Whole Exome Sequencing for Mutation Screening in Hemophagocytic Lymphohistiocytosis

Edris Sharif Rahmani MSc[1], Majid Fathi MSc[1], Mohammad Foad Abazari MSc[2], Hojat Shahraki MSc[3], Vahid Ziaee Fellow[4,5], Hamzeh Rahimi PhD[6], Arshad Hosseini PhD[1,*]

1. Department of Medical Biotechnology, School of Allied Medicine, Iran University of Medical Science. Tehran- Iran.
2. Department of Genetics, Tehran Medical Sciences Branch, Islamic Azad University, Tehran- Iran.
3. Department of Laboratory Sciences, School of Allied Medical Sciences, Zahedan University of Medical Sciences, Zahedan- Iran.
4. Pediatric Rheumatology Research Group, Rheumatology Research Center, Tehran University of Medical Sciences. Tehran- Iran.
5. Department of Pediatrics, Children's Medical Center, Pediatrics Center of Excellence, Tehran University of Medical Sciences. Tehran- Iran.
6. Department of Molecular Medicine, Biotechnology Research Center, Pasteur Institute of Iran. Tehran-Iran.
*Corresponding author: Dr Arshad Hosseini, Department of Medical Biotechnology, School of Allied Medicine, Iran University of Medical Sciences, Tehran, IR Iran. E-mail: hoseini.a@iums.ac.ir. ORCID: 0000-0002-2077-0817.

Abstract
Background: Hemophagocytic lymphohistiocytosis (HLH) is an immune system disorder characterized by uncontrolled hyper-inflammation owing to hypercytokinemia from the activated but ineffective cytotoxic cells. Establishing a correct diagnosis for HLH patients due to the similarity of this disease with other conditions like malignant lymphoma and leukemia and similarity among its two forms is difficult and not always a successful procedure. Besides, the molecular characterization of HLH due to the locus and allelic heterogeneity is a challenging issue.
Materials and Methods: In this experimental study, whole exome sequencing (WES) was used for mutation detection in a four-member Iranian family with children suffering from signs and symptoms of HLH disease. Data analysis was performed by using a multi-step in-house WES approach on Linux OS.
Result: In this study, a homozygous nucleotide substitution mutation (c.551G>A:p.W184*) was detected in exon number six of the *UNC13D* gene. W184* drives to a premature stop codon, so produce a truncated protein. This mutation inherited from parents to a four-month female infant with an autosomal recessive pattern. Parents were carrying out the heterozygous form of W184* without any symptoms. The patient showed clinical signs such as fever, diarrhea, hepatosplenomegaly, high level of ferritin, and a positive family history of HLH disease. W184* has a damaging effect on cytotoxic T lymphocytes, and natural killer cells. These two types of immune system cells without a healthy product of the *UNC13D* gene will be unable to discharge toxic granules into the synaptic space, so the inflammation in the immune response does not disappear.
Conclusion: According to this study, WES can be a reliable, fast, and cost-effective approach for the molecular characterization of HLH patients. Plus, WES specific data analysis platform introduced by this study potentially offers a high-speed analysis step. This cost-free platform doesn't require online data submission.
Keywords: Hemophagocytic Lymphohistiocytosis, Sequencing, UNC13D

Introduction

Progress in controlling communicable diseases increases the relative importance of non-infectious diseases, including genetic disorders (1). Hemophagocytic lymphohistiocytosis (HL H) is a type of immunodeficiency syndromes characterized by uncontrolled hyper-inflammation due to the high secretion of cytokines from the activated but ineffective cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (2). Clinical symptoms and laboratory findings associated with HLH include prolonged fever, hepatosplenomegaly, cytopenia, hemophagocytosis, decreasing level of fibrinogen, and increasing level of ferritin

and triglycerides (3). The etiology of HLH is now considered as a genetic defect and cause failures in the immune response against infectious agents. So far, 12 distinct genetic defects in responsible genes for the killing ability of CTLs and NK cells have been identified, which are divided into familial HLH (FHL) and syndromes associated with HLH (4). FHL is a rare autosomal recessive disease with an incidence rate of 1.2 births per 100,000, which is more prevalent in children's infancy period (5). Four out of five types of FHL (FHL 2-5) have been determined as a responsible gene associated with FHL. FHL-1 has no specific gene candidate except for a cytogenetic location (9q21.3-22). Perforin deficiency which described by Stepp et al. as the first genetic defect involving in familial HLH, later classified as type 2 of FHL (FHL-2) (6). Table I shows that deficiencies in some other genes are responsible for syndromes associated with HLH, including *GS2*, *CHS*, and *HPS2*. Except for the perforin gene, all other genes are accountable for vesicle priming, fusion, docking, or trafficking of cytotoxic granules in CTLs and NK cells. Other genetic diseases that predispose patients to HLH, mainly induced by Epstein bar virus (EBV) infection, including X-linked proliferative syndromes 1 and 2, CD27 deficiency, Interleukin-2-Inducible T-Cell Kinase (ITK) deficiency, and XMEN syndrome (4, 7). Infectious agents, including EBV, protozoa, bacteria, and fungi, can induce HLH symptoms. However, identifying these agents in HLH patients does not rule genetic HLH out (8).

