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Genetic mutations and immunological features of severe combined immunodeficiency patients in Iran

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ABSTRACT

Background: Severe combined immunodeficiency (SCID) is the most severe form of primary immunodeficiency disorders that is characterized by impaired early T lymphocyte differentiation and is variably associated with abnormal development of other lymphocyte lineages. SCID can be caused by mutations in more than 20 different genes. Molecular diagnosis in SCID patients contributes to genetic counseling, prenatal diagnosis, treatment modalities, and overall prognosis. In this cohort, the clinical, laboratory and genetic data related to Iranian SCID patients were comprehensively evaluated and efficiency of stepwise sequencing methods approach based on immunophenotype grouping was investigated

Methods: Clinical and laboratory data from 242 patients with SCID phenotype were evaluated. Molecular genetic analysis methods including Sanger sequencing, targeted gene panel and whole exome sequencing were performed on 62 patients.

Results: Mortality rate was 78.9% in the cohort with a median follow-up of four months. The majority of the patients had a phenotype of T-NK-B+ (34.3%) and the most severe clinical manifestation and highest mortality rate were observed in T-NK-B- SCID cases. Genetic mutations were confirmed in 50 patients (80.6%), of which defects in recombination-activating genes (RAG1 and RAG2) were found in 16 patients (32.0%). The lowest level of CD4+ and CD8+ cells were observed in patients with ADA deficiency ($p = 0.026$) and IL2RG deficiency ($p = 0.019$), respectively.

Conclusion: Current findings suggest that candidate gene approach based on patient's immunophenotype might

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accelerate molecular diagnosis of SCID patients. Candidate gene selection should be done according to the frequency of disease-causing genes in different populations. Targeted gene panel, WES and WGS methods can be used for the cases which are not diagnosed using this method.

1. Introduction

Severe combined immunodeficiency (SCID) is a heterogeneous group of primary immunodeficiency diseases (PIDs) characterized by severe reduction in T and/or B lymphocytes, and its overall prevalence estimated 1 in 50 000–100 000 live births worldwide [1]. However, the actual number of PIDs especially in the populations with a high rate of consanguineous marriage such as Iran is higher than previously reported [2]. Among PIDs, SCID patients have the most severe form of clinical manifestations. They commonly present severe and repeated infections by opportunistic microorganisms, and share typical clinical presentations such as early onset skin rashes, cutaneous complications, persistent diarrhea, pneumonitis, oral candidiasis and failure to thrive (FTT) during the first year of life [3,4]. Moreover, rare side effects of vaccination like Bacillus Calmette–Guérin (BCG)-osis and immunodeficiency-associated vaccine-derived polioviruses (iVDPVs) shedding, may occur in SCID patients [3,5]. In the absence of immune reconstitution, SCID patients rarely survive beyond 6–12 months [6], however, they usually respond successfully to allogeneic hematopoietic stem cell transplantation (HSCT) [7].

Several different genetic defects resulting in SCID have been described so far [8]. According to basic immunological defects, SCID patients are classified into 4 main groups including [1] defective function of pre–T-cell receptors such as, *CD3ε/δ/ζ,* [2] impaired signaling pathways, such as defects in the interleukin 2 receptor common γ-chain (*IL2RG*), interleukin 7 receptor α-chain (*IL7R*), and Janus kinase3 (*JAK3*); [3] V(Drecombination defects, including Artemis (*DCLRE1C*) and recombination-activating genes (*RAG1/ RAG2*)deficiency; and [4] early lymphocyte death mediated by purine metabolic dysfunction such as *ADA* and *PNP* deficiency [9]. Furthermore, based on the abnormalities in T and B cell frequency and functions, SCID patients are categorized into T-B TB − + and T-B- phenotypes according to the International Union of Immunological Societies (IUIS) guideline. Both groups include forms with or without defect in natural killer (NK) cells [10].

As an indicator of quality of PID care, depiction of the prevalence and characteristics of SCID patients is critical not only to increasing awareness of physicians but also health policy makers. An improvement in timely diagnosis and effective treatment of SCID is essential especially in the countries lacking the newborn screening system and developed public cord blood banks. In this study, the clinical, laboratory and genetic data related to SCID patients were comprehensively evaluated. We aimed to evaluate if the clinical manifestation would be prognostic in the different immunophenotypic subsets and whether the genetic bases of the disease in our SCID patients would be predictable based on clinical manifestation and immunophenotypic grouping. Moreover, we compared the clinical, laboratory and genetic data of Iranian SCID patients in different immunophenotyic or genetic groups.

2. Patients and methods

2.1. Patients' characteristics

The present study included all registered patients with SCID whose data were submitted to the Iranian national registry for PIDs established by the National PID Network under the supervision of the Research Center for Immunodeficiencies [11]. The diagnosis of SCID was defined according to the updated diagnostic criteria of the European Society for Immunodeficiencies (https://esid.org/Working-Parties/Registry/Diagnosis-criteria). Relevant informed consent was obtained from the parents of patients before participating in this study. A questionnaire surveyed the patients' demographic data, parental consanguinity, family history of immunodeficiency, the first clinical presentation, infectious and non-infectious clinical complications, presenting any disseminated infection following BCG or oral polio vaccinations and laboratory data for immunological diagnosis as described previously [12–14]. This study was approved by the Ethics Committee of Tehran University of Medical Sciences.

