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Application of blood and immunodeficiency gene detection in the diagnosis of hemophagocytic lymphohistiocytosis patients

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To investigate the value of genetic mutations in the pathogenesis and differential diagnosis of hemophagocytic lymphohistiocytosis (HLH), mutations related to blood and immune deficiency genes were analyzed in patients with HLH. Peripheral blood samples from 33 children diagnosed with HLH on the basis of the 2004 diagnostic criteria were collected, and 317 genes related to blood system diseases and 562 genes related to immunodeficiency were detected by second-generation targeted sequencing technology, bioinformatic analysis, and parental verification analysis. A total of 159 mutations related to blood system diseases and immunodeficiency were found in 33 patients, including 7 HLH-related gene mutations (UNC13D, XIAP, LYST, STX11, ITK, PRF1, and SRGN) in 12 patients. UNC13D was found in 6 patients, with the highest frequency. Two cases (6.1%, 2/33) were diagnosed as primary hemophagocytic lymphohistiocytosis (pHLH), and 6 cases (18.2%, 6/33) were diagnosed as primary immunodeficiency disease (PID) or hereditary hematopathy; the remainder were diagnosed as secondary hemophagocytic lymphohistiocytosis (sHLH). It is necessary to detect blood and immunodeficiency genes to exclude the possibility of pHLH, PID, or hereditary hematopathy associated with HLH for children.

Hemophagocytic lymphohistiocytosis (HLH) is caused by excessive activation of lymphocytes and histiocytes, secretion of a large number of cytokines, which eventually leads to fatal inflammatory damage, characterized by damages to multiple organs, rapid disease progression and high mortality. HLH can be divided into primary and secondary HLH according to etiology. Primary HLH includes familial HLH (FLH) and immunodeficiency-related HLH. The former can be classified into five types: FHL1–FHL5. The latter include Chediak–Higashi syndrome 1, Glicelli syndrome II, Hermansky–Pudlak syndrome II, X-linked lymphoid tissue proliferation syndrome 1 and X-linked lymphoproliferative syndrome 2, and interleukin (IL)-2-mediated T-cell kinase deficiency-related lymphoblastic syndrome. Primary HLH is a hereditary disease with high recurrence and poor prognosis, and it is difficult to identify early. Most children die before the primary type is identified, at a mortality rate ranging from 58% to75% [1]. One study reported that patients with inherited HLH mutations survived only 2 months without treatment [2]. In another follow-up study of 249 patients who received the HLH-94 regimen, the researchers found that the mortality rate of familial HLH patients who did not receive hematopoietic stem cell transplantation (HSCT) was

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100%, while 124 patients who received allogeneic hematopoietic stem cell transplantation (allo-HSCT) had a 5-year survival rate of 66% [3]. In addition, asymptomatic carriers of HLH gene mutations also have potential risks. Lucchini et al. [4] reported that asymptomatic carriers of HLH gene mutations could also develop HLH, and that if these carriers undergo allo-HCT treatment in advance, their disease-free survival rate could improve. It was also suggested that the age and prognosis of patients with different gene mutations differ; for example, those with PRF1 mutation had earlier onset, and those with MUNC18-2 dysfunction had lighter onset and longer survival. It has also been reported that HLH patients may have genetic defects associated with immunodeficiency or blood genetic diseases. For example, Chi et al. [5] detected one case of pHLH in immunodeficiency patients, and Wang et al. [6] reported a case of congenital factor VII deficiency with HLH in China. Therefore, early diagnosis and differential diagnosis of HLH patients and timely selection of treatment options are very important for the prognosis of patients. Gene detection is helpful in the diagnosis of primary HLH [7]. By detecting the related genes of HLH patients and understanding the genetic information, we can identify the primary HLH or PID and the blood hereditary diseases combined with HLH in time, which is of great significance in prevention and treatment of the disease. In this study, 33 patients with HLH were tested for blood- and immunodeficiency-related genes to investigate their roles in the pathogenesis and diagnosis of HLH.

Methods

Cases and methods

From January 2016 to December 2018, data on 33 children with HLH and their biological families were collected from the First Hospital of Peking University and the Department of Hematology and Pediatrics of Beijing Children's Hospital. The patients or their parents signed the informed consent, which was approved by the Medical Ethics Committee of the First Hospital of Peking University.

