

# Genetic and clinical characteristics of 54 pediatric lymphoma patients with variant mutation sites of *UNC13D*

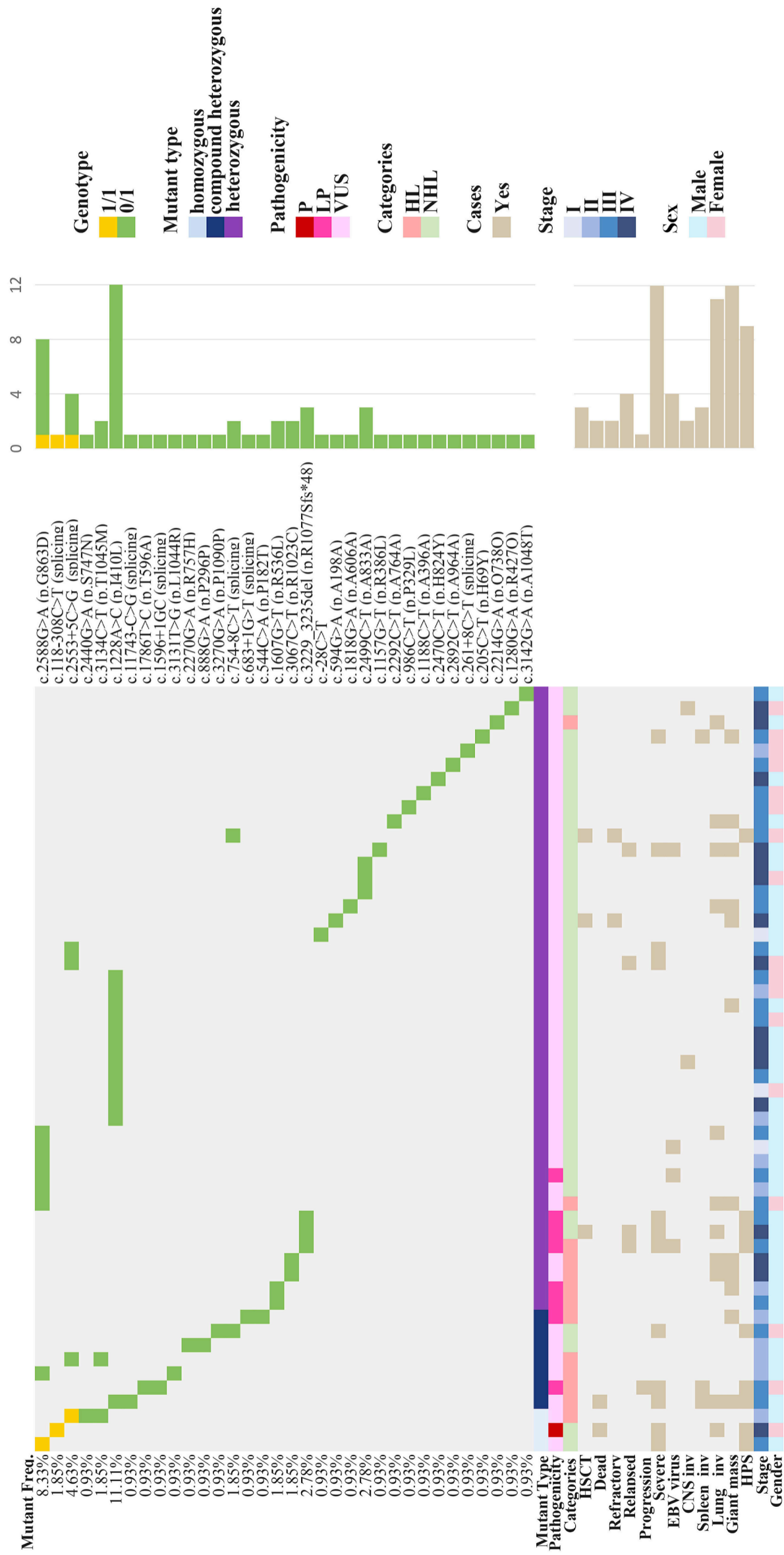
Mutations in the *UNC13D* gene lead to functional defects in cytotoxic lymphocytes (CL) and cause familial hemophagocytic lymphohistiocytosis type 3 (FHL3).<sup>1,2</sup> A proportion of patients with lymphoma have been reported to harbor mutations in the *PRF1*, *UNC13D*, *STX11*, *STXBP2* or *SH2D1A* genes,<sup>3</sup> which cause functional defects in CL, and recent studies<sup>4,5</sup> have shown that defects in these FHL-related genes may increase the risk of lymphoma and Epstein-Barr virus (EBV)-associated T/NK-cell lymphoproliferative disorders, indicating that genetic defects in CL may increase susceptibility to lymphomagenesis. Data regarding the association between genetic defects and the development of lymphoma in pediatric patients are limited to date. Based on emerging evidence that a certain relationship exists between genetic defects in FHL-related genes and lymphoma pathogenesis, in this retrospective analysis, we comprehensively reported the clinical characteristics of 54 pediatric lymphoma patients as well as their genetic features.