2.2. Genetic sequencing and molecular diagnosis

According to the pilot study in our patients [11], defect in seven genes including interleukin 2 receptor subunit gamma (*IL2RG)*, Janus kinase 3 (*JAK3)*, Interleukin-7 receptor subunit alpha (*IL7RA)*, adenosine deaminase (*ADA)*, purine nucleoside phosphorylase (*PNP)*, recombination activating gene 1 and 2 (*RAG1* and *RAG2*) constituted the most common mutations among SCID patients. Therefore, the targeted evaluation of these particular genes was prioritized for the molecular diagnosis of patients with available samples who consented to participate in the present study. Briefly, for the patients with T- B- phenotype (*ADA, PNP, RAG1*and*RAG2*) and T- B + phenotype (*IL2RG, JAK3* and *IL7RA*) genes were investigated, respectively. Genetic analysis was performed using Sanger sequencing of all exons and exon/intron junctions of these 7 genes in patients with available samples. In this regard, DNA was extracted from peripheral blood leukocytes by salting out method. High quality DNA samples, with the OD260/280 ratio of 1.8–2.0, at the concentration of 100 ng/μl were used for further analysis. PCR amplification of each DNA sample was carried out with 53 primer pairs for the selected seven genes (Table S1) in a final volume of 30 μl PCR master mix (Ampliqon) containing 100–200 ng genomic DNA, and 10 pmol of each primer. Initial denaturation of genomic DNA was performed for 5 min at 94 °C, followed by 30 cycles of amplification as follows: 30 s at 94 °C, 30–45 s at 62–67 °C and 60 s at 72 °C. Evaluation of polymerase chain reaction (PCR) products was done by electrophoresis on 1% agarose gel. Finally, the PCR products were sequenced at Macrogene Company (South Korea). The sequencing results were analyzed by Chromas and CLC workbench software. Also to identify disease causing variants in unsolved patients, targeted gene panel (TGP) and whole exome sequencing (WES) were performed as described previously [15,16]. The pathogenicity of disease variants was re-evaluated using the updated guideline for interpretation of molecular sequencing by the American College of Medical Genetics and Genomics (ACMG) considering the allele frequency in the population database, immunological/functional data, familial segregation and parental genotype (https://www.acmg.net/).

Mutations were defined as either mild or severe based on their effect on protein production or structure. Based on the criteria of the ACMG, we considered mutations with strong evidence of computational and predictive data (PVS1, e.g. nonsense, frame shift and splicing site mutations) as severe and the remaining mutations were classified as non-severe (mild). The clinically severe phenotype was defined by having two out of the following criteria: early onset symptoms (< 1 month), death before the age of one year, absence of CD3, CD4 or CD8 T cells, development of opportunistic infections, and development of severe infectious complications during the course of the disease (i.e., sepsis, central nervous system infections, osteomyelitis, and bacterial arthritis). In the present study, the correlation between the genotype and phenotype was examined.

2.3. Statistical analysis

Statistical analysis was performed using a commercially available software package (SPSS Statistics 22.0.0, SPSS, Chicago, Illinois). Kolmogorov-Smirnov test was applied to estimate whether data distribution was normal. Parametric and nonparametric analyses were performed based on the findings of this evaluation. Diagnosis delay was defined as a period of time between the age at onset and age at diagnosis. Overall Survival (OS) rate with 95% confidence intervals was estimated using the Kaplan-Meier plot and two groups were compared with the log-rank test. A p-value of < 0.05 was considered to be statistically significant for all tests.

3. Results

3.1. Clinical data of the patients

From 271 patients who were registered as SCID cases, 29 patients were excluded from the study due to the lack of sufficient clinical or laboratory data. A total of 242 patients including 113 females (47%) were enrolled in the present study and were followed-up for a median time of four months. Consanguinity was found in 78.5% (n = 190) of the cases and a positive family history of SCID was recorded in 90 patients (37.1%). The median age at diagnosis was 5 months (IQR of 3–8 months) with the median period of delayed diagnosis up to two months (IQR of 1–4 months). Majority of the patients (60.7%, n = 147) were diagnosed during a period of four months following their first presentation. The main demographic data of the patients are summarized in Table S2.

The first clinical manifestations were divided into two major groups: respiratory and non-respiratory symptoms. As shown in Table S2, first presentation with non-respiratory complications was more frequent than respiratory symptoms in our patients' cohort (82.2% vs. 17.8%, respectively). In contrast, during the course of disease, pneumonia was the most common clinical manifestation in patients (50.8%), followed by chronic diarrhea (42.5%), BCG-osis (38%), and persistent fever (37%, Table 1). Moreover, regarding the severity of clinical complications, 62.8% (n = 152) of the patients had a severe phenotype. Mortality was recorded in 191 patients at the end point of this study (78.9%), possibly due to delay in diagnosis and lack of equipment required for hematopoietic stem cell transplantation (HSCT). Moreover survival analysis showed significant poor outcomes of male patients ($p = 0.04$) and those without parental consanguinity ($p = 0.01$, Fig. S1).

The complications following BCG vaccination was reported in 97 out of 242 studied cases. 78 cases (31.4%) manifested BCG-osis as first that was the most common first presenting manifestation in our patients. Our data showed that the patients with BCGosis had a significantly decreased overall survival (OS) compared with the patients without BCGosis (8.9 months vs. 14.5 months, Long rank test, $p = 0.037$) (Fig. S2). Information about the iVDPV shedding test was available for 20 patients. Among these patients, 13 cases (65%) had negative poliovirus shedding result and in 7 (35%) cases the test was positive. Antigenic serotypes found in the patients are indicated in Table S3.

