

# Exome sequencing reveals germline *NPAT* mutation as a candidate risk factor for Hodgkin lymphoma

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A strong clustering of Hodgkin lymphoma in certain families has been long acknowledged. However, the genetic factors in the background of familial Hodgkin lymphoma are largely unknown. We have studied a family of 4 cousins with a rare subtype of the disease, nodular lymphocyte predominant Hodgkin lymphoma. We applied exome sequencing together with genome-wide linkage analysis to this family and identified a truncating germline mutation in *nuclear protein, ataxiatelangiectasia locus (NPAT)* gene, which segregated in the family. We also studied a large number of samples from other patients with Hodgkin lymphoma, and a germline variation leading to the deletion of serine 724 was found in several cases suggesting an elevated risk for the disease (odds ratio = 4.11; P = .018). NPAT is thus far the first gene implicated in nodular lymphocyte predominant Hodgkin lymphoma predisposition. (*Blood*. 2011;118(3):493-498)

# Introduction

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is a rare subtype of Hodgkin lymphoma (HL) accounting for approximately 5% of HL. It is distinct from classic Hodgkin lymphoma (cHL), which is the commonest HL subtype. Tumor cells in both NLPHL and cHL originate from germinal center B cells. These cells compose only approximately 1% of the tumor mass the rest consisting mostly of benign cellular infiltrate of reactive lymphocytes and other inflammatory cells.<sup>1</sup> In NLPHL, the malignant lymphocyte predominant (previously called lymphocytic and histocytic) CD20-positive cells grow in a nodular, sometimes diffuse, pattern representing transformed germinal centers. They do not usually express CD30 and CD15, which typifies the Hodgkin and Reed-Sternberg cells in cHL. Expression studies on lymphocyte predominant cells have shown down-regulation of several B-cell markers, although not as abundantly as in cHL, as well as active expression of nuclear factor-kB target genes.<sup>2</sup>

Epidemiologic studies of HL have suggested that certain infections, genetic factors, and deficits in the immune system increase the risk of developing the disease.<sup>3,4</sup> However, it is uncertain whether this applies to NLPHL. A hallmark of cHL epidemiology is the bimodal age-specific incidence and the disease in young adults and older adults are probably etiologically different; in particular, there is a low prevalence of Epstein-Barr virus (EBV) in younger cHL cases.<sup>3</sup> Evidence for a strong heritable basis to HL is provided by the elevated risk in first-degree relatives and high concordance in monozygotic twins.<sup>5,6</sup> The distribution of different HL subtypes seems to be similar in sporadic and familial cases.<sup>7</sup> A recent genome-wide association study of HL has confirmed the strong relationship between the major histocompatibility complex region and HL risk and identified 3 additional loci to which common risk variants map.<sup>8</sup> Collectively, such loci do not impact significantly on the familial HL risk, and the strong familial clustering in some families suggests the existence of high to moderately penetrant susceptibility. In keeping with this model of susceptibility is the observation that variations of the *KLHDC8B* gene were found to segregate with cHL in 4 families.<sup>9</sup>

We have recently reported a family of 4 Finnish cousins with NLPHL.<sup>10</sup> All patients had been diagnosed at the ages between 22 and 26 years. We studied KLHDC8B and excluded it as a predisposing gene to NLPHL in this family. In this study, our aim was to further clarify the genetic background of familial NLPHL susceptibility by applying linkage analysis in combination with exome sequencing to this Finnish family. Whole exome sequencing is a new, powerful method that is used to sequence all the protein-coding regions in the genome of a person. It is considered cost-effective and unbiased, and it has been recently used successfully in unraveling genetic defects in familial conditions, including predisposition to cancer.<sup>11</sup> In our approach, we combined exome sequencing of one affected family member with genome-wide linkage data and identified a truncating germline deletion of 2 bp in the nuclear protein, ataxia-telangiectasia locus (NPAT) gene, which segregated in the family. This is the first gene implicated in NLPHL predisposition.

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The online version of this article contains a data supplement.



