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Whole exome sequencing reveals a missense mutation in the ADA gene causing severe combined immune deficiency in a Pakistani family

Zara Khalid Khan[a], Sadaf Jafar[b], Sheeba Shabbir[c], Muhammad Zeeshan Anwar[d], Rabea Nasir[e], Attea Zaman[f], Momin Iqbal[g], Syed Irfan Raza[h]

[a]*Assistant Professor, Department of Biochemistry, Rawal Institute of Health Sciences.*
[b]*Professor, Department of Biochemistry, Islamabad Medical & Dental College.*
[c]*Assistant Professor, Department of Forensic Medicine, HBS Medical & Dental College, Islamabad.*
[d]*Associate Professor, Department of Biochemistry, CMH Kharian Medical College, Punjab.*
[e]*Assistant Professor, Department of Physiology, M. Islam Medical College Gujranwala.*
[f]*Assistant Professor, Department of Biochemistry, Federal Medical & Dental College.*
[g]*Resident, Department of Histopathology Aga Khan University Hospital.*
[h]*Professor, Department of Biochemistry, HBS Medical & Dental College, Islamabad.*
Correspondence: siraza.pk@gmail.com*

ABSTRACT

BACKGROUND & OBJECTIVE: Human adenosine deaminase deficiency, OMIM 102700 (ADA deficiency) is a genetic disorder which causes severe combined immunodeficiency (SCID). The enzyme adenosine deaminase is a housekeeping in nature and is involved in purine catabolism by catalyzing irreversible deamination of adenosine and deoxyadenosine. The SCID patients' immune system is unable to fight off most of the bacterial and fungal infections due to profound lymphopenia (T-B-NK$^+$). About 20% of the SCID patients are genetically homozygous for defective ADA gene. In our study we aimed to find out genetic variant in ADA gene in a family carrying severe combined immune deficiency. Objective in the current study is to unravel and characterize the molecular cause of the patient suffering from by birth SCID.

METHODOLOGY: In the present study we enrolled a 6-month-old female SCID patient belonging to highly consanguineous Pakistani family. Patient clinical features included repeated chest infection with failure to thrive, fever and chronic diarrhea. Whole blood samples from patient, parents and healthy siblings were acquired in EDTA tubes. DNA was extracted from all the blood samples.

RESULTS: Flowcytometry revealed lymphopenia (T-B-NK$^+$) type of SCID. Whole Exome Sequencing (WES) identified a one nucleotide change (c.716G>A) in ADA gene exon 8. The segregation of the identified variant in the family was confirmed through Sanger Sequencing.

CONCLUSION: In this study, we presented detailed clinical and genetic description of patient suffering from severe combined immune deficiency. The immunological and genetic findings presented in this study will facilitate early diagnosis of the disease. Segregation of the identified variant in the family members will also aid in genetic counseling the family.

KEYWORDS: Adenosine deaminase deficiency, Flow-cytometery, Exome sequencing.

INTRODUCTION

Adenosine deaminase (ADA) is a monomeric enzyme which is involved in purine metabolism. It performs the deamination of toxic adenosine and 2′-deoxyadenosine to non-toxic inosine and 2′- deoxyinosine. This enzyme is coded by ADA gene located on chromosome 20q12-q13.11 and is expressed ubiquitously in all the cells, with highest expression observed in lymphocytes, which develop in lymphoid tissues. Patients with ADA deficiency buildup adenosine and deoxyadenosine metabolites which results in intracellular accumulation of toxic adenosine and 2-deoxyadenosines which disturb cellular functions in all the lymphocyte lineages. The functional defects in lymphocytes lead to adenosine deaminase mediated severe combined immunodeficiency (ADA – SCID) [1-3].

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Partial or complete ADA deficiency lead to severe combined immunodeficiency (SCID) syndrome. More than 85% of the ADA mediated SCID patients suffer from recurrent and opportunistic fungal, viral, and bacterial infections, failure to thrive, and lymphopenia. These manifestations are milder in late onset of disease [4-5]. Lymphopenia with T-, B- and NK+ cells is observed in patients with late onset of ADA-SCID [6].

Globally, the incidence rate of ADA-SCID is estimated to be 1 per in 40,000 – 75,000 births. Only in USA the incident rate is about 10–20% of all SCID cases [7-8]. Pakistan, with highest rate of consanguine marriages is at risk of Primary Immune Deficiencies, including ADA-SCID[9]. Consanguinity increases the risk of rare inherited disorders [10].