3.2. Immunophenotype characteristics

Patients were categorized into four groups based on their immunophenotypes at the time of diagnosis, including T−B + NK+, T-B + NK-, T-B-NK+, and T-B-NK-. Regarding the comparison of demographic data of these four groups, we have not found any significant difference. The patients in B- groups manifested their first presentation slightly ealier comparing to B + groups (2.5[0–4] months vs. 3[0–5] months). Infants with the T-B-NK- showed the lowest age of onset with a median of 1.5 (0.75–4) months after their birth. Respiratory complications as the first clinical manifestations were significantly more frequent in T-B-NK- (42.9%) compared to other immunophenotypes ($p = 0.011$, Table S2). In line with this observation, pneumonia was the most common clinical manifestation in 2 phenotypes including T-B-NK + (58.4%) and T-B-NK- (71.1%). In contrast, BCG-osis and chronic diarrhea were the most frequent clinical complications in T−B + NK + and TB− + NK- phenotypes, affecting more than 49% and 45% of the patients, respectively. There was a significant difference between the four immunophenotype groups regarding pneumonia (evaluated in T-B-NK-, OR = 2.9, $p = 0.035$). Among non-infectious complications, splenomegaly was slightly more prevalent in the T-B + NK+ immunophenotype group (29.5%; OR = 2.5, $p = 0.079$, Table 1). Regarding comparison of laboratory data, the frequency of lymphocyte subsets and immunoglobulin levels reflected the immunophenotyping of groups expectedly, however, CD + T cells were significantly higher in patients with T-B + NK + ($p = 0.01$, Table S4). Of note, the survival rate at 1 year increased to 63% overall, in which the 1-year mortality rate was 47% in T-B- and only 20% in T-B + groups ($p = 0.042$, Fig. S3).

3.3. Genetic diagnosis results

Of the 242 patients enrolled in this study, the genetic examination was carried out for 62 patients. Given that 62 patients with available samples who consented to participate in the present study, we have to performed genetic analysis only on this group of patients. Mutations

Table 1
Clinical manifestations of the SCID patients with different phenotypic features.

Clinical presentations	Total (n = 242)	T-B + NK+ (n = 61)	T-B + NK- (n = 83)	T-B-NK+ (n = 77)	T-B-NK- (n = 21)	P-value
Pneumonia, Number (%)	123 (50.8)	28 (45.9)	35 (42.2)	45 (58.4)	15 (71.4)	**0.035***
BCG-Osis, Number (%)	93 (38.4)	30 (49.2)	32 (38.6)	22 (28.6)	9 (42.9)	0.072
Oral candidiasis, Number (%)	70 (28.9)	15 (24.6)	26 (31.3)	22 (28.6)	7 (33.3)	0.842
FTT (Failure to thrive), Number (%)	52 (21.5)	7 (11.5)	21 (25.3)	19 (24.7)	5 (23.8)	0.209
Diarrhea, Number (%)	103 (42.6)	22 (36.1)	38 (45.8)	34 (44.2)	9 (42.9)	0.775
Skin infection, Number (%)	38 (15.7)	10 (16.4)	10 (12)	16 (20.8)	2 (9.5)	0.368
Hives, Number (%)	11 (4.5)	4 (6.6)	1 (1.2)	5 (6.5)	1 (4.8)	0.299
Rash, Number (%)	17 (7)	4 (6.6)	4 (4.8)	8 (10.4)	1 (4.8)	0.564
Eczema, Number (%)	9 (3.7)	4 (6.6)	5 (6)	1 (1.3)	1 (4.8)	0.416
Otitis, Number (%)	17 (7)	4 (6.6)	9 (10.8)	4 (5.2)	0 (0)	0.291
Urinary Tract Infections, Number (%)	14 (5.8)	4 (6.6)	5 (6)	5 (6.5)	0 (0)	0.709
Fever, Number (%)	90 (37.2)	19 (31.1)	30 (36.1)	35 (45.5)	6 (28.6)	0.282
Inguinal hernia, Number (%)	3 (1.2)	1 (1.6)	1 (1.2)	1 (1.3)	0 (0)	1.00
LAP (Lymphadenopathy), Number (%)	50 (20.7)	16 (26.2)	16 (19.3)	15 (19.5)	3 (14.3)	0.579
Hepatomegaly, Number (%)	60 (24.8)	17 (27.9)	24 (28.9)	17 (22.1)	2 (9.5)	0.241
Splenomegaly, Number (%)	54 (22.3)	18 (29.5)	22 (26.5)	11 (14.3)	3 (14.3)	0.079

* Statistically significant P-value.

Table 2
Characteristics of the genetic mutations found in the genetically diagnosed SCID patients.

