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Spectrum mutations of *PRF1*, *UNC13D*, *STX11*, and *STXBP2* genes in Vietnamese patients with hemophagocytic lymphohistiocytosis

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Abstract

Introduction: The prevalence of gene mutations in hemophagocytic lymphohistiocytosis (HLH) varied between studies. Thus far, data on the genetic background of HLH in Vietnamese patients are limited.

Methods: We recruited 94 HLH patients and analyzed for the 4 genes using Sanger sequencing technology.

Results: Pathogenic variants were observed in 36 (38.29%) patients, including 27 in *UNC13D*, 5 in *STXBP2*, 3 in *PRF1*, and 2 in *STX11* (one patient with digenic variants in both *UNC13D* and *STX11)*. Monoallelic variants accounted for 77.8% of all cases with mutation. A total of 23 different types of pathogenic variants were documented in the 4 genes tested, including 15 in *UNC13D*, 3 in *PRF1*, 3 in *STXBP2*, and 2 in *STX11*. Interestingly, the novel splicing variant c.3151G>A in *UNC13D* was recurrently identified in 8 unrelated patients.

Conclusion: Vietnamese patients with HLH showed a distinct genetic variant spectrum, in which *UNC13D* is the predominant genetic lesion associated with HLH.

KEYWORDS

PRF1, *STX11*, *STXBP2*, *UNC13D*, vietnamese

1 | INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is an uncommon, dismal disorder resulted from dysregulation of the immune system. It manifests with symptoms such as persistent fever, cytopenias, hepatosplenomegaly, hepatitis, hemophagocytosis, and natural killer (NK) cell dysfunction.[1] It is believed that excessive and ineffective immune responses result in multi-organ dysfunction accompanying by series of severe clinical presentation.[2] There are two categories of HLH, namely primary (inherited) and secondary (acquired) HLH, based on the presence or absence of genetic alteration, respectively. Inherited HLH consists of familial (FHL) and is caused by genetic defects. FHL is autosomal recessive, subdivided into 5 types, FHL1 to FHL5. The causes of FHL2, FHL3, FHL4, and FHL5 are pathogenic variants in the genes encoding perforin 1 (*PRF1*), Munc13-4 (*UNC13D*), syntaxin 11 (*STX11*), and syntaxin-binding protein 2 (*STXBP2*), respectively.

The genetic defect in FHL1 remains unidentified. Cytotoxic T and NK cell activity is consistently low or absent in FHL patients.[3]

The combination of etoposide and dexamethasone has been established as standard treatment for FHL while awaiting an allogeneic hematopoietic stem cell transplantation (HSCT) that provides the cure for most patients.[4] Clinically, secondary HLH patients manifest a similar picture and respond to standard treatment the same as in patients with FHL. However, unlike patients with FHL, secondary HLH patients are left without any further effective therapy once relapse. Thus, it is critical to distinguish FHL from HLH because HSCT is only compatible with FHL, while it is ineffective for secondary HLH.[5]

The variants in *PRF1*, *UNC13D*, *STX11*, and *STXBP2* genes are the most common cause of HLH, and detection rate differs depending on the ethnicity and country of origin. In the American population, *PRF1* accounted for 50.86% of mutated patients, followed by *UNC13D* (39.31%) and *STXBP2* (9.83%) as causative genes.[6] In European countries, *PRF1* mutations were found in 10%-60% of Caucasian HLH patients, *UNC13D* mutations in 8% of Turkish and 17% of German patients.[7] In Asian countries, *UNC13D* was the most gene mutated in Korean patients (89%),[8] while in Japanese patients, *PRF1* and *UNC13D* accounted for 19% and 20% of mutations.[9] To the best of our knowledge, thus far, data on the genetic background of HLH in Vietnamese patients are limited.

In this study, we performed mutational analyses of *PRF1*, *UNC13D*, *STX11*, and *STXBP2* genes in a cohort of 94 Vietnamese patients with HLH. We report mutation spectrum, with some novel variants in the four genes, and thus provide an overview of the genetic background involved in Vietnamese HLH.