# Methods

## Patient samples and clinical data

In the Finnish NLPHL family, 4 maternal cousins (3 males and 1 female) had all been affected by early-onset NLPHL (Figure 1). All cases were reexamined by a hematopathologist (K.F.), and the diagnoses were confirmed both in histologic and immunohistochemical studies. Cases 1 to 4 have been diagnosed at the ages of 26, 24, 22, and 23 years with the stages of IIIB, IVB, IIA, and IIIA, respectively (Figure 1). Cases 2 to 4 have achieved remission. Full clinical details of the patients have been previously reported.<sup>10</sup> Blood-derived genomic DNA was available from all 4 NLPHL patients and from 2 mothers who are obligate carriers of the putative genetic defect, as well as from 5 healthy family members (Figure 1).

A karyotype analysis with the resolution of 400 to 500 bands was performed from the blood sample of one NLPHL patient (case 1, Figure 1). Possible EBV infection was studied using EBV-encoded RNA in situ hybridization, which was performed from paraffin-embedded, NLPHLaffected lymph node tissue received from the 4 NLPHL patients.

#### Table 1. NLPHL and HL samples used in NPAT sequencing

Genomic DNA extracted from paraffin-embedded tissue was obtained from 73 Finnish HL patients. Twenty-seven were from families with 1 NLPHL patient and at least 1 close relative with NLPHL, cHL, or non-Hodgkin lymphoma and 38 were < 30 years old at diagnosis. Eight of the cases were from the same geographic region as the original Finnish family of 4 NLPHL patients. The patients were ascertained from a systematic search for related or early-onset NLPHL patients within the Finnish cancer registry. Blood-derived genomic DNA from 282 healthy Finnish blood donors served as a source of controls.

In addition to Finnish samples, we analyzed samples from the United Kingdom. Genomic DNA was obtained from blood samples of 93 patients with HL of whom 26 had a close relative with a lymphoproliferative disorder, and 177 healthy persons with no personal history of malignancy ascertained through the National Study of Colorectal Cancer Genetics study.<sup>12</sup> We also screened genomic DNA available from 3 previously reported NLPHL families from United Kingdom/India, France, and Turkey and from an NLPHL-derived cell line, DEV.<sup>13-16</sup> A summary of the available samples is shown in Table 1.

Lymphoblast cell lines were established from all 11 Finnish family members, 4 of the French family members, and from 1 patient in the United Kingdom/India NLPHL family.

Description of patients	No. of cases available	Deletion of c. 2437-2438delAG	Deletion of serine 724			
Finland						
Finnish NLPHL family	4	4	0			
HL patients from other Finnish NLPHL families	27	0	0			
NLPHL patients with early-onset disease (< 30 years)	38	0	1			
NLPHL patients from same geographic region as the original family	8	0	0			
Healthy controls	282	0	2			
United Kingdom						
HL patients with a family history of a lymphoproliferative disorder	26	0	2			
Sporadic HL patients	67	0	4			
Healthy controls	177	0	3			
Previously reported NLPHL cases						
United Kingdom/India: 2 siblings with NLPHL	2	0	0			
France: a family of 3 NLPHL patients	3	0	0			
Turkey: mother and son with NLPHL	2	0	0			
DEV cell line	1	0	0			

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The number of cases with a deletion in NPAT is shown.

The study has been approved by the Ethics Committee of the Hospital district of Helsinki and Uusimaa. All samples were derived either after signed informed consent (blood samples) or authorization from Valvira (National Supervisory Authority for Welfare and Health; paraffin blocks) in accordance with the Declaration of Helsinki. United Kingdom samples were obtained with Ethical board approval and written consent.

#### Linkage analysis and fine mapping

Whole genome genotype data were obtained from the 11 members of the Finnish family using Affymetrix 50K Xba SNP array. Genotyping was performed according to the manufacturer's standard protocol in the Institute for Molecular Medicine Finland (FIMM) Genome and Technology Center Finland. The array data were analyzed with Merlin,<sup>17</sup> using an affected only approach in which penetrance is assumed to be 100%, phenocopy rate 0%, and healthy persons are coded with unknown disease status. Both parametric models, recessive and dominant, were analyzed. Linked regions were haplotyped with Merlin "best" haplotyping function and further mapped with microsatellite markers. For this purpose, we used 40 microsatellite markers, which were either known dinucleotide repeats available in the www.Ensembl.org database or repeats found using the RepeatFinderprogram.<sup>18</sup> The microsatellite markers are available in supplemental Data (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

# Exome sequencing and sequence analysis

The exome of one NLPHL patient (case 1, Figure 1) was sequenced. Exomic regions of the genome were enriched using Agilent SureSelect Human All Exon Kit totaling approximately 38 Mb of the genome (Agilent Technologies). Paired end short read sequencing with a read length of 56 bp was performed with Illumina Genome Analyzer II in FIMM Genome and Technology Center Finland (Illumina). The sequence data were analyzed with NextGENe- analysis software Version 1.94 (Softgenetics) using NCBI36/Hg18 as reference build. Exomic regions as well as sequence within 2 bp of the exon-intron boundary were analyzed. A minimum sequencing coverage of 5 reads was required for variation analysis; variations existing in only one read or < 20% of reads were considered sequencing errors, and variations resulting to a synonymous amino acid were excluded from the results.

