

## Immunological and molecular study in children with combined immunodeficiency

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### Summary

**Background.** Severe combined immunodeficiency (SCID) is a form of immunodeficiencies (PID), caused by molecular defects. These defects can restrict the development and function of lymphocytes. Early diagnosis and treatment of SCID can lead to disease-free survival. **Objective.** This study aims to investigate some of the possible underlying genetic defects in a group of Egyptian infants and children with clinical and immunological profiles suggestive of SCID. **Methods.** This study included eighty patients who showed clinical warning signs of immunodeficiency. Subjects were thoroughly examined clinically. Laboratory evaluation included immunoglobulins serum levels and flow cytometric assessment of immune cells. This testing showed an altered immune profile in thirty patients. They had decreased T and/or B lymphocytes or natural killer cells. DNA extraction was done for those cases. The coding regions of the RAG1 gene and RAG2 gene was investigated for hot spot mutations by sequencing technique guided by the patient clinical evaluation, inheritance pattern, immunophenotyping by flow cytometric analysis of lymphocyte subsets, and serum immunoglobulins level detection. **Results.** Results showed novel and previously reported variants (mutation, polymorphism), they were found in 18 cases which include variants in the RAG1 gene (E880K, A960A, H249R, S913R, K820R, V782G), and variants in the RAG2 gene (P501T, L514M, rs10836573, cDNA.2129A>T). **Conclusions.** To evaluate SCID patients completely; mutation gene analysis is highly required and recommended.

### Introduction

Primary immunodeficiencies (PID) are a heterogeneous group of disorders with a broad range of clinical symptoms. Most of the primary immunodeficiency diseases are monogenic disorders and inherited as autosomal recessive forms. The prevalence of the various PIDs varies in different countries (1). In the Middle East and North Africa, the rate of registered patients ranges between 0.02 and 7.58 per 100,000 population with a total of 17,120 patients registered. The prevalence rate is 0.68 per 100,000 population in Egypt (2). Another 343 patients with

monogenic diseases of immune dysregulation were registered in the same region where Egypt constituted 5% of patients (3). Severe combined immunodeficiency (SCID) is a very severe form of PID, and early diagnosis can be lifesaving (4). SCID syndromes occur more frequently in males; the prevalence of which are nearly 1:50,000 live births. This reflects the excessive representation of X-linked SCID. Despite this, when high consanguinity populations are studied; autosomal recessive SCID occurs at a higher frequency than previously reported (5). SCID occurs when molecular defects are encountered and affect lymphocyte development and function. These defects may also restrict the differentiation and proliferation of T cells, B cells as well as natural killer (NK) cells. Although mature B cells may be normally present; antibody production is markedly compromised due to a lack of T-cell help. Moreover, NK cells are also affected which renders the innate immunity to threats as well (6). At birth, SCID may not be explicit clinically. Thus, infants may present to medical clinics with complications of infections that are indistinguishable from repetitive childhood infections. This accordingly delays diagnosis in a significant manner. During the first year of life, these infants may die of infectious complications (7). Nevertheless, routine administration of live vaccines may put them at risk of developing “Vaccine Associated Paralytic Poliomyelitis” (VAPP) and BCGosis following OPV and BCG administration (8).

Early diagnosis of SCID is highly recommended. Starting treatment and avoiding infections and complications will reduce hospitalization costs and draw attention to early stem cell transplantation. This will lead to disease-free survival in immune-compromised infants. Testing immediately after birth can be done by sequencing DNA if the family mutation is known or by estimation of the numbers of T, B, and NK cells. On the routine evaluation of SCID patients, lymphopenia and altered absolute lymphocyte count (ALC) at birth help in pre-symptomatic detection and early medical interference. However, ALC may show normal in some cases as it is not as sensitive and specific compared to molecular testing (9). In SCID evaluation, mutation analysis is gaining more importance especially because the list of disease-associated genes is continuously increasing (10). Moreover, recognizing the disease-associated genes of this potentially life-threatening disease will have an intense influence on several disease aspects as the value of genetic counseling and prenatal diagnosis. In addition, prognosis and treatment modalities will remarkably advance (11).