2 | MATERIALS AND METHODS

2.1 | Subjects

Patients with clinical diagnosis of HLH were referred to Center for Molecular Biomedicine, University of Medicine and Pharmacy, at Ho Chi Minh City for genetic testing from June 2016 to February 2021. All patients met the HLH-2004 diagnostic criteria, including hemophagocytosis in bone marrow, as described by the Histiocyte Society.[10] Because all specimens were from outside hospitals, we did not have access to clinical data and treatment information, including primary or secondary feature of HLH. The protocol for this study was approved by the Ethics Committee of the University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam (approval number 307/HDDD-DHYD).

2.2 | Sample collection and DNA extraction

We collected 2 mL of peripheral blood from patients with EDTA anticoagulant. DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) and stored at −30°C.

2.3 | Mutational analyses

PCR and sequencing primers were designed for the sequencing of exons and flanking introns of 4 genes using the CLC Main Workbench v.5.5. Referenced genomic and coding sequences of *PRF1* (NG_009615.1 and NM_001083116.3), *UNC13D* (NG_007266.1 and NM_199242.2), *STX11* (NG_007613.1 and NM_003764.4), and *STXBP2* (NG_007613.1 and NM_001127396.3) were obtained from the National Center for Biotechnology Information Consensus CDS database (https://www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.cgi).

Primers for amplification of 4 genes are listed in Table 1. For all primer pairs, the annealing temperature was set to 60°C. PCR was done in separate 25 μl reactions consisting of 1X PCR Buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 U Taq Hot Start Polymerase (Takara Bio, Shiga, Japan), 0.1 μM each forward and reverse primers, and 25-50 ng of genomic DNA. The PCR products were analyzed on agarose gel electrophoresis and then were purified with the ExoSAP-IT reagent (Thermo Scientific, USA) and direct sequenced with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were analyzed on an ABI 3500 Genetic Analyzer (Applied Biosystems, USA).

3 | RESULTS

A total of 94 patients with HLH (61 males and 33 females) were recruited in this study. The median age at presentation was 13.4 years (range, 1 to 86 years) with a male predominance (male:female ratio, 1.8:1). All patients fulfilled the HLH-2004 diagnostic criteria.

Of these patients, 36 had pathogenic variants, including 28 patients with 1 mutated allele, and 8 patients with 2 mutated alleles. A total of 23 different types of pathogenic variants were documented in the 4 genes tested, including 15 (65.2%) in *UNC13D*, 3 (13.0%) in *PRF1*, 3 (13.0%) in *STXBP2*, and 2 (8.8%) in *STX11* (Table 2).

3.1 | Mutations in UNC13D

UNC13D was the most frequently mutated gene in this study. A total of 34 mutated alleles were detected in 27 patients. Splicing variants were most common, which accounted for 52.9% (18/34). Missense, deep intronic, and nonsense variants accounted for 20.6%, 14.7%, and 11.8% of mutated alleles, respectively. Most patients (19/27, 70.4%) had only one *UNC13D* mutant allele, while 5 patients were homozygous and two patients were compound heterozygous for *UNC13D*. Of note, we identified one patient (HLH-87) who harbored digenic variants in both *UNC13D* and *STX11* genes.