Clinical signs of genetic and acquired form of HLH are indistinguishable. In other words, because of phenotype similarity between two types and another condition like malignant lymphoma and leukemia (7), the diagnosis of HLH is not always an easy-going procedure (9). Therefore, all of the patients with HLH symptoms are receiving a recommendation for performing a genetic test (10). However, allelic and locus heterogeneity doubled diagnosis difficulty. Generally, the application of conventional direct DNA sequencing methods such as Sanger sequencing (11) for molecular characterization of affected patients, which involves many genes, is a time-consuming and expensive method. Hence, many patients may remain undiagnosed or died due to the delay in initiating a suitable treatment, especially in developing countries. The mean life expectancy for patients who are not receiving proper therapy is about two months, whereas, more than 55% of patients have a positive outcome by suitable treatment (10). Next-generation sequencing (NGS) technologies, especially whole exome sequencing (WES), provide a reliable, fast, and cost-effective approach for clinicians and researchers through the re-sequencing of all coding regions (exome) in one test instead of the gene by gene approach (12, 13). For example, in a study by Mukda et al., evaluation of 25 HLH samples led to the identifying disease-causing mutations in genes, including *PFR1*, *UNC13D, STXBP2, LYST,* and *XIAP* (14). In another study, WES has implemented on 16 HLH samples to the identification of harboring mutations in *PFR1* and *LYST* genes (15). This study aimed to use WES to underlie the mutational landscape of a patient from a four-member family manifesting clinical symptoms of HLH.

Materials and Methods
Patient information
A four-member Iranian family with two children, a four years old boy and a four-month infant girl, participated in this study. Parents were the first cousins. The infant, due to some complications, was hospitalized in the Children Medical Center, Pediatrics Center of Excellence, Tehran, Iran. Specialized clinicians reported clinical symptoms such as fever,

diarrhea, and hepatosplenomegaly. Laboratory investigation had revealed anemia (hemoglobin = 5.9 mg/l) and cytopenia (White blood cell (WBC)=2.12 10^3/µl, Platelet (PLT)=25000 /µl, Lymphocyte (Lymph)=71.3%, Neutrophil (Neut)=15.7%, Monocyte (Mono)=8.5%, and Eosinophil (Eos)=4.4%). The investigation of infectious diseases throughout the culture of blood, stool, urine, and bone marrow aspiration was negative. The result of prothrombin time (PT), partial thromboplastin time (PTT), and international normalized ratio (INR) was 17 secs, 50 secs, and 1.6, respectively. Besides, by first blood evaluation, the C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) levels were 67.2 mg/l and 5 mm/h, respectively. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were 205 and 124 U/L, respectively. Serology for viral infection, including human immunodeficiency virus (HIV), EBV, and viral hepatitis (A, B, C), was negative. Serum immunoglobulin G (IgG) against Cytomegalovirus (CMV) was positive; whereas, immunoglobulin M (IgM) was negative, which means there is no recent viral infection in the patient. Based on the explanation above, probably IgG antibodies of the mother, which transferred into the child placental, caused this incident. Further lab findings showed a decreased level of fibrinogen (58 mg/dl), increased level of ferritin (6200 ng/ml), and an increased level of triglyceride (404 mg/dl). Analysis of bone marrow by flow cytometry showed a lymphocyte population of 84% of total cells that was normal for T and B cells (CD 3= 58%, CD 4= 31%, CD 5= 20%, CD 7= 22%, CD 8= 28%, CD 19= 2%, CD 20=5%, and CD 22= 2%). The expert clinicians confirmed the HLH diagnosis based on existing symptoms and diagnostic criteria (HLH-2004) (16). Additionally, this family lost their previous infant with similar symptoms that was suspected of HLH disease. After initial treatment, WBC and ESR increased to 6700 and 65, respectively, and serum ferritin, ALT, and AST decreased gradually to 450, 76, and 48, respectively.

Ethical Consideration
This study was performed under the ethical permission code of No: IR.IUMS.REC.1394.27436 from Ethical Committees of Iran University of Medical Sciences and earlier, informed consent signed by infant's parent.

