Clinical, Laboratory and Molecular Findings of 63 Patients with Severe Combined Immunodeficiency: A Decade's Experience

Running title: Severe combined immunodeficiency in Iran

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ABSTRACT

Introduction: Severe combined immunodeficiency (SCID) is known as a pediatric emergency disease with life threatening conditions. This is an exclusive report of clinical evaluation, immunological assessment, molecular analysis and outcomes of SCID patients in a tertiary referral center in Iran.

Methods: During January 2006 and December 2015, in a prospective cohort study, initial screening and advanced immunological tests were carried out on patients suspected of having SCID. Genetic analysis was also performed to confirm the disease.

Results: Sixty-three patients were diagnosed with SCID, forty-three (68.3%) being male. The median age of disease onset, diagnosis and delay diagnostic time were 40, 110 and 60 days respectively. Forty-nine patients (77.8%) had a history of BCG vaccination and one-third of them showed BCG associated complications. The most common clinical manifestations of all patients were pneumonia, recurrent oral candidiasis, chronic diarrhea and failure to thrive. Of the thirteen patients who underwent hematopoietic stem cell transplantation (HSCT), 8 survived and unfortunately the others died before they could receive an HSCT. Most patients (34.9%) were classified as T-B-NK+ SCID and the majority of the patients had a mutation in RAG2 or RAG1 genes.

Conclusion: Autosomal recessive SCID is the most inherited type in Iranian patients. It should be considered high priority to provide high quality training to physicians and patients' families to reduce the delay time in diagnosis. It is also important to raise the awareness of live vaccination as well as to expand the stem cell donor registries to speed up the transplantation process.

Key Words: Severe combined immunodeficiency. Newborn screening. BCG complications. Stem cell transplantation.

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RESUMEN

Introducción: La inmunodeficiencia combinada severa (SCID) es una grave enfermedad pediátrica que puede comprometer la vida del paciente. El artículo recoge la evaluación clínica e inmunológica, el análisis molecular y la supervivencia de los pacientes con SCID atendidos en un Hospital de referencia de Irán.

Métodos: Desde enero de 2006 a diciembre de 2015, se realizó un estudio prospectivo en los pacientes con SCID en el que se realizó un screening inicial junto a diferentes análisis inmunológicos. Se realizó un análisis genético para confirmar el diagnóstico.

Resultados: Sesenta y tres pacientes fueron diagnosticados de SCID, cuarenta y tres (63,8%) de los mismos eran varones. La mediana de la edad de inicio de la enfermedad, diagnóstico y retraso en su diagnóstico, fueron de 40, 110 y 60 días respectivamente. Cuarenta y nueve pacientes (77.8%) recibieron vacunación con BCG y un tercio de los mismos presentó complicaciones como consecuencia de la misma. Las manifestaciones clínicas más frecuentes de estos pacientes fueron: neumonía, candidiasis oral recidivante, diarrea crónica y retraso en el crecimiento. Ocho de los treces pacientes que recibieron trasplante de progenitores hematopoyéticos, lograron sobrevivir. Los restantes pacientes fallecieron antes de poder recibir dicho trasplante. El 34.9% de los pacientes tuvieron T-B-NK+ SCID y la mayoría de los pacientes eran portadores de mutaciones en los genes RAG2 o RAG1.

Conclusión: La variante autosómica recesiva de la SCID es la forma más común en los pacientes Iraníes. Se debe considerar prioritario proporcionar una formación adecuada a los médicos y las familias para reducir el retraso en el diagnóstico. Es igualmente importante concienciar para evitar la vacunación con gérmenes vivos y expandir los registros de donantes de células madre para agilizar el trasplante de estos pacientes.

Palabras clave: Inmunodeficiencia combinada severa. Screening del recién nacido. Complicaciones BCG. Transplante de células madre.

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Introduction

Severe combined immunodeficiency (SCID) is a prototype of primary immunodeficiency diseases (PIDs). It is known as a heterogeneous, life-threatening syndrome representing a group of rare and monogenic diseases[1].

The prevalence of SCID worldwide is estimated to be 1 in 50,000 to 100,000 live births [2]. However, the actual number of primary immunodeficiency disorders has been shown to be higher than that previously reported especially in countries like Iran with a high rate of consanguinity [3] and the other countries with a large number of unknown early deaths [4]. It is so difficult to precisely estimate the number of cases in Iran before conducting the newborn screening for SCID, but it seems to be greater than that we thought [5].

SCID patients generally present severe and repeated cycles of infections with opportunistic microorganisms (like Pneumocystis jiroveci, Candida albicans, and Cytomegalovirus),skin rashes, persistent diarrhea and failure to thrive within the first year of life. SCID patients often need an immediate pediatric emergency care [1, 6].