The pathogenic variants were distributed widely in different exons and their adjacent splice site regions, as well as in the highly conserved region of intron 1 of *UNC13D* (Figure 1). Among them, the novel splicing mutation c.3151G>A was the most frequent variant, which was identified in 8 patients (10 mutated alleles, including 2 homozygous cases). Of the 6 patients with a single c.3151G>A allele,

TABLE 1 Primer sequences for PCR amplification

Gene	Primer name	Region	Primer sequence (5' 3')	Length (bp)
PRF1	PRF1-2F	Exon 2	AAGGGAGCAGTCATCCTCCA	699
	PRF1-2R		ACACACAAAGGTTCCTGCGG	
	PRF1-3F	Exon 3	ATGGGGGAAATACTCCCCTG	1230
	PRF1-3R		TGGACTGAAGGGGTTCTCAC	
UNC13D	UNC-g1F	Exons 1 - 6	ATAATCCTGTGGCTTCGCTG	2059
	UNC-g6R		TGAGATGAGTAGGTCGTCCT	
	UNC-g7F	Exons 7 - 12	TGTGGTCACTTACTGCTTCG	1343
	UNC-g12R		TCTCAGCAGAGTTCCCTTTG	
	UNC-g13F	Exons 13 - 20	TCAGCCTGTACTGGTGGATG	1700
	UNC-g20R		CTGGACCTCCAAAGCGTAGT	
	UNC-g21F	Exons 21 - 25	TCTGTGCCTGGTGATGGTAG	2288
	UNC-g25R		CATCCGTTCTGAGGGGTGTA	
	UNC-g26F	Exons 26 - 32	CGTCTTTGCTTCCTCCTCCG	3562
	UNC-R6		TGTAGGGTCTGGGGCTTCCC	
STX11	STX11-g2F	Exon 2	TGCCCACACCGAGGAATACA	1104
	STX11-g2R		CAACCCTTTCCGGAACTCAG	
STXBP2	STXBP2-g1F	Exons 1 - 4	ACCTTGGGACACACCCGGAA	2860
	STXBP2-g4R		CATGTGTGCATGTGTATACG	
	STXBP2-g5F	Exons 5 - 6	TGTTTGCACATGGTGGCAGA	488
	STXBP2-g6R		CTCATACCTGTAATCCCAGC	
	STXBP2-g7F	Exons 7 - 13	ATTCGGCAAAGCAGGCTTCA	1754
	STXBP2-g13R		CTGTAGAGATGGGGGGTTCT	
	STXBP2-g14F	Exons 14 - 16	ATCCCCTTCCCTGACACATA	1900
	STXBP2-g16R		CTCATTGCTGGCATTCCCTT	
	STXBP2-g17F	Exons 17 - 19	AGGATCTTGGCCCCTGAATA	801
	STXBP2-g19R		TAAACTCTTCCCGTCGCTCT	

two patients were compound heterozygous due to carrying other *UNC13D* mutant alleles (c.1055+1G>A or c.118-307G>A). To clarify how the novel splicing mutation c.3151G>A effect on RNA processing, we performed reverse transcriptase PCR using primers encompassing *UNC13D* exons 30 to 32. As shown in Figure 2, sequence analysis revealed a deletion of exon 31 from the mRNA transcript, leading to a frameshift (p.F985fsX14).

The second most common variant was c.118-307G>A in intron 1, with a total of 5 mutated alleles, detected in four patients including one homozygous case. This mutation impaired transcription by disrupting a highly conserved transcription factor binding site or enhancer element[1] and abolish Munc13-4 expression.[11]

More interestingly, there were 3 patients with an identically novel insertion-deletion mutation c.965_967>68bp occurring at exon 12, which had not been reported before. The 65-bp inserted fragment, when using the BLAST tool, matches with the *COX1* gene from the mitochondrial DNA sequence. With RNA analysis, we further showed that this mutation resulted in a stop codon at 318 position in *UNC13D* gene (Figure 3).

Out of 15 different types of variants detected in *UNC13D*, we found 7 missense mutations. Three of them (c.1232G>A: p.R411Q, c.2180G>A: p.R727Q, and c.2243C>T: p.A748V) were reported elsewhere.[12-14] All of the remaining 4 novel missense mutations had a PolyPhen-2 score of 1.0, predicted to be pathogenic on HLH (c.1159C>G: p.L387V, c.1283T>G: p.L428R, c.2039G>A: p.R680Q, and c.3181C>T: p.R1061W).