DNA extraction and quality control
Peripheral blood collected into a 5 ml EDTA coated vacuum, and then DNA extraction from leukocytes performed with QIAamp DNA Blood Mini kit (Qiagen, Germany, Cat. No. 51104) according to the manufacturer's instructions. The purity of DNA was assessed by spectrophotometry using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of DNA was evaluated by agarose gel electrophoresis.

Whole exome sequencing
Sequencing was performed with Illumina HiSeq 2000/2500 sequencer (Macrogen, Seoul, South Korea) by subjecting two µg high-quality gDNA to Agilent SureSelect Target Enrichment Kit with an average coverage of 100-fold and desired read-length of 101 bp. For increasing sensitivity and reliability detection of duplicate and possible fusions regions, paired-end sequencing was employed. After sequencing, raw data were converted into the Fastq file format by running the bcl2fastq tool (support.illumina.com/downloads/bcl2fastq-conversion-software-v2-20.html).

Bioinformatics analysis
All steps for NGS data analysis was performed on Linux OS. Accordingly, quality control was performed on Fastq files by using the FastQC tool (www.bioinformatics.babraham.ac.uk), then further preparation, such as trimming issues, was performed by the Fastx-toolkit (hannonlab.cshl.edu/fastx_toolkit/). The

alignment of reads against the human reference genome (GRch38) was processed by BWA-MEM (17) and result stored in the SAM (Sequence Alignment/Map) file format. Some post-alignment steps, such as converting SAM file to BAM (Binary Alignment/Map), de-duplicating, and sorting the BAM file, were processed by the Picard tools (broadinstitute.github.io/picard/). Read coverage percentage was performed by Bed tools (v 2.26) (bedtools.readthedocs.io/en/latest/) and integrative genomics viewer (IGV) (v 3) (18), the latter has the capability of direct bam file visualization. GATK pipeline (19, 20) was settled for the base quality score recalibration, SNP (Single nucleotide polymorphism), and INDEL (Insertion and Deletion) calling, INDEL, and SNP realignment, and variants score recalibration by using Genome Analysis Toolkit (GATK v 3.7) (21). Raw variants were stored in a text-based file format, namely VCF (variant call format). A biological knowledge-based mining platform from Kggseq (v 1) tool was used to identify genetic loci responsible for human diseases/traits. According to this multi-step platform, quality assessment, filtration, annotation, and pathogenicity prediction were applied to the variants. For example, allele frequency-based filtering was performed against the 1000 genome project, dbsnp 141, Exome Aggregation Consortium (ExAC r 0.3.1), and ESP6500AA (NHLBI GO Exome Sequencing Project). Then, the Mouse phenotype (22), Deciphering Developmental Disorders (DDD) study (23), OMIM and PubMed database were used for annotation of the remained variants. In addition, dbSNFP (24) was used for functional prediction and annotation of all potential non-synonymous single-nucleotide variants (nsSNVs) by compiling prediction scores from 26 prediction algorithms such as SIFT (25), PolyPhen-2 (26), and CADD. Additionally, the DAVID database was used for further comprehensive functional annotation (27). Pathway and protein-protein interaction analysis were performed by using the KEGG pathway database (28) and STRING database (https://string-db.org), respectively. Plus, Interpro Scan was used for detecting possible domains in the proteins (29).

Sanger sequencing validation

All variants were identified by the WES approach confirmed using Sanger sequencing, which was performed on corresponding regions. PCR primers were designed by Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and checked by primer-blast webtool (Table II). PCR condition was done as follows: 95°C/4min, followed by 30 cycles with 95°C/30 s, 66°C/30 s, and 72°C/45 s. Finally, the result of sequencing was analyzed using the Chromas software v 2.6 (http://technelysium.com.au).

The limitation of this study includes required sophisticated bioinformatics systems and extensive data storage capabilities, which can be costly. Moreover, the amount of produced data, heterozygosity, high repeat content, short DNA read lengths, and the high error rate can impact the outcome.

Results

A four-member Iranian family with children suffering from signs and symptoms of HLH disease was investigated in this study for genetics diagnosis by WES, which is crucial for them. The family pedigree was made after genetic consulting (Figure 1). After performing exome sequencing, quality assessment of raw Fastq files showed that total reads were 66,582,362, with GC constancy of 51%. Besides, more than 99% of reads were above 20 Phred quality score (>Q2O). Moreover, alignment statistics showed that 79% of all reads mapped to the human reference genome (GRch38) with a mean depth of 70 at the target regions. The total number of SNPs and INDELs fond in the