According to their fundamental defects, SCID patients are classified into four major groups:

1) imperfect function of pre-Tcell antigen receptor (TCR) complex (e.g., CD3ε/δ/ζ and CD45);

2) impaired signaling function based on common γ- chain-dependent cytokine receptors such as defects in interleukin-2 receptor γ- chain, interleukin-7 receptor α- chain, and Janus kinase 3;

3) V(D)J recombination defects (e.g., Artemis and Rag1/2 deficiency); and 4) Early lymphocyte antecedent cell death mediated by the purine metabolic dysfunction (ADA deficiency) [7].

There are a few publications on Iranian newborns with SCID. So, the aim of this study was to present the clinical features of SCID patients and their available laboratory findings as well as their genetic analyses.

Methods

Data Collection

This study was carried out on 63 SCID patients being referred to Immunology, Asthma and Allergy Research Institute (IAARI), Tehran University of Medical Sciences (TUMS) from different professional centers across the country. During January 2006 and December 2015, a prospective cohort study was conducted over a period of 10 years.

A questionnaire was provided and filled out for each patient consisting of questions about demographic information, history of complicated infections in the past and associated symptoms, family history of immunodeficiency, disseminated infection following BCG vaccination and patients' outcomes. This study was approved by ethics committee of IAARI.

Early screening and advanced immunological workup were investigated, which included CBC (complete blood count), serum immunoglobulin levels of IgG, IgA, IgM and IgE measured by nephelometery technique, lymphocyte enumeration and immunophenotype analysis by flow cytometry (CD3, CD4, CD8, CD19 and CD16/56), Lymphocyte proliferation response to Phytohemagglutinin (PHA), nitro-blue tetrazolium (NBT) test, and the total hemolytic complement (CH50) assay.

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According to the guidelines of Pan-American Group for Immunodeficiency (PAGID) and European Society for Immunodeficiencies (ESID) [8], clinical diagnostic criteria include at least one invasive bacterial, viral or fungal/opportunistic infection and/or persistent diarrhea, recurrent pneumonia, persistent thrush as well as failure to thrive and/or affect family members. Also first year manifestation of symptoms to rule out the HIV infection were obligatory. In addition, patients participating in this study had low or zero numbers of CD3 or CD4 or CD8 T (except for atypical SCID). Based on normal lymphocyte subsets in healthy children [9] and lymphocyte immunophenotyping of our patients, they were classified into five groups including: T-B-NK+ SCID; T-B-NK- SCID, T–B+NK+ SCID, T-B+NK- SCID and finally as atypical T+ SCID.

Genetic analysis:

Regarding the establishment of gene mutation analysis in IAARI, molecular evaluations for some specific genes known to be involved in SCID (including: IL-2R, IL-7RA, ADA, and RAG1/RAG2) were done for available patients. Briefly, for patients with (T- B- NK+), (T- B- NK-), and (T- B+ NK+) phenotypes, genetic study for RAG1/RAG2, ADA, and IL-7RA genes were performed, respectively. In addition, for 3 atypical patients with (T+ B- NK+), genetic study for RAG1/RAG2 was also executed. Male individuals with (T- B+ NK-) phenotype were evaluated regarding the mutations of IL-2R. Extraction of genomic DNA was performed from peripheral blood leukocytes. The exons and exon-intron boundaries were amplified by PCR runs with a final volume of 25 µl containing 200 ng genomic DNA, 10 pmol of each primer, 100 µM dNTP, various concentrations of MgCl2, 1x Taq DNA polymerase buffer and 2 U Taq DNA polymerase (Cinagene, Tehran, Iran). Initial denaturation of genomic DNA was performed for 5 min at 95°C, followed by 35 cycles of amplification as follows: 35 s at 95°C, 35 s at 54-64°C and 30 s at 72°C. After evaluating on 1.5% agarose gel, PCR products were sequenced.

Accordingly, definite diagnosis was made by cumulating data of clinical findings, immunological assessments and molecular analysis.

Statistical Analysis:

All data extracted from questionnaires as well as laboratory test results were statistically analyzed by SPSS statistical software package version 16 (SPSS Inc, Chicago IL, USA). Descriptive statistics (including mean. median, 25th percentile (Q1), 75th percentile (Q3) and standard deviation) were used to describe the data. Normality of quantitative variables was determined using Kolmogorov-Smirnov test. Independent Sample T test (or Mann–Whitney U test for variables with non- normal distribution) was applied to compare means of two quantitative variables. $P<0.05$ was considered as a statistically significant level.