## **Methods**

This study was approved by Medical Ethics Committee no (16/108). Informed consent from the parents or guardians of patients included in our study was obtained according to the rules of the Medical Ethics Committees in the National Research Centre (NRC). 80 patients were included in this study. They were subjected to;

### **I- Clinical evaluation: -**

- Demographic data (name, age, sex, social class).
- Full history taking stressing on:
  - a) Birth history; length of gestation, birth weight, perinatal and neonatal problems, such as jaundice, respiratory distress, or need for intensive care.
  - b) Transfusions in the neonatal period
  - c) Delayed detachment of the umbilical cord beyond 30 days.
  - d) Feeding history including food intolerance and duration of breastfeeding.
  - e) Any adverse effects from a vaccine particularly live virus vaccines.

- f) The presence of family members with similar diseases, recurrent infections, unexplained death, or autoimmune disease suggests the possibility of a genetic illness.
- g) The infection history; the age of onset, duration, frequency, sites, organisms, treatment, and response to therapy.
- h) Thorough clinical examination: Any abnormal clinical finding elicited during the clinical examination was recorded as well as any of the ten warning signs of primary immunodeficiency in children (12).

## **II - Laboratory analysis:**

Complete blood count: total leucocytic count (TLC) and differential counts were recorded, and absolute lymphocyte number was calculated

-Measurement of serum immunoglobulins (IgG, IgA, and IgM) were done by nephelometry using a binding site Kit. (13).

-Measurement of peripheral blood lymphocyte subsets percentage including the T-cell subset (CD3, CD4, CD8), B-cell (CD19), and natural killer cell (CD 16) were done by flowcytometry (14).

After flow cytometry analysis cases studied (n =80) were categorized into 2 groups according to T lymphocytes % and/ or B lymphocytes concerning their age range (15).

Group I n=50 with normal T and B lymphocytes

Group II n=30 with decreased T &/or B lymphocytes. This group was further subjected to molecular studies

## **III-Molecular Studies:**

Eleven sets of primers were used for the amplification of the whole RAG1 gene and RAG2 gene. (Table 1)

The coding regions of the suggested genes were sequenced using ABI PRISM® 3130 Genetic Analyzer.

Three fragments were purified for each case, and then purified fragments were sequenced.

### *Mutational analysis*

Mutational analysis of the suspected gene defect was performed guided by the patient clinical evaluation and flow-cytometric phenotype of the lymphocyte subset.

### **Statistical analysis**

Statistically analyzed data was performed by using SPSS version 16.0 software. A nonparametric Mann-Whitney U test was used to compare concentrations of parameters between patients and healthy controls. Association between parameter expressions with age, gender, and total leucocytic count of patients was tested using Spearman rank correlation. Data were presented as mean±SD. A P-value <0.05 was deemed statistically significant.

The detected variants were subjected to bioinformatics analysis by a proper bioinformatics algorithm.

## **Results**

This study included eighty patients who had warning signs of primary immunodeficiency. Patients were categorized into 2 groups according to the results of flow cytometric analysis into 2 groups: group I whose results revealed a normal count of T and B lymphocytes and group II which had decreased T &/or B lymphocytes. Group II was subjected to further molecular studies. **(Table 2)** shows a comparison between both groups regarding age and gender.

A statistically significant decrease in absolute lymphocyte count was shown in group II, the P-value was 0.0107 as compared to group I. Also, A statistically significant decrease in CD3%, CD19%, and CD8% was detected in group II as compared to group I, the p-value were 0.0001, 0.038, and 0.0022, respectively **(Table 3)**.

A statistically significant decrease in both IgM and IgG in group II as compared to group I is detected, p-value were 0.0072 & 0.036 respectively **(Table 4)**.

The coding regions of genes (RAG1 and RAG2 genes) were investigated for hot spot mutations by sequencing technique guided by the patient clinical evaluation, inheritance pattern, flow-cytometric phenotype of lymphocyte subsets and serum immunoglobulin subtypes level detection.

Successful PCR amplification of seven fragments in the RAG1 gene (exon 1, exon 2a, exon 2b, exon 2c, exon 2d, exon 2e, exon 2f) and four fragments in the RAG2 gene (exon 1, exon 2a, exon 2b, exon 2c) in affected cases **(Table 5)**.