3.2 | Mutations in *PRF1*, *STX11*, and *STXBP2*

STXBP2 was the second most common mutated gene in our cohort. Five mutant alleles were detected in 5 patients. They were two reported missense mutations and one novel large deletion of exons 7 to 13. Three patients (HLH-38, HLH-52, and HLH-67) had the same heterozygous missense mutation c.1430C>T (p.P477L) at a conserved residue, which has been shown to reduce amounts of *STXBP2* and decrease levels of syntaxin-11.[15] The other missense mutation was c.953C>T (p.T318 M) at exon 11, previously reported in Chinese patients.[14,16] RNA analysis from patient HLH-29, who was heterozygous for a deletion of exons 7 to 13 in genomic DNA, revealed the c.430_1107del which resulted in a frameshift p.V144fsX225 (data not shown).

TABLE 2 Pathogenic variants found in the 4 HLH-related genes among 36 mutated patients

No	Code	Gender/Age (years)	Gene	Pathogenic variants	Zygosity
1	HLH-2	F/7	UNC13D	c.3151G>A (p.F985fsX14)	Het.
2	HLH-3	F/7	UNC13D	c.965_967>68bp (p.A318X)	Het.
3	HLH-6	F/1	UNC13D	c.1283T>G (p.L428R)	Het.
4	HLH-7	F/19	UNC13D	c.3151G>A (p.F985fsX14)	Het.
5	HLH-16	M/6	UNC13D	c.1055+1G>A	Het.
				c.3151G>A (p.F985fsX14)	Het.
6	HLH-34	M/2	UNC13D	c.3151G>A (p.F985fsX14)	Het.
7	HLH-35	F/1	UNC13D	c.1159C>G (p.L387V)	Het.
8	HLH-39	M/1	UNC13D	c.118-307G>A	Hom.
9	HLH-40	M/2	UNC13D	c.2039G>A (p.R680Q)	Het.
10	HLH-42	M/7	UNC13D	c.3151G>A (p.F985fsX14)	Hom.
11	HLH-44	M/34	UNC13D	c.3151G>A (p.F985fsX14)	Hom.
12	HLH-45	M/16	UNC13D	c.118-307G>A	Het.
13	HLH-47	F/1	UNC13D	c.3152-12delC	Het.
14	HLH-48	F/36	UNC13D	c.3151G>A (p.F985fsX14)	Het.
15	HLH-54	F/42	UNC13D	c.965_967>68bp (p.A318X)	Het.
16	HLH-63	M/1	UNC13D	c.2180G>A (p.R727Q)	Het.
17	HLH-64	M/23	UNC13D	c.2243C>T (p.A748V)	Het.
18	HLH-66	M/2	UNC13D	c.2183-13G>A	Hom.
19	HLH-72	M/37	UNC13D	c.1055+1G>A	Hom.
20	HLH-74	F/16	UNC13D	c.3181C>T (p.R1061W)	Het.
21	HLH-75	M/21	UNC13D	c.859-3C>T	Het.
22	HLH-76	M/19	UNC13D	c.859-3C>T	Het.
23	HLH-82	F/29	UNC13D	c.2296C>T (p.Q766X)	Het.
24	HLH-83	M/31	UNC13D	c.118-307G>A	Het.
25	HLH-86	M/2	UNC13D	c.1232G>A (p.R411Q)	Het.
26	HLH-88	M/3	UNC13D	c.118-307G>A	Het.
				c.3151G>A (p.F985fsX14)	Het.
27	HLH-10	M/1	PRF1	c.1312T>C (p.Y438H)	Het.
28	HLH-24	M/9	PRF1	c.272C>T (p.A91V)	Het.
29	HLH-77	F/18	PRF1	c.503G>A (p.S168N)	Het.
30	HLH-29	M/6	STXBP2	c.430_1107del (p.V144fsX225)	Het.
31	HLH-30	M/37	STXBP2	c.953C>T (p.T318 M)	Het.
32	HLH-38	M/1	STXBP2	c.1430C>T (p.P477L)	Het.
33	HLH-52	M/1	STXBP2	c.1430C>T (p.P477L)	Het.
34	HLH-67	M/1	STXBP2	c.1430C>T (p.P477L)	Het.
35	HLH-13	F/5	STX11	c.568_569insT (p.S190fsX100)	Het.
36	HLH-87	M/1	UNC13D	c.965_967>68bp (p.A318X)	Het.
			STX11	c.122T>C (p.L41P)	Het.