Results

During the study, 63 patients with SCID from 61 families were diagnosed and followed up. The most patients (n=52, 82.5%) with a typical feature of SCID had CD3+ T cell counts less than 500 cells/mL. furthermore, CD3+ T cell <300/µl was found to be positive in 77.8% of the patients. As shown in Table 1, findings of immunological laboratory assessments and cell counts were indicated

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among different phenotypes of the patients. Lymphocytopenia (less than 3000 cells/mL) was present in 82.5% of patients. The median lymphocyte count was 1380 (Q1, Q3=602, 2304) for all patients, including atypical SCID patients. The other immunological assessments of phagocytic function (NBT) and the complement activity (CH50) were normal in all patients. According to typical immunologic classifications based on the immunophenotyping findings, twenty-two patients (34.9%) were classified as T-B-NK+ SCID, fifteen patients (23.8%) as T-B-NK-, ten patients (15.9%) as T – B+NK +, nine patients (14.3%) as T-B+NK-, which is known as x-linked SCID, and finally seven patients (11.1%) as atypical SCID (T+ SCID).

Among them, forty-three (68.3%) patients were male. Consanguinity was found in 87.3% (n=55) of patients' families. A positive family history of SCID was noticed in 34 patients (54%) and the majority of families had just one SCID child. The mean ± SD and median age of disease onset and diagnosis were 52±48, 40 (Q1, Q3= 15, 60) and 113±90, 110 (Q1, Q3= 45,150) days respectively. Interestingly, the mean ± SD and median of diagnosis time decreased to 97 ± 85 and 60 (Q1, Q3=35, 150) days in the second half period of the study.

Except for 2 patients who were early diagnosed before beginning the symptoms based on the death of a previous sibling, the median diagnostic delay for the remaining patients (61 patients) was about 60 (Q1, Q3=15.5, 90) days. Similarly, the median diagnostic delay decreased to 45 (Q1, Q3=13.7, 60) days in the second half period of the study. In addition, the difference in diagnosis delay time between the patients with a positive family history of SCID [45 (Q1, Q3= 5.5, 101)] and the patients with a negative family history of SCID [60(Q1, Q3= 33.5, 90)] was only fifteen days.

Forty-nine patients (77.8%) had a history of BCG vaccination and subsequent localized complications (adenitis) were observed in 22 out of 49 (44.9%); from which 14 patients showed disseminated infection (BCGosis). Unfortunately, among patients with a positive family history of PID, about 65% had received BCG vaccines. Since the complications were not seen in all vaccinated patients, the median absolute numbers of T, B, NK cells at the time of diagnosis in patients with BCG complications (except for atypical patients) were evaluated. The respective median absolute counts of CD3, CD19 and CD16+56 cells in SCID patients with BCG complications were 329 (Q1, Q3= 124, 748), 73 (Q1, Q3= 7.5, 711) and 69 (Q1, Q3= 14, 139); while SCID patients without localized or disseminated BCG complications theses parameters were 23 (Q1, Q3= 9, 114), 8 (Q1, Q3= 3, 426) and 117 (Q1, Q3= 31, 336), respectively. However, no significant differences were found between these two groups of patients.

In the present study, the most common clinical manifestations of all patients were pneumonia (n= 49, 77.8%), recurrent oral candidiasis (n=29, 46%), chronic diarrhea (n=15, 23.8%), failure to thrive (n=13, 20.6%) and oral ulcer (n= 10, 15.4%). Anti-tuberculosis, -bacterial, -viral and -fungal prophylaxis were used in 14 (22.2%), 36 (57.1%), 13 (20.6%), and 22 (34.9%) of all patients, respectively. Also, IVIG was administered to 43 (68.3%) of the patients.

During ten years, 21 patients found appropriate donors and 13 of them underwent hematopoietic stem cell transplantation (HSCT). However, among these patients, HSCT could rescue just eight (12.7%) who are still alive, but the remaining five patients died of GVHD or sepsis following the

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transplantation. Patients' mean age at the time of transplantation was about 8.3 months (range 3-13 months).The mean survival time after transplantation until the end of this study was 17.7 months (range 2-63 months). Unfortunately, 50 out of 63 patients (87.3%) expired due to not having found suitable donors.

Lymphocyte proliferation response to Phytohemagglutinin (PHA) was assessed in 11 patients. A low response was found in 10 cases. In spite of a mutation found in the RAG2 gene in the remaining 1 patient, the response was normal (>50%).