Our results showed novel and previously reported variants (mutation, polymorphism), they were found through 18 cases which include variants in RAG1 gene (E880K, A960A, H249R, S913R, K820R, V782G), and variants in RAG2 gene (P501T, L514M, rs10836573, cDNA.2129A>T).

The clinical manifestations of SCID patients was represented in **(Table 6)**, furthermore, in 18 cases diagnosed molecularly as SCID, 9 patients (50%) were of positive consanguineous parents.

## **Discussion**

This study included eighty patients who showed clinical warning signs of immunodeficiency. After flow-cytometric analysis, the cases studied (n = 80) were classified into two groups according to T lymphocytes and/ or B lymphocytes percentages concerning their age range. Group I (n = 50) with normal T and B lymphocytes and group II (n = 30) with reduced T and/or B lymphocytes. Group II underwent further molecular studies that yielded 18 cases of SCID from 30 samples sequenced with identified variants (mutation or polymorphism) in the RAG1 and RAG2.

T lymphocyte differentiation blockade is a main feature of SCID. Irregular development of lymphocyte lineages (whether B and/or natural killer cells) may inconsistently accompany the disease. Unless a hematopoietic stem cell transplant is offered; early death is usually inevitable (16). SCID includes heterogeneous cases; genotypically and phenotypically. The genetic origins of nearly 85% of the original immunologic defects have been lately clarified. The fact that diverse mutations in a single gene may cause different clinical conditions renders interpreting SCID pathogenesis a major obstacle. Moreover, similar clinical phenotypes can result from different gene mutations (17).

Severe combined immunodeficiency is characterized by severe and persistent infections from early life, which are due to profound impairment of both cellular and humoral immune function. Classically, the disease usually presents with an absence of both T and B cells. This is probably related to lymphocytic stem cell affection. Deficiencies in some enzymes as adenosine deaminase or purine nucleoside phosphorylase may lead to autosomal recessive forms of SCID. These types are either of yet unknown cause or due to IL-2 deficiency or related to the deficiency in major histocompatibility complex class II (bare lymphocyte syndrome) (18).

Accurate counseling and identification of the genetic defect are necessary for SCID to help parents to take rational decisions following pregnancies. Different ages of onset were revealed among patients in previous studies in addition to diverse molecular causes. However, this spots that in high-risk families; newborn screening is crucial (19). As to Meshaal et al. in 2015, RAG mutations were proved to be common among SCID cases in Egypt (20).

Early diagnosis is recommended in in PID patients order to decrease the risk of infectious and non-infectious conditions. Not only does this improve the disease outcome and quality of life, but also prevents serious complications (21). Aggressive respiratory and gastrointestinal tract infections are typical presenting symptoms that occur in their first year of life. Nevertheless, older children; up to 15 years may present with milder symptoms.

Initial evaluation of immunodeficiency cases after presenting to the clinic; includes a few screening tests. Ranging from basic complete blood count (CBC), together with an immunologic profile including estimation of immunoglobulins and lymphocyte subsets (22).

Results of this study revealed a statistically significant decrease in IgM and IgG levels in group II as compared to group I, this agrees with Stepensky *et al.*, (2018) who also detected a decrease in both immunoglobulins levels. They stated that in primary immunodeficiency, antibody deficiencies were the most common, constituting 53% of the patients (23).

Flow cytometry is a highly sensitive tool for evaluating the immune system and supporting the diagnosis of (24). This study showed a statistically significant decrease in CD3%, CD8% and CD19% in group II as compared to group I. Stepensky *et al.*, (2018) found a decrease in CD4% and not CD16% in their study done on severe combined immunodeficiency (23).

There is significant heterogeneity in circulating subsets in blood as per Bisgin *et al.*, (2018) (25). Immunophenotyping of lymphocyte subsets by flow cytometry is of great utility in the diagnosis and prognosis of many PIDs (24). In addition to immunological phenotype, more specific gene panels may be targeted for diagnosis when immune profiling is not informative enough.

In this study, the most common immunophenotyping patterns found in (group II) in thirty cases were T – B –NK+ and T – B + NK+ patterns. This agrees with Aluri *et al.*, (2019) who found the same immune phenotyping patterns in their studied cases (17).

