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# Clinical and Genetic Analysis of Nine Suspected Familial Haemophagocytic Lymphohistiocytosis Patients for MUNC13-4 Deficiency and Introducing Four Novel Mutations in *UNC13D*

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**ABSTRACT**

Familial haemophagocytic lymphohistiocytosis (FHL) is a rare disorder of immune dysregulation. FHL inherited in an autosomal recessive pattern is classified into five subtypes based on underlying genetic defects. Mutations in four genes including *PRF1*, *UNC13D*, *STX11* and *STXBP2* are responsible for FHL2 to FHL5 respectively. The cause of FHL1 is associated with mutations in an unknown gene located at 9q21.3-22. This study aims to report the clinical features and genetic results of nine Iranian patients suffering from - haemophagocytic lymphohistiocytosis.

Nine patients (five males and four females) suspected to FHL whose genetic evaluation of *PRF1* and *STX11* revealed no mutations, were entered the study to investigate *UNC13D* mutations. Primers were designed to amplify all coding regions and exon-intron boundaries of the gene. PCR products were then sequenced and analyzed by sequence analysis tools including BLAST.

The most frequent clinical manifestations observed in the patients were fever and hepatosplenomegaly. In this study, five mutations were detected in *UNC13D* including four novel mutations (c.1434_1446delACCCATGGTGCAGinsTGGTGCT, c.1933C>T, c.1389+1G>C and c.2091+1G>A) besides to a previously reported deletion (c.627delT). The pathogenicity of the missense mutation was assessed using online prediction tools including SIFT and PolyPhen2.

The study results may provide valuable information for genetic counseling especially for those who have a history of immunodeficiency diseases in their family and can be used for prenatal diagnosis.

**Keywords:** FHL3; Haemophagocytic lymphohistiocytosis; *UNC13D*

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## INTRODUCTION

Familial haemophagocytic lymphohistiocytosis (FHL) or primary HLH is a rare fatal disorder of the immune system with predisposition to HLH which predominantly characterized byrecurrent episodes of prolonged fever, hepatosplenomegaly, and cytopenia.[1] In FHL, a genetic defect disrupts the cytotoxic activity of cytotoxic T cells (CTLs) and natural killer (NK) cells as two major cell types of the immune system in defending against cancerous and infected cells. To compensate this disruption, T cells increase the production of IFN-γ which is leads to hyperactivation of CTLs and macrophage and hyperproduction of proinflammatory cytokines.[2] Consequently, this uncontrolled immune activity triggers the development of symptoms observed in FHL patients.[3]

Several genes have been identified to be associated with FHL. The gene responsible for FHL type 2, *PRF1*,[4] is located on chromosome 10 (10q22.1) and encodes perforin. Perforin by forming pores in the membrane of target cells paves the way for granzyme proteins to transfer into those cells and induce apoptosis. FHL type 3 is caused by mutations in *UNC13D*.[5] *UNC13D* located on chromosome 17 (17q25.1) encodes a 1090-amino acid protein known as MUNC13-4. MUNC13-4 plays a role in priming of cytolytic granules containing perforin and granzyme proteins. FHL type 4 and 5 are consequences of defects in *STX11* (6q24.2)[6] and *STXBP2* (19p13.2)[7,8] respectively. The products of these two genes are involved in docking cytolytic granules to the plasma membrane. The cause of FHL1 is associated with mutations in an unknown gene located at 9q21.3-22.[9]

FHL predominantly occurs in the first years of life[10] and usually end up to an early death if not treated by hematopoietic stem cell transplantation (HSCT).[11,12] Therefore, early and definitive diagnosis for timely HSCT is of the essence. MUNC13-4 deficiency (OMIM#608898) cannot be distinguished from other genetic causes of FHL and missense or splice site mutations can lead to an atypical clinical presentation, predominantly with hypogammaglobulinemia, recurrent infections and liver and lung granulomatous lesions. FHL3 accounts for about one-third of FHL cases.[13] By expanding our knowledge of this genetically heterogeneous disorder, we can reduce the diagnosis time especially in those patients who have no family history of FHL. This study aims to report the clinical and genetic features of nine FHL patients referred to Immunology, Asthma, and Allergy Research Institute (IAARI).