discovery section was 52456. Prioritization step resulted in 110 potentials disease-causing variants (Figure 2). Among these damaging variants and according to the clinical symptoms, known genes, and locus responsible for HLH disease, one homozygous nucleotide substitution in the exon six of *UNC13* homolog D gene (munc13-4 protein) was found that caused a premature stop codon at Tryptophan 184 position (NM_199242: c.551G>A:p.W184*). The minor allele frequency (MAF) for this variant based on the ExAC database was 0.000008. The CADD score was 30, and the LRT prediction score was a deleterious effect. The corresponding region was amplified by a set of primers and specific PCR conditions. Sanger sequencing result showed that candidate variant from WES analysis was a homozygous substitution for the patient, and her parent was carrying a heterozygous form (Figure 3). According to the DAVID server and KEGG database, the gene products of the *UNC13* family (Pathway ID: hsa04721) are the transmembrane protein that functions as the regulators of docking, priming, and fusion of vesicles to the plasma membrane. In the immunologic synapse, munc13-4 motives secretion of lytic granules, and the occurrence of FHL-3 is due to the defect in this protein. The result of the InterPro Scan showed that this protein had two conserved Munc13 homology domain (MHD 1 & 2) flanked by two calcium-binding domains (C2) (Figure 4A). Additionally, protein-protein interaction analysis revealed that the *UNC13D* product participated in the leucocyte mediated toxicity process by interacting with *STXBP2, STX11,* and *RAB27A* (Figure 4B). The finding of this study highlighted a particular platform in whole exome sequencing experiments and speculated on both clinical and research applications of these sequencing platforms for rare complex disorders in the future. This study indicated that WES

experiments were able to identify some rare variants of diseases, including HLH, which consider a life-threating illness.
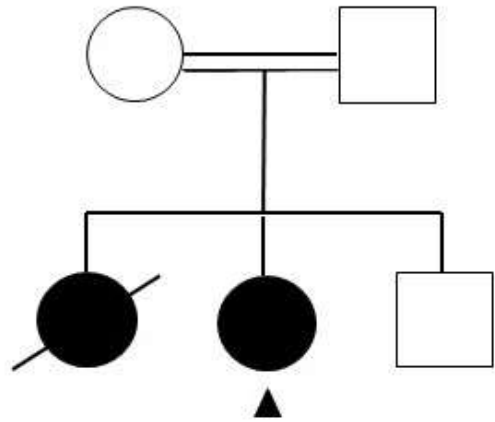

Figure 1. Family Pedigree. Squares and circles indicate males and females, respectively; open symbols indicate unaffected individuals; whereas, filled symbols indicate affected individuals; Proband (triangle) has lost her sister due to the HLH.
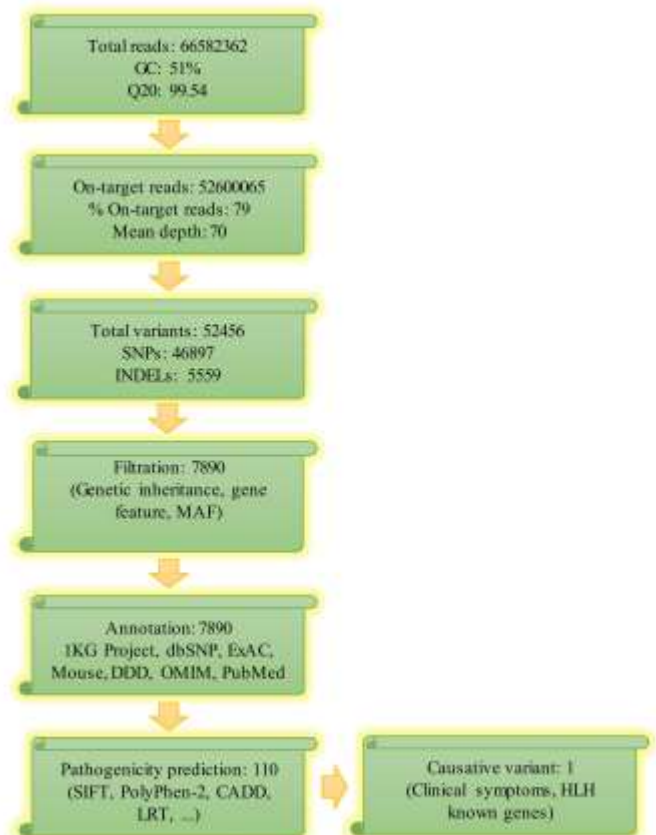
Figure 2. WES data analysis statistics from
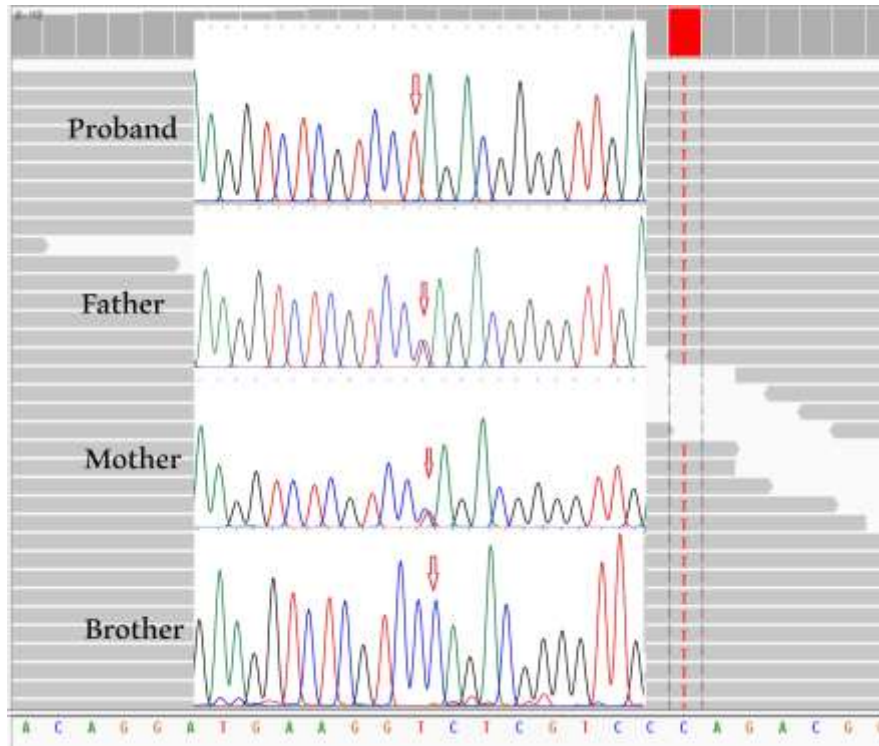raw data to the causative variant