Picard *et al.*, (2015) stated that difficulties are met in these conditions regarding the clinical and immunological presentation. This is due to extensive phenotypic variability for individual gene defects. This suggests that host and/or environmental modifying factors affect phenotype between individuals who share the same mutation within the same gene. Nevertheless, a variety of mutations within the same gene add to disease complexity (26).

Genetic, clinical, and immunological heterogeneity add to the complexity of PID diagnosis. However, multiplex gene testing in a single assay helps solve these complexities. This came with the introduction of next-generation sequencing that provided better screening and diagnosis for conditions with genetically heterogeneous backgrounds (8). Babies diagnosed with SCID and treated through transplantation, enzyme replacement, and/or gene therapy survived in 92% of the cases (27). Newborn screening for SCID enables early diagnosis in the asymptomatic phase (28).

This study revealed molecular variants in lymphoid-specific recombinase activating genes RAG1 and RAG2 genes in 18 of 30 cases (60%) while De-Pagter *et al.*, (2015) found molecular variants in RAG1 and RAG2 genes in 26% of their studied cases. In the current study, Sanger sequencing was used to detect disease-causing mutations. E880K, A960A, H249R, and K820R were previously reported variants in the RAG1 gene; they were recognized in a heterozygous state through SCID patients. E880K was reported as a disease-causing variant, while A960A, H249R, and K820R were reported as polymorphisms (29).

S913R and V782G are new variants in the RAG1 gene that were recognized in heterozygous states in SCID patients; both S913R and V782G are predicted to be disease-causing variants.

P501T and rs10836573 were previously reported variations in the RAG2 gene which were recognized through SCID patients. P501T was reported as a disease-causing variant, while rs10836573 is a polymorphism (29).

L514M and cDNA.2129A>T are new variants in the RAG2 gene that were recognized in SCID patients, they are predicted to be polymorphisms. (29).

This study revealed 18 cases (from 30 sequenced patients' samples) with identified variants (mutation or polymorphism) in RAG1 and RAG2 genes, this agrees with Bisgin *et al.*, (2018) who studied 37 suspected PID patients and identified the causative mutations in 17 of them (25). However, some suspected subjects were undiagnosed by immunophenotyping and immunoglobulin profiles. Researchers recommended whole exome sequencing to reveal uncharacterized mutations in other genes and highlight other genetic etiologies. Also, Woon and Ameratunga (2016) found that 23% of suspected cases had a positive genetic diagnosis (30).

The study has some limitations. PIDs are rare and suspicions depend on warning signs (12). These signs are not necessarily recognized in infancy but start appearing with age (31). This is the reason why, in the postnatal diagnosis group, many patients experienced serious infections. Due

to inadequate prophylaxis and treatment; irreversible complications took place which increased morbidity and mortality.

Positive family history for consanguinity in 57% of cases was recorded in this study. In the immune deficiency disorder research field, populations with high consanguinity create a high-potential area that facilitates the detection of new genetic causes. Causative genes for many types of SCID have been identified along the path of the post-genome era. It is expected that more is yet to come. Using such knowledge and expanding the understanding of disease pathogenesis remains the ultimate objective. An objective that assures research continuity is to improve diagnostic methods and develop an effective treatment for affected individuals (32).

## **Conclusion**

This study demonstrated 18 SCID cases from 30 sequenced samples with identified variants (mutation or polymorphism) in the RAG1 and RAG2 genes which revealed the importance of full evaluation of patients with SCID.

The main challenge remains in documenting genetic defects in patients with SCID. This study affirms the importance of the RAG1 and RAG2 genes for enhancing the efficiency of the recombination reaction. However, further studies involving other genes that could be the disease-causing of SCID patients are recommended.

## **Acknowledgments**

This study was funded by National Research Centre.

## **Conflict of interest**

The authors declare no conflict of interest.