A total of 680 children were diagnosed with lymphoma at Beijing Children's Hospital from January 2020 to January 2024, 54 of whom carried *UNC13D* gene mutations. Pathology samples were reviewed and classified based on the World Health Organization (WHO) guidelines.<sup>6</sup> Staging was based on clinical evaluation and was defined by the Ann Arbor or St. Jude staging system. Patients were divided into three groups according to the different risk factors and responses to treatment: low-risk, intermediate-risk, and high-risk. Medical records, including clinical features and genetic findings, were retrieved. Hemophagocytic lymphohistiocytosis (HLH) was diagnosed based on HLH-2004 guidelines.<sup>7</sup> Genomic DNA was isolated from peripheral blood mononuclear cells for whole-exome sequencing, and exfoliated cells of the oral mucosa were matched to determine the germline origin of the variants. Classification of variants were performed per the American College of Medical Genetics and Genomics (ACMG)<sup>8</sup> and the Association for Molecular Pathology (AMP) criteria. Severe cases were defined as patients having been diagnosed with HLH or having manifestations of high tumor burdens, or when patients have superior vena cava compression symptoms or airway obstruction, or symptoms of central nervous system (CNS) invasion or compression. This study has been approved by Institutional Review Board of Beijing Children's Hospital, and written informed consent was obtained from the patients' guardians.

Figure 1 and *Online Supplementary Table S1* show the clinical characteristics of the patients in our study group. The male-to-female ratio was 1.7:1. The median age at clinical diagnosis was 9 years (range, 1-14 years). Thirteen patients

were diagnosed with Hodgkin lymphoma (HL), and 41 patients were diagnosed with non-Hodgkin lymphoma (NHL). A total of 40 patients (74.1%) were diagnosed at stage III and IV, with severe cases accounting for 22.2% of the patients. HLH was diagnosed in nine patients, among which seven cases were concurrent at the time of diagnosis, while another two patients developed secondary HLH during disease progression or relapse. Analysis revealed that patients carrying the *UNC13D* gene with pathogenic/likely pathogenic variants were correlated with the severity of their clinical features and the occurrence of HLH ( $\chi^2=6.943$ ;  $P=0.008$ ;  $\chi^2=11.76$ ;  $P=0.001$ ). However, no correlations were found with mortality or clinical disease staging. The follow-up times of the two groups with and without *UNC13D* mutations (54 patients vs. 626 patients) were 31 (range, 1-60) months and 38 (range, 1-60) months, respectively. The 2-year overall survival (OS) rates of the two groups were 96.3% and 99.5%, respectively, while the 2-year event-free survival (EFS) rates were 83.3% and 96.3%, respectively, with statistically significant differences ( $P=0.0076$ ;  $P<0.0001$ ). Furthermore, by further analyzing the pathogenic/likely pathogenic (P/LP) variants group and uncertain significance (VUS) variants group separately against the wild-type (WT) group, we found that the prognosis of the P/LP group was significantly worse than that of the WT group in terms of both EFS and OS (Figure 2).

Our study revealed that approximately 7.9% of patients carried the *UNC13D* gene mutations. All mutations were confirmed to be germline-derived. A total of 34 different *UNC13D* (NM\_199242.2) mutations were identified in 54 unrelated patients (*Online Supplementary Table S1*), including 44 with monoallelic mutations, three with homozygous mutations and seven with compound heterozygous mutations. The *UNC13D* gene contains 32 exons and 31 introns.<sup>1</sup> In this group of patients, 77.7% and 11.1% of the *UNC13D* gene mutations occurred in exons and introns, respectively; 9.3% of the patients had mutations in both exons and introns, and one patient (1.9%) had an unclear mutation region. The most prevalent *UNC13D* mutations were missense mutations (57.4%), followed by synonymous mutations (16.7%), splicing errors (11.1%) and deletion mutations (5.5%), and 7.4% of patients had both a missense mutation and a splicing mutation. The Munc13-4 protein has four domains (C2A, MHD1, MHD2, and C2B) and a region that interacts with RAB27A. Protein domain analysis revealed that the main affected domain of the Munc13-4 protein in lymphoma patients was the RAB27A domain (29.6%), followed by non-domain involvement (22.4%); C2B and MHD2 each accounted for 16.6%, and C2A and MHD1 each

