ARTICLE

Whole exome sequencing in families at high risk for Hodgkin lymphoma: identification of a predisposing mutation in the *KDR* gene

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ABSTRACT

Hodgkin lymphoma shows strong familial aggregation but no major susceptibility genes have been identified to date. The goal of this study was to identify high-penetrance variants using whole exome sequencing in 17 Hodgkin lymphoma prone families with three or more affected cases or obligate carriers (69 individuals), followed by targeted sequencing in an additional 48 smaller HL families (80 individuals). Alignment and variant calling were performed using standard methods. Dominantly segregating, rare, coding or potentially functional variants were further prioritized based on predicted deleteriousness, conservation, and potential importance in lymphoid malignancy pathways. We selected 23 genes for targeted sequencing. Only the p.A1065T variant in *KDR* (*kinase insert domain receptor*) also known as *VEGFR2* (vascular endothelial growth factor receptor 2) was replicated in two independent Hodgkin lymphoma families. *KDR* is a type III receptor tyrosine kinase, the main mediator of vascular endothelial growth factor induced proliferation, survival, and migration. Its activity is associated with several diseases including lymphoma. Functional experiments have shown that p.A1065T, located in the activation loop, can promote constitutive autophosphorylation on tyrosine in the absence of vascular endothelial growth factor and that the kinase activity was abrogated after exposure to kinase inhibitors. A few other promising mutations were identified but appear to be "private". In conclusion, in the largest sequenced cohort of Hodgkin lymphoma families to date, we identified a causal mutation in the *KDR* gene. While independent validation is needed, this mutation may increase downstream tumor cell proliferation activity and might be a candidate for targeted therapy.

Introduction

Classical Hodgkin lymphoma (HL) is a lymphoproliferative malignancy of B-cell origin with an age-adjusted incidence in 2011 in the United States of 2.8/100000.[1] The number of new HL cases in the United States diagnosed in 2014 was estimated to be 9190 with 1180 estimated deaths. Etiologic clues about HL have been suggested by several observations including: 1) the bimodal age distribution with one peak occurring in the third decade of life and a second peak after 50 years of age; 2) an elevated risk in males; 3) an elevated risk in individuals with higher socioeconomic

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status and in smaller families; 4) the occurrence of Epstein-Barr virus (EBV) in HL tumor cells and 5) a strong familial risk.[2] Specifically, there is strong evidence for genetic factors based on evidence from multiply affected families from case series, twin, case-control, and population-based registry studies.[3-9] Furthermore, our group analyzed data from registries in Scandinavia and found significant familial aggregation of HL (RR = 3.1) and other lymphoproliferative tumors. Relative risks were higher in men compared with women, in siblings of cases compared with parents and offspring, and in relatives of patients with diagnosis under the age of 40.[4] In addition, we showed that non-Hodgkin lymphoma (NHL) also aggregates in HL families. The human leukocyte antigen (HLA) region on chromosome 6 has been associated with HL in several studies.[10-12] Other non-HLA susceptibility loci have been identified through GWAS.[13-15] The identification of genes with major susceptibility effects has been more difficult. A study of a family with a reciprocal translocation between chromosomes 2 and 3 segregating with HL led to the identification of a disruption in the gene *KLDHC8B*, which codes for a midbody kelch protein, hypothesized to disrupt cytokinesis thereby promoting tumorigenesis. Additional familial patients were found to carry an uncommon 5' UTR variant in this gene.[16] Recently, a mutation in the *NPAT* gene was identified in an extended pedigree segregating for the less common nodular lymphocytic predominant subtype of HL.[17] A homozygous deletion in the *ACAN* gene was identified in 3 siblings with classical HL.[18] We conducted a genome-wide linkage study in 44 HL families that revealed several regions of the genome possibly linked to HL,[19] but no susceptibility genes were subsequently identified. In this study, we have attempted to identify high risk genes causing susceptibility to HL using whole exome sequencing and follow-up targeted sequencing in families segregating HL.

