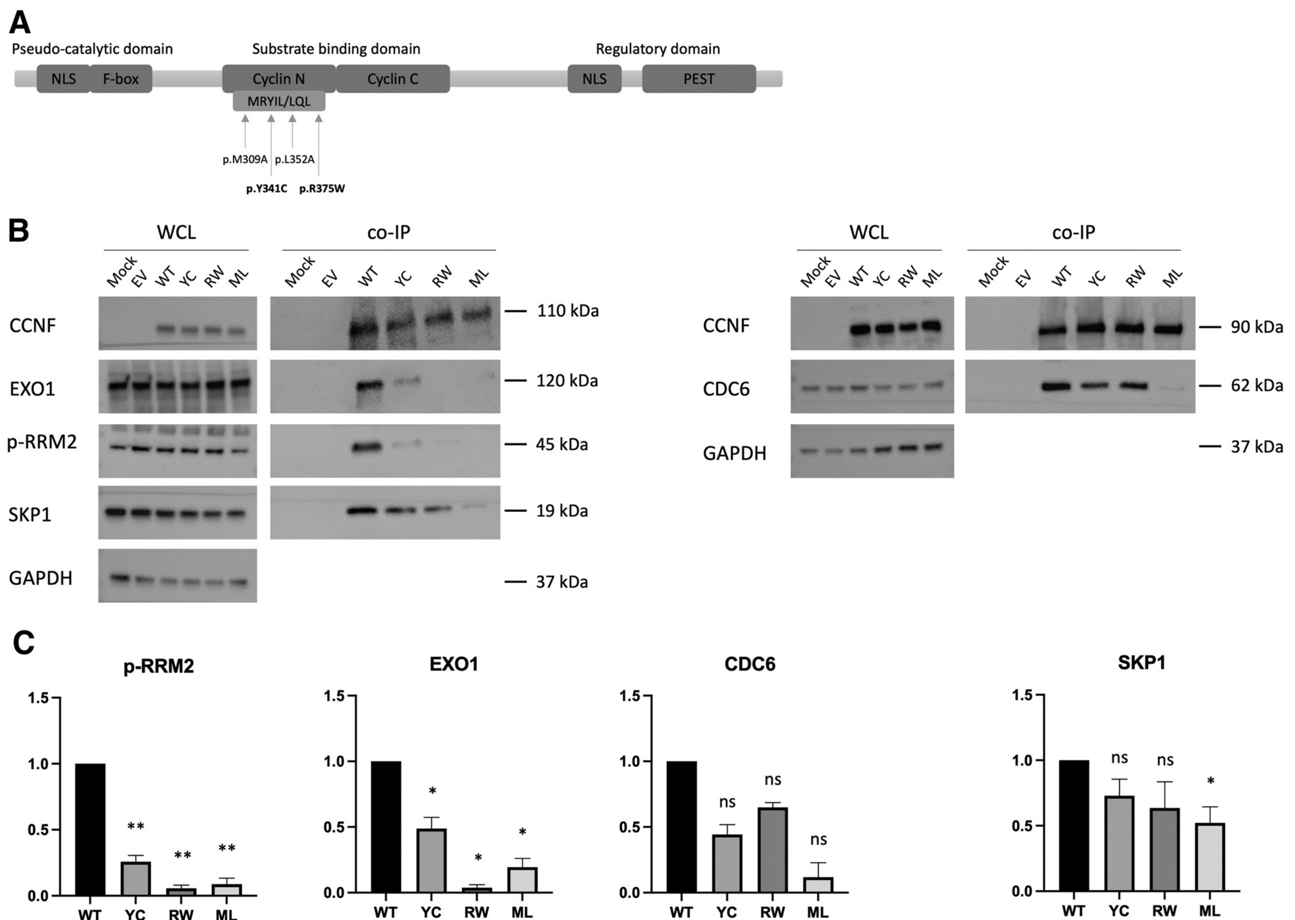

Figure 2. Decreased binding of familial Hodgkin Lymphoma (fHL)-associated *CCNF* variant forms to ubiquitination targets. (A) CCNF protein domains showing localization of fHL-associated variants p.Y341C (YC) and p.R375W (RW), and synthetic double mutant p.M309A-L352A (ML). (B) HEK293T cells were mock transfected (mock) or transfected with an empty vector (EV), FLAG-tagged wild-type *CCNF* (WT), fHL-associated variants YC or RW, or synthetic double mutant ML. Cells were lysed and whole cell lysates (WCL) and coimmunoprecipitated fractions (co-IP) were immunoblotted as indicated. (C) Graphs showing quantification of intensity of bands on Western blot (n = 7 [phosho-RRM2]; n = 6 [EXO1 and SKP1]; n = 3 [CDC6]) (mean ± SD). * = $P < 0.05$; ** = $P < 0.01$; ns = nonsignificant (each vs WT, Bonferroni-corrected Mann-Whitney). CCNF = cyclin F; co-IP = coimmunoprecipitated fractions; EV = empty vector; fHL = familial Hodgkin lymphoma; ML = synthetic double mutant p.M309A-L352A; RW = p.R375W; WCL= whole cell lysates; WT = FLAG-tagged wild-type CCNF; YC = p.Y341C.