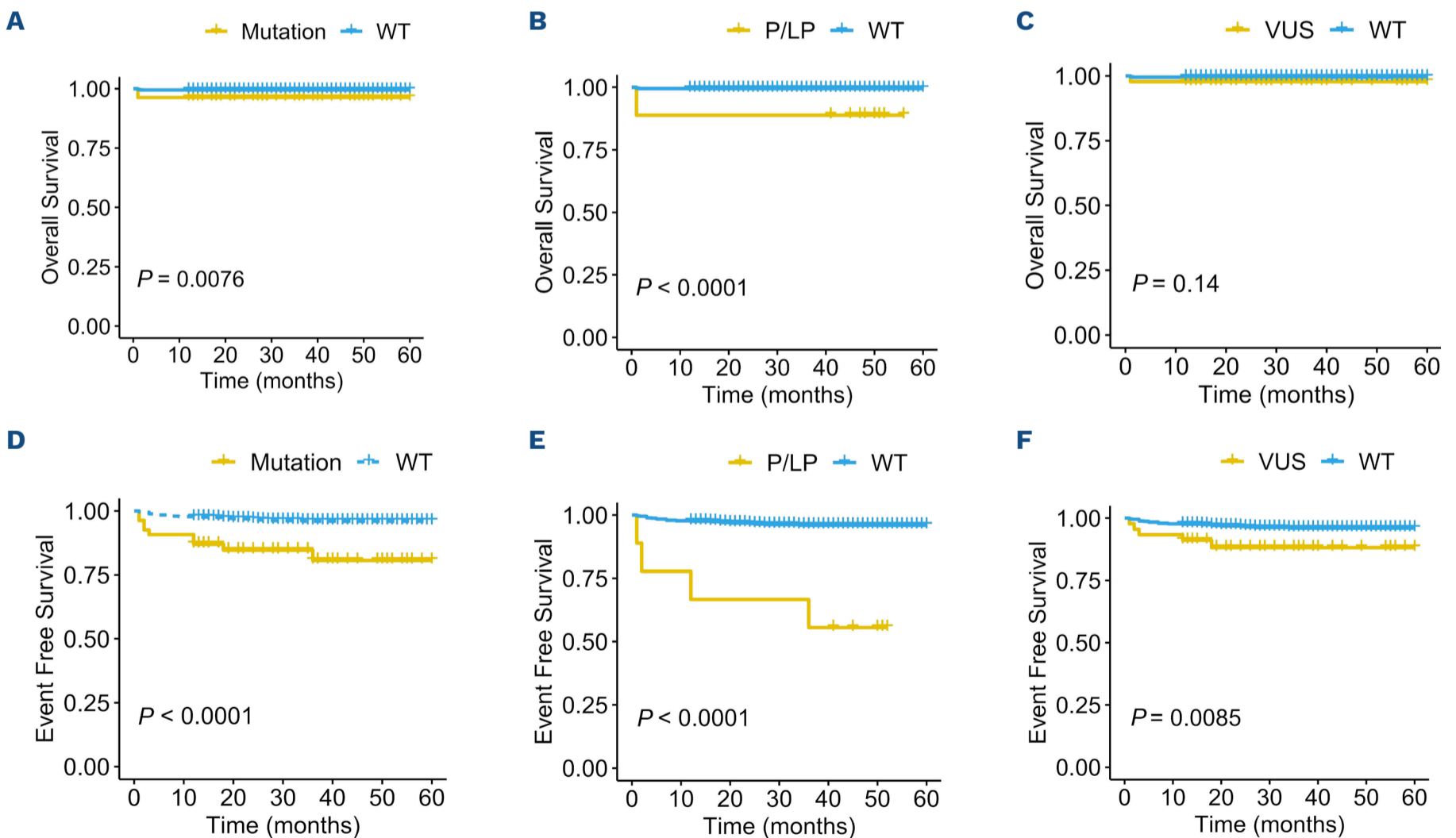


**Figure 1. Distribution of UNC13D mutations and clinical characteristics in pediatric lymphoma patients.** The upper part of the graph shows the frequencies of each mutation site in the present study (green), the lower part displays the main clinical characteristics of patients carrying UNC13D mutations (gray). Each column shows the clinical information of a patient and their type of mutation site. HPS: hemophagocytic syndrome; HSC T: hematopoietic stem cell transplantation; HL: Hodgkin lymphoma; NHL: non-Hodgkin lymphoma; 0/1 represents a heterozygous genotype, where 1 reference allele and 1 alternate allele are present; 1/1 represents a homozygous genotype for the alternate allele, where both alleles at that locus are the alternate form. Freq.: frequency; EBV: Epstein-Barr virus; CNS: central nervous system; inv: involvement; P: pathogenic; LP: likely pathogenic; VUS: variants of uncertain significance.

accounted for 7.4% (Figure 3). Among the 12 patients with severe cases, two had homozygous mutations (c.2588G>A, c.118-308C>T), three had compound heterozygous mutations, and the remaining patients had heterozygous mutations, including three deletion mutations involving the same site (c.3229\_3235del/p.R1077Sfs\*48), two splicing mutations in the same intron region (c.2553+5C>G), and two mutations in the same exon region. Data analysis revealed that the biallelic mutation rate of *UNC13D* in severe cases was 41.7%, which was significantly higher than that in mild cases (11.9%), with a statistically significant difference ( $P=0.019$ ), which may suggest that *UNC13D* biallelic mutations in lymphoma patients are associated with severe clinical manifestations.