Methods

Description of Families and Study Design

The 65 HL-prone families included in this study are participants in an IRB approved NCI study (NCI-02-C-0210) who were accrued through healthcare professionals or self-referral[19] and had at least 2 confirmed HL patients. The sequencing study design is shown in Table 1. In the first phase, 63 affected (including 2 NHL patients) or obligate carrier individuals and 6 unaffected spouses from 17 'discovery families' (required to have at least 3 affected members or obligate carriers with available DNA) were whole exome sequenced to identify candidate HL susceptibility variants. In the second phase, selected candidate genes from Phase 1 were (a) re-sequenced in the original 17 families to validate the variants and to determine the mutation status of 27 additional unaffected members first-degree relatives of cases and (b) tested for replication in an additional 48 'replication families' corresponding to 80 sequenced individuals (64 from 32 HL siblings or child-parent pairs, 16 from multiplex families with only one patient with DNA available for sequencing). The distribution of sequenced individuals among all families is shown in *Online Supplementary Table S1*.

Sequencing and Analyses of Sequence Data

Whole exome sequencing (WES) was performed at the Cancer Genomics Research Laboratory, National Cancer Institute (CGR, NCI), as previously described.[20] Details of the bioinformatics pipeline for variant alignment and calling used in this study appear in the *Online Supplementary Methods*.

Annotation of each variant locus was made *via* a custom software pipeline based on public data sources described in the *Online Supplementary Methods*.

Filtering of WES variants called in discovery families was based on the following criteria: 1) present in all affected (HL and NHL) or obligate carrier individuals in the pedigree and absent in unaffected married-in individuals; 2) present in ≤ 1% of families from an in-house database (700 families), in ≤ 1% of the NHLBI Exome Sequencing Project (ESP) European American population (4300 individuals) and in ≤ 1% of the phase 1 1000 Genomes Project European population (379 individuals) and 3) occurring in exonic or UTR regions or in locations linked to epigenetic findings from ENCODE.

Prioritization of the filtered-in variants was based on mutation type, low minor allele frequency and functional predictions using the programs described in the *Online Supplementary Methods*. We also considered the genes' links to cancer or immune-related processes from the literature and whether the variant or gene was present in multiple HL families.

Validation and replication of a set of genes selected was based on the above prioritized list and on amplicon length to optimize available resources. Validation was performed in all available families using Ion Torrent (see *Online Supplementary Methods*). We checked for technical validation in whole exome sequenced samples, for segregation in additional family members, and for replication in additional families. Re-sequencing the whole genes allowed us to test the hypothesis that different variants from the same gene might occur in different families.

In silico analysis was performed for the variants that replicated in multiple families (see *Online Supplementary Methods*).

Results

As noted previously, and consistent with other studies of familial HL, patients in the families we studied are skewed toward younger age at diagnosis. The average age at diagnosis of 169 patients in these families is 27.4, with more than 90% being less than 45 years old at diagnosis. The ratio of male to female patients in the families is 1.25, which is consistent with population incidence rates. As expected from the age distribution of patients, among 141 HL patients with subtype information, 82% had nodular sclerosis and 15% had mixed cell.

Based on our filtering criteria between 9 and 406 variants were shared by all cases or obligate carriers within each discovery family, making a total of 2699 variants (listed in the *Online Supplementary Table S2*) whose characteristics are described in Table 2.

The 2699 variants and the corresponding 2383 genes were prioritized based on mutation types, low minor allele frequency, functional prediction, possible role in epigenetic regulation, literature linked to cancer or immune-related processes, and presence in multiple HL families. In general, the highest priority was given to the 650 non-synonymous variants and, among those, to the 180 variants identified in families with 4 or 5 affected members. These genes were further prioritized based on the information available in literature. Based on these criteria, 23 genes were selected to be followed up through Ion Torrent targeted sequencing (see Table 3). Additional genes could have been included based on our criteria but we were limited by the number and size of genes that we could investigate.

As shown in the *Online Supplementary Table S3*, WES

Table 1. Summary of study design.

Family pedigrees	Discovery of variants 17 (≥3 affected or obligate carriers sequenced each)	Validation of 23 genes Same 17 discovery families	Replication of 23 genes 48 new families (1 or 2 affected sequenced each)
Hodgkin Lymphoma	44	45	80
Non-Hodgkin Lymphoma	2	2	0
Obligate Carrier	17	14	0
Spouse unaffected	6	3	0
Related unaffected	0	27	0
Total subjects	69	91	80
Results	2699 variants, 2383 genes	All variants validated	One variant replicated

