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Genetic Analysis Reveals A Missense Mutation In Reactivation Gene-2 (RAG2) Causing Severe Combined Immunodeficiency Disease In A Pakistani Family

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Abstract

Objective: To perform clinical and genetic analysis in a family suffering from lymphopenia (low B-lymphocyte, T-lymphocyte and healthy Natural Killer cells).

Method: In the current research project we enrolled a female child from Rawalpindi district. Laboratory investigations including lymphocyte subset analysis were performed using anti-antibodies (CD19, CD3, CD4, CD8 and CD16/56). Estimation of serum immunoglobulins was performed. DNA Sanger sequencing of the *RAG2* gene was performed to identify the gene mutation.

Results: A thorough clinical examination by a team of expert physicians revealed severe nappy rash, interstitial pneumonitis and intractable diarrhoea leading to failure to thrive. Lymphopenia with CD3+CD8 cells (0.1%), CD19 cells (0.1%), CD19/CD56 cells (27%) and CD4/CD8 cells (0.0%) resulting in $B^-T^-NK^+$ deficiency. The patient's serum immunoglobulins showed low levels of IgG, IgA and IgM levels. Exons-specific polymerase chain reaction (PCR) was performed using oligonucleotide primers and subsequently DNA Sanger sequenced which resulted in a missense variant in the gene *RAG2*.

Conclusion: In human reactivation genes 1 and 2 encode proteins with endonuclease activity which are involved in V(D)J rearrangement to generate a variety of different types of T- and B- cell receptors. Genetic analysis revealed a missense mutation in RAG2 genes in female SCID patients.

Keywords: Lymphocyte subset, flow cytometry, DNA Sequencing.

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1. Introduction

The prevalence of Inherited primary Immunodeficiency diseases (PID) is significantly high in populations with a high rate of consanguinity.[1] Among PIDs, severe combined immunodeficiency (SCID) is characterized by a high rate of life-threatening infections in early infancy due to defective or diminished functional T/B cells. Such immunological deregulations are a function of numerous genetic mutations responsible for the production of functional T/B cells. Genes such as recombination activation genes 1 & 2 (*RAG1* and *RAG2*) play pivotal roles in variable (V), diversity (D) and joining (J) segments recombination to produce antigen receptor repertoire. Defected recombination due to mutations in *RAG1* (OMIM 179615) and *RAG2* (OMIM 179616) genes leads to limited functional antigen receptors in the adaptive immune system.[2] Patients with *RAG* gene mutations exhibit $B^-T^-NK^+$ type of SCID.[3] Patients with RAG deficiency exhibit a variety of clinical and immunological phenotypes.[4,5] Severe combined immunodeficiency patients exhibit life-threatening opportunistic viral and fungal infections and failure to thrive just after birth. The spectrum of diseases includes granuloma, Omenn syndrome, chronic cytomegalovirus or EBV infection, early onset autoimmunity, idiopathic CD4 lymphopenia and a phenotype resembling common variable immunodeficiency.[6,7] Patients with $T^-B^-NK^+$ type of SCID with RAG1/2 null mutations display a variety of recurrent sinopulmonary infections including persistent diarrhoea, oral candidiasis, intestinal pneumonitis and growth impairments, while patients with hypomorphic mutations are free of skin manifestations with normal or lightly low B-cell counts and immunoglobulin (Ig) levels. If not treated conditions become fatal within the first 2 years of life.[8,9] Atypical SCID is a subtype of severe combined immunodeficiency that does not present classical clinical disease phenotype. Patients with atypical SCID (OMIM 601457) present poor clinical as well as immunological phenotypes in the later stage of life (adulthood).[8]

The current research study describes the detailed clinical and immunological status of an infant from the tertiary care hospital of Rawalpindi diagnosed with SCID. Genetic analysis revealed a missense mutation in the Reactivation Gene-2 (*RAG2*).[2]

2. Materials & Methods

A female patient of age four months from a distinct family from a remote area of Gilgit and Baltistan was admitted to the Combined Military Hospital (CMH) Rawalpindi with complaints of recurrent nappy rash, oral thrush and erythroderma. While taking family history mother of the patient informed me that this is the second child with such complaints. The first male child with similar disease manifestations died at the age of 9 months. Detailed clinical and immunological investigations were performed by a group of expert paediatricians and immunologists. She was diagnosed with a case of severe combined immunodeficiency (SCID) based on criteria issued by the International Union of Immunological Societies (IUIS).[10]

Before a detailed clinical, immunological investigations and family history of the patient informed oral and written consent was acquired from parents to publish the patient clinical and genetic analysis findings. The Father of the patient was reluctant to publish the patient facial features as well as affected skin parts. Approval to conduct this research study was obtained from the Institutional Review Board (IRB) of HBS Medical College, Islamabad, Pakistan. Halinski