In addition, we also identified concomitant mutated genes in pediatric lymphoma patients (*Online Supplementary Figure S1*), most of which were associated with immunodeficient phenotypes. The most common co-mutated gene in this study was the *PRF1* gene (12.9%), followed by *BRCA2* (11.1%). Notably, in two patients with the same intronic mutation (c.2553+5C>G) in the *UNC13D* gene, one experienced clinical recurrence, and the other presented with severe manifestations. This finding also suggests that intronic variants are significant contributors to human disease.

In this study, we first reported the clinical characteristics of pediatric lymphoma patients with different *UNC13D* gene mutations. A total of 34 different mutations were identified in 54 unrelated patients (13 mutations have been reported, and 21 mutations have not been reported). The majority of mutations detected in the current study were heterozygous missense mutations, which was consistent with previous reports;<sup>4,9</sup> this may explain why these patients developed lymphoma later in life rather than having an outbreak of fatal FHL during infancy, which suggests that such monoallelic mutations may contribute to the pathogenesis of the disease. Notably, one patient carried a homozygous disease-causing mutation in intron one of the *UNC13D* gene (NM\_199242:c.118-308C>T), although the initial presentation was an outbreak of fatal hemophagocytic syndrome-like manifestations, the final biopsy pathology revealed EBV-positive diffuse large B-cell lymphoma. Due to the rapid progression of the disease, the patient lost the opportunity for further treatment. Deep intronic variants in *UNC13D* have also been identified in HLH patients from Europe and North America.<sup>10,11</sup> These variants frequently involve the first intron of *UNC13D*, which appears to serve as a key regulatory region, and one variant, c.118-308C>T, has been shown to disrupt transcription factor binding to a

