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IDENTIFICATION OF THE CAUSATIVE MUTATION IN THE *ITGB2* GENE IN A LAD1 PATIENT BY WHOLE EXOME SEQUENCING

Nguyen Thi Kim Lien[1,✉], Nguyen Thi Van Anh[2], Nguyen Van Tung[1,3], Duong Anh Linh[1], Nguyen Thi Phuong Mai[4], Nguyen Huy Hoang[1,3]

[1]*Institute of Genome Research, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[2]*Allergy, Immunology and Rheumatology Department, Vietnam National Hospital of Pediatrics, 18/879 La Thanh, Dong Da District, Hanoi, Vietnam*
[3]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[4]*Human Genetics Department, Vietnam National Hospital of Pediatrics, 18/879 La Thanh, Dong Da District, Hanoi, Hanoi, Vietnam*

✉To whom correspondence should be addressed. E-mail: ntkimlienibt@gmail.com

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SUMMARY

Leukocyte adhesion deficiency type 1 (LAD1) is a rare congenital immunodeficiency disease. The cause of disease is determined to be the mutations in the *ITGB2* gene that codes for CD18, the beta chain of beta-2 integrins, leads to decreased expression or functioning of CD18. This deficiency leads to severe impairment of leukocyte adhesion to the vascular wall and leukocyte migration to sites of infection and inflammation. LAD1 has also been associated with inhibition of interleukin-23 and interleukin-17 resulting in a hyperinflammatory and chronic inflammation. Patients with LAD1 typically present in early infancy with recurrent, life threatening infections that are frequently fatal before 2 years of age without hematopoietic stem cell transplant (HSCT). However, LAD1 is difficult to diagnose and many LAD1 patients die at a young age despite intensive antibiotic therapy. Accurate diagnosis requires detailed clinical information (delayed umbilical cord loss, severe periodontitis, delayed wound healing and sores, skin abscesses, and recurrent infection), and confirmation the absence of integrins by flow cytometric analysis. A better understanding of the molecular characteristics of this disease is needed to raise awareness and definitive diagnosis infants with LAD1. To definitive diagnosis, whole exome sequencing and Sanger sequencing were performed in an eighteen-month-old boy with severe leukocytosis, recurrent infections, delayed wound healing, and hepatosplenomegaly associated with an acquired cytomegalovirus infection. Two variants: One previously reported mutation (c.533C>T, p.Pro178Leu) and one novel variant (c.59-1G>A), in the *ITGB2* gene were detected. These results can be used for definitive genetic diagnosis, genetic counseling, as well as a prenatal diagnosis in LAD1 patients.

Keywords: LAD1 patient, mutation, the *ITGB2* gene, immunodeficiency, infant, WES

INTRODUCTION

The leukocyte adhesion cascade system allows for leukocyte accumulation at sites of tissue inflammation and infection. Leukocyte adhesion molecules including selectins, integrins, and proteins of the immunoglobulin superfamily play an important role in the

movement of leukocytes in the vessels and into tissues (Schmidt *et al*., 2013). The leukocyte adhesion deficiency disease (LAD) is divided into 3 subgroups including LAD1 (beta-2 integrin defect), LAD2 (fucosylated carbohydrate ligands for selectins are absent), and LAD3 (activation of all beta integrins is defective) (Al-Herz *et al*., 2011).

Leukocyte adhesion deficiency type 1 - LAD1 (OMIM 600065) is a rare type of primary immunodeficiency disease with a prevalence of 1 in 1,000,000 live birth (Hanna & Etzioni, 2012). LAD1 is characterized by delayed umbilical cord separation, recurrent severe bacterial infections, absence of pus formation, periodontitis, delayed wound healing, and often persistent leukocytosis, especially neutrophilia (Etzioni, 2009). LAD1 is the consequence of mutations in the *ITGB2* gene that is located on chromosome 21 (21q22.3), encodes the β2 subunit of the integrin molecule CD18 protein (Schmidt *et al*., 2013), and shows an autosomal recessive pattern of inheritance (Thakur *et al*., 2013). β2 integrins family has critical roles on leukocyte adhesion, functions in immune and inflammatory reactions such as adhesion of leukocyte to the endothelial cell, transendothelial migration, and chemotaxis (Yashoda-Devi *et al*., 2011). These mutations influence the function of neutrophils and lymphocytes such as proliferation, cytotoxic T lymphocyte response, and natural killer cell (NK) activity (Kuijpers *et al*., 1997). And these mutations lead to a deficiency and/or defect of the CD18 resulting in leukocytes being unable to attach to the endothelium and to migrate into the tissues (van de Vijver *et al*., 2012). LAD1 has also been associated with inhibition of interleukin-23 and interleukin-17 resulting in a hyperinflammatory and chronic inflammation (Moutsopoulos *et al*., 2014).