Among the common disease manifestations due to primary immune deficiencies are fever, chest, and gastrointestinal tract (GIT) infections. The GIT infections are mediated due to poor hygienic living standards. Communicable infections such as Tuberculosis (TB) may cause doubts in proper diagnosis of the disease. The key clinical manifestation of ADA-SCID is recurrent infections, including pneumonia, diarrhea, and chronic and widespread skin rashes. Developmental delays and rarely neurological symptoms are also observed in patients with ADA deficiency [8-12]. Onset of the disease manifestations in most cases is in the first 6 months of life and if not treated, patients do not survive after age 2 year. According to literature survey genetic defects in 15 genes have been associated to SCID and still there are many cases in which genetic defects are still unknown. The present study intended to report the identification of missense variant in adenosine deaminase (ADA) gene in a 6 months old female patient belonging from remote village of Punjab Pakistan.

METHODOLOGY

The female patient, age 6 month, belong to a highly consanguine family (Figure-I) was referred to Armed forces Institute of Pathology (AFIP), Rawalpindi from Pakistan Institute of Medical Sciences (PIMS) hospital Islamabad, Pakistan. This project was carried out partially at (AFIP), Rawalpindi, and Department of Biochemistry Quaid-i-Azam University (QAU) Islamabad, Pakistan. A detailed clinical history using pre-designed proforma was obtained from Parents/ guardian. Before the start of this research project, ethical approval was acquired from Quaid-i-Azam University, Review Board (QAU/BCH/2023). Informed and written consent was obtained from healthy parents. The patient was admitted in a federal government hospital. The detailed clinical evaluation of the patient's skin, gastrointestinal tract infections (including omphalitis) was performed by the group of physicians in the Pediatrics department of PIMS.

In order to perform patient complete picture test and flow-cytometery, 3-5 ml of whole blood samples were acquired in K-EDTA tubes. To evaluate number of B,T and NK cells flow cytometery (FCM) analysis was performed in Immunology department of (AFIP), Rawalpindi. Flow cytometry using anti CD11b PE, anti CD18 FITC, anti CD11c PE and CD19, CD3, CD4, CD8, CD16/56 (BD Biosciences, San Jose, CA, USA) was performed on Becton Dickinson (BD) FACSCalibur. Blood Complete picture was performed on sysmex KX21 automated hematology analyzer.

According to a recommendation by the manufacturer, patient's white blood cells (10,000/ul) along with 100 ul of ethylenediaminetetraacetic acid (EDTA) and recommended antibody was incubated for about 30 minutes. Each time a control sample was run along with a patient sample. After 30 minutes incubation, Samples were run on flow-cytometer and was analyzed using BD FACSDiva software.

Whole blood samples (5CC each) of patient, available parents, and healthy siblings were collected K-EDTA tubes (BD Vacutainer® EDTA Tubes - 366643). Genomic DNA from all the blood samples were extracted using the GenEluteTM whole blood genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). The genomic DNA samples were quantified on Nanodrop1000 spectrophotometer (Thermal scientific, Wilmington, MA, USA). In order to detect pathogenic variant causing underlying disease etiology we perform Whole Exome Sequencing (WES). The exome sequencing revealed a pathogenic variant in the gene ADA gene (20q13.12). Pathogenicity score (out of 5) was identified using MutationTaster (http://www.mutationtaster.org/), CADD score (https://cadd.gs.washington.edu/), and Polymorphism Phenotyping V2 (PolyPhen 2). The segregation of the pathogenic variant was tested through DNA Sanger sequencing. The sequences of all ADA gene specific Primers to sequence exons, exon-intron boundaries as reported earlier [13,14]. To amplify the required DNA segment polymerase chain reaction (PCR) was performed in 200ul PCR tube containing 25ul reaction mixture as described previously [14]. To check the shearing and to measure exact size of each PCR product was resolved through electrophoresis on 2% agarose gel and was examined in GelDoc UV illuminator. Later amplified PCR products were purified using a commercially available kit (Axygen, CA, USA). The purified products were Sanger sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit on Beckman Coulter CEQ-8000 Analyzer (Stanwood, Washington, USA).

RESULTS

Six-month-old female patient from a first-degree cousin was admitted to pediatric ward of PIMS hospital, Islamabad with a complaint of frequent upper respiratory tract infection, chronic diarrhea, failure to thrive and neurodevelopmental delay was also observed. According to mother the family has already lost one male child at the age of 6 month with similar pathological symptoms (Figure-I). Patient suffered multiple episodes of fever and chest infection since birth.