No	Causal Gene†	Inheritance Pattern	Chromosome	Exon/ Intron	DNA Change *	Protein Change	Affected Domain	Pathogenicity (ACMG)**	Patient report/ novel	Variant HGMD,ENSEML Report ID/novel	Severity ***	
											Mutation	Medical
1	*ADA*[2]	AR	20	5	c.G415T^{A2}	p.E139X	DDP4	P	(11)	New	S	S
2	*ADA*[3]	AR	20	8	c.704 G > A^{A1}	p.R235Q	Deaminase	LP	(11)	CM011751	M	S
3	*ADA*[1]	AR	20	6	c.556 G > A^{A1}	p.E186K	Deaminase	LP	New patient	New	M	S
4	*ADA*[3]	AR	20	6	c.541 G > A^{A1}	p.D181N	Deaminase	LP	New patient	New	M	S
5	*ADA*[1]	AR	20	8	c.736C > T^{A2}	p.Q246X	Deaminase	P	New patient	CM114610	S	M
6	*CD3D*[2]	AR	11	2	c.247 G > T^{A2}	p.E83X	Extracellular	P	(11)	New	S	S
7	*CD3E*[2]	AR	11	6	c.208 G > T^{A2}	p.E70X	Ig Like	P	(11)	New	S	S
8	*CD3E*[3]	AR	11	IVS6	c.353-2A > C^{A6} (IVS6 AS -2A > C)		Helical	P	New patient	New	S	S
9	*CD3E*[3]	AR	11	6	c.173delT^{A3}	L58H fs X67	Ig Like	P	New patient	New	S	M
10	*CD3E*[3]	AR	11	6	c.173delT^{A3}	L58H fs X67	Ig Like	P	New patient	New	S	S
11	*CD3E*[3]	AR	11	6	c.173delT^{A3}	L58H fs X67	Ig Like	P	New patient	New	S	S
12	*CIITA*[2]	AR	16	18	c.3242-3244 del^{A4}	p.N1082del	LRR4	P	(11)	rs767284761	M	M
13	*DCLRE1C*[2]	AR	10	14	c.1250-1260 del^{A3}	p.S417C fs X422	DNAPK	P	(11)	New	S	M
14	*DCLRE1C*[2]	AR	10	1	c.41 G > T^{A1}	p.G14V	Nuclease	LP	(11)	New	M	M
15	*DCLRE1C*[3]	AR	10	IVS5	c.362 + 1G > T^{A6} (IVS5 ds +1 G > T)		Nuclease	P	New patient	CS011851	S	S
16	*DCLRE1C*[3]	AR	10	5	c.329 T > G^{A1}	p.L110R	Nuclease	LP	New patient	New	M	S
17	*IL17RA*[3]	AR	22	13	c.1867delC^{A3}	p.L623W fs X634	Cytoplasmic	P	New patient	New	S	M
18	*IL2RG*[1]	XLR	X	6	c.850 G > T^{C2}	p.E284X	Cytoplasmic	P	(11)	New	S	M
19	*IL2RG*[1]	XLR	X	2	c.181-182 del^{C3}	p.Q61 V fs X79	Extracellular	P	(11)	New	S	S
20	*IL2RG*[1]	XLR	X	2	c.267 T > G^{C2}	p.Y89X	Extracellular	P	New patient	New	S	S
21	*IL2RG*[1]	XLR	X	1	c.1A > G^{C1}	p.M1V	Extracellular	P	New patient	CM990751	S	S
22	*IL2RG*[1]	XLR	X	3	c.273C > G^{C2}	p.Y91X	Extracellular	P	New patient	CM011375	S	S
23	*IL7RA*[1]	AR	5	2	c.152C > A^{A2}	p.S51X	Extracellular	P	New patient	New	S	M
24	*JAK3*[1]	AR	19	16	c.2164 G > A^{A1}	p.V722I	Protein kinase 1	LP	(11)	CM000172	M	M
25	*JAK3*[1]	AR	19	16	c.2125 T > A^{A1}	p.W709R	Protein kinase 1	LP	(11)	New	M	S
26	*JAK3*[3]	AR	19	17	c.2324 G > A^{A1}	p.R775H	Protein kinase 1	LP	(11)	New	M	M
27	*JAK3*[1]	AR	19	12	c.1645C > T^{A2}	p.R549X	Protein kinase 1	P	New patient	rs1011307501	S	M
28	*JAK3*[1]	AR	19	15	c.1951C > T^{A1}	p.R651W	Protein kinase 1	LP	New patient	CM012983	M	S
29	*JAK3*[1]	AR	19	10	c.1383insG^{A5}	L462A fs X518	SH2	P	New patient	New	S	M
30	*MALT1*[2]	AR	18	12	c.1454A > G^{A1}	p.N485S	Caspase-like	LP	(11)	New	M	S
31	*NHEJ1*[2]	AR	2	4	c.526C > T^{A2}	p.R176X	Coiled coili	P	(11)	CM105781	S	S
32	*RAG1*[1]	AR	11	2	c.2564A > G^{A1}	p.N855S	ZnH2	LP	(11)	New	M	M
33	*RAG1*[1]	AR	11	2	c.1073 G > A^{A1}	p.C358Y	ZFA	LP	(11)	New	M	M
34	*RAG1*[1]	AR	11	2	c.2570C > A^{A1}	p.A857D	ZnH2	LP	(11)	rs753534445	M	M
35	*RAG1*[1]	AR	11	2	c.2985 G > A^{A2}	p.W995X	CTD	P	(11)	rs760674116	S	M
36	*RAG1*[1]	AR	11	2	c.1405 G > C/ c.322C > T^{B0}	p.V469 L/ p.R108X	DDBD	P	(11)	New/ CM114804	S	S
37	*RAG1*[1]	AR	11	2	c.1180C > T^{A1}	p.R394W	NBD	P	(11)	CM065417	M	S
38	*RAG1*[1]	AR	11	2	c.1180C > T^{A1}	p.R394W	NBD	P	(11)	CM065417	M	M
39	*RAG1*[1]	AR	11	2	c.1180C > T^{A1}	p.R394W	NBD	P	New patient	CM065417	M	M
40	*RAG*[1]	AR	11	2	c.2689C > T^{A2}	p.R897X	ZnH2	P	New patient	CM961217	S	S
41	*RAG1*[1]	AR	11	2	c.834delC^{A3}	p.A280Q fs X87	ZDD	P	New patient	New	S	M
42	*RAG2*[1]	AR	11	2	c.130 G > A^{A1}	p.G44R	Core	LP	(11)	New	M	M
43	*RAG2*[1]	AR	11	2	c.734C > T^{A1}	p.P245L	Core	LP	New patient	New	M	M