Physicians prescribed lymphocyte subset analysis to evaluate the immune cell status in patients including circulatory T-cells, B-cells as well natural killer cells (NK-cells). Using butterfly whole blood samples (3CC) were obtained in 5 CC sodium ethylene diamine tetra acetate (Na-EDTA) tubes (BD Vacutainer® EDTA Tubes, Becton Dickinson, UK). Lymphocyte subset analysis was performed using anti-antibodies (CD19, CD3, CD4, CD8 and CD16/56) from (BD Biosciences, San Jose, CA, USA). The test was performed on a FACSCanto II instrument (Becton Dickinson, San Jose, USA) installed in the Armed Forces of Immunology CMH Rawalpindi. Another 2 CC blood was acquired in a separate tube to perform a blood complete picture test using an automated Sysmex KX21 Hematology Analyzer, Japan, installed in Armed Forces Institute pathology Rawalpindi.

The current research study is a "familial-based designed genetic association study". To find the inheritance pattern of the disease considering the shared genetic background among family members, the DNA sequencing data was analyzed. To perform genetic analysis 3 CC peripheral blood samples were acquired from the patient, and parents and a control sample as a healthy sibling was not available. Nucleic acid (genomic DNA) was extracted from nucleated blood cells (WBCs) using a GenEluteTM blood genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). The concentration of the extracted DNA was calculated using a Nanodrop1000 spectrophotometer (Thermal Scientific, Wilmington, MA, USA). Based on clinical diagnosis (Omen syndrome - SCID) by expert clinicians, we designed *RAG1 & RAG2* genes selected exon-specific primers to PCR amplify. The exon-specific primers were constructed using PRIMER 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). PCR amplification was carried out in 25ul volume according to standard protocol using a commercially available kit (Axygen, CA, USA). All the amplified and purified PCR products of patients, parents and control were Sanger sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The data obtained by Sanger sequencing of the selected exons of *RAG1* and *RAG2* genes of patients and parents along with a control sample was analyzed and matched with the corresponding human control gene sequence available on the Ensemble Genome Browser database (http://ensembl.org/index.html). Mutational analysis was conducted using nucleotide sequence variant BioEdit sequence alignment editor version 6.0.7. Two bioinformatics software Phenotyping V2 (PolyPhen 2) and MutationTaster (http://www.mutationtaster.org/) were used to measure the disease-causing ability of the identified variants.

According to pedigree analysis disease (SCID) follows the autosomal recessive pattern of inheritance. Clear squares and circles represent healthy/carrier subjects while filled symbols show patients. The block colour diagonal line represents deceased family members. (B) *RAG2* gene mutational analysis. *RAG2* Sanger sequencing analysis shows a substitution of [c.1247G>A; (p.Trp416Leu)]. In the electropherogram, a red colour arrowhead in the patient (IV-3) shows the homozygous mutant nucleotide position while (Carrier – III-2) and a healthy control DNA sequence.

3. Results

Clinical Findings:

In the current research project, we registered a four-month-old female child. The family history was positive with already a deceased male child with similar disease manifestations (Figure 1). This female patient is third in birth number after a healthy male child of 5 years.

Patients was exhibiting severe nappy rashes, oral thrush and erythroderma. The deceased male SCID child died at the age of 9.5 months due to recurrent pneumonia and body rashes (Figure 1). The female patient was admitted to the Department of Pediatrics, MH-Rawalpindi with complaints of severe nappy rash and oral thrush. Later she developed interstitial pneumonitis and intractable diarrhea leading to failure to thrive. Supportive care along with symptomatic relief was immediately provided by expert physicians with cyclosporine and corticosteroids.

Laboratory findings:

Lymphocyte subset and immunoglobulin analysis findings:

Flowcytometery-mediated lymphocyte subset analysis showed lymphopenia with total absence of CD19 and B-lymphocyte while CD3 and CD4 lymphocytes were also absent. CD3 and CD3+CD4 lymphocytes constitute total T-lymphocytes. CD8 + T-lymphocytes were lacking CD3 expression. The NK cell markers including CD16 and CD56 were normal. All the T-lymphocyte, B-lymphocyte and NK cell concentrations are shown in (Table 1). These findings are suggestive of humoral immune deficiency. Estimation of immunoglobulin levels revealed hypogammaglobulinemia (low IgA, IgG and IgM). As a control, we measure immunoglobulin levels in the mother. The detailed results are shown in (Table 1).

Genetic Finding:

The patient's disease manifestations in association with laboratory investigations were clearly showing the association of defective *RAG1* or *RAG2*. DNA Sanger sequencing of selected exons revealed a missense variant [c.1247G>A; p.(Trp416Leu)] in exon 2 of the *RAG2* gene in Patient (IV-3) shown in Figure 1.