## MATERIALS AND METHODS

### Patients

Nine patients suspected to have FHL were referred to IAARI between 2006 and 2018. A questionnaire to obtain demographic, clinical and laboratory data according to the diagnostic criteria of HLH (2004-HLH guideline)[12] was completed for each patient, including full cell blood count (CBC), CD markers (CD3, CD4, CD8, CD19 and CD16/56) measurement, liver function tests (alanine transaminase (ALT) and aspartate aminotransferase (AST) levels) and the evaluation of triglycerides, ferritin and coagulation profile[1]. For genetic analysis, initially, the genetic screening of *PRF1* and *STX11* was performed for all patients and patient six's parents samples. Patient 6 died of HLH with unknown etiology prior to providing blood sample for genetic analysis and her parents had been referred to our center to search for possible genetic causes of HLH in their child. Afterward, those patients whose genetic defects in *PRF1* or *STX11* had been identified were excluded and other remaining patients entered this study to be evaluated for mutations in *UNC13D*. The study was approved by the Ethics Committee of IAARI (412/p/375). After taking informed written consents, blood samples were obtained from patients and their parents.

### Genetic studies

Genomic DNA was extracted from whole blood samples according to the manufacturer instructions (High Pure PCR Template Preparation Kit, Roche, Germany). The quality of extracted DNA was assessed using gel electrophoresis and spectrophotometry. PCR primers were designed to amplify all 32 exons and exon-intron boundaries of *UNC13D* (NM_199242). (The primer sequences are available upon request). Amplifications were performed in an Astec thermal cycler for an initial 2-minutes denaturation at 95°C followed by 35 cycles of 95°C for 30 s, 56-60°C (different annealing temperatures were set for various primer pairs) for 30

s and 72°C for 30 s. All PCR products were separated by electrophoresis on 1.5% w/v agarose gels in 1X TAE buffer, stained and finally visualized under ultraviolet light. All PCR products were then purified and sequenced. The sequencing results were viewed by FinchTV program and analyzed by Basic Local Alignment Search Tool (BLAST) in order to characterize mutations.

## RESULTS

### Clinical Results

Nine suspected FHL patients (five males and four females) with no mutations in *PRF1* or *STX11* entered the study for further genetic evaluation in *UNC13D*. All patients were from unrelated families and seven patients had consanguineous parents. The median age of disease onset and diagnosis were 9 months and 16 months respectively. All patients are alive except three including two patients (P2 and P8) who died before HSCT due to the lack of acceptable donors and P7 who died three months after HSCT because of pneumonia. Positive family history of suspected immunodeficiency was detected in two patients, including P1 whose sibling died at age of 2.5 years (due to encephalitis) and P2 whose sibling died at age of 10 months (because of fever, hepatosplenomegaly, and jaundice).

The most frequently reported clinical HLH symptoms in the patients in order were fever (9/9), splenomegaly (8/9), hepatomegaly (6/9), pancytopenia (5/9) and pneumonia (3/9). Central nervous system (CNS) involvement was observed only in one patient (P8) including seizure and cranial neuropathy, aphasia. In MRI study of brain, there was moderate cerebellar atrophy and remarkable decrease in meningeal enhancement in the base of skull. Hypertriglyceridemia was observed almost in all patients. Except for P4, all patients showed hyperferritinemia. Elevated liver enzymes (AST and ALT) were seen in half of the patients. The absolute numbers of CD markers particularly CD16/56 NK cells and CD8 T cells were normal in all patients. Summary of clinical manifestations and laboratory data are shown in Table 1.

### Genetic Results

Genetic studies of patients revealed four novel mutations. Patient 1 had a G to A transition (c.2091+1G>A) in the splicing site of intron 22. Patient 3 had a C to T transition (c.1933C>T) in exon 21. In P6's parents samples, a delins (c.1434_1446delACCCATGGTGCAGinsTGGTGCT) was detected in exon 16. In P8 a G to C transversion (c.1389+1G>C) occurred in splicing site of intron 15. A previously reported frame shift deletion (c.627delT) was also found in P3. For other patients, no mutations of *UNC13D* were identified (Table 2). All mutations were confirmed in heterozygous status in the patients' parents. Genetic findings are shown in details in Table 2.