results were technically validated by Ion Torrent for all tested variants. Targeted sequencing in the additional families produced none or little additional evidence in support of the variants and genes investigated. Specifically, the variants generally did not segregate with HL in the new families. However, of the 23 genes selected for follow-up in the discovery set, only one variant was found in two additional HL patients in one family in our replication set, therefore co-segregating with HL/NHL in two independent families (indicated as F6 and F30 in Figure 1 and *Online Supplementary Tables S1 and S4*). This is a C-to-T missense mutation located at chr4:55955969 (rs56302315) in the *Kinase Insert Domain Receptor* (*KDR*) gene, a type III receptor tyrosine kinase. *KDR* produces a 1356 amino acid protein transcript (NP_002244.1) called VEGFR2. The identified variant results in a single amino acid residue change (UniProt: P35968; p.A1065T) of the wild-type VEGFR2, has an allele frequency of 7.8x10-4 in the ExAC European (non-Finnish) database and is highly conserved. Consistent with this low frequency, we found it to be present in only one individual among the 1700 in approximately 700 non-lymphoid cancer families sequenced in our lab.

Because the same *KDR* variant was identified in two families, it was further investigated through additional in-depth functional *in silico* analysis. The *KDR* gene contains 30 exons and the missense variant NM_002253.2:c.3193C>T occurs in the first nucleotide position of exon-24. The mutated residue is part of the kinase domain activation loop (kinase domain: 806-1171 AA; activation loop: 1046-1075 AA),[21] that undergoes major conformational changes upon phosphorylation to allow for phosphate transfer. The fully activated kinase serves as the signaling center for further downstream events such as cell proliferation and growth. The wild-type residue, alanine, is non-polar and hydrophobic, whereas the modified residue, threonine, is polar and can form up to three hydrogen bonds.[22] Since the VEGFR2 variant p.A1065T yields a polar residue with an acquired ability to get phosphorylated, this modification could make VEGFR2 prone to take more open and solvent accessible conformations, similar to those observed during activation. Structure-based impact assessment analysis was based on forty-seven partial VEGFR2 structures available (27 May 2015) from RCSB PDB. All the structures exhibited inactive-like folds and were co-crystallized with different inhibitors. Ten of these PDB structures were selected for our structure-based impact assessment analysis based on the structure availability of kinase domain, the structure quality (e.g., resolution and R-value) and the presence of kinase activation loop residues (D1046-E1075).[21] A sequence alignment based structure overlay was carried out in a Discovery Studio Visualizer using the PDB structure ID 3VO3[23] as the template. The template (see Figure 2) was selected based on both structure release date and quality (i.e., R-value), and the alignment resulted in two unique inactive fold groups, represented in our study by PDB IDs 3VO3 (group 1) and 2OH4[24] (group 2). Structurally aligned conformations of group representative structures are shown in the *Online Supplementary Figure S1*. Conformational analysis using the ten selected PDB structures of VEGFR2 showed that the activation loop containing p.A1065 had definite mobility (*Online Supplementary Figure S1*). Using the representative group structures we were also able to identify the group affiliations of all the remaining VEGFR2 structures. Group 2 (e.g., PDB ID 2OH4 and 1VR2) structure analysis showed that the p.A1065 containing segment is closer to the catalytic residues p.D1028 and p.R1032, and the neighboring residues R1066-P1068 in 1VR2 adopt an inhibitory conformation that can impact the substrate binding.[21] With the p.A1065T modification (*data not shown* in Figure 2), the inhibitory conformation could be weakened, therefore promoting a more open, active-like conformation and possibly leading to a constitutively active state. Most impact assessment software predicted the p.1065T variant to be damaging or deleterious to the VEGFR2 function (*see Online Supplementary Table S5*).

Other promising variants confirmed in single large (i.e., with more than 3 affected or carrier members) families were missense T-to-C changes at chr20:54956620 in *AURKA*, and at chr6:133073884 in *VNN2*, and missense C-to-T changes at chr13:49281386 in *CYSLTR2*, at chr20:39989937 in *EMILIN3*, and at chr16:82033722 in *SDR42E1*.