Clinical characteristics of children with HLH

There were 14 males and 19 females, for a ratio of 1:1.3. The youngest was only 34 days, the oldest was 23.67 years, and the median age was 1.75 years. Two cases (6.1%) were within 3 months, 24 cases (72.2%) were between 3 months and 5 years, and 7 cases (21.2%) were older than 5 years. HLH diagnoses were in accordance with the International Organization Cell Association HLH-2004 diagnostic criteria [8] (at least five of eight criteria) (Table 1).

Sample collection and DNA extraction

We collected 4-mL samples of peripheral blood from patients and 2-mL samples from their biological family members with EDTA anticoagulant. DNA was extracted using the FlexiGene DNA Kit (No. 512206, Qiagen, Germany), and stored at −80°C.

Table 1. HLH—2004 diagnostic criteria

Fever
1. Splenomegaly
2. Cytopenias (affecting two of three lineages in the peripheral blood):
Hemoglobin: <90 g/L (in infants <4 wk: <100 g/L)
Platelets: $<100 \times 10^9$/L
Neutrophils: $<1.0 \times 10^9$/L
3. Hypertriglyceridemia and/or hypofibrinogenemia:
Fasting triglycerides: ≥3.0 mmol/L (i.e., ≥265 mg/dl)
Fibrinogen: ≤1.5 g/L
4. Hemophagocytosis in bone marrow or spleen or lymph nodes
No evidence of malignancy
5. Low or absent NK-cell activity (according to local laboratory reference)
6. Ferritin: ≥500 mg/L
7. Soluble CD25 (i.e., soluble IL-2 receptor): ≥2,400 U/mL

Selection of target genes and design of probes

Sequencing primers were designed for the sequencing of the exons and flanking introns of 562 hematological disease-related genes and 317 immunodeficiency-related genes using the Agilent SureDesign (Agilent,USA) online design tool. The number of probes for blood disease-related genes was 19,978, and the size was 1.386 Mbp; there were 15,207 immunodeficiency-related gene probes, and the probe size was 1.061 Mbp. The NEXTSEQ500 sequencer (Illumina, USA) was used for sequencing.

Analysis of sequencing results

The original data obtained with the NEXTSEQ500 sequencer were analyzed by real-time analysis (RTA, Illumina), the Burrows–Wheeler Alignment Tool (BWA), and Genome Analysis Toolkit (GATK) bioinformatics software. The sequencing depth of high-throughput blood and immunodeficiency disease pathogenic gene panels is about 260 × .The coverage with a sequencing depth of 20 × is >93%, and the base with a quality score of 30 is >93%. Sequencing quality was satisfied with the requirements. Variation annotations were made using bioinformatics methods (PolyPhen-2.2.2 software, SIFT, Mutation Taster, NetGenes, ANNOVAR, HGMD, dbSNP, 1000 Genomes). Mutations and variations of the patients and their family members in this article were verified by polymerase chain reaction (PCR)–Sanger sequencing [5].

Results

A number of variants in blood- and immune deficiency-related genes were detected in HLH patients

A total of 159 mutant genes related to blood system diseases and immune deficiencies were detected in 33 children. The frequency of mutant genes was 215. The frequencies of mutations of UNC13D and KMT2D genes were the highest, up to sixfold. Mutations of genes including BRAF, FANCA, VWF, POLE, KMT2A, SPTA1, DTNBP1, TFR2, ANKRD26, and TCN2 appeared three or more times. There were 25 genes that appeared at twice the mutation frequency and 122 genes that appeared once. Among these, 69 genes related to immunodeficiency diseases demonstrated 88 mutations [9], accounting for 40.9%. The main mutation type was missense