**Table 1. Clinical manifestations and laboratory findings of familial haemophagocytic lymphohistiocytosis (FHL) patients**

| P. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Gender | M | M | F | M | F | F | M | F | M |
| Onset age (m) | 2 | 7 | 1 | 3 | NA | 17 | 7 | 2 | 3 |
| Diagnosis age (m) | 2 | 7 | 3 | 12 | 24 | 22 | 9 | 60 | 5 |
| Current status | A | D | A | A | A | A | D | D | A |
| Consanguinity | + | + | + | + | + | - | NA | + | + |
| Fever | + | + | + | + | + | + | + | + | + |
| Splenomegaly | + | + | + | + | - | + | + | + | + |
| Hepatomegaly | + | + | - | - | - | + | + | + | + |
| Pancytopenia | + | - | + | + | - | + | - | + | - |
| CNS involvement | - | - | - | - | - | - | - | + | - |
| Lymphadenopathy | - | - | - | - | - | - | - | - | + |
| Pneumonia | - | - | - | + | + | + | - | - | - |
| Increased liver enzymes | + | + | - | - | - | - | + | + | NA |
| Hypertriglyceridaemia | + | + | + | + | + | + | + | NA | NA |
| Hypofibrinogenaemia | - | - | - | - | + | - | - | NA | NA |
| Hyperferritinaemia | + | + | + | - | + | + | + | NA | NA |

M: Male, F: Female, m: month, NA: Not available, A: Alive, D: Dead, CNS: Central nervous system


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**Table 2. Genetic results of familial haemophagocytic lymphohistiocytosis (FHL) patients**

| P. | location | cDNA mutations | Protein alternation | Protein domain | Mutation type | Ref. |
|---|---|---|---|---|---|---|
| 1 | Intron 22 | c.2091+1G>A | - | - | Splice-site | Novel |
| 3 | Exon 8 | c.627delT | p.Val210fsTer39 | C2-1 | Deletion | (19) |
| | Exon 21 | c.1933C>T | p.Arg645Trp | MHD1 | Missense | Novel |
| 6 | Exon 16 | c.1434_1446delACCCATGGTGCAGinsTGGTGCT | p.478_482delGlnProMetValGlninsHisGlyAla | - | delins | Novel |
| 8 | Intron 15 | c.1389+1G>C | - | - | Splice-site | Novel |

C2: Calcium-binding domain, MHD: Munc13-homology domains

## DISCUSSION

Here we have focused on the clinical and genetic features of nine suspected FHL patients referred to IAARI. In familial haemophagocytic lymphohistiocytosis (FHL) which is the primary type of HLH, initial symptoms usually occur soon after birth. According to the study of Sieni et al the most common clinical manifestations of patients with FHL and *UNC13D* mutations were splenomegaly and fever (96% and 89%, respectively).[14] This finding is quite similar to the results of the current study with fever as the most common manifestation being found in all reported patients. Lymphadenopathy is also observed with less frequency. Bicytopenia including anemia and thrombocytopenia is a common laboratory finding in FHL patients, while pancytopenia is reported in untreated cases.[15] Typical biochemical findings are hypertriglyceridemia, hyperferritinemia and reduced level of fibrinogen. Hyperferritinemia provides physicians with a key clue for the diagnosis of FHL.[1]

All patients of the current study developed typical symptoms of HLH in their first two years of life. A short diagnosis time (less than one month), which observed in P1 and P2, is due to the positive family history of HLH observed in these two patients.