The patient was found to have lymphopenia with Mycobacterium intracellular infection with a suspect of cytomegalovirus (CVM) gastroenteritis. Fine skin rashes

with no lymphadenopathy was also observed. Her abdomen was soft with palpable hepatosplenomegaly. The rest of her physical exam was normal. Past medical history revealed she had at least four severe episodes of respiratory tract infection with bronchospasm. She also suffered diarrhea with loose stools 3 to 5 times per day. In view of these pathological conditions, physicians suspected a case of severe combined immunodeficiency (SCID).

Blood Complete Picture (CP) test revealed total WBC count 9.28 x 103/ul, RBC count 4.06 x 106/ul and platelet count 65 x 103/ul. Neutrophil count was 53.7%, lymphocyte count was 0.4% while Reticulocyte count was 0.5%. Patient hemoglobin level was 9.5 g/dl which is slightly anemic (Table-I).

Table-I: Blood complete picture test.

S. No	Patient Value	Results	Reference Range Age Specific
1.	WBC	9.28 x 10^3/ul	4 -12 10^3/ul
2.	RBC	4.06 x 10^6/ul	4.2 – 6.3 10^6/ul
3.	Platelet count	65 x 10^3/ul	10 -400 10^3/ul
4.	Hemoglobin	9.5 g/dl	11 – 13 g/dl
5.	Reticulocyte	0.5%	0 %
6.	Neutrophil count	53.7%	45 – 74 %
7.	Lymphocyte count	0.4%	22 – 50 %

In order to check the patient's immunoglobulin status, we performed measured selected immunoglobulin levels. Tests showed Immunoglobulin A (IgA) level was 0.2 g/L, immunoglobulin G (IgG) was 4.0 g/L and immunoglobulin D (IgD) 0.2 g/L (Table-II).

Table-II: Immunoglobulin's level.

S. No	Patient Value	Results	Reference Range Age Specific
1.	Immunoglobulin A	0.2g/L	0.10 – 0.50g/L
2.	Immunoglobulin G	4.5g/L	2.4 – 8.8g/L
3.	Immunoglobulin D	0.1g/L	0 – 0.1g/L

FCM results showed near absences of lymphocytes with lymphocytes count 0%, CD4 to CD8 ratio of 0; and CD3 to CD8 ration 0%, CD19 cell 0% (Table-III). Patients characteristically was diagnosed with T-B-NK^+ type of SCID. This type of phenotype is likely to have ADA deficiency and hence we immediately focused on ADA gene mutation and performed Sanger Sequencing[15].

The WES in female patient (IV-3) revealed a previously reported disease-causing missense variant in exon 8 of the gene ADA gene (20q13.12). The gene Adenosine deaminase (ADA) comprises of total 12 coding exons. Sanger sequencing of all the coding exons revealed a missense mutation (c.716G>A; p.239Gly>Asp) in exon 8 in the index patient. This mutation resulted in a change of amino acid of Glycine to Aspartate at position 239 of the resulting protein Figure-I.

Table-III: Lymphocyte subset count.

S. No	Patient Value	Results	Reference Range Age Specific
1.	WBC	9200/ul	4000 – 1400/ul
2.	Lymphocyte Percentage	0%	44 – 76 %
3.	Lymphocyte count	0 /ul	1760 – 10640
4.	CD^{3+} cells	0% (0)	49 – 76 % (1900 – 5900)
5.	CD3+CD4+ cells	0% (0)	31-56% (1400-4300)
6.	CD3+CD8+ cells	0% (0)	12-24%(500-1700)
7.	CD19+ cells	0% (0)	14-37%(610-2600)
8.	CD16+56+ cells	12% (0)	3-15%(160-950)
9.	CD4+CD8	0	1.48-3.77

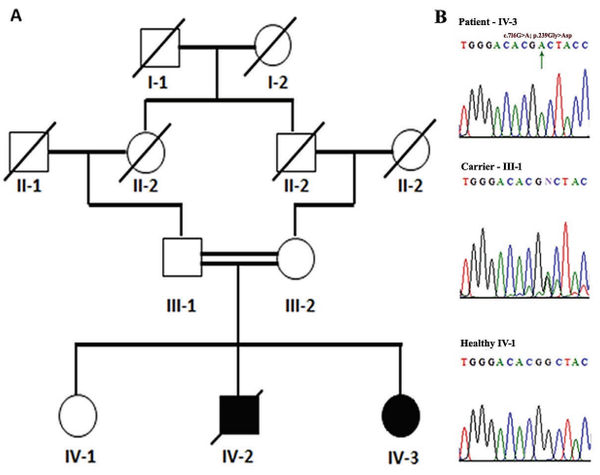

Figure-I: Pedigree & ADA gene mutation analysis.