(*continued on next page*)

Table 2 (*continued*)

No	Causal Gene†	Inheritance Pattern	Chromosome	Exon/ Intron	DNA Change *	Protein Change	Affected Domain	Pathogenicity (ACMG)**	Patient report/ novel	Variant HGMD,ENSEML Report ID/novel	Severity ***	
											Mutation	Medical
44	*RAG2*[1]	AR	11	2	c.581 C > A[A2]	p.S194X	Core	P	New patient	New	S	M
45	*RAG2*[1]	AR	11	2	c.685C > T[A1]	p.R229W	Core	P	(11)	CM010087	M	S
46	*RAG2*[1]	AR	11	2	c.685C > T[A1]	p.R229W	Core	P	New patient	CM010087	M	S
47	*RAG2*[1]	AR	11	2	c.C685C > T[A1]	p.R229W	Core	P	New patient	CM010087	M	S
48	*RFXANK*[2]	AR	19	3	c.163delG[A3]	p.D55 M fs X67	ANK 1	P	New patient	New	S	M
49	*RFXANK*[2]	AR	19	3	c.162delG[A3]	p.D55 M fs X67	ANK 1	P	New patient	New	S	M
50	*ZAP70*[3]	AR	2	12	c.1561 G > A[A1]	p.D521N	Protein Kinase	LP	(11)	rs748318780	M	S

AR: Autosomal recessive, XLR: X chromosome-linked recessive.† Diagnostic methods used 1:Sanger Sequencing, 2: Targeted Gene Panel, 3: Whole Exome Sequencing* A: Homozygous, B: CompoundHeterozygous, C: Hemizygous, 1: Missense, 2: Nonsense, 3: Frame shift deletion-stop gain, 4: In frame deletion, 5: Frame shift duplication-stop gain, 6: Splicing. ** P: Pathogenic, LP: Likely pathogenic. *** S: Severe, M: Mild XLR: X chromosome- linked recessive, WES: whole exome sequencing, TGS: third generation sequencing.

have found in 50 cases (80.6%), while 12 patients (19%) were not diagnosed by genetic analysis. Out of these 50 patients, 29 (58%) cases were diagnosed through immunophenotypic grouping and sequencing of the seven-gene panel by Sanger sequencing. The characteristics of the genetic analysis have been shown in Table 2. As shown in the pie chart, *RAG1*was the most common causative gene in 20% of the cases, followed by *RAG2* (12%) and *JAK3* (12%).Overall, missense mutations were the most common mutation type, affecting 23% of the patients (Fig. 1).

We found 44 variants in our patients, of which 17 variants have been reported in genetic online databases (http://www.hgmd.cf.ac.uk, https://asia.ensembl.org) and other 27 are novel variations. Of these 27 new variants, 17 was nonsense, frame shift Ins/Del and splicing changes leading to a truncated protein, and were classified as pathogenic changes, while the remaining 10 variants were missense changes and were classified as likely pathogenic variations. Altogether, 68% (n = 34) of the mutations were pathogenic and 32% of them were likely pathogenic variations. In terms of mutation severity, 54% (n = 27) of the mutations were severe according to PSV1 category of ACMG criteria. Moreover, regarding the severity of clinical complications, 52% of the patients with molecular diagnosis had a severe phenotype (n = 26, Table 2). In general, in 50% of the patients, we observed the discordant severity between clinical and genetic data (Fig. 2).

3.4. Classification of the patients based on the disease causing genes

In order to make a comparison, we categorized the most observed casual genes into 7 different groups (Tables 3 and 4). The clinical features of the seven groups are mentioned in Table 3 and their demographic and laboratory data are presented in Tables S5 and S6.Pneumonia was the most common clinical manifestation in patients with *RAG2* mutations. In contrast, patients with mutations in *JAK3*, *ADA*, and *IL2RG* genes showed fever as the most frequent common complication. In the patients with *CD3E* mutations, common clinical manifestation was chronci diarrhea. Patients with the RAG1 deficeincy, showed pneumonia and fever as major clinical manifestations, while both diarrhea and fever were more common in the DCLRE1C deficient group. However, we did not observe any significant difference among these 7 groups regarding clinical complications. The genotype–phenotype correlation was considered to be positive when the disease causing gene was one of the expected genes in the related phenotypic group (Table 5). We observed that genotype- immune phenotype was significantly correlated in 35 out of 41 patients in the 7 groups (85.4%, $p = 0.009$). Moreover, the data about other identified genes (with low frequency) is summarized in Table 6. Among these 50 genetically diagnosed patients, the causal mutation was hypomorphic in four cases (3 cases with *ADA* mutations and 1 case with *NHEJ* mutation) (Tables 4 and 6). The genotype- clinical phenotype correlation was positive in 72% of our total patients (n = 36).Our results showed no significant differences in overall survival rate between the patients with mutations in 3 most observed casual genes *RAG1*, *RAG2* and *JAK3*. The Kaplan Meier plots for different comparison modes of these three most common gene groups are presented in Fig. S4-7.