The diagnosis of FHL3 was confirmed in four patients as their genetic defects were revealed. FHL3 which resulted from mutations in *UNC13D* accounts for 30-35% of FHL cases.[16] *UNC13D* encodes a peripheral membrane protein known as MUNC13-4. MUNC13-4 consists of four structural domains including calcium-binding domain 1 (C2-1) (residues 98–221), Munc13-homology domain 1 (MHD1) (residues 557–677) Munc13-homology domain 2 (MHD2) (residues 788–895) and calcium-binding domain 2 (C2-2) (residues 912–1019). As the mutation c.2091+1G>A happened at the junction between exon 22 and intron 22, we assumed that it could disrupt the splice donor site and cause an aberrant splicing event. The similar prediction was made for the second splice-site mutation (c.1389+1G>C) found in the junction between exon 15 and intron 15 of P8. The novel missense mutation (c.1933C>T, p.Arg645Trp) found in P3 changes arginine, a positively charged amino acid, to tryptophan, a non-polar, aromatic amino acid, at MHD1 domain. The pathogenicity of this mutation was assessed using two online prediction tools including SIFT (http://sift.jcvi.org/)[17] and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/).[18] This mutation was confirmed as deleterious (score: 0.01) by SIFT, however it was predicted as benign using Polyphen2. Another mutation found in P3 is a previously reported single-nucleotide deletion (c.627delT) which is predicted to result in an early termination codon (p.Val210fsTer39) and generate a truncated nonfunctional protein.[19] The c.1434_1446delACCCATGGTGCAGinsTGGTGCT mutation consists of thirteen-bp deletion, ACCCATGGTGCAG, and seven-bp insertion, TGGTGCT, leads to deletion of five amino acids and synchronically insertion of three other amino acids (p.478_482delGlnProMetValGlninsHisGlyAla) (Figure 1).

This delins occurs in a sequence (residues between 240 and 543) that is essential for direct interaction between MUNC13-4 and RAB27A. It has been demonstrated that disruption of MUNC13-4/ RAB27A interaction prevents cytolytic granules to fuse with the plasma membrane.[20] It should be noted that according to the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/), only one small delins mutation has been reported in *UNC13D* up to date.[14]

The present study investigated the clinical and genetic analysis of nine Iranian suspected FHL patients

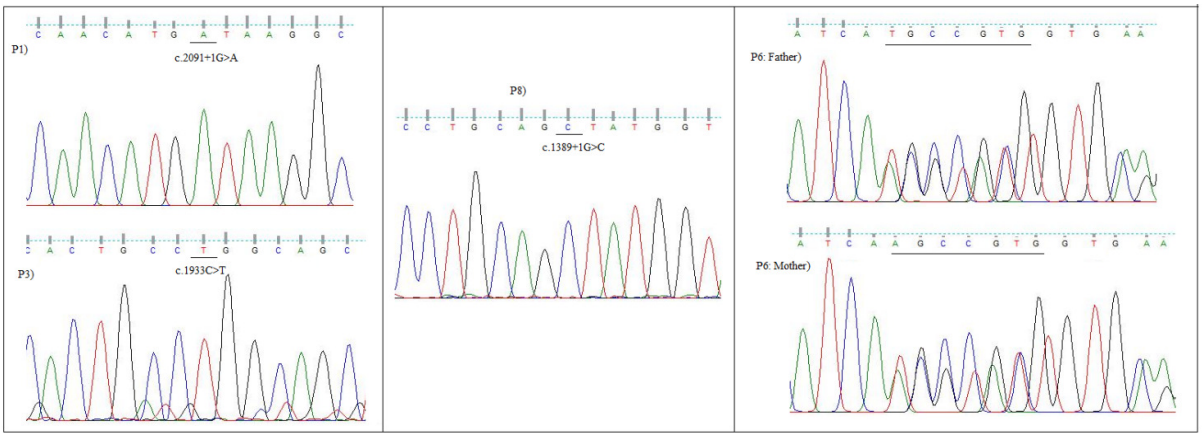


**Figure 1. The novel mutations found in the study of nine suspected familial haemophagocytic lymphohistiocytosis patients, the splicing site defect c.2019+1G>A in P1, the splicing site defect c.1389+1G>C in P8, the missense mutation c.1933C>T in P3and a delins (c.1434_1446delACCCATGGTGCAGinsTGGTGCT) in P6's parents.**

followed up in Immunology, Asthma and Allergy Research Institute. Since in Iran, the consanguineous marriage is common, the study results can provide valuable information for prenatal testing and genetic counseling especially for those who have a history of immunodeficiency diseases in their family.

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