Figure 3. Mutational analysis (WES and Sanger sequencing) of the *UNC13D* gene in the proband and her family. This illustration shows an IGV screenshot of a homozygous substitution (c.551C>T) in the proband and further Sanger sequencing confirmation for the patient (arrow) and her parents (as heterozygous); whereas, her brother is in a healthy state (chromatogram is a result of the reverse strand).
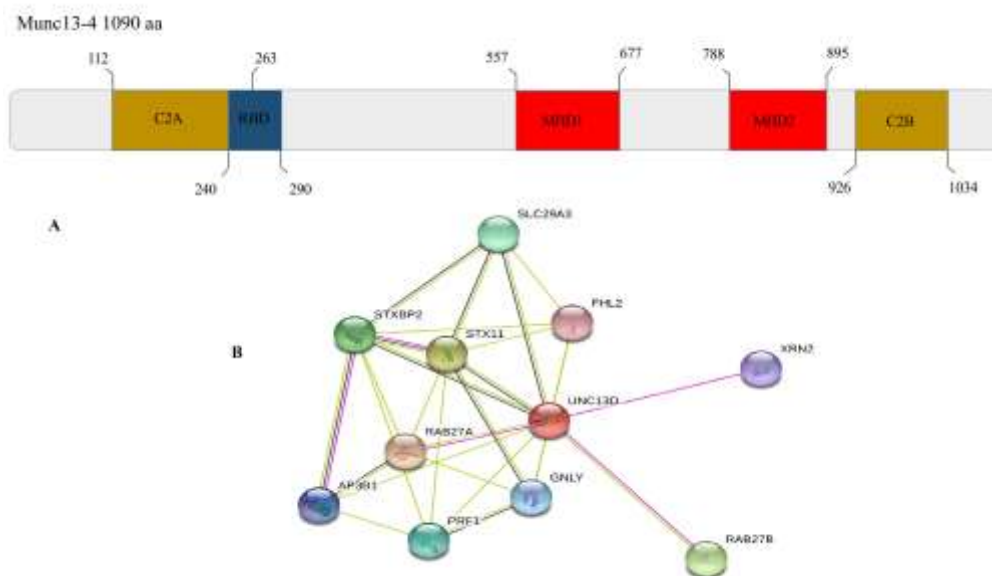

Figure 4. Schematic illustration of munc13-4 domains and specified residue number (A, Based on InterproScan result). The unc13d network revealed that this protein had interaction with ten other

gene products and participated in the leucocyte mediated toxicity process (B, Based on string database).

Table I. The responsible genes for HLH and syndromes associated HLH.