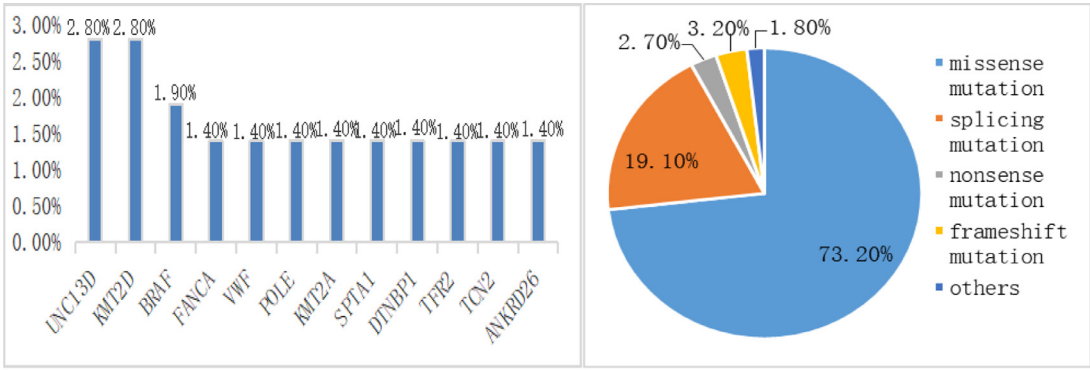


Figure 1. A total of 159 blood system- and immunodeficiency-related genes and the mutation types in 33 patients with HLH. **(A)** Twelve genes with three or more occurrences among 215 gene frequencies, including UNC13D and KMT2D six times, BRAF four times, and the other 9 genes three times. (B) Proportion of mutation types in which the missense mutation accounted for 73.2%, and the splicing mutation, 19.1%.

mutation, accounting for 73.2%, followed by splicing mutation at 19.1%. The percentage of missense mutations predicted by PolyPhen-2 software as ''probably damaging'' or ''possibly damaging'' was 42.9% and that predicted as ''benign'' was 54.7% (Fig. 1).

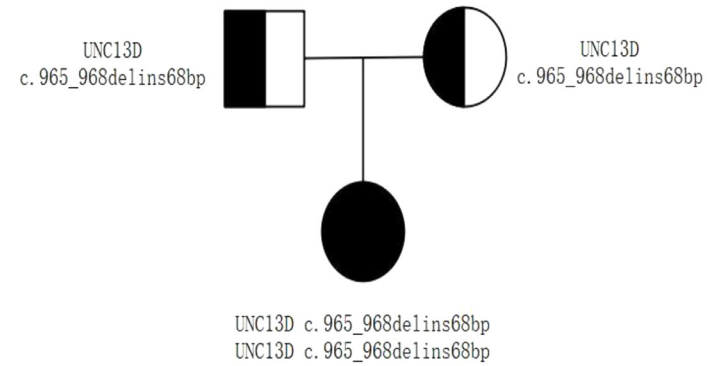
HLH-related gene mutations were detected in many patients, and 2 cases of primary HLH were confirmed by molecular diagnosis

Seven mutant genes related to HLH were detected in 12 children, and the detection rate was 36.4%. UNC13D had the highest frequency of mutation, up to sixfold. Mutations of XIAP, LYST, STX11, ITK, PRF1, and SRGN [10] were detected once respectively. There were 6 missense mutations, 1 nonsense mutation, 1 deletion mutation, 2 frameshift mutations, and 2 splicing mutations. A missense mutation was predicted to be ''probably damaging'' or ''possibly damaging'' in 3 cases by PolyPhen-2 software (Table 2).

HLH-related gene mutations were mostly heterozygous in 12 children. Two of them were in accord with familial inheritance of HLH. In one girl 1 year 2 months of age, the UNC13D gene c.965_968 delins68bp/p.Ser322 IlefsTer96 code-shifting mutation was found. It was a novel homozygous mutation that had not been reported before, and was inherited from parents with heterozygous mutations (non-close relatives married); the patient was diagnosed with "hemophagocytic lymphohistiocytosis, familial,

Table 2. Mutation genes and loci in 10 cases of sHLH

Gender/age	Mutant genes and loci	Inherited from	Amino acid change variation type	PolyPhen-2 predicted	Molecular diagnosis/ genetic method /OMIM
F/2Y1M	NM_199242 UNC13D c.3273+326C>T	Father	Splicing mutation	—	FHL3/AR/608897
F/1Y7M	NM_199242 UNC13D c.175G>A	Father	p.Ala59Thr missense mutation	Benign	FHL3/AR/608897
M/3M	NM_199242 UNC13D c.1978_1979insATTACCG	Father	p.Val660AspfsTer47 frameshift mutation	—	FHL3/AR/608897
M/1Y6M	NM_000081 LYST c.2635_2637del	Mother	p.879del deletion mutation	—	Chediak–Higashi syndrome/AR/606897
F/1Y2M	NM_199242 UNC13D c.766C>T	Mother	p.Arg256Ter nonsense mutation	—	FHL3/AR/608897
M/10Y	NM_199242 UNC13D c.261+8C>T	Mother	Splicing mutation	—	FHL3/AR/608897
F/4Y9M	NM_003764 STX11 c.401C>G	Mother	p.Ala134Gly missense mutation	Possibly damaging	FHL4/AR/
M/3Y5M	NM_002727 SRGN c.295G>A	Mother	p.Gly99Ser missense mutation	Probably damaging	HLH/-/177040
F/23Y8M	NM_005546 ITK c.1741C>T	Father	p.Arg581Trp missense mutation	Probably damaging	Lymphoproliferative syndrome 1/AR/186973
F/1M	NM_005041 PRF1 c.1066C>T	Father	p.Arg356Trp missense mutation	Benign	FHL2/AR/170280