A: shows the detailed family pedigree. Squares and circles show male and females successively. Filled circle and square show patient while on them means deceased. B: Sanger Sequencing of ADA gene. Upper column (IV-3) represents homozygous patient. Arrow head denotes the position of missense mutation (c.716G>A p.239Gly>Asp) Middle portion showing carrier peak in father (III-1) while the lower portion denotes the homozygous healthy (IV-1).

DISCUSSION

Severe combined immunodeficiency (SCID) is a heterogeneous group of disorder that arises as a result of profound defects of immune system development and function. The SCID patients suffer severe to mild disease manifestations. More than 20 different genes have been identified till date [15-17].

The ADA-SCID is type of severe combined immune deficiency (T-B-NK+) caused due to deficiency of gene adenosine deaminase. The human ADA gene spans almost 32kb on the long arm of chromosome 20 and harbors 12

exons[3,18]. The gene ADA encodes approximately 41 kDa protein which is enzymatic in nature [19]. So far more than 70 disease causing mutations have been reported in the gene ADA. According to Human Genome Mutation Databank (HGMD) majority of these mutations (about 63%) are missense, while 18% are splice site, 13% are deletions, and only 6% are nonsense mutations.

Almost 10-15% of ADA-SCID cases present delayed onset (6-24 months) of or even until adulthood with less severe pathologies. Clinical manifestations in such cases include hemolytic anemia, diabetes mellitus, autoimmune hypothyroidism, and immune thrombocytopenia [20].

In the current study, we recruited a 6-month-old female patient who was admitted in PIMS Hospital Islamabad. Flow cytometry confirmed T-B- and NK+ type of severe ADA-SCID. The key clinical manifestations included respiratory tract infection, chronic diarrhea, failure to thrive and neurodevelopmental delay. The disease features observed in the patients are coherent to those reported earlier with similar types of SCID [13, 21-23].

Genetic analysis of entire ADA gene revealed an already reported single nucleotide substitution (c.716G>A) in exon 8 shown in Figure-I. This missense variant have already been observed in a Pakistani and Indian patients reported from UK and India in two separate studies [24,25]. This substitution mutation resulted in change of neutrally charged aliphatic and hydrophobic glycine to a negatively charged acidic aspartic acid (p.239Gly>Asp). Previously, in two separate studies it is confirmed that p.239Gly>Asp results in low or absent RBC-ADA levels and high dATP levels at diagnosis, which confirms the pathogenicity of this missense variant [24,25]. The identified mutation located in the active domain of the protein which possibly affects the deaminase activity. As the parents are heterozygous (AG) while healthy sibling is homozygous (GG) while patient is homozygous (AA) for the disease allele confirms the complete segregation of the identified missense variant. Moreover, pathogenicity measuring tools such as Sorting Intolerance to Tolerance (SIFT) and Combined Annotation Dependent Depletion (CADD) predicted that the AA change at this location could be highly pathogenic in nature.

ADA-SCID remains a highly challenging and complex disease condition. A greater understanding and underlying molecular defects leading to immunological defects has led to better disease management options such as Bone marrow Transplant (BMT) and stem cell therapy. This study will not only provide a better understanding of the disease but also the missense mutation in the current study will be added to the mutation data bank, which will help physicians and molecular biologists diagnose the disease at molecular level. The study also helped in providing genetic counseling to affected families and identified carriers for the mutation in the family.

CONCLUSION

In the current study ADA –SCID patient showed lymphopenia with T-B- deficiency. Genetic analysis revealed an already reported missense variant in exon 8 of the ADA gene. Primary care physicians should suspect SCID by lymphopenia in a patient suffering from recurrent chest infections. Neutropenia in blood complete picture test can help in suspecting SCID. This missense mutation should be added to the national mutation databank for immune deficiencies.

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Author's Contribution:

Zara Khalid Khan: Substantial contributions to the conception or design of the work.
Sadaf Jafar: Drafting the work.
Sheeba Shabbir: Interpretation of data for the work.
Muhammad Zeeshan Anwar:
Rabea Nasir: Acquisition of the data for the work.
Attea Zaman: Reviewing the manuscript critically for important intellectual content.
Syed Irfan Raza: Final approval of the version to be published.
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