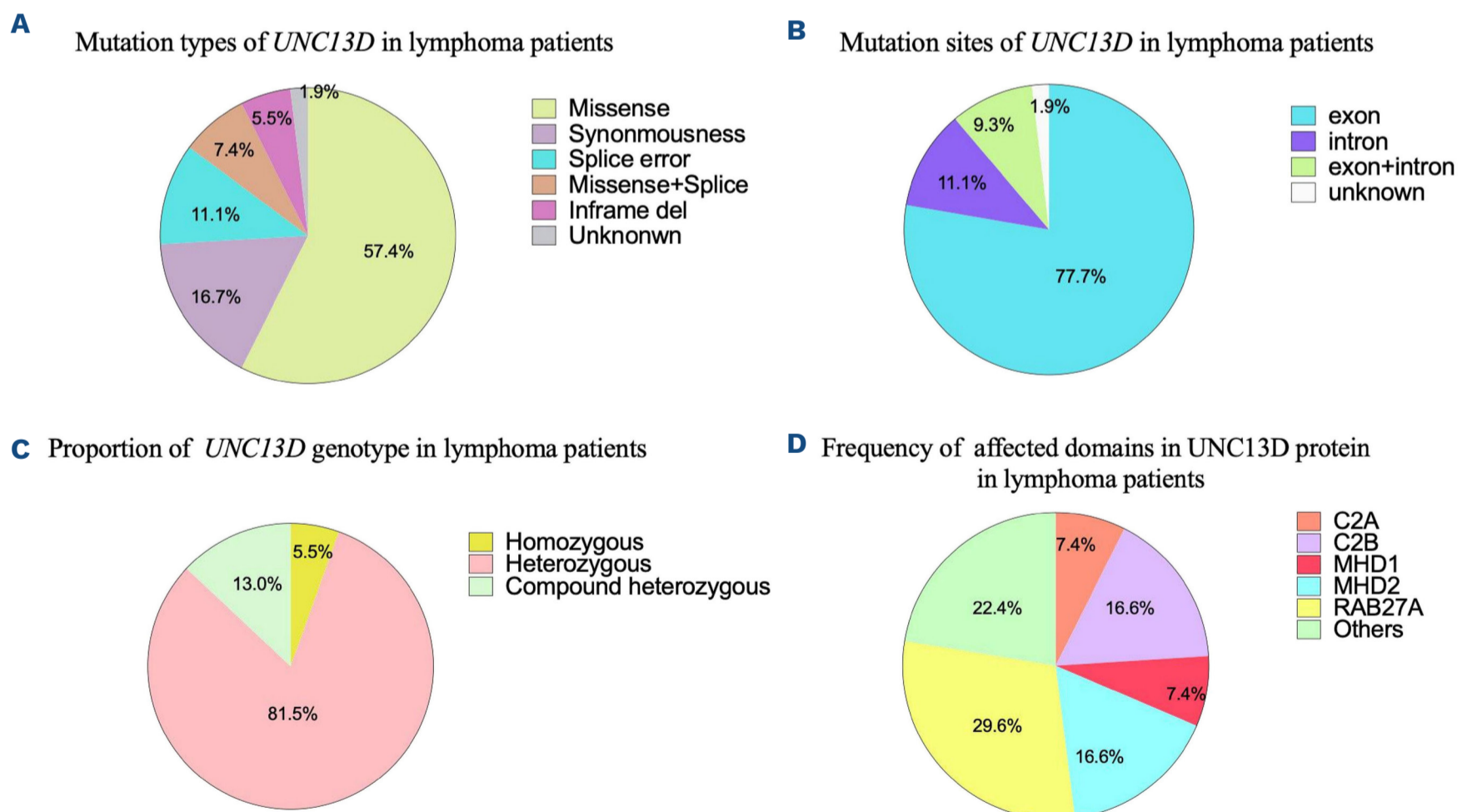


**Figure 2. Kaplan-Meier curves for overall survival and event-free survival.** (A) Overall survival (OS) of the whole study population without *UNC13D* mutations/wide-type group (WT, N=626) and the target group (N=54). (B) Event-free survival (EFS) of the WT group and the target group. (C) OS of the WT group and patients carrying pathogenic/likely pathogenic (P/LP) variants (N=9). (D) OS of the WT group and patients carrying variants of uncertain significance (VUS) (N=46). (E) EFS of the WT group and patients carrying P/LP variants. (F) EFS of the WT group and patients carrying VUS.

cytotoxic lymphocyte-specific alternative promotor, thereby selectively diminishing *UNC13D* expression in cytolytic cells.<sup>12</sup> It is speculated that the genetic defects in CL may increase susceptibility to lymphomagenesis.

This study revealed that a subset of pediatric lymphoma patients carried monoallelic missense mutations in the *UNC13D* gene. Unlike the study by Chen *et al.*,<sup>4</sup> which reported that the variant *UNC13D* c.2588G>A was the founder mutation for lymphoma in the Chinese population, our study demonstrated a higher prevalence of c.1228A>C/p. I410L compared with c.2588G>A/p. G863D in pediatric lymphoma patients (22.2% vs. 14.8%). Twelve c.1228A>C/p. I410L mutations included one compound heterozygous mutation, 11 monoallelic mutations, and eight c.2588G>A/p. G863D mutations included one homozygous mutation, one compound heterozygous mutation and six monoallelic mutations. This single amino acid substitution occurred in an evolutionarily conserved position and was predicted to be pathogenic via the sorting-intolerant form tolerant (SIFT) sorting algorithm. The mutation site of c.1228A>C/p. I410L occurred in the interaction region with RAB27A, and SIFT predicted that its pathogenicity was tolerable. Previous studies<sup>13</sup> have shown that the most prevalent *UNC13D* mutations in FHL3 patients are splice errors (35%) and missense mutations (20.5%), and the rarest mutations are insertions and deletions (1%). In our study, we found that the most common *UNC13D* mutation in pediatric lymphoma patients