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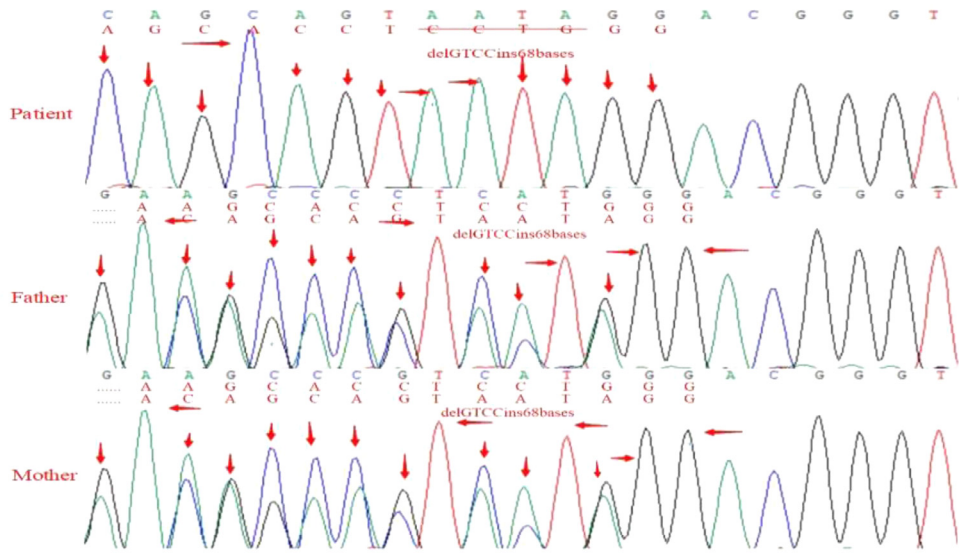

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Figure 2. A girl 1 year 2 months of age diagnosed with FHL3. **(A, B)** Both of her unaffected parents have a heterozygous mutation of c.965_968delins68bp in UNC13D. The girl has a homozygous mutation of c.965_968delins68bp in UNC13D, which she inherited from her parents.

3(FHL3)" (Fig. 2). Another child with a half-zygote variation of the XIAP gene c.1196T > G/p.Ile399Arg was 1 year 8 months old. There was heterozygous variation in this gene locus in the child's mother, so the mutation of the child was inherited from the mother in accord with the inheritance rule of XR. The child was diagnosed with "lymphoproliferative syndrome, X-linked, 2 (XLP2)" (Table 3).

Several patients with PID were diagnosed with HLH

Six children with HLH were found to be in accord with the genetic rules of PID or hematological diseases, 3 with XR and 3 with AR. The mutated genes were FANCB, BLM, ERCC4 (Fig. 3), WAS (2 cases), and LRBA respectively. The mutation sites were all newly discovered mutations that had not been reported before. Three different software (SIFT, PolyPhen-2, and Mutation Taster) were used to predict whether the protein function was damaged (Table 3).

Discussion

Twelve genes related to HLH have been reported: PRF1, UNC13D, STX11, STXBP2, XIAP, SH2D1A, RAB27A, AP3B1, LYST, ITK, CD27, and MAGT1 [11]. Mutations of SRGN [10] have also been reported to be associated with HLH. The distribution of gene mutations differs in different ethnic groups. It has been reported that the UNC13D mutation is the most common mutation in