Familial HLH			
Type	Gene	Protein	Location
FHL-1	Unknown	Unknown	9q21.3-q22
FHL-2	PFR1	Perforin	10q21-22
FHL-3	UNC13D	Munc13-4	17q25
FHL-4	STX11	Syntaxin11	6q24
FHL-5	STXBP2	Munc18-2	19p13.2-3
Syndromes associated HLH			
Syndrome	*Gene*	*Protein*	*Location*
Griscelli syndrome type II (GS-2)	RAB27A	Rab27a	15q21
Ché diak-Higashi syndrome (CHS)	LYST	Lyst	1q42.1-q42.2
Hermansky-Pudlak syndrome type II (HPS-2)	AP3B1	**Ap3b1**	5q14.1
EBV associated syndromes			
X-linked proliferative syndrome 1 (XLP-1)	SH2D1A	Sh2d1a	Xq25
X-linked proliferative syndrome 2 (XLP-2)	BIRC4	Birc4	Xq25
CD27 deficiency	CD27	Cd27	12p13.31
ITK deficiency	ITK	Itk	5q33.3
XMEN syndrome	MAGT1	Magt1	Xq21.1

Table II. Forward (F) and reverse (R) PCR primers designed by Primer 3.

Primer	Tm (°C)	Length	Product size (bp)
F 5'-TGACCCTCCTTGCCCCATCAG-3'	63	21	431
R 5'- TGCCTCGTCTTCCCCACTCCT-3'	64	21	

Discussion

The molecular characterization of HLH patients owing to the heterogenic nature of HLH is a challenging issue. Patients also need an early and correct molecular diagnosis. In this study, to understand the possible genetic damage related to the patient, whole exome sequencing on the Illumina HiSeq2500 machine was performed using Agilent SureSelect Human All Exon kit. The familial HLH type 3 (FHL-3, OMIM # 608898) was confirmed in the patient according to the clinical manifestation, pedigree, WES, and Sanger sequencing result. FHL-3 is a genetic defect with autosomal recessive inheritance and responsible for 10%-32% of genetic HLH cases. FHL-3 considered the second most common familial HLH subtype (the first one is FHL-2). This life-threating disease requires an early and

correct diagnosis to prevent compliance and mortality (9, 30). Genetic defects in the *UNC13D* gene (OMIM # 608897) cause the FHL-3. This gene is located on chromosome 17, comprises of 32 exons, which translated to a 120 KD protein, namely munc-13 homolog D or munc13-4 (UniProtID: Q70J99), and functions in the vesicle maturation, docking, priming, and fusion during secretary process (30). It is demonstrated that MHDs region (MHD1 and MHD2) are present in a variety of proteins from human, C.elegans, Drosophila melanogaster, mouse, rat, some of which function as membrane's trafficking regulators. Munc13A and munc13C are brain-specific, while other family members express ubiquitously (31). Human munc13-4 has an expression at a high level in thymus, spleen, peripheral leukocytes, and mast cells (30, 32).

According to a model proposed by Willenbring and Johnson (2017), this protein is involved in the regulation of cytolytic granules secretion in the cytotoxic cells, such as CTLs and NK ones. In other words, munc13-4 plays a vital role in intracellular trafficking. In the immunologic synapses, exocytosis of perforin and granzyme is a responsibility of munc13-4. This event trigger apoptosis in the target cells (33). The hypothetical molecular mechanism behind how munc13-4 works introduced by Elstak et al. in 2011. They explained that secretory event induces by triggering effector cells (such as T lymphocytes) and followed by the formation of a complex consist of small GTPase rab27 and munc13d in the phagocytic cells. Co-localization of these two proteins recruit other effectors and facilitates the tethering of secretory lysosome to the plasma membrane. Tethering can occur when munc13 binds to syntaxin11 or Doc2α, which causes interaction with SNAP-23. Then, the secretory lysosome can fuse to the plasma membrane (34). Genetic defects in the munc13-4 and other effectors engaged in exocytosis machinery dramatically eliminate the killing efficacy of cytotoxic cells, so it provides an environment for insurgent infections, such as HIV, influenza virus, EBV, Cytomegalovirus (CMV), and Streptococcus pneumonia (35, 36). Human Gene Mutation Database (HGMD) contains 113 mutations related to the *UNC13D* gene (www.hgmd.cf.ac.uk), and it confirmed that mutations in this gene lead to FHL-3 (30). In addition, some studies have described an association between variants of the *UNC13D* gene and systemic juvenile idiopathic arthritis (sJIA) (37, 38). The *UNC13D* gene belongs to the patient has a premature stop codon (W184*) producing a truncated munc13-4, which is incompatible with functional protein and finally subjected to cell degradation machinery. Therefore, in the immunologic synapse, stimulated, but ineffective cytotoxic cells secrets high amount cytokines, including Interleukin-1 (IL-1), IL-6, and Tumor Necrosis Factor α (TNFα). These released cytokines cause prolonged fever. Furthermore, the TNFα, along with Interferon γ (INFγ), prevents the proliferation of blood cells and the hydrolysis of triglycerides. Activated macrophages cause an increased level of ferritin, whereas the level of fibrinogen decreased by them. Finally, the clinical picture of familial HLH disease present in the patient (9). To reduce the mortality rate associated with HLH treatment should initiate the elimination of possible infectious agents and activated CTLs. Then administrate drugs such as Dexamethasone, Etoposide, Cyclosporine A. In familial forms, the restoration of the immune system requires Hematopoietic stem cell (HSCs) transplantation (3, 39).