Abbreviations: F, female; Het., heterozygous; Hom., homozygous; M, male.

Three distinctly heterozygous missense mutations in *PRF1* were identified in unrelated patients. The c.272C>T (p.A91V) and c.503G>A (p.S168N) mutations were previously reported in HLH.[12,14] The novel c.1312T>C (p.Y438H) mutation had a PolyPhen-2 score of 1.0, predicted to be pathogenic.

Finally, there were two patients with novel mutations in *STX11*. The first one was heterozygous for a 1-bp insertion, leading to a frameshift (p.S190fsX100). The other patient (HLH-87) carried digenic variants: a novel heterozygous mutation in *STX11* (p.L41P, predicted to be pathogenic by PolyPhen-2 with a score of

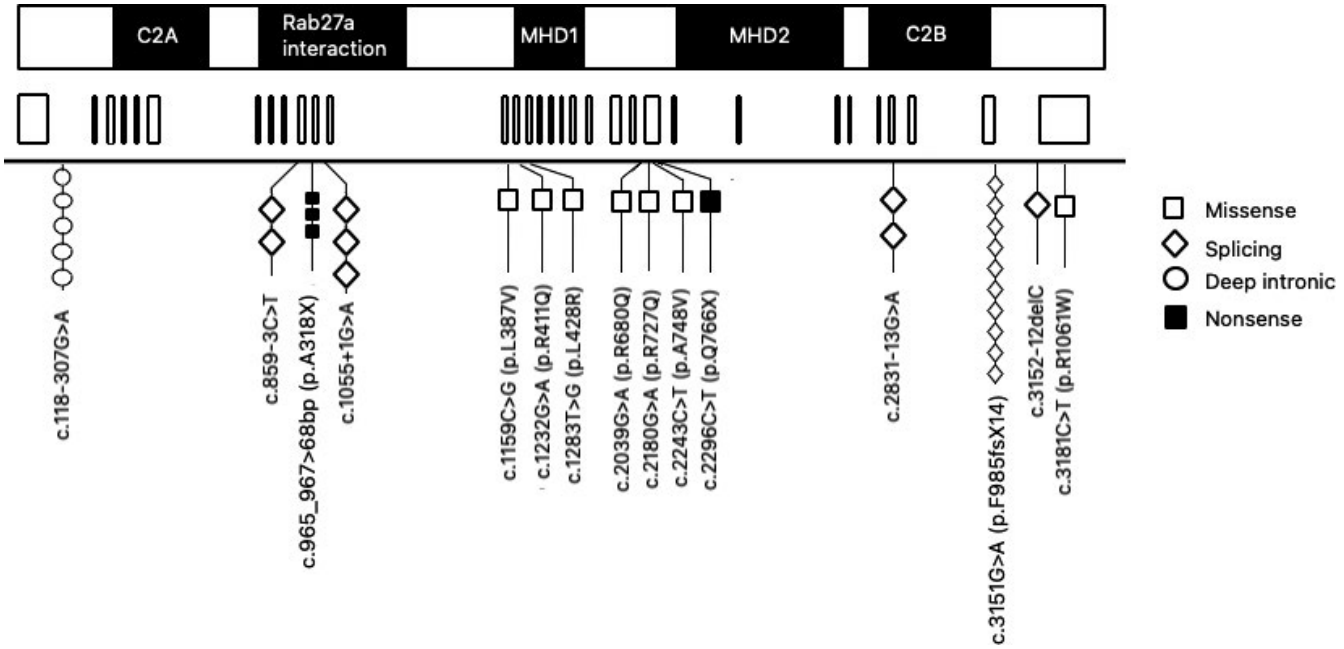
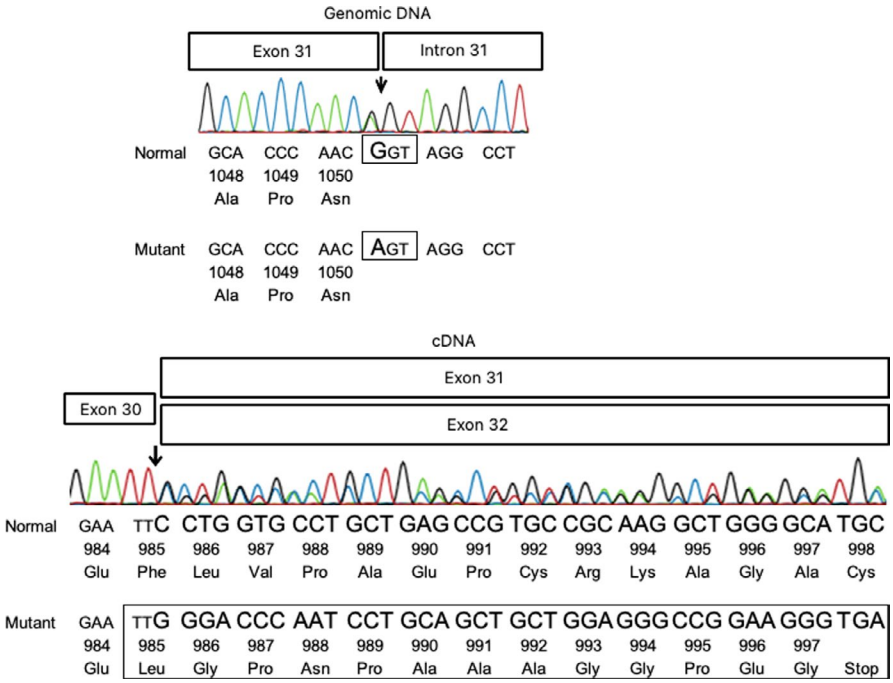