Table 3. Mutation genes and loci in 2 cases of pHLH and 6 cases of PID with HLH

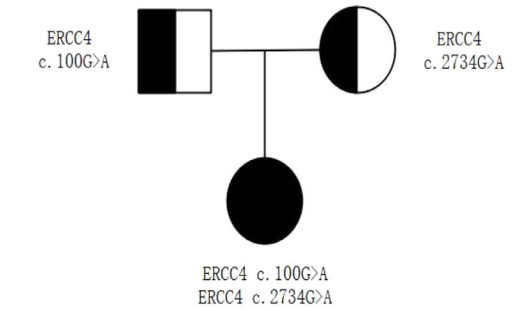
Gender/age	Mutant genes and loci	Inherited from	Amino acid change variation type	Sift/polyphen-2/ mutationtaster predicted	Molecular diagnosis/genetic method/omim
M/1Y8M	NM_001204401 XIAP c.1196T>G	Mother	p.Ile399Arg missense mutation	Neutral/benign/ polymorphism	XLP2/XR/300079
F/1Y2M	NM_199242 UNC13D c.965_968delins68bp c.965_968delins68bp	Mother Father	p.Ser322IlefsTer96 frameshift mutant	—	FHL3/AR/608897
M/10M	NM_000377 WAS c.273+4_273+5insCC	Mother	Splicing mutation	–	Wiskott–Aldrich syndrome/ XR/300392
M/10Y	NM_000057 BLM c.2515A>G c.4163C>A	Mother Father	p.Lys839Glu missense mutation p.Ala1388Glu missense mutation	Neutral/benign/disease causing Neutral/probably damaging /polymorphism	Bloom syndrome/AR/604610
F/3Y	NM_005236 ERCC4 c.100G>A c.2734G>A	Father Mother	p.Val34Met missense mutation p.Gly912Arg missense mutation	Neutral/possibly damaging/disease causing	Fanconi anemia/AR/133520
M/3Y	NM_000377 WAS c.273+4_273+5insCC	Mother	splicing mutation	—	Wiskott–Aldrich syndrome/ XR/300392
M/1Y4M	NM_152633 FANCB c.560A>G	Mother	p.Lys187Arg missense mutation	Neutral/benign/ polymorphism	Fanconi anemia B/XR/ 300515
F/6M	NM_006726 LRBA c.2828T>C c.5149G>A	Mother Father	p.Val943Ala missense mutation p.Val1717Met missense mutation	Neutral/benign/ polymorphism Neutral/benign/disease causing	Common variant immune deficiency with autoimmune type 8/AR/606453

whites, and the PRF1 mutation is common in black and Japanese people [12,13]. In the current study, 12 children with hemophagocytic syndrome-related gene mutations were identified, of whom 6 had UNC13D mutations, and 6 had XIAP, LYST, STX11, ITK, PRF1, and SRGN mutations. Two children were diagnosed with primary HLH with novel mutations that had not been reported before. One had a splicing mutation of UNC13D, diagnosed as FHL3. The other had X-linked lymphoproliferative syndrome caused by a missense mutation of the XIAP gene.

Another 10 cases of HLH gene heterozygous mutations included 1 nonsense mutation, 1 deletion mutation, 1 frameshift mutation, and 7 missense mutations. Three of the 7 missense mutations were predicted as ''probably damaging'' or ''possibly damaging'' by PolyPhen-2 software. The functions of the mutations in the pathogenesis of patients require further study.

In the past, it was thought that a single gene with double allele mutations, that is, a single gene with homozygous or compound heterozygous mutation, could cause primary HLH in children. However, with our deepening understanding of HLH, primary HLH may also be caused by double heterozygous mutations or double-gene inheritance, or may even be related to single-allele mutation. Cetica et al. [14] believe that because of the lack of haploid dosage, patients with heterozygous mutations could show a decrease in the cell killing function. In a retrospective study, Zhang et al. [15] reported that heterozygous defects in 2 genes involved in the degranulation pathway had synergistic harmful effects, suggesting that a dual genetic mutation could lead to FHL. In another study, it was found that mutations of the HLH gene might be associated with delayed HLH, and these heterozygous mutations were classified as subtype mutations [16]. It has been found that heterozygous mutations in adult HLH genes partially impaired the functions of related proteins, which might be associated with the pathogenesis of HLH [17]. Zhang et al. [18] reported that heterozygous Rab27A mutations could reduce cytolytic activity and lead to degranulation of NK cells, and then Rab27A protein could reduce the binding with Munc13-4 and delay the polarization of granulase B to immune synapses. This partial dominant negative effect contributed to HLH, blurring the genetic difference between primary HLH and secondary HLH. In our study, the heterozygous mutations of HLH-related genes may have increased genetic susceptibility to HLH and played important roles in the pathogenesis of HLH.