Reports on the implementation of NGS technologies, especially WES, for detecting the harmful mutation in the HLH patients showed the accuracy and effectiveness of these methods. Ferarri et al. (2017) revealed causative mutations in the *UNC13D, STX11*, and *STXBP2* genes in 11 out of 12 patients by using WES (40). Moreover, Qian et al., (2014), by evaluating 1709 HLH samples, reported a 253 Kilo base pair inversion and deep intronic mutations in the *UNC13D* gene. They also discovered the W184* mutation harbored in the two Caucasian patients (41).

Conclusion

In conclusion, one of the more significant findings of this study was that the use of WES for determining the molecular diagnosis of a complex genetic disease like HLH could be a fast, reliable, and cost-effective approach for clinicians and families. Additionally, designed a local multi-step, WES specific data analysis platform, and cost-free introduced by this study potentially offer a high-speed analysis step. No need for online data submission. Users are capable of replicating analysis in all stages.

Conflict of interest
All authors contributed equally to this work and declare that there is no conflict of interest regarding the publication of this article.

References
1. Rahim F, Kaikhaei B, Zandian K, Hoseini A. Co-inheritance of alpha-and beta-thalassemia in Khuzestan Province, Iran. Hematology 2008;13(1):59-64.
2. Mehta RS, Smith RE. Hemophagocytic lymphohistiocytosis (HLH): a review of literature. Med Oncol 2013; 30(4): 740-743.
3. Janka GE. Familial and acquired hemophagocytic lymphohistiocytosis. Eur J Pediatr 2007; 166(2): 95-109.
4. Filipovich AH, Chandrakasan S. Pathogenesis of hemophagocytic lymphohistiocytosis. Hematol Oncol Clin North Am 2015; 29(5): 895-902.
5. Filipovich AH. Hemophagocytic lymphohistiocytosis (HLH) and related disorders. ASH Education Program Book 2009; 2009(1): 127-131.
6. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, et al. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. Science 1999; 286(5446): 1957-1959.
7. Janka GE, Lehmberg K. Hemophagocytic syndromes—an update. Blood Rev 2014; 28(4): 135-142.
8. Janka-Schaub G. Hemophagocytic Lymphohistiocytosis—When The Immune System Runs Amok. Pediatr Blood Cancer 2009; 53(5): 278-285.
9. Janka G. Familial and acquired hemophagocytic lymphohistiocytosis. Annu Rev Med 2012; 63: 233-246.
10. Jordan MB, Allen CE, Weitzman S, Filipovich AH, McClain KL. How I treat hemophagocytic lymphohistiocytosis. Blood 2011; 118(15): 4041-4052.
11. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci1977; 74(12): 5463-5467.
12. Rabbani B, Mahdieh N, Hosomichi K, Nakaoka H, Inoue I. Next-generation sequencing: impact of exome sequencing in characterizing Mendselian disorders. J Hum Genet 2012; 57(10): 621-632.
13. Xuan J, Yu Y, Qing T, Guo L, Shi L. Next-generation sequencing in the clinic: promises and challenges. Cancer Lett 2013; 340(2): 284-295.
14. Mukda E, Trachoo O, Pasomsub E, Tiyasirichokchai R, Iemwimangsa N, Sosothikul D, et al. Exome sequencing for simultaneous mutation screening in children with hemophagocytic lymphohistiocytosis. Int J Hematol 2017;106(2): 282-290
15. Schulert GS, Zhang M, Fall N, Husami A, Kissell D, Hanosh A, et al. Whole-exome sequencing reveals mutations in genes linked to hemophagocytic lymphohistiocytosis and macrophage activation syndrome in fatal cases of H1N1 influenza. J Infect Dis 2015; 213(7): 1180-1188.
16. Henter JI, Horne A, Aricó M, Egeler RM, Filipovich AH, Imashuku S, et al. HLH 2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. Pediatr Blood Cancer 2007; 48(2): 124-131.
17. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 2009; 25(14): 1754-1760.
18. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol 2011; 29(1): 24-26.
19. DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, et al. A framework for variation discovery and genotyping using next-generation DNA

sequencing data. Nat Genet 2011; 43(5): 491-498.