Some patients with severe forms may die due to delayed diagnosis so an accurate and early diagnosis becomes very important. The early diagnosis of immunodeficiency is essential for treatment with hematopoietic stem cell transplantation (HSCT). Diagnosis of LAD1 based on typical clinical manifestations, combined with laboratory evidence of leukocytosis and reduction of CD18 expression, and molecular characteristic to confirm the diagnosis. However, a definitive diagnosis of LAD1 in infancy is challenging because the lack of typical clinical presentations and heterogeneity in the *ITGB2* mutations results in different clinical features of this disease (Shaw *et al*., 2001). Better understanding the molecular characterization of this disease is necessary to increase awareness and identification of infants with LAD1. Though LAD1 has been studied, so far only 500 cases and 126 mutations in *ITGB2* have been reported (Madkaikar *et al*., 2015). And now, whole exome sequencing (WES) has become a more cost-effective and faster tool for analysis of the disease-causing mutations in many genetic diseases including LAD1.

In this study, whole exome sequencing and Sanger sequencing were performed to confirm the definitive diagnosis of LAD1 for an 18-month-old boy with severe leukocytosis, recurrent infections, delayed wound healing, and hepatosplenomegaly associated with an acquired cytomegalovirus infection.

MATERIALS AND METHODS

Patient

An 18-month-old boy with severe leukocytosis, recurrent infections, delayed wound healing, and hepatosplenomegaly associated with an acquired cytomegalovirus infection was initially diagnosed with LAD at The Allergy, Immunology and Rheumatology Department, Vietnam National Hospital of Pediatrics.

Blood samples from the patient and the members of patient's family were collected in blood collection tubes and stored at -20ºC until use.

Ethics

All experiments performed by relevant guidelines and regulations based on the experimental protocol on human subjects which was approved by the Scientific Committee of Institute of Genome Research, Vietnam

Academy of Science and Technology under reference number 01/QD-NCHG.

Whole exome sequencing analysis

Genomic DNA was isolated from peripheral blood samples (including sample from patient and members in family) using a QIAamp DNA Blood Mini preparation kits (QIAGEN, German) following the manufacturer's guidelines. Whole exome sequencing (WES) was performed on the Illumina sequencing machine (Illumina, CA, USA) using the Agilent SureSelect Target Enrichment kit (Illumina, CA, USA) for preparation the library and the SureSelect V7-Post kit (Illumina, CA, USA) for sequencing. The reads were mapped on the genome reference (GRCh38) and then were analyzed by BWA, Picard, GATK, SnpEff softwares for determination the variants and annotation and prediction of the effects of variants on genes.

Mutation analysis in the *ITGB2* gene

The exons and exon-intron boundaries of the *ITGB2* gene were amplified and analyzed by direct sequencing (Mortezaee *et al*., 2015). Primers were synthesized and purchased from PhusaBiochem Company (Cantho, Vietnam) for PCR amplification that was carried out on an Eppendorf Mastercycler EP gradient (USA Scientific, Inc). DNA sequencing was performed on ABI PRISM 3500 Genetic Analyzer machine (USA). Sequencing data were analysed and compared with the *ITGB2* gene sequence published in Ensembl (ENSG00000160255) using BioEdit software version 7.2.5 to detect mutations.

***In silico* analysis**

To study the effect of mutations on the splicing signals, we used MutationTaster (Schwarz *et al*., 2010) and MaxEntScan (Yeo & Burge, 2004).