In addition, 159 kinds of gene mutations related to immune deficiency and blood system diseases were detected. Six children had genetic variations that were in accord with the hereditary rules of hematopathy or PID. Two had WAS gene shearing variations. Four patients had BLM, ERCC4, LRBA, and FANCB gene missense variations respectively. The effects of mutations on protein structure were predicted by SIFT, PolyPhen-2 and



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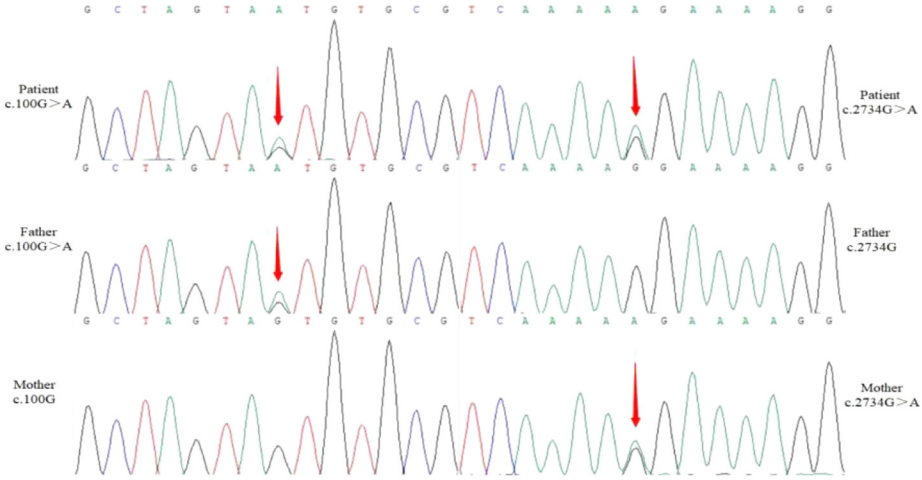

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Figure 3. A 3-year-old girl diagnosed with Fanconi anemia with sHLH. **(A, B)** Her unaffected father has a heterozygous mutation of c.100G>A in ERCC4, and her unaffected mother has a heterozygous mutation of c.2734G>A in ERCC4. The patient, who does not have an HLH-related gene mutation, but has compound heterozygous mutations in ERCC4, inherited the c.100G>A allele from her father and the c.2734G>A allele from her mother. **(C)** The two missense mutations in ERCC4 in the patient are predicted by PolyPhen-2 software, the results indicate that both mutations are ''possibly damaging.''

Mutation software. Three cases showed harmful variation. Previous studies have found that genetic mutations in hematological or immunodeficiency diseases may increase the risk of HLH. Bode et al. [19] found that HLH occurred before PIDs such as Wiskott–Aldrich syndrome were diagnosed. Chinn et al. [20] detected 28 potential pathogenic gene defects in 48 patients without HLH-related gene mutations, including double-allele mutations of NLRC4, NLRP12, and NLRP4, which were associated with PID or immune activation or value-added disorders. Karapinar et al. [21] reported four attacks of HLH in 3 children with congenital neutropenia. In this group, a boy was diagnosed with Bloom syndrome caused by BLM gene mutation. BLM is a gene encoding RecQ helicase, which plays an important role in the development and function of T lymphocytes. Lack of the BLM gene can lead to a decrease in T-cell number and function, including TCR-β dysfunction and damage toCD4+ and CD8+ T-cell responses after antigen attack [22,23]. It is speculated that HLH pathogenesis is related to T-cell dysfunction caused by a BLM gene mutation. The other two cases were complex heterozygous mutations in ERCC4 and LRBA, which were also predicted to be harmful mutations. Patients with hereditary blood diseases and immune deficiency are prone to infection, tumors, immune disorders, and so forth [24,25], which are high risk factors for secondary HLH. In addition, 2.8% of patients have been found to have heterozygous mutations in KMT2D and 1.9% in BRAF. Do the mutations increase the risk of HLH? Our data suggest that mutations in WAS, FANCB, BLM, ERCC4, LRBA, KMT2D, and BRAF genes could increase the risk of HLH, but to date we do not have more experimental evidence.

In this study, 72.2% of the children were younger than 5 years, 8 of the 12 patients with HLH-related gene mutations were younger than 1 year, and 5 of the 6 children diagnosed with hereditary hematopathy or primary immunodeficiency were younger than 3 years. Chinn et al. [20] reported that among the 101 cases, in 19 with familial HLH gene deficiency, onset of the disease occurred within 1 year old, suggesting that patients with gene mutations were younger than those without HLH gene mutation. Therefore, in clinical work, HLH patients under 3 years of age should undergo genetic testing to exclude the possibility of primary HLH, hereditary hematological diseases, or primary immunodeficiency diseases combined with HLH.

Acknowledgements

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