The exome and linkage data were integrated, and potential disease causing variations found in the linked regions were verified with Sanger sequencing implemented on ABI3100xl technology (Applied Biosystems). Segregation of the verified variants was studied in additional family members with direct sequencing using the available samples from the Finnish family. Sequencing primers were designed with ExonPrimer and Primer3 programs (www.ihg.gsf.de/ihg/ExonPrimer.html and www.frodo. wi.mit.edu/primer3) using NCBI36/Hg18 as a reference. Primer sequences and polymerase chain reaction (PCR) conditions are available on request. The PCR products were purified using ExoSAP-IT PCR purification kit (USB Corporation), and the sequencing reactions were performed using the Big Dye Terminator, Version 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Electrophoresis was performed on an ABI3730 Automatic DNA sequencer (Applied Biosystems at FIMM Genome and Technology Center Finland). Sequences were analyzed using Mutation Surveyor (Softgenetics).

#### NPAT sequencing

All exons and exon-intron boundaries of *NPAT* were directly sequenced from the genomic DNA extracted from all available HL samples. We also sequenced the entire *NPAT* cDNA (CCDS41710) from those samples where we had established lymphoblast cell lines. Separate primers were designed for paraffin, cDNA, and blood-derived samples. Primer design, direct sequencing, and sequence analysis were performed as described in "Exome sequencing and sequence analysis."

# Table 2. The linked regions in the Finnish NLPHL family according to the dominant model of inheritance (reference build: NCBI36/Hg18)

Chromosome	Region start dbSNP ID	Region end dbSNP ID	Region size, bp
1	rs3007734	rs950922	3077046
3	rs10510592	rs864391	18114197
5	rs1161343	rs2311735	6016348
8	rs3735915	rs4388475	2001845
10	rs10508295	rs2025465	1521857
11	rs7120017	rs633619	12464624
12	rs4133070	rs1920706	12276245
13	rs493041	rs9300629	25624357
16	rs2347089	rs9319570	2440380
18	rs727929	rs4797211	2070050
20	rs6140795	rs2876404	6465648
21	rs198057	rs2831528	4988897
Х	rs830249	rs768198	15833438

#### **Expression analysis**

Gene expression was studied from the blood-derived RNAs of the Finnish family members as well as from 10 controls using GeneChip Human Genome U133 Plus2 (Affymetrix; GSE28536). The genome-wide data were robust multichip average normalized in R using custom chip definition files (ENTREZG, Version 11.0.1).<sup>19</sup> Two-tailed *t* test for *NPAT* (8 mutation carriers vs 12 nonmutation carriers and controls) was performed with the equal variance assumption.

# Results

## Karyotyping and EBV status

Before genetic studies, a karyotype analysis was performed in 1 NLPHL patient of the family (case 1, Figure 1). The karyotype was normal, suggesting that chromosomal abnormalities are not associated with NLPHL in the family. The EBV status of the tumor cells in lymph node samples of the patients was evaluated, and all were EBV-negative.

#### Linkage analysis

According to a dominant model, 13 genomic regions (> 112 Mb) were found to segregate with NLPHL. These were further fine mapped with microsatellite markers. Fine mapping confirmed linkage in all 13 regions. The linked regions are shown in Table 2.

# Exome sequencing

The exome of 1 NLPHL patient of the Finnish family was sequenced (case 1, Figure 1) and 7737 variations fulfilling our predefined criteria were found. Common known SNPs and variations found in 13 control samples available from other projects were excluded from the study. In the linked regions, 14 variations unique to the NLPHL patient's exome were identified, and these were directly sequenced using samples from all 4 NLPHL patients, the 2 mothers, and a preliminary set of 3 healthy controls. Seven of the sequence changes were verified, and 6 of them segregated. Three of those were found in at least 1 of the 3 controls. The 3 remaining variations were further sequenced from additional healthy Finnish controls (Table 3). Only 1 was not detectable in the controls. This was a heterozygous deletion of 2 bp, AG (c. 2437-2438), resulting to frame shift and premature stop codon at serine 819 in exon 13 of NPAT gene. Three of 5 healthy family members were shown to carry the deletion (Figure 1).