Although mutation analysis was done for 33 (53%) patients, defects, due to mutations, were found in only 17 patients. Fourteen different mutations, including 5 novel mutations, were identified. As shown in table 2, the most alterations were detected in RAG2 and RAG1 genes.

Discussion

In our knowledge, this is the first comprehensive national report from Iran to show clinical manifestations, laboratory and molecular findings of sixty-three patients with SCID. In our study, most of the patients (34.9%) were positive for NK cells but negative for both T and B cells (T-B-NK+), The genetic analysis was done for 53% of the patients which revealed the most alterations in RAG1 or RAG2 genes while the lowest number of patients belonged to XL-SCID group (14%). That was in contrast with the reports of European and American countries [10] which might be related to genetic variations between different populations and a higher rate of consanguinity in Iran.

It seems that it is similar to another PID disorder, namely chronic granulomatosis disease (CGD), in Iran that has been previously reported with a higher incidence of autosomal recessive than X-linked type [11]. The dominant phenotype of SCID varies in different countries. For example, 25 out of 44 reported SCID patients were X-SCID in China [12], 12 out of 21 SCID and Omenn's syndrome patients were T-B-NK+ SCID phenotype, 11 of which had RAG1or RAG2 and 1 Artemis gene mutations in a report of Serbia and Montenegro [3] and 12 out of 30 SCID patients (40%) were T−B−NK+ in a report from Greece [13].

In this study, the median age at the onset of symptoms and the age of diagnosis were 40 and 110 days, respectively. According to the previous report from Iran in 2008 [5] the mean age of diagnosis in Iranian SCID patients was 150±20 days which has been shortened to less than 4 months during the last decade. Therefore, it was a valuable achievement to reduce the diagnosis age to lower than 4 months in our country and also in comparison with other countries such as Canada (4.2 months of age), Brazil (6.1 months) and Serbia (4 months) [14-16]. Our results also indicated that the median diagnostic delay time reached 60 days (2 months) which was in accordance with other reports from China (2.6 months), Serbia (2 months), Netherlands (2 months) [12, 15, 17], and was better than Brazil (4.2 months) [16].

According to the study of Roifman et al., CD3+ T cells <500 /μl can be considered as the highest specificity and sensitivity item for separating patients with SCID from those with combined immunodeficiency (CID) [18]. They also explained that a significant decrease in response to PHA

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test was found in all patients with CD3+ T cells <500/µl; while CD3 count may be normal in patients with profound T cell dysfunction. Similarly, most of our patients were considered to have typical SCID regarding the relevant feature of presenting CD3+ T cell counts of less than 500/µl. Nevertheless, new diagnostic criteria for SCID proposing the CD3+ T cell < 300/µl as the index of typical SCID [19], have raised some controversies in determining the precise index. Therefore, we also evaluated our patients with CD3+ T cell < 300/µl index that did not show any significant difference compared to CD3+ T cell < 500/µl index (77.8% vs. 82.5%).

Although most patients in our study had typical clinical manifestations and laboratory findings including lymphopenia and low levels of immunoglobulins except for maternal IgG, some of them indicated elevated value of absolute lymphocyte count (ALC) and normal levels of immunoglobulins. These patients are known to have an atypical presentation of lymphocyte subsets and normal serum immunoglobulins suggesting to be a subgroup of SCID patients with normal B lymphocytes, having a potential capacity to produce measurable amounts of serum IgE and eosinophilia [2, 7]. In atypical cases different clinical and immunological features, even within one family, were described [20].
Pulmonary diseases were the most complicated problem (80%) in our patients. The other affected organs were ENT, reticuloendothelial, GI, skin, bone and joint, in turn. However, autoimmune diseases were not found in them.

In this study, we could observe BCG associated complications in one-third of the patients (not all of them) which can be related to some factors including the genetic differences, the immunological repertoire, the vaccine ingredients and possibly lower T cell count [21] (the last one was not confirmed in our study needing more investigation).

BCG vaccine as other live attenuated vaccines is extremely contraindicated in SCID infants [21]. To avoid inoculating the patients with a positive family history (as 65% of patients in our study), physicians are needed and patients' families also need to be educated.

One of the recent approaches to the early diagnosis of SCID would be the establishment of a newborn screening program (TREC assay) to hasten the treatment and avoid using live vaccines for immunocompromised children [22-24]. .

Limited laboratory facilities for performing genetic studies and lymphocyte proliferation test for all participants are the limitations of the current study. In spite of these limitations, the findings of the present long term study with a considerable number of patients may be of great value.