Discussion

The most important finding based on the sequencing of 65 HL families (17 discovery and 48 replication families) is the presence of a rare non-synonymous c.3193G>A mutation in the *KDR* gene shared by patients and obligate carriers in two families. It appears that NHL in these families also shares the same genetic susceptibility, which is consistent with our previous familial aggregation data based

on Scandinavian registries that showed significant co-segregation of HL and NHL.[4] The first family (F6) is particularly informative as it contains HL patients who are first cousins. One of the obligate carriers in this family was not available for direct testing but did have a diagnosis of diffuse large B-cell lymphoma (DLBCL) based on a slide review. The second family (F30) is consistent with the mutation causing susceptibility, but the family structure is somewhat more complex. The father and daughter with confirmed HL both have the mutation. However, two unaffected siblings of the daughter, who were tested at a relatively young age, also have the variant. This is not surprising because of the siblings' young age and the expected incomplete penetrance of mutations predisposing to familial HL based on the observed proportion of patients in high-risk HL families. In addition, either parent of the father in this second family, F30, (II.3 and II.2, Figure 1) could be a gene carrier and thus the genetic information is limited.

KDR (also known as *VEGFR2*) is a tyrosine-protein kinase that acts as a receptor for VEGFA, VEGFC, and VEGFD. It is an important gene in tumor angiogenesis, but is also involved in cell proliferation and survival. Our structural analyses of the p.A1065T mutation show that it is located in the activation loop, close to key active site residues, and able to impact protein function by interrupting the inactive conformation of VEGFR2. The codon for the A1065 amino acid residue is also in a splice region at the border of intron-exon 24, and based on the known donor/acceptor patterns[25] the c.3193C>T change might cause a splice variant. However the acceptor site is unlikely altered, since alignments of homologous VEGFR sequences show that the VEGFR1 protein (*FLT1* gene) contains a naturally occurring threonine residue, p.T1059, also found in exon 24, and has the same sequence context in the splicing region. Moreover, the p.A1065T germline mutation found in the HL families has also been studied somatically in an angiosarcoma tumor and found to be associated with high mRNA and protein KDR expression. Transfection of the mutant into COS-7 cells showed that p.A1065T could be responsible for ligand-independent activation of the kinase.[26] This same mutation was also identified as germline in a study that explored the somatic mutation patterns of kinase genes in cancer genomes.[27] Based on the experimental information and our sequence-structure analysis, we believe that the p.A1065T variant could impact the VEGFR2 function *via* structural (conformational) effects. Thus, we have good evidence that the (p.A1065T) mutation observed in the two families is functionally significant. There is also evidence supporting the potential functional significance of KDR and VEGF in lymphomas, including HL.[28] VEGF and constitutively active KDR have been found to be expressed in Hodgkin Reed-Sternberg (RS) cell lines and in RS cells from patients, as well as in serum from patients.[29,30] KDR also has high expression in DLBCL tumors and is associated with poor treatment response.[31] EBV is involved in the pathogenesis of both HL and NHL tumors. In one study, EBV positivity in NHL tumors was associated with VEGFA expression.[32] VEGF inhibitors are used in cancer treatment, including lymphomas, and have shown some efficacy in a small study of HL patients and in a HL preclinical model.[33] *KDR* mRNA was not expressed in the peripheral blood samples from our two HL families with the p.A1065T mutation (*data not shown*). Based on publicly available data from the

Table 2. Characteristics of 2699 variants identified in 17 HL discovery families after filtering.

Characteristic	No Variants
Reference/Variant	
A/T & T/A	124
C/T & T/C	867
G/T & T/G	192
A/C & C/A	203
G/C & C/G	253
A/G & G/A	887
Indels	173
Family size (affected)	
3	1991
4	425
5	283
Number of non LPD* families	
0	992
1,2,3	1021
4,5,6,7	686
ESP EA	
0	1890
(0-0.001]	442
(0.001-0.005]	319
(0.005-0.01]	48
Genomic region	
exonic	1183
intronic	868
UTR3	376
UTR5	119
downstream	60
upstream	49
intergenic	21
splicing	5
missing	18
1000 Genome	
0	2467
(0-0.001]	139
(0.001-0.005]	86
(0.005-0.01]	7
GERP	
≥2	422
<2	238
FATHMM (SCORE)	
<-0.75	123
≥-0.75	432
MUTATIONTASTER (PRED)	
D or A	222
N or P	325
Mutation type	
non-exonic	1671
non-synonymous	650
synonymous	337
frameshift	31
non-frameshift	10
MUTATIONASSESSOR (PRED)	
high	21
medium	158
low	191
neutral	202
SIFT (SCORE)	
≤ 0.05	217
> 0.05	391
POLYPHEN2 HVAR (PRED)	
D	139
P	106
B	329
Epigenetic Finding (ENCODE)	
Yes	1527
No	1172

**LPD indicates Lymphoproliferative diseases.*

NIH Roadmap Epigenomics projects,[34] *KDR* mRNA is not expressed in blood cells, but it is expressed in the epithelial tissues of HL target organs, such as the liver and the spleen. We could not study the effect of the mutation directly in HL tumor cells from carrier patients since we did not have fresh-frozen tumor available. We speculate that an early prerequisite epigenetic event is needed in order for p.A1065T carriers to develop anomalous KDR expression that then promotes enhanced proliferation.