Table 2

Somatic Variants Identified in 19 Sporadic Hodgkin Lymphoma Tumors

Gene	Sample	Chr	Pos	Codon (DNA)	AA (Protein)	Total Reads	Proportion Variant Reads	Proportion Reference Reads	Predicted Damaging by
ATM	CAHL-AC1898	11	108163369	c.4460C>T	p.Ser1487Leu	19497	3%	97%	12
POT1	CAHL-AC10331	7	124537226	c.2T>A	p.Met1?	4539	2%	95%	Start codon lost

Chr, Pos = genomic position (GRCh37), codon change (DNA), and corresponding amino acid (AA) change (Protein); total reads = total number of reads covering the variant position, and proportion of reads carrying the variant vs reference allele; predicted damaging by = number of programs (out of 20) that predict the missense substitution would affect protein function. Both variants were absent in the local Whole Exome Sequencing (WES) and Catalogue of Somatic Mutations in Cancer (COSMIC) databases.
AA = amino acid.

FLAG-tagged wild-type versus fHL-associated forms of CCNF, expressed in HEK293T cells, showed a marked reduction in coimmunoprecipitated target proteins phospho-RRM2 and EXO1. A nonsignificant decrease was observed in coimmunoprecipitated CDC6 and the SCF$^{\text{CyclinF}}$ complex component SKP1. The synthetic double mutant p.M309A-L352A, previously shown to abolish target-binding by CCNF,[25] was included for comparison (Figure 2B and 2C). This implies that both (heterozygous) fHL-linked variants likely cause loss of CCNF function, suggesting that CCNF may act as a tumor-suppressor gene in a small proportion of fHL.

The identification of somatic second hits in tumor tissue can be a convincing argument for the candidacy of germline variants in inherited cancers. The need for local loss of the normal allele has been widely demonstrated, particularly for inherited tumor-suppressor gene mutations. Lack of tumor material (including archival samples) from this series of families unfortunately precluded the use of deep-sequencing approaches to assess for somatic second hits in candidate genes. Genes linked to inherited forms of cancer often also cause sporadic (ie, non-inherited) forms, due to the acquisition of de novo or somatic/mosaic mutations. We assessed for (germline or somatic) mutations in *CCNF* and other selected candidate genes, by targeted deep-sequencing of frozen tumor cell-positive biopsies from 19 patients with sporadic HL (Suppl. Table S8). While no *CCNF* variants were identified in this limited series, somatic mutations were identified in *ATM* (ataxia-telangiectasia mutated gene) and *POT1* (protection of telomeres 1) (Table 2), in 1 sample each. Germline *POT1* mutations that lead to increased telomere length and fragility have been previously reported in fHL.[13] *ATM* plays a major role in the repair of double-stranded breaks in DNA, thereby controlling genome stability and cell survival; it has long been associated with cancer predisposition, particularly lymphoid malignancies.[26]

Collectively, our results indicate that notwithstanding its apparent genetic heterogeneity, fHL may be underpinned by a common disease mechanism: genome instability and mitosis/mitotic checkpoint defects. This in itself may be insufficient for disease causation. It is tempting to speculate that loss of Cyclin F function may allow B cells to progress through mitosis, while accumulating DNA damage, with EBV infection enabling them to escape apoptosis. As EBV status in our (familial and limited sporadic) series was not ascertained, it would be of interest to assess for variants in *CCNF* in a larger series of EBV-positive versus EBV-negative HL tumor tissues, as performed in Burkitt lymphoma.[16] Genomic background, that is, the combination of risk and protective alleles of common polymorphisms associated with HL (catalogued at ebi.ac.uk/gwas) no doubt participates in disease inheritance in fHL. We also propose that, in at least a proportion of families, fHL may be inherited as a di- or oligogenic trait; random segregation of unlinked disease-genes may in part explain the paucity of large, multigenerational affected pedigrees in this disease. In this context, it would be of interest to test additional candidate gene variants identified in the same families as the *CCNF* variants, and should they prove to affect protein function, test for combined effects in vitro and/or in vivo. This has the potential to provide fundamental new insights into tumorigenesis, as well as guide the construction of animal models true to the genetics of fHL in patients.

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AUTHOR CONTRIBUTIONS

EK and HM performed experiments and analyzed data, prepared figures, and helped write the manuscript; AM helped perform experiments and analyze data; SM implemented, applied, and interpreted data from pathway analysis tools; AC contributed to pathology data collection and interpretation; VD'A contributed to design, execution, and interpretation of in vitro experiments on *CCNF*; JL-P, CB, and SC recruited patients, provided samples, and contributed to study design and data analysis; HAP and NL designed the study, analyzed data, and wrote the manuscript.

DATA AVAILABILITY

For reasons of ethics and patient confidentiality, we are not able to deposit WES data in a public data repository. These data are available from the corresponding author upon request.

DISCLOSURES

The authors declare no conflicts of interest.

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