4. Discussion

In the present study, we tried to investigate the molecular diagnosis of our SCID patients by examining their medical and immunological manifestations and implementation of stepwise sequencing methods. In this cohort, the prevalence of different immunophenotypic groups among SCID patients showed a higher prevalence of T−B + NK- (34.3%) and T-B-NK+ (31.8%) phenotypes in our region. This pattern has not been consistent with the reports from other Asian populations such as India [17], China [18] Turkey [19] and Saudi Arabia [20].In India the prevalence has been indicated as follows: more than half of SCID infants are T−B + NK + andmore than one-third SCID infants are

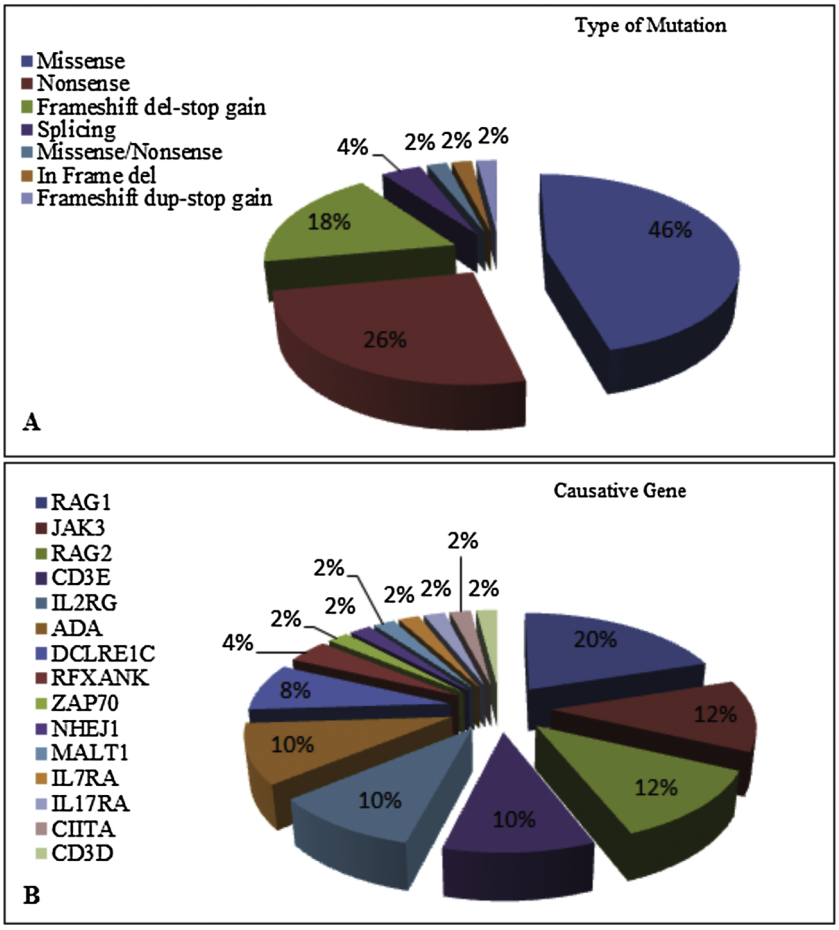

Fig. 1. A: The gene mutation types in the genetically examined SCID patients, B: The causative genes in genetically examined SCID patients.

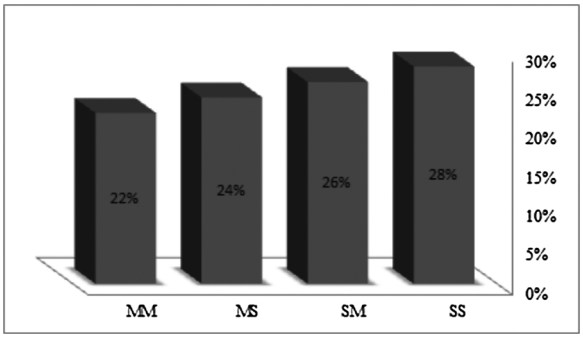

Fig. 2. Severity of mutations and clinical manifestations in 50 genetically diagnosed SCID patients. The first and second letters of each two letter state abbreviation in the X-axis indicated the severity of the mutation and the severity of the clinical symptoms, respectively. S: Severe, M: Mild.

T-B-NK+ [17]. In a Chinese registry, 66.7% ofpatients had B + SCID and B- SCID constituted 7.1% of the cohort [18]. In the registry ofSaudi Arabia, SCID T-B- was the most commontype in 17% [20] and in registry of Turkey the T-B-NK+ 47% of SCID patients [19].In our study, BCG-osis and diarrhea represented the most common clinical manifestations among T−B + SCID patients whereas pneumonia was more frequently diagnosed in T-B- SCID patients. Although similar studies are not available in this area, it seems that at least in our experience respiratory and non-respiratory manifestations occurred differently in T-B- and T−B + group.

The diagnostic yield of our genetic approach reached approximately 80% of the evaluated patients, indicating one of the most efficient approaches toward molecular diagnosis within our PID registry [21] and also compared to other SCID registry worldwide [12]. Most of the disease-causing genetic alterations were detected in *RAG1* and *RAG2* genes. This finding is consistent with previous reports in Iranian SCID patients [3,22] as well as genetic studies from regional registries from Saudi [20], Turkey [19] and Kuwait [23]. Also, missense variations were the most common alteration type, followed by nonsense mutations. These findings were consistent with our expectations according to the HGMD (http://www.hgmd.cf.ac.uk) data as well as previous studies [3,22]. Regarding the protein domains affected by gene mutations, geneswith enzymatic roles such as *ADA*, *DCLRE1C*, *JAK3*, *RAG1*, *RAG2* and *ZAP70* are often mutated in catalytic or core enzyme domains (81%), while genes that act as receptors such as *CD3D*, *CD3E*, *IL7RA* and *IL2RG* are often mutated in ligand binding or extracellular domains (62%). These findings were consistent with our expectations according to the HGMD (http://www.hgmd.cf.ac.uk) and Atlas-Genetics-Oncology (http://atlasgeneticsoncology.org) databases as well as previous studies [24–27].