20. Van der Auwera GA, Carneiro MO, Hartl C, Poplin R, del Angel G, Levy Moonshine A, et al. From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. Curr Protoc Bioinformatics 2013;43:11.10. 1-33.

21. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res 2010; 20(9): 1297-1303.

22. Eppig JT, Blake JA, Bult CJ, Kadin JA, Richardson JE, Group MGD. The Mouse Genome Database (MGD): facilitating mouse as a model for human biology and disease. Nucleic Acids Res 2014; 43(D1): D726-D736.

23. Firth HV, Richards SM, Bevan AP, Clayton S, Corpas M, Rajan D, et al. DECIPHER: database of chromosomal imbalance and phenotype in humans using ensembl resources. Am J Hum Genet 2009; 84(4): 524-533.

24. Liu X, Wu C, Li C, Boerwinkle E. dbNSFP v3. A One stop database of functional predictions and annotations for human nonsynonymous and Splice Site SNVs. Hum Mutat 2016; 37(3): 235-241.

25. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res 2003; 31(13): 3812-3814.

26. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Meth 2010; 7(4): 248-249.

27. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2008; 37(1): 1-13.

28. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000; 28(1): 27-30.

29. Finn RD, Attwood TK, Babbitt PC, Bateman A, Bork P, Bridge AJ, et al. InterPro in 2017—beyond protein family and domain annotations. Nucleic Acids Res 2016; 45(D1): D190-D199.

30. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, et al. Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). Cell 2003; 115(4): 461-473.

31. Henriette K, Hofmann K, Brose N. Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform. Biochem J 2000; 349(1): 247-253.

32. Neeft M, Wieffer M, De Jong AS, Negroiu G, Metz CH, Van Loon A, et al. Munc13-4 is an effector of rab27a and controls secretion of lysosomes in hematopoietic cells. Mol Biol Cell 2005; 16(2): 731-741.

33. Willenbring RC, Johnson AJ. Finding a Balance between Protection and Pathology: The Dual Role of Perforin in Human Disease. Int J Mol Sci 2017; 18(8): 1-18.

34. Elstak ED, Neeft M, Nehme NT, Voortman J, Cheung M, Goodarzifard M, et al. The munc13-4–rab27 complex is specifically required for tethering secretory lysosomes at the plasma membrane. Blood 2011; 118(6): 1570-1578.

35. Beutel G, Wiesner O, Eder M, Hafer C, Schneider AS, Kielstein JT, et al. Virus-associated hemophagocytic syndrome as a major contributor to death in patients with 2009 influenza A (H1N1) infection. Crit Care 2011; 15(2): R80-R85.

36. Janka G, Imashuku S, Elinder G, Schneider M, Henter J-I. Infection-and malignancy-associated hemophagocytic syndromes: secondary hemophagocytic lymphohistiocytosis. Hematol Oncol Clin North Am 1998; 12(2): 435-444.

37. Zhang K, Biroschak J, Glass DN, Thompson SD, Finkel T, Passo MH, et al. Macrophage activation syndrome in patients with systemic juvenile idiopathic

arthritis is associated with MUNC13-4 polymorphisms. Arthritis Rheumatol 2008; 58(9): 2892-2896.

38. Hazen MM, Woodward AL, Hofmann I, Degar BA, Grom A, Filipovich AH, et al. Mutations of the hemophagocytic lymphohistiocytosis–associated gene UNC13D in a patient with systemic juvenile idiopathic arthritis. Arthritis Rheumatol 2008; 58(2): 567-570.

39. Henter J-I, Samuelsson-Horne A, Arico M, Egeler RM, Elinder G, Filipovich AH, et al. Treatment of hemophagocytic lymphohistiocytosis with HLH-94 immunochemotherapy and bone marrow transplantation. Blood 2002; 100(7): 2367-2373.

40. Fager Ferrari M, Leinoe E, Rossing M, Norström E, Strandberg K, Steen Sejersen T, et al. Germline heterozygous variants in genes associated with familial hemophagocytic lymphohistiocytosis as a cause of increased bleeding. Platelets2018;29(1):56-64.

41. Qian Y, Johnson JA, Connor JA, Valencia CA, Barasa N, Schubert J, et al. The 253-kb inversion and deep intronic mutations in UNC13D are present in North American patients with familial hemophagocytic lymphohistiocytosis 3. Pediatr Blood Cancer 2014; 61(6): 1034-1040.