RESULTS

We sequenced and analyzed the exome of the patient who diagnosed with LAD disease. After target enrichment, whole exome DNA libraries from the patient was sequenced in 150 bp paired-end reads. A total of 9.36 Gb data was obtained and the coverage of the target region for the sample was over 99.7% (Table 1).

Table 1. Data summary of exome sequencing.

Total read bases (bp)	**9.355.350.554**
GC (%)	49.5
Q20 (%)	98.0
Q30 (%)	94.2
Total reads	62.639.402
Average read length (bp)	149.35
Initial mappable reads (%)	62.570.824 (99,8)
Non-redundant reads (%)	55.260.116 (88,3)
Coverage of target region (50X)	73.7%

First of all, the reads were mapped onto the reference genome (the GRCh38 version) using the BWA and Picard software. After that, only uniquely mapped sequences (target and adjacent regions) were used for variants detection. Variations (SNPs, Indels...) in the coding region were determined by the GATK software, the genetic variations were annotated and predicted the impact of these genetic variations by SnpEff software. In total, 82,288 SNPs including 12,182 synonymous variants, 11,626 missense variants, 334 frameshift variants and 11,120 indels were detected in the patient (Table 2). Among that, 6 SNPs were detected in the *ITGB2* gene. Two variants that may be the cause of disease in the patient in the *ITGB2* gene were identified: one previously reported mutation (c.533C>T, p.Pro178Leu) and one novel variant (c.59-1G>A).

The dbSNP142 Database (https://ftp.ncbi.nlm.nih.gov/snp/) was used to determine that the variant was novel. Besides, the impact possibility of the novel variant was predicted by using *in silico* tools such as Mutation Taster and MaxEntScan. Protein function prediction results (Table 3) showed that

the novel variant (c.59-1G>A) was a novel polymorphism (with Mutation Taster analysis) but as a novel splice acceptor variant (with MaxEntScan analysis) in LAD1 patient.

Table 2. Summary of variants that found in patient (in the *ITGB2* gene).

SNP (in the *ITGB2* gene)	82,288 (6)
Synonymous variants (in the *ITGB2* gene)	12,182 (1)
Missense variants (in the *ITGB2* gene)	11,626 (2)
Frameshift variants (in the *ITGB2* gene)	334 (1)
Stop gained/stop lost (in the *ITGB2* gene)	109/41 (0)
Inframe insertion/inframe deletion (in the *ITGB2* gene)	197/212 (0)

Table 3. The prediction results of the mutation *in silico* analysis.

Mutation	MutationTaster score	Prediction	MaxEntScan score	Prediction
c.59-1G>A	0.99	Polymorphism	Wild type: -23.55; Mutation: -32.30	Splice acceptor