In the present study, there was no definitive relationship between the severity of clinical symptoms and the severity of mutations as discrepancies observed in 50% of cases, This can be due to the different molecular mechanisms and unique complexities of each gene, mutation and other modifying genetic or environmental factors [28], that should be considered in examining different SCID patients. Based on our results, 68% of variations were pathogenic and 32% of them were likely pathogenic genetic alterations. Interestingly, all variations found in *CD3E*, *IL2RG*and*RFXANK* genes were pathogenic while variations found in other genes were both pathogenic and likely pathogenic mutations. Since there is no similar study, we could not compare our results with other studies. However, these findings could be the result of statistical

Table 3
Clinical features of the genetically diagnosed SCID patients and statistical tests results for seven comparable phenotypic groups.

Clinical manifestations	RAG1 (n = 10)	RAG2 (n = 6)	JAK3 (n = 6)	ADA (n = 5)	IL2RG (n = 5)	CD3E (n = 5)	DCLRE1C (n = 4)	P-value
Pneumonia, Number (%)	4 (40)	4 (66.7)	3 (50)	2 (40)	1 (20)	1 (20)	2 (50)	0.76
BCG-osis, Number (%)	3 (30)	2 (33.3)	3 (50)	1 (20)	2 (40)	2 (40)	1 (25)	0.97
Oral candidiasis, Number (%)	3 (30)	1 (16.7)	3 (50)	2 (40)	1 (20)	1 (20)	1 (25)	0.91
Failure to thrive, Number (%)	3 (30)	1 (16.7)	3 (50)	1 (20)	1 (20)	0 (0)	2 (50)	0.46
Diarrhea, Number (%)	5 (50)	3 (50)	3 (50)	1 (20)	1 (20)	3 (60)	3 (75)	0.64
Skin infection, Number (%)	0 (0)	1 (16.7)	1 (16.7)	2 (40)	0 (0)	1 (20)	0 (0)	0.33
Hives, Number (%)	0 (0)	0 (0)	0 (0)	1 (20)	0 (0)	1 (20)	0 (0)	0.35
Rash, Number (%)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1.00
Otitis, Number (%)	0 (0)	1 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (25)	0.20
Urinary Tract Infections, Number (%)	1 (10)	0 (0)	0 (0)	1 (20)	0 (0)	0 (0)	0 (0)	0.60
Fever, Number (%)	4 (40)	3 (50)	4 (66.7)	3 (60)	4 (80)	2 (40)	3 (75)	0.62
Lymphadenopathy, Number (%)	3 (30)	1 (16.7)	0 (0)	1 (20)	1 (20)	3 (60)	2 (50)	0.52
Hepatomegaly, Number (%)	2 (20)	2 (33.3)	2 (33.3)	2 (40)	3 (60)	0 (0)	1 (25)	0.44
Splenomegaly, Number (%)	1 (10)	1 (16.7)	1 (16.7)	1 (20)	2 (40)	0 (0)	1 (25)	0.71

Table 4
The frequency and the distribution of the genetic variations in the genetically diagnosed SCID patients.

Title	RAG1 (n = 10)	RAG2 (n = 6)	JAK3 (n = 6)	ADA (n = 5)	IL2RG (n = 5)	CD3E (n = 5)	DCLRE1C (n = 4)	P-value
Immunophenotype								
T-B + NK+	0	0	0	0	0	3	0	
T-B + NK-	0	0	5	0	5	1	0	
T-B-NK+	10	6	1	3	0	0	4	
T-B-NK-	0	0	0	2	0	1	0	
Type of mutation								
Missense (21)	6	5	4	3	1	0	2	0.17
Nonsense (11)	2	1	1	2	3	1	0	
Frame shift ins (1)	0	0	1	0	0	0	0	
Frame shift del (6)	1	0	0	0	1	3	1	
In frame del (0)	0	0	0	0	0	0	0	
Splicing site (2)	0	0	0	0	0	1	1	
Missense/nonsense (1)	1	0	0	0	0	0	0	
Zygosity								
Homozygote	9	6	6	5	0	5	4	–
Compound Heterozygote	1	0	0	0	0	0	0	
Hemi zygote	0	0	0	0	5	0	0	
Hypomorphic mutation								
No	10	6	6	2	5	5	4	–
Yes	0	0	0	3	0	0	0	
Genotype-Phenotype Correlation								
No (6)	0	0	1	3	0	2	0	
Yes (35)	10	6	5	2	5	3	4	0.009*

*sign indicates significant difference.

Table 5
The expected causative genes in different immunophenotype groups versus the observed causative genes in the genetically diagnosed patients.