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**Table 1:** The Sequence of PCR primers

Fragment (bp)	*Sequence (5'-3')	primer	
690 bp	CAAGCCAACCTTCGACATCT	F	RAG1 E2a <sub>[SEP]</sub>
	GATATCGGCAAGAGGGACAA	R	
502 bp	CGCCAACTGCAGTAAGATACA	F	RAG1 E2b <sub>[SEP]</sub>
	ACACGGACTTCACATCTCCACCT	R	
600 bp	CCACATCTCAAGTCACAAGGAA	F	RAG1 E2c <sub>[SEP]</sub>
	TCCATGTCCATCAAAGCAGA	R	
819bp	GCCATCACAGGGAGACAGAT	F	RAG1 E2 d <sub>[SEP]</sub>
	GGAACGCCAGACCTCATAAC	R	
594bp	GCACCGGCTATGATGAAAAA	F	RAG1 E2 e <sub>[SEP]</sub>
	CACTCTTTAGCAGGGCATGA	R	
800	TCATGAGGATGAATGGCAAC	F	RAG1 E2 f <sub>[SEP]</sub>
	TCCTGTTGCTTTTCGGAAC	R	
693	CCATAAACACTGTCAGAAGAGGAA	F	<b>RAG1 Exon 1</b>
	ACATCGATCCGGAAAACCTT	R	

494	AGCGTGTGGGAGGACTTAAAA	F	RAG2
	AACCTTTTTGTTGTTCTTGCAAAC	R	<b>Exon 1</b>
497	GGAGGGAAAACACCAAACAA	F	RAG2
	TGGAGACAGAGATTCCCTCCTG	R	<b>Exon 2a</b>
812	ATAATATCCGGCCTGCCAAC	F	RAG2
	GTAGGACTCTTTGGGGAGTGTG	R	<b>Exon 2b</b>
700	TCTACTGCTCTCATGGGGATG	F	RAG2
	GCCAACTGCCGAATTGTTAT	R	<b>Exon 2c</b>

\*Primers are listed from 5' to 3' ends and the second of each pair is the reverse or antisense sequence. F: forward, R: reverse. (14).

**Table 2:** Age & gender for Group I and II

Parameter		Group I n=50	Group II n=30
Age range		10 days- 3 years	7 days- 2.5 years
Gender (%)	M	25 (50%)	16 (53%)
	F	25 (50 %)	14 (47%)
Consanguinity	+ve	24 (48%)	17 (57%)
	-ve	26 (52%)	13 (43%)

**Table 3:** White blood cell characteristics & Lymphocyte subsets by Flowcytometry for Group I and II

parameter	Group, I n =50 Mean $\pm$ SD	Group II n=30 Mean $\pm$ SD	P value
TLC10 <sup>3</sup> /mm <sup>3</sup>	11.44 $\pm$ 1.46	11.31 $\pm$ 1.67	0.7289
Absolute lymphocyte count/mm <sup>3</sup>	6.01 $\pm$ 1.61	4.44 $\pm$ 1.53	0.0107*
CD3%	52.34 $\pm$ 9.57	37.98 $\pm$ 16.26	0.0001*
CD19%	16.01 $\pm$ 6.08	12.33 $\pm$ 6.37	0.038*
CD16%	16.97 $\pm$ 5.91	19.47 $\pm$ 7.75	0.206
CD4%	27.45 $\pm$ 7.19	23.64 $\pm$ 10.01	0.1135
CD8%	22.33 $\pm$ 6.66	15.94 $\pm$ 8.1	0.0022*

\* Significant difference at P-value <0.05

- TLC: Total Leukocytic count

**Table 4:** Immunoglobulins levels in Group I and II

parameter	Group, I n =50 Mean $\pm$ SD	Group II n=30 Mean $\pm$ SD	P value
IgA (g/L)	0.91 $\pm$ 0.62	0.66 $\pm$ 0.58	0.1591
IgM (g/L)	1.04 $\pm$ 0.54	0.72 $\pm$ 0.37	0.0072*
IgG (g/L)	11.46 $\pm$ 5.05	9.04 $\pm$ 2.92	0.036*