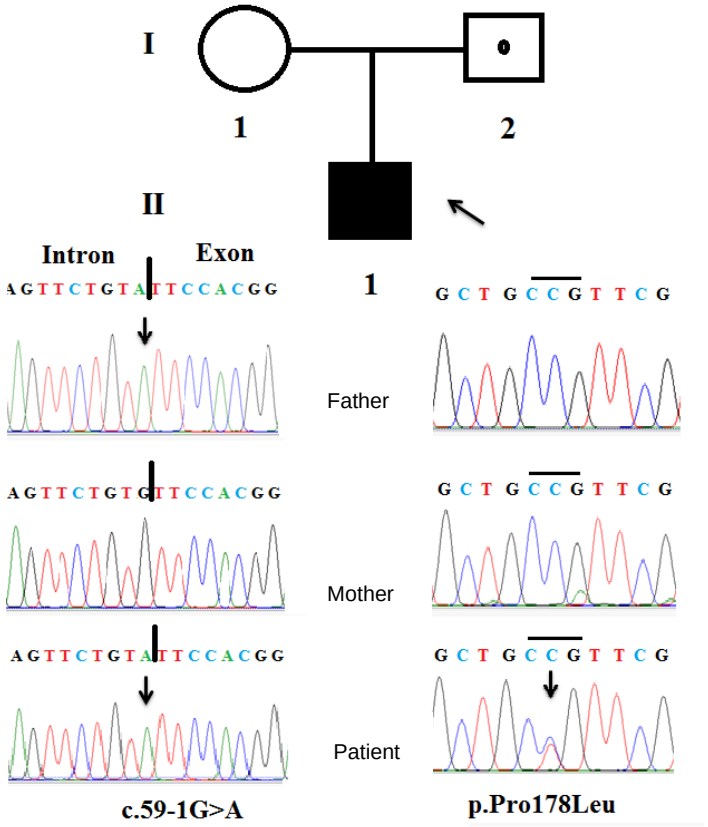

Figure 1. Genealogical diagram and results of genetic analysis at two mutation points in patient and members of the family.

Based on literature review and the function prediction results, the c.533C>T mutation is a known pathogenic mutation that has been published in the HGMD database (http://www.hgmd.cf.ac.uk/ac/index.php). Thus, the patient carried one known pathogenic mutation (c.533C>T) and one novel variant (c.59-1G>A) in the *ITGB2* gene. These changes were selected for further PCR and Sanger sequencing validation (Figure 1). As shown in Figure 1, the c.59-1G>A variant was inherited from the father and the c.533C>T mutation was the *de novo* mutation in the patient.

DISCUSSION

We performed whole exome sequencing analysis for one LAD1 patient to identify key genetic lesions contributing to the disease. Further PCR and Sanger sequencing for selected variants in the patient and members of the family were carried out for validation. Two variants, including one known missense mutation and one novel variant in the *ITGB2* gene were confirmed to exist in LAD1 patient. Defect of the *ITGB2* gene has been considered to be the cause of LAD1 (Springer *et al*., 1984; Anderson *et al*., 1985). The *ITGB2* gene encoded the integrins, the transmembrane receptors composed of α and β subunits, that mediate cellular adhesive interactions throughout the body. Patient with LAD1 have remarkable leukocytosis with neutrophilia. The neutrophils are released normally from bone marrow into blood stream but emigration of leukocytes from the blood vessels to the sites of infection is impaired (Tipu *et al*., 2008). As a result, patients with LAD1 suffer from severe bacterial infections and impaired wound healing, accompanied by neutrophilia (van de Vijver *et al*., 2012).

We reported a new homozygous variant (c.59-1G>A) in the *ITGB2* gene which is inherited from healthy father of the patient (Figure 1). This variant was considered to be a polymorphism with MutationTaster analysis. However, this variant was appreciated to be a splice acceptor variant with MaxEntScan analysis and may affect the splicing of the pre-mRNA in the maturation process. The severe phenotype of patient can be explained by the patient also carried another pathogenic mutation (c.533C>T) in the *ITGB2* gene. The c.533C>T mutation was a *de novo* mutation in the patient (Figure 1). It is a known pathogenic mutation that has been published in the HGMD database (http://www.hgmd.cf.ac.uk/ac/index.php).

Previous studies showed that most of the point mutations were found in a ~240-residue domain that was highly conserved in all β integrin subunits and coded for by exons 5 – 9 of *ITGB2* (van de Vijver et al., 2012). It is the von Willebrand Factor type A (VWFA) domain that forms the extracellular domain of CD18 protein and is critical for the structural association of α and β integrin subunits for heterodimer formation on the cell surface and functional activity. It suggests that any significant alterations in the amino acid sequence in this region will have a deleterious effect on the expression and functional activity of CD18 antigen (Madkaikar *et al*., 2015).

CONCLUSION

In summary, whole exome sequencing analysis of the LAD1 patient and further Sanger sequencing validation in other members from the family were carried out to identify mutations in the *ITGB2* gene which contribute to the pathogenesis of the disease. Two variants, including one *de novo* missense mutation (c.533C>T) and one novel variant (c.59-1G>A) of the *ITGB2* gene, which related to the phenotype of the patients were identified. Our results suggested that the whole exome sequencing analysis provides us a new insight and a new tool in investigation of the molecular mechanism of LAD1 disease.

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