Conclusion

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Based on the results of this study, autosomal recessive SCID is the most inherited type in Iranian patients. However, delay in diagnosis and also a lack of suitable donor are the main causes of high mortality rate among the SCID patients. Therefore, the awareness of physicians about the onset of serious infections and irreversible outcomes of BCG vaccination would be of interest to better manage the patients.

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Table 1. SCID Patients' clinical characteristics and immunologic laboratory findings

Title	($T^-B^+NK^-$)	($T^-B^+NK^+$)	($T^-B^-NK^-$)	($T^-B^-NK^+$)	Atypical	Total
	N=9	N=10	N=15	N=22	N=7	N=63
Gender (M/F)	7/2	6/4	11/4	13/9	6/1	43/20
Presentation age (day)	30 (0-150)	65 (0-150)	45 (8-150)	48 (0-180)	14 (0-60)	40 (0-180)
Diagnostic time (day)	45 (7-270)	97 (10-300)	90 (27-360)	120 (0-390)	60 (30-240)	110 (0-390)
WBC(cell/ul)	8000 (4470-14200)	10100 (4540-17500)	5500 (1300-25610)	6320 (2990-23850)	12680 (4480-44360)	7060(1300-46360)
Absolute Granulocyte Count (AGC) (cell/ul)	4209 (2080-11218)	4355(2180-10841)	3720 (410-23610)	3350 (250-17453)	3810 (6894-11110)	3800(250-30736)
Absolute Lymphocyte Count (ALC) (cell/ul)	1910 (960-3880)	1625 (40-5590)	360 (90-2108)	1330 (300-2584)	5600 (3136-25520)	1380(40-25520)
Eosinophil(cell/ul)	295 (0-760)	20 (100-950)	120 (0-1210)	395 (0-6970)	71(0-11440)	235(0-11440)
CD3(cell /ul)	23 (3-512)	57 (3-492)	26 (1-553)	43 (2-582)	2999 (1255-23478)	42(1-23478)
CD4(cell /ul)	6 (3-307)	31 (0-711)	16 (1-387)	35 (0-784)	2576 (68-5869)	31(0-5869)
CD8(cell /ul)	31 (4-153)	55 (0-1422)	7 (0-518)	77(0-784)	2507 (60-16588)	32(0-16588)
CD19(cell /ul)	1382 (365-2754)	1057 (487-14081)	8(2-100)	7(0-187)	44 (5-2848)	44(0-14081)
CD16+56(cell /ul)	117 (9-156)	342 (183-2800)	33 (6-120)	722 (169-3181)	1020 (266-3379)	321(6-3379)
IgA(mg/dl)	10 (0-70)	4 (0-26)	4 (0-66)	10 (1-68)	7 (6-47)	7(0-70)
IgM(mg/dl)	26.5 (0-56)	42 (3-66)	13 (2-120)	21 (3-304)	27 (1-65)	24(0-304)
IgG(mg/dl)	79.5 (30-1164)	358 (60-2041)	216 (70-1340)	225 (25-740)	167 (27-370)	202(25-2041)
IgE(IU/ml)	1 (0-1608)	1 (0-10)	2 (0-45)	1 (0-1612)	285 (0-1250)	1(0-1612)

Median (Min-Max) is shown for quantitative variables.

Table 2. Genetic analysis of SCID patients

Immunophenoypes (No.)	Patients being evaluated with genetic tests (No.)	Evaluated Genes	No. (%) of cases with positive test	Positive cases	Mutations	Results	Reported/Nonreported
T- B- NK+ (22)	14	RAG1	2 (11.8)	1	c.2687G>A	p.W896X	Non-reported
				1	c.835Cdel	p.A280fsX9	Non-reported
		RAG2	7 (41)	1	c.749_750CAdel		Reported
				1	c.686G>A	p.T250fsX17	Reported
				2	c.685C>T		Reported
				1	c.415G>A	p.R229Q	Non-reported
				2	c.1432T>C	p.R229W p.G139S p.C478R	Non-reported
Atypical (7)	3	RAG1	1 (5.8)	1	c.2192C>T	p.T731I	Reported
		RAG2	1 (5.8)	1	c.685C>T	p.R229W	Reported
T- B – NK - (15)	7	ADA	3 (17.6)	1	c.956_960AAGAG del c.219-2A>G	p.E319fsX3 Splicing site defect	Reported
				1			Reported
				1	c.704G>A	p.R235Q	Reported
T- B+ NK - (9)	4	IL-2R	2 (11.8)	1	c.670C>T	p.R224W	Reported
				1	c.675C>A	p.S225R	Reported
T- B+ NK+ (10)	5	IL-7R	1 (5.8)	1	c.31Gdel	p.V11fsX38	Non-reported