\* Significant difference at P-value <0.05

- Ig: Immunoglobulin

**Table 5:** Different in silico prediction algorithms: SIFT, PolyPhen-2, and condel for the detected variants.

DNA changes	AA changes *	Status	MT** / Grantham Matrix score (ref range; 0-215)	splice sites modifications (wt:mu scores) ***	SNP pred. score ****		condel
					SIFT	PolyPhen	
cDNA.2750G>A rs4151033	E880K	Reported	Disease-causing / 56	Donor increased (wt: 0.47 / mu: 0.83)	0.04	0.991	0.793
cDNA.2992A>G rs1980131	A960A	Reported	polymorphism	None	--	--	--
cDNA.858A>G rs3740955	H249R	Reported	Polymorphism/ 29	None	0.38	0	--

cDNA.2851T> A RAG1	S913R	Novel	Disease-causing / 110	Donors gained (0.44) Acc increased (wt: 0.21 / mu: 0.47)	0.03	0.999	
NM_00124378 5.1:c.1540T>A  RAG2 Exon 2c	L514M	Novel	polymorphism/15	Donor gained (0.61)	1	0.001	--
NM_00124378 6.1:c.1501C>A  RAG2 Exon 2c  rs781104028	P501T	reported	Disease-causing /38 moderate	Donor gained ( 0.37)  Donor increased (wt: 0.47 / mu: 0.62)	0.03	0.073	--
cDNA.2129A> T RAG2	3'UTR	Novel	Predicted Polymorphism	Donor marginally increased (wt: 0.8292 / mu: 0.8545)			
NM_000536.3( RAG2):c.*328 A>G  rs10836573	-	reported	Polymorphism	-	-	-	-
NC_000011.9: g.36597313A> G  cDNA.2571A> G  rs2227973 RAG1	Lys820 Arg K820R	reported	polymorphism/26	Donor gained (0.72)	0.13	0.006	
cDNA.2457T> G  NM_000448.2: c.2345T>G RAG1	V782G	Novel	Disease-causing / 109	-	0.01	0.994	

\* AA changes: Amino Acid changes

\*\* MT: Mutation Taster, this score is taken from an amino acid substitution matrix (Grantham Matrix) which considers the Physio-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid. Scores may range from 0.0 to 215.

\*\*\* wt: Wild & mu: Mutant

\*\*\*\*SIFT: Amino acids with probabilities < .05 are predicted to be deleterious.

- PolyPhen-2: Polymorphism Phenotyping v2 for annotating coding nonsynonymous SNPs, variants scores between 0.0 to 0.15 are predicted to be benign. 0.15 to 1.0 are possibly damaging. 0.85 to 1.0 are more confidently predicted to be damaging.
- condel: CONsensus DELeteriousness score of missense mutations.

**Table6:** Clinical manifestations of SCID patients.

No	Gender	AOO	AOD	Consanguinity	Main presenting features	Sibling death or abortion
1	M	3.5m	5m	-ve	FTT,skin lesions,hypopigmented areas, pneumonia	His sister died at 3m by pneumonia
2	M	7 days	1m	+ve	FTT,chest infection	Two sisters' death



3	Twin M/F	3m	4m	-ve	FTT, pneumonia	First pregnancy
4	M	53 days	2m	+ve	Pneumonia, chest infection, FTT	History of 4 neonatal death and IUFD
5	M	10 days	1m	-ve	FTT	History of abortion
6	F	1.5y	1.7y	+ve	FTT, pneumonia	Patient was first child
7	F	1w	1m	+ve	FTT, pneumonia	1 neonatal death
8	M	11da ys	1m	+ve	RDS	2 neonatal deaths
9	M	5 days twin	1m	-ve	pneumonia	Abortion once
10	F	1.5y	2y	+ve	FTT, pneumonia	1 neonatal death
11	F	8 ys	9y	-ve	severe pneumonia	First child
12	F	1.2 y	2y	-ve	severe pneumonia	1 neonatal death
13	F	19 days	1m	-ve	severe pneumonia	First child
14	F	8m	10m	-ve	FTT	First child
15	M	1m	2m	+ve	FTT, pneumonia	1 neonatal death
16	M	2m	4m	-ve	Recurrent pneumonia	Abortion twice
17	M	1m	2m	+ve	FTT, skin lesions, pneumonia	2 neonatal deaths
18	F	2w	1m	+ve	FTT, Pneumonia	Abortion once

- AOO: Age of Onset
- AOD: Age of Diagnosis
- FTT: Failure to thrive.
- RDS: Respiratory distress syndrome
- IUFD: Intrauterine fetal death