FIGURE 1 Mutation spectrum of *UNC13D* gene in this study

FIGURE 2 The mutation c.3151G>A in genomic DNA of the *UNC13D* gene resulted in exon 31 skipping at the mRNA level



0.999), in combination with a heterozygous mutation in *UNC13D* (c.965_967>68bp).

4 | DISCUSSION

Identifying the causative genes in HLH is crucial for diagnosis, to help choose the most appropriate treatment, including HSCT. We investigated mutations of 4 FHL-related genes in 94 Vietnamese patients and found that 38.29% (36/94) of patients carried the disease-causing mutation. Previous studies have shown that the frequencies of variants in FHL-related genes vary among different ethnic groups, such as 22.5% in Korean patients[8] or 32.83% in Chinese cohort.[14]

Our data on 94 Vietnamese patients showed that *UNC13D* gene mutation was the most common genetic lesion associated with HLH. This result is very different from reports from studies in Caucasian patients, where *PRF1* was most commonly recognized in HLH, possibly up to 50%.[17,18] We only detected 3 out of 94 patients carrying *PRF1* mutations (3.19%) in this study. However, our results are completely consistent with studies in Asian patients, in which *UNC13D* was also the leading genetic lesion associated with HLH.[8,14,16] Interestingly, we found some novel mutations in Vietnamese patients, which have