Table 3. The unique variation	s found in the linke	d regions using exom	e sequencing of one	NLPHL patient
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0	Amino acid	Mall darks an adapted	0	Example controls (c(0)	Formation additional combinets
Gene	cnange	Validation status	Segregation in the family	Found in controls (of 3)	Found in additional controls
DLEC1	1305E > EG	False			
KIF5A	342W > LW	False			
KRT85	189D > DN	True	Yes	1	
MIP	107V > VI	True	Yes	2	
NBPF3	170Q > QR	False			
NPAT	c. 2437–2438delAG	True	Yes	0	0/239
PAN2	247N > NT	False			
PFKFB3	72G > WG	False			
PFKFB3	440C > SC	False			
POF1B	315R > C	True	Yes	0	2/251
SLC22A14	c. 713–716delTGTT	True	Yes	1	
UGCGL2	983Q > HQ	False			
DDIT3	157R > RQ	True	No		
DGKA	296T > TA	True	Yes	ND	2/110

The results of the verification by direct sequencing and the segregation of the variation in the family are also shown. ND indicates not determined.

#### **NPAT** sequencing

After identification of the truncating deletion in the NPAT gene in the Finnish NLPHL family, we examined whether NPAT mutations were carried by other patients with HL. NPAT contains 18 exons totaling 1427 amino acids. The exons and exon-intron boundaries of NPAT were sequenced from genomic DNA of the 73 Finnish HL patients, 93 HL patients from United Kingdom, 3 previously published NLPHL families, and from an NLPHL-derived cell line DEV.<sup>13-16</sup> A deletion of 3 bp resulting to the loss of serine 724 (S724) was detected in 7 patients (Table 1). One of them was a Finnish early-onset NLPHL patient, and 4 were sporadic HL patients from United Kingdom who had all been diagnosed between ages of 14 and 30 years. In addition, the S724 deletion was detected in 2 HL patients from the United Kingdom of whom one had a relative with non-HL and one with HL. The deletion was sequenced from 282 healthy Finnish and 177 United Kingdom persons, and was found in 5 persons.

We estimated the impact of the S724 deletion on HL risk through unconditional logistic regression calculating odds ratios and 95% confidence interval using STATA (Version 10.0, State College). On the basis of frequencies of S724 deletion seen in the United Kingdom and Finnish populations, the odds ratio for HL associated with this sequence change is 4.11 (95% confidence interval, 1.27-13.35, P = .018). The success rate of sequencing was 93% for the HL patients and 97% for the controls at the site of S724 deletion.

We also screened the *NPAT* cDNAs of the NLPHL patients in the original Finnish family as well as in the French and English families and the DEV cell line for possible mutations, but none was identified. The *NPAT* deletion c. 2437-2438delAG product was seen with clearly lowered intensity in the cDNA sequences (approximately one-third of the wt allele) of the Finnish mutation carriers.

#### Expression analysis

Significant decrease in *NPAT* mRNA level (fold change = 0.80, *t* test, *P* = .016) was also observed in the patients and the carriers of the deletion c. 2437-2438delAG compared with the controls and the nonmutation carriers, suggesting that the mutated product is directed to the nonsense mediated decay.

# Discussion

Genetic factors have long been recognized to contribute to the risk of developing HL, and familial cases, including all HL subtypes, have been estimated to represent 4.5% of HL.20 Association to human leukocyte antigen and linkage to non-human leukocyte antigen loci have been suggested in familial HL, and the genetic susceptibility seems mainly to be related to the disease in young adults.<sup>6,7,21-23</sup> The interaction of genes and environmental determinants, such as viral infections, have also been implicated, but, for example, EBV is detected in the tumor cells of less than one-third of familial HL patients compared with 40% to 50% in sporadic cases.<sup>24,25</sup> In a few families, a variant in KLHDC8B gene resulting in a decreased protein expression has been reported to associate with cHL, a finding awaiting confirmation.9 The remarkable majority of the patients in the studied HL families have been diagnosed with cHL, most often with the nodular sclerosis or mixed cellularity subtype, and it is possible that the etiologic factors are different in different lymphoma subtypes. Four NLPHL families from United Kingdom/India, France, Turkey, and Finland have thus far been published.<sup>10,13-15</sup> EBV, which is infrequently found in sporadic NLPHL, was not detected in any of the patients' tumors, and screening of KLHDC8B did not reveal diseaseassociated mutations.<sup>10</sup> Consequently, the cause of familial NLPHL has thus far remained unknown.