We have identified a few other potential susceptibility variants for Hodgkin lymphoma in the families that we have surveyed. However, these additional potential candidate mutations appear to be private, at least in this cohort. In particular, the largest HL family (F4, 4 HL cases and 1 obligate carrier), in addition to having changes in 2 genes from our follow-up study (*GPT* and *SMTNL2*), presented with an A to G missense change at chr20:54956620 corresponding to the N192D amino acid change in Aurora Kinase A (AURKA). This mutation, which is highly conserved and predicted to be deleterious, is located in the kinase domain of the *AURKA* gene and absent in the ESP, 1000 Genome, and ExAC databases. AURKA is a kinase regulating mitosis, aberrantly expressed in mouse and human lymphoma cells,[35] HL cell lines, and lymph nodes.[36] AURKA inhibitors are used in cancer treatment, including lymphomas.[37]

Interestingly, we did not find shared variants in the three genes (*KLHDC8B, NPAT,* and *ACAN*) previously identified in high-risk HL families,[16-18] while we observed the *POT1* rs202187871 variant also reported in a high-risk melanoma family.[20] Given the large total number of genes (2383) with family-shared variants that we identified, we also conducted pathway analyses using two independent tools, Ingenuity Variant Analysis (IVA)[38] and the program, GoMiner[39] (*Online Supplementary Tables S6A-6F*). As seen in *Online Supplementary Table S6A*, the most enriched IVA pathways included Macrophages, T cell and B Cell Signaling in Rheumatoid Arthritis, Hematopoiesis, Allograft Rejection Signaling, OX40 Signaling, Innate and Adaptive Immune Cells, IL-10 Signaling, and Autoimmune Thyroid Disease Signaling. The most enriched GoMiner gene ontologies involved cell adhesion biological processes (*Online Supplementary Table S6E*) and protein binding molecular functions (*Online Supplementary Table S6F*). We did not observe significant enrichment in kinase activities or in cell division processes or in Kelch gene families (*Online Supplementary Table S6B*).

It has been suggested that, based on increased sex concordance of HL sibling pairs seen in some studies,[40,41] there could be a susceptibility gene in the pseudoautosomal region (PAR). Our overall sample of HL families do not show increased sex concordance, although our families were selected for greater density of cases occurring in multiple generations. Still, there could be rare families with susceptibility due to this mechanism. We do not see an enrichment of gene variants that may be in the PAR among our families (*Online Supplementary Table S2*). However, we do have one 3-generation family with 3 HL