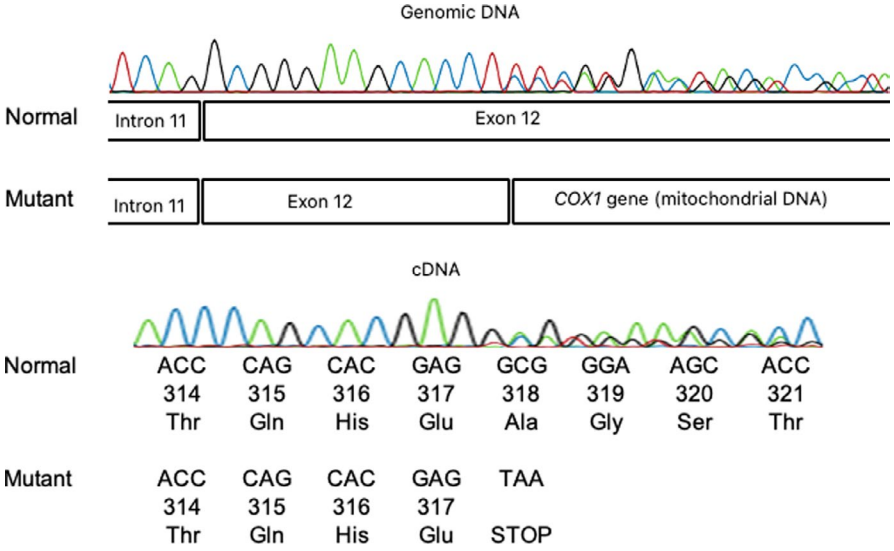


FIGURE 3 The mutation c.965_967>68bp in genomic DNA of the UNC13D gene resulted in a stop codon at 318 position at mRNA level (p.A318X)

never been reported in the literature. The c.3151G>A mutation occurred in the last nucleotide of exon 31, eventually resulted in exon 31 skipping from the mRNA with a premature stop codon. It was also the most noted mutation among the studied patients. In addition, insertion of the *COX1* gene of mitochondrial DNA into the middle of *UNC13D* exon 12 has never been reported before. Of the 7 missense mutations, 3 have been reported previously, while the remaining 4 are novel. All four of these mutations have a PolyPhen-2 score of 1.0, supporting these mutations as pathogenic. Our finding extends the spectrum of possible causative mutations in FHL3.

STXBP2 is the second most frequently mutated gene in our patients, in part due to 3 patients carried the same mutation c.1430C>T (p.P477L). It should be noted that this well-known mutation has been documented mainly in the Middle East,[12] but not in China.[14] Particularly, the large deletion from exons 7 to 13 was discovered thanks to our use of primers designed on introns 6 and 13 for a 1754-bp PCR. If using short amplicons, this heterozygous mutation would not be detected because primers for short PCR could be normally paired up to the remaining wild-type allele.

Among 36 patients with gene mutations, only 8 cases were in homozygous or compound heterozygous state (including 1 case of digenic variants). The remaining cases (77.8%) were monoallelic mutations. Zhang et al found that among 2701 patients referred for genetic testing, only 225 (8%) were homozygous or compound heterozygous for mutations, while 28 (1%) showed digenic inheritance.[13] Similarly, among 281 patients classified as having "sporadic" HLH, Cetica et al confirmed monoallelic mutations in 43, suggesting that this disorder is not a simple recessive one.[5] It seems that additional unidentified genetic defects or possibly even environmental factors may contribute to the development of HLH.[6,19]

In conclusion, this is the first report of spectrum mutations in *PRF1*, *UNC13D*, *STX11*, and *STXBP2* from Vietnamese patients with HLH. Our data showed that *UNC13D* is the predominant genetic lesion associated with HLH in the Vietnamese population. We provided the unique variant spectrum in Vietnamese patients which is important for understanding genetic background of HLH and supporting genetic counseling.

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CONFLICT OF INTEREST

The authors have no competing interests.

AUTHOR CONTRIBUTIONS

PX, HC, and HV designed the study and wrote the manuscript. TD, TN, NV, NA, HN, and PX recruited the patients. PX, HC, TD, and HV designed and performed the experiments. All authors read and approved the manuscript.

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