was a missense mutation (57.4%). Protein domain analysis revealed that the main affected domains of the Munc13-4 protein in FHL3 patients were the C2A and C2B domains,<sup>13</sup> whereas in pediatric lymphoma patients, the RAB27A interaction domain was the primary domain involved. These findings suggest that the type of mutation in the *UNC13D* gene and the functional domains of the affected protein may influence the clinical phenotype and disease severity. In this study, biallelic mutations in *UNC13D* were found in five of 12 severe cases, all of which were associated with hemophagocytic manifestations, which is consistent with previous reports that compound heterozygous mutations in the *UNC13D* gene may be detrimental to FHL.<sup>14,15</sup> *UNC13D* c.3229\_3235del occurred in three children from unrelated families, all of whom exhibited hemophagocytic manifestations. *UNC13D* c.3229\_3235del was classified as likely pathogenic for lymphoma according to the ACMG guidelines. The deletion mutation results in a frameshift mutation starting from amino acid 1,077 and leads to a premature termination codon in the terminal region. All three patients were co-morbid with HLH, presenting with severe clinical manifestations and poor clinical treatment outcomes. To the best of our knowledge, the recurrent monoallelic deletion mutation c.3229\_3235del/p. R1077Sfs\*48 inducing hemophagocytosis and developing into lymphoma has been reported for the first time, and its underlying pathogenesis remains to be further explored.



**Figure 3. Frequency and type of variants in the *UNC13D* gene.** (A) Type of mutations in pediatric lymphoma patients within exons and introns of the *UNC13D* gene. (B) Distribution of mutations in pediatric lymphoma patients within exons and introns of the *UNC13D* gene. (C) Proportion of *UNC13D* genotype in pediatric lymphoma patients. (D) Proportion of affected domain of *UNC13D* protein in pediatric lymphoma patients.

Yang *et al.*<sup>16</sup> described a case of peripheral T-cell lymphoma and HLH complicated by multiple germline heterozygous mutations, including HLH-specific variants (*UNC13D* and *CD27*), which emphasized that a thorough understanding of both the functional and genetic facets of genetic defects is essential.

In summary, *UNC13D* gene mutations are relatively common among pediatric patients with lymphoma. We found that the prevalence of c.1228A>C/p. I410L was the highest in pediatric lymphoma patients. Lymphoma patients with the c.3229\_3235del/p.R1077Sfs\*48 monoallelic deletion mutation all presented with HLH and showed poor clinical treatment outcomes. Patients carrying the *UNC13D* gene with P/LP variants were correlated with the severity of their clinical features and the occurrence of HLH, furthermore, the prognosis of the P/LP group was significantly worse than that of the WT group in terms of both EFS and OS, and we recommend screening for *UNC13D* gene mutations in newly diagnosed pediatric lymphoma cases. The findings of this study hopefully will prompt more thorough investigations into the relationship between germline defects in cytotoxic lymphocytes and lymphoma pathogenesis.

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<https://doi.org/10.3324/haematol.2025.287782>

Received: March 17, 2025.

Accepted: February 20, 2026.

Early view: March 5, 2026.

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### Disclosures

No conflicts of interests to disclose.

### Contributions

YD designed the research and performed the primary data retrieval. HG analyzed results and wrote the manuscript. HG provided data analysis and plotting. HX analyzed clinical and genetic data for patients at Beijing Children's Hospital. TW supervised the study. LJ, JY, SH, MZ, NL and YL provided clinical data. All authors edited and approved the final manuscript.

### Acknowledgments

Thanks goes to all patients for their consent to participate and to our colleagues for data collection. Revised manuscript language editing was provided by Bethune Pediatric Young and the Middle-aged Doctors' Capacity Enhancement Program.

### Funding

Funding support was provided by the Special Funding Project for Research on Children's Clinical Nutrition (2022E17-B4).

### Data-sharing statement

All data are available on request from the corresponding author.

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