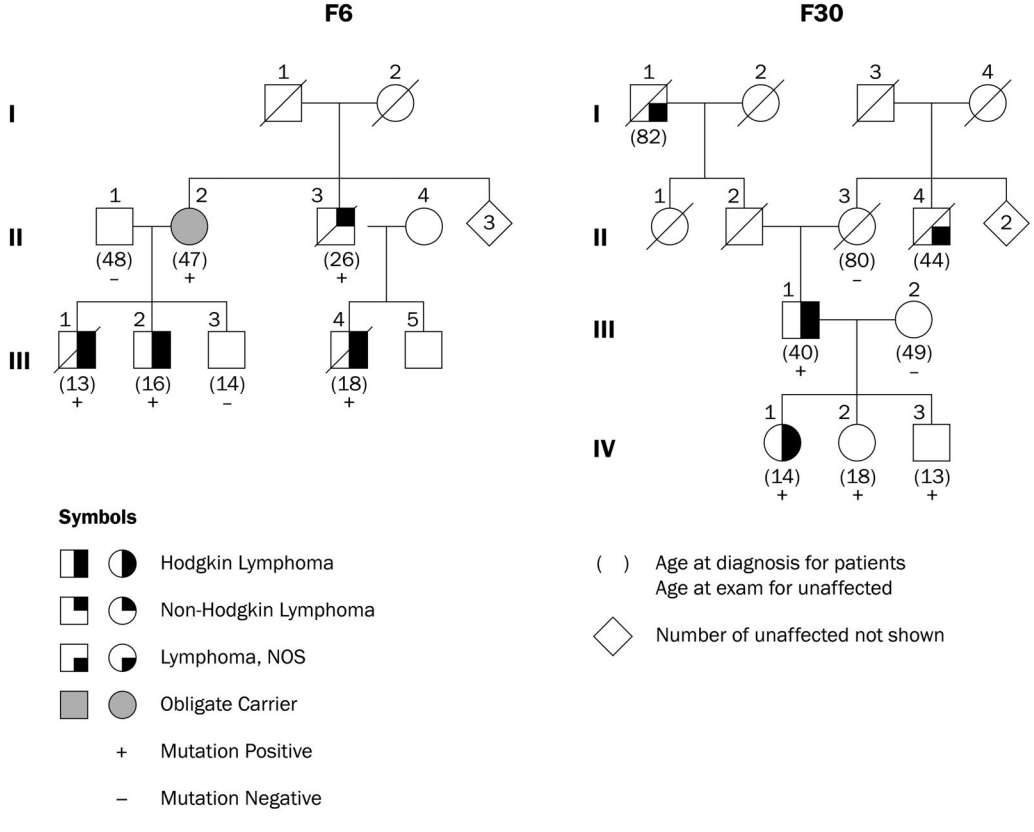

Figure 1. Family pedigrees in the KDR mutation segregating families.

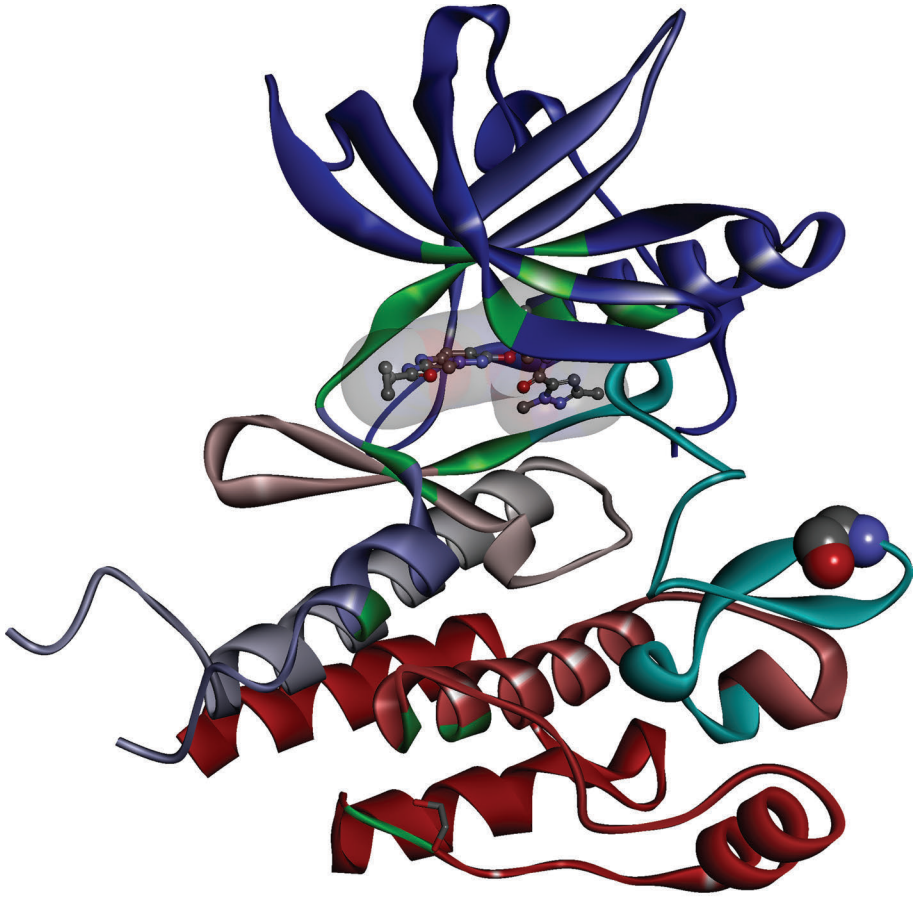

Figure 2. Inhibitor bound VEGFR-2 structure (PDB ID: 3V03). The wild-type protein structure display is set to solid ribbon and colored using N(blue)-to-C(red) terminal coloring style. The active site residue locations are colored in green and the activation loop segment in cyan. The inhibitor molecule is shown in ball-and-stick style and highlighted with a transparent closed surface. The amino acid residue A1065 is shown in CPK (solid spheres) style.