Immunophenotype (number of patients)	Expected genes	Observed genes (number of patients)
T-B + NK+ (7)	*IL7R, CD3E, PTPRC, CD3Z, CD3D*	*IL7RA* (1), CD3E (3), NHEJ1 (1), RFXANK (1), ZAP70 (1)
T-B + NK- (15)	*JAK3, IL2RG*	JAK3 (5), IL2RG (5), *CD3E* (1), CD3D (1), CIITA (1), IL17RA (1), MALT1 (1)
T-B-NK+ (25)	*RAG1, RAG2, DCLRE1C*	*RAG1* (10), *RAG2* (6), *DCLRE1C* (4), *JAK3* (1), ADA (3), RFXANK (1)
T-B-NK- (3)	*ADA, PNP, AK2*	ADA (2), *CD3E (1)*

bias and it should be tested with more sample size examined in different gene groups [29].

The clinical presentations of SCID may vary among the patients, reflecting various molecular bases such as hypomorphic changes of key modifier genes mutations and epigenetic alterations [30–35]. Here, we have reported 4 patients with hypomorphic mutations in genes that showed milder phenotype than expected according to their causal genes (leaky SCID phenotype) mainly in patient with ADA deficiency consisting with the previous observation on adult onset of this diseases. Moreover, six of our SCID patients had gene variants in *RFXANK*, *CIITA*,*ZAP-70*, *IL17RA*and*MALT1* genes, which defect in these genes could result in combined immunodeficiency (CID) or less severe forms of CID [36,37]. Overall, the presence of such gene variants may justify the lack of correlation between genotype-phenotype correlations observed in 14 cases of our SCID patients. Therefore the possibility of mutation in unexpected genes should not be ignored in the genetic evaluation of SCID patients. This issue should be considered in choosing the type of sequencing method and targeted gene panel.

The reports from the United States, Europe, and China [10,38] implicated that SCID is more common in male patients reflecting the overrepresentation of X-linked SCID (XL-SCID) due to IL2RG deficiency. The rate of this mutation in our cohort was 10%, whereas the remaining cases showed autosomal recessive (AR) pattern of inheritance and there was no significant difference between the frequency of male and female patients. This could be due to a relatively high rate of consanguinity (78.5%) in our patients' families that is much higher than the normal

Table 6
Genetic data in SCID patients with mutations in low frequent causal genes.

Title/Rare genes 0	RFXANK (n = 2)	ZAP70 (n = 1)	NHEJ1 (n = 1)	MALT1 (n = 1)	IL17RA (n = 1)	CIITA (n = 1)	IL7RA (n = 1)	CD3D (n = 1)
Immunophenotype (4groups)								
T-B + NK+	1	1	1	0	0	0	1	0
T-B + NK-	0	0	0	1	1	1	0	1
T-B-NK+	1	0	0	0	0	0	0	0
T-B-NK-	0	0	0	0	0	0	0	0
Type of mutation	Frame shift deletion	Missense mutation	Nonsense mutation	Missense mutation	Frame shift deletion	In-frame deletion	Nonsense mutation	mutation Nonsense
Zygosity	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote	Homozygote
Hypomorphic mutation								
No	2	1	0	1	1	1	1	1
Yes	0	0	1	0	0	0	0	0
Genotype-Phenotype Correlation								
No (8)	2	1	1	1	1	1	0	1
Yes (1)	0	0	0	0	0	0	1	0

*Statistically significant P-value.

population (˜30–40%) [39].

Complications following vaccinationhave also been reported as one of the life threatening manifestation in SCID patients [11,40]. Our data showed that the patients with BCG-osis had a significantly decreased overall survival compared with the patients without BCG-osis. These findings are in agreement with previous investigations in Iran [39,40]. Another vaccination complication is shedding after poliovirus vaccination. Although widespread use of oral poliovirus vaccine has led to an approximate 99.9% decrease in the global incidence of poliomyelitis [41,42], patients with SCID might shed iVDPVs for a long period, which could pose a remarkable threat to polio eradication programs. In the present study, 7 patients had positive results in polioviruses shedding test. We have recently reviewed this point regarding SCID patients that they showed lower rates of vaccine-associated paralytic poliomyelitis but also lower polio infection clearance, compared to other PID patients [43]. As a result, delaying BCG vaccination for a few months and efficient iVDPV surveillance to screen PID patients for the shedding of polioviruses could be suggested in those families with a history of recurrent infections or early death [5,40].

Overall, out of the total 62 patients enrolled in this study, the rate of positive and negative results by Sanger sequencing was 47% and 53%, respectively. By adding DCLRE1C and CD3E genes to this seven-gene panel, the rate of positive results for the immunophenotypic grouping-Sanger sequencing method changed to 72% in the population under study. This stepwise approach can lead to lower costs for families in the process of genetic diagnosis. Out of the patients were candidate for NGS, 64% were diagnosed (21 out of 33), while twelve (19%) patients were not diagnosed by NGS sequencing.. The results related to efficiency of the NGS method are consistent with the previously reported studies [5,40].

In conclusion, molecular diagnosis is a critical step for genetic counseling, carrier detection, and prenatal diagnosis in SCID patients, which are essential in countries with limited resources for HSCT and gene therapy specially.Applying rational ways based on immunological and clinical findings, in order to shorten the path to genetic diagnosis and reduce costs, can be very effective in managing the disease. We were able to detect genetic defects in a large number of our patients, using the immunophenotype grouping and Sanger sequencing based on the candidate gene approach. This approach can be the front line in molecular diagnosis of SCID.Despite all efforts until now, more comprehensive studies are needed, especially for better knowing etiology of SCID disorder, in order to better diagnosis and management of the disease. This perspective makes the database and a valuable tool for future investigations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imlet.2019.10.001.

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