We have identified a germline deletion c. 2437-2438delAG of 2 bp, resulting in frame shift in the *NPAT* gene in the Finnish family with NLPHL susceptibility using linkage analysis and exome sequencing, a new, powerful and unbiased tool for large-scale genome-wide detection of genetic variations. The deletion in *NPAT* was the only segregating variation located in the linked regions that was neither a previously known polymorphism nor detected in healthy controls. The *NPAT* mRNA expression was significantly decreased in the carriers of the deletion. The deletion segregated with the disease in the family members, and also 3 healthy relatives were found to have the mutation suggesting that the penetrance for NLPHL susceptibility is low or additional genetic or environmental factors modify disease risk.

We screened a large number of additional HL patients and another variation leading to a deletion of a serine 724 of NPAT was found. This was significantly overrepresented in the patients with HL, further indicating a role for *NPAT* in the genetic background of HL. It is, however, acknowledged that our estimate of the impact of this rare variant on HL is imprecise because it is based on a relatively small dataset and a subset of cases were enriched for genetic susceptibility by virtue of having a family history of a lymphoproliferative disease.

NPAT is located in chromosome 11q22.3 next to ataxiatelangiectasia mutated gene (ATM) with which it shares a putative promoter region. It is expressed in all human tissues.<sup>26,27</sup> NPAT is phosphorylated by cyclin E-cyclin-dependent kinase 2 (CDK2) complex, and its expression peaks in accordance with cyclin E-CDK2 complex activity at the G<sub>1</sub> to S transition of the cell cycle promoting entry to the S-phase.28 NPAT interacts with histone nuclear factor P (HiFN-P) and mediates the activation of histone gene transcription in a cyclin E-CDK2-regulated manner.<sup>29,30</sup> The HiFN-P/NPAT complex also affects the transcription of other target genes, such as NPAT and ATM; and in B-cell chronic lymphocytic leukemia cells, NPAT has been shown to be down-regulated.<sup>31,32</sup> These data suggest that NPAT has an important role in the regulation of the cell cycle; and, together with HiFN-P, it contributes to the activation of the promoter of ATM, a gene in which mutations have been found in different lymphoid malignancies.<sup>31,33-35</sup> In addition, the ataxia-telangiectasia patients with germline mutations in ATM are predisposed to different cancers, particularly leukemias and lymphomas.<sup>36</sup>

This is the first study in which a candidate germline mutation has been identified in familial NLPHL, providing potential insights to the molecular basis of the disease. Although our findings are intuitively interesting, replication in an independent material is highly desirable. Considering that *NPAT* shares the promoter region with *ATM*, mediates important functions in the cell cycle, and possibly has an effect on *ATM* transcription, there are various cellular mechanisms to which a defect in *NPAT* could putatively cause dysregulation and thus cause cancer susceptibility. It is also possible that *NPAT*-related pathways are involved in the pathogenesis of sporadic NLPHL or other hematologic malignancies. In familial HL cases where mutations of *NPAT* were not found, it is possible that mutations in other genes of the same pathway are involved.

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# Authorship

Contribution: S.S. was responsible for the study, acquired, analyzed, and interpreted the data, and wrote the manuscript; M.A. performed sequencing and expression analyses and wrote the manuscript; V.L. contributed to the study design and supervised the study; R.L. performed linkage analysis and wrote the respective sections of the manuscript; K.F. performed pathologic evaluation; H.J.L. performed laboratory experiments; E.K. analyzed expression data; K.A. acquired patients and contributed to the study design; P.B. performed laboratory experiments; J.T. performed pathologic evaluation of EBV-encoded RNA in situ hybridization samples; B.J.B., F.B., A.U., A.J.S., R.C., and M.J.M. acquired patients and provided samples; R.H. performed statistical analysis and wrote the manuscript; P.V. contributed to the study design and supervised the study; L.A.A. was the principal investigator of the study; and all authors read and contributed to the final version of the manuscript.

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