Table 3. Results in genes selected for Ion Torrent validation and follow-up study.

Count	Gene symbol	Chromosome	Position	Reference Allele	Sample Allele	Gene Region	Translation Impact	Protein Variant
1	*AURKA*	20	54956620	T	C	Exonic	missense	p.N192D
2	*BCAR1*	16	75268923	G	A	Exonic	missense	p.P671L
3	*CD33*	19	51742917	G	A	Exonic	missense	p.E230K; p.E357K
4	*COX7A2L*	2	42578483	G	A	Exonic	missense	p.P74L
5	*CYSLTR2*	13	49281386	C	T	Exonic	missense	p.R145W
6	*EMILIN3*	20	39989937	C	T	Exonic	missense	p.E758K
7	*FAM86A*	16	5140298	T	A	Exonic	stop gain	p.K143*; p.K177*
8	*FSTL5*	4	162841700	A	G	Exonic	missense	p.C89R; p.C88R
9	*GPT*	8	145730071	C	T	Exonic	missense	p.R83C
10	*HLTF*	3	148757909	A	G	Exonic	missense	p.I804T
11	*IL17RA*	22	17589509	T	C	Exonic	missense	p.L467P
12	*ITGB2*	21	46308800	C	T	Exonic	missense	p.E630K
13	*KDR*	4	55955969	C	T	Exonic	missense	p.A1065T
14	*KRT82*	12	52789643	C	T	ncRNA	ncRNA	none
15	*MYL6*	12	56553920	C	T	Exonic	missense	p.L113F
16	*NKD2*	5	1033498	G	A	Exonic	missense	p.A72T
17	*PPP1R32*	11	61249376	A	G	Exonic	missense	p.Y32C
18	*RHOG*	11	3849350	C	A	Exonic	missense	p.V7L
19	*SDR42E1*	16	82033735	G	C	Exonic	missense	p.Q55E
20	*SMTNL2*	17	4500575	A	G	Exonic	missense	p.T406A; p.T262A
21	*SUDS3*	12	118821819	T	C	Exonic	missense	p.M72T
22	*VNN2*	6	133073884	T	C	Exonic	missense	p.H181R; p.H128R
23	*ZGPAT; LIME1*	20	62367256	G	T	Exonic	missense	p.K507N; p.K498

patients and 1 obligate carrier that are all male. Future deep sequencing of the Y chromosome or PAR genes would be appropriate in this family.

The strength of our study is the ability to search comprehensively for coding variants causing susceptibility to HL in a number of highly informative families. Nonetheless, there are limitations in applying WES to find germline susceptibility genes. First, it is possible that the key susceptibility loci are outside of exonic regions and we would therefore not be able to detect them. Some genes and some regions within genes are not well covered by exome targets. Our filtering may be too stringent and thus we could have missed important mutations. It is also possible that not all top potential candidate genes were selected for the replication study. Alternatively, heritable epigenetic marks, rather than specific gene variants, may cause disease susceptibility. The underlying genetics of HL may be more complex than assumed in this study, even in high-risk families. There could be extensive genetic heterogeneity so that some causative mutations are "private". It is also possible that more than one susceptibility gene is involved, even in a single family. These factors generally make it more difficult to detect critical loci and ultimately large consortia may be required to combine the sequencing of an even larger number of families.

Future studies could include whole genome sequencing as well as integrated epigenetic and RNA-Seq expression studies in order to narrow down critical loci. Although our finding in the *KDR* gene is promising, this must be verified in independent studies. The identified germline mutation in the *KDR* gene has been shown to encode a constitutively active form of KDR that may have the capacity to enhance cell proliferation activity in the absence of ligand. Importantly, the phosphorylation was decreased and the kinase activity was abrogated after exposure to sunitinib and sorafenib drugs,[26] suggesting that the p.A1065T *KDR* mutation could be targeted for therapy in HL and NHL patients carrying this mutation. In addition, our study is the largest sequenced cohort of HL families to date and provides the scientific community with potentially important variants readily available for replication in independent family studies.

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