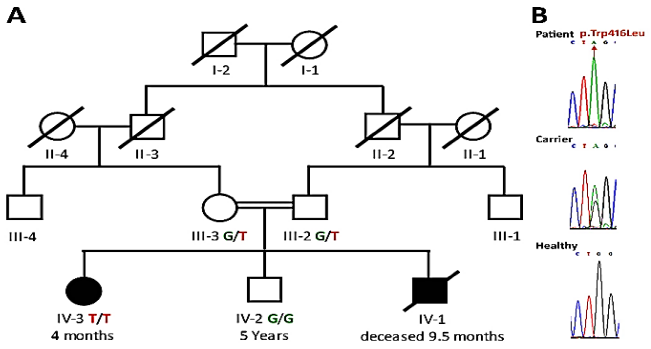

Figure 1: Pedigree of Family and Mutational Analysis: (A) Family pedigree. Four-generation consanguine family belonging to Pakistan.

Table 1: Patient Lymphocyte Subset Analysis and Immunoglobulin Levels

S. No	Parameters	Results	Reference Range 1 day – 11 months
Peripheral blood lymphocyte subset analysis			
	TLC	12600/ul	6400-11000/ul
	Lymphocyte Percentage	33%	38-59%
	Lymphocyte Count	4158/ul	2700-5400
	$CD3^+$ cells	57% (2370)	58-67%(1700-3600)
	$CD3^+$ $CD4^+$cells	57% (2370)	38-50%(1700-2800)
	$CD3^+$ $CD8^+$cells	0.1 % (0)	18-25%(800-1200)
	$CD19^+$ cells	0.1 % (0)	19-31%(500-1500)
	$CD19^+$ $CD56^+$cells	27% (1123)	8-17%(300-700)
	CD4:CD8	0.0	1.5-2.9
Immunoglobulin Levels			
	IgG	11.4	670–1530 mg/dl
	IgA	1.6	52–274mg/dl
	IgM	0.6	48–179mg/dl

4. Discussion

In human recombination activating genes 1 & 2 (*RAG1* & *RAG2*) are involved in the production of diverse antigen receptors repertoire via early stage V(D)J recombination process.[2] The receptor deficiency on T/B cell lymphocytes leads to the most common immunodeficiency syndrome, $T^-B^-NK^+$ type of SCID.[2,3,11] *RAG1* or *RAG2* missense mutations severely affect the recombine activity but permit occasional rearrangement which results in fractional production of V(D)J events and production of oligoclonal T-lymphocyte population.[3,12]

The human RAG 2 (OMIM 179616) is localized on chromosome 11p12 which encodes a 527 amino acid protein which is involved in double-stranded break through its endonuclease activity. The RAG2 protein comprises of total five domains that are: N-terminal Domain (1-174 amino acids), core domain (175-387 amino acids), Plant Homeodomain (PHD) Finger Domain (388-460 amino acids), C-terminal Domain (CTD) (461-527 amino acids), Transactivation Domain (528-587 amino acids).[12]

Mutations in the RAG2 gene lead to severe combined immunodeficiency syndrome. In the current study, we enrolled a female patient belonging to Rawalpindi, Pakistan. Genetic analysis of the RAG2 gene identified an already reported missense mutation [c.1247G>A;

p.(Trp416Leu)].[13] The mutation p.(Trp416Leu) lies in the C-terminal Domain of the RAG2 protein. This domain harbours a nuclear localization signal (NLS) which directs the protein to the nucleus where the protein performs recombination activity through its endonuclease activity. This domain is also involved in protein-protein interaction and RAG complex regulatory functions.

Patients in the current study display typical clinical symptoms including nappy rash oral thrush and intractable diarrhoea which are coherent with findings reported earlier.[13] Lab investigation in the current study showed lymphopenia with low levels of T- lymphocytes, and B- lymphocytes while NK-cells were with in normal range. Hypoimmunoglobulenmia is observed in the current study, these findings match with studies reported earlier with similar RAG2 mutation.[13-15]

5. Conclusion

Autosomal recessive type of severe combined immunodeficiency disease (SCID) is a rare inherited disorder mainly classified into omen syndrome and classical SCID which share disease phenotypes. In the present study, we clinically diagnosed a case of SCID in a 3-month-old female child. Genetic analysis revealed a missense mutation in the *RAG2* gene. The finding will help clinicians in making a proper diagnosis of the disease while the mutation identified will assist geneticists in exclusion analysis. The presence of a recurrent gene mutation in the *RAG2* gene is showing an increased frequency of this variation. Based on the identified variant in the *RAG2* gene, genetic counselling will be provided to the affected family.

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W.O - Conception of study
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S.A, W.O - Manuscript Writing
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- Facilitation and Material analysis

All authors approved the final version to be published & agreed to be accountable for all aspects of the work.

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