Newborn Screening for Severe T and B cell Immunodeficiency in Israel: A Pilot Study

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ABSTRACT:

Background: Enumeration of T cell receptor excision circles (TREC) was recently adopted as a neonatal screening assay for severe combined immunodeficiency (SCID). Enumeration of kappa-deleting recombination excision circle (KREC) copy numbers can be similarly used for early assessment of B cell lymphopenia.

Objective: To assess the ability of TREC and KREC counts to identify patients with combined T and B cell immunodeficiency in a pilot study in Israel.

Methods: We studied seven children born in Israel during the years 2010–2011 and later diagnosed with SCID, and an additional patient with pure B cell immunodeficiency. TREC and KREC in peripheral blood upon diagnosis and in their neonatal Guthrie cards were analyzed using real-time quantitative polymerase chain reaction, as were Guthrie cards with dried blood spots from healthy newborns and from normal and SCID-like controls.

Results: The first features suggestive of SCID presented at age 3.1 ± 2.4 months in all patients. Yet, the diagnosis was made 4.1 ± 2.9 months later. Their TREC were undetectable or significantly low at their clinical diagnosis and in their originally stored Guthrie cards, irrespective of the amount of their circulating T cells. KREC were undetectable in six SCID patients who displayed B cell lymphopenia in addition to T cell lymphopenia. KREC were also undetectable in one patient with pure B cell immunodeficiency.

Conclusions: TREC and KREC quantification are useful screening tests for severe T and B cell immunodeficiency. Implementation of these tests is highly important especially in countries such as Israel where a high frequency of consanguinity is known to exist.

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KEY WORDS: Guthrie cards, hematopoietic stem cell transplantation (HSCT), immunodeficiency, kappa-deleting recombination excision circles (KREC), neonatal screening, severe combined immunodeficiency (SCID), T cell receptor excision circles (TREC)

Certain genetic disorders have been found at a relatively high frequency because of consanguineous marriages in some communities [1]. One of these disorders is severe combined immunodeficiency. SCID encompasses a heterogeneous group of genetic disorders characterized by thymic dysplasia and arrest of T lymphocyte maturation. Patients also experience variable associations with an abnormal differentiation of B and natural killer cells. Affected infants are born healthy and usually present with severe infections, failure to thrive and thrush during the first months of life [2]. They will all have a fatal outcome unless their immune system is promptly restored by hematopoietic stem cell transplantation or gene therapy [3]. Early diagnosis is imperative. It was shown that SCID babies diagnosed at birth because of a positive family history will have a significantly improved outcome compared with the first-ever presenting family member [4]. Similarly, affected infants diagnosed with SCID as neonates had better survival than those whose disease detection was delayed [5]. Early diagnosis of SCID was also shown to be relatively cost-effective in spite of the low incidence of the disease [6]. SCID recently became the first genetic disorder of the immune system to be amenable to newborn blood screening.

In 2010, the quantification of T cell receptor excision circle levels emerged as an economical, sensitive and specific screening test for SCID [7]. The TREC assay enumerates the number of TREC by using real-time quantitative polymerase chain reaction on DNA extracted from newborn blood screening cards. The process of TCR maturation consists of random rearrangement of different DNA segments of the variable TCR chains ($\alpha,\beta,\gamma,\delta$) to form a unique and functional broad-spectrum repertoire. Both coding and signal joints are produced during the TCR-delta deletion rearrangements

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SCID = severe combined immunodeficiency
TREC = T cell receptor excision circle
TCR = T cell receptor

from the TCR-alpha locus. The latter is a specific circular DNA byproduct, termed TREC, that is present in cells exported from the thymus but not in peripheral blood replicating cells [8]. Their levels in human peripheral blood were shown to reflect thymus activity [9]. Quantitative studies have shown that approximately 70% of the TCRD deletion rearrangements produce this circular DNA. Moreover, thymocyte proliferation between TCRD deletions and TCRA rearrangements is limited; therefore, the dilution of this specific rearrangement secondary to thymocytes expansion is minimal as well. Thus, TREC quantification became a widely accepted, accurate and non-invasive tool to detect T cell immune disorders, and serves as a surrogate marker for the number of circulating T cells.

Two pioneering studies using TREC depicted by RQPCR on DNA extracted from routine newborn blood screening cards confirmed its accuracy in identifying infants with SCID. McGhee et al. [10] suggested a two-tiered strategy combining TREC and interleukin-7 levels as having sufficient accuracy to justify universal SCID screening. TREC alone had a specificity of 92.3% and a sensitivity of 100% in diagnosing SCID. Chan and Puck [11] showed that unlike filters obtained from normal newborns, which had a mean of 1020 TREC in two 3 mm punches, samples obtained from 23 infants with SCID had < 30 TREC, indicating that TREC are a stable analyte that can identify T cell developmental defects in newborn blood screening cards. Two years ago, TREC testing was incorporated as part of the newborn screening panels in several U.S. states, including Wisconsin, Massachusetts and California [12,13].

Similar to TREC, quantification of the kappa-deleting recombination excision circles from Guthrie cards can be used for early assessment of B cell development in primary B cell immunodeficiencies. Thus, simultaneous measurement of TREC and KREC copy numbers in Guthrie card samples readily identified patients in whom T or B cells were absent [14].

SCID is estimated to occur in 1 per 50,000 to 1 per 100,000 births [2]. The relative frequency of the disease and the relative frequency of different forms of SCID may vary in different countries and ethnic groups. For example, the most common SCID genotype worldwide is the T-B+NK- X-linked deficiency. The genetic defect is within the cytokine receptor subunit gamma-c (the interleukin receptor common gamma chain, IL2RG). This receptor subunit is shared by at least six different cytokine receptor complexes: the receptors for IL-2, 4, 7, 9, 15, and 21. In contrast, the most frequent type of SCID genotype in Israel is autosomal-recessive T-B-NK+ RAG1/2 mutations, while the X-linked SCID is rare [15]. The frequency of SCID in Israel is expected to be higher than in most western countries due to the high rate of consanguinity in certain communities

TCRD = TCR delta
TCRA = TCR alpha
RQPCR = real-time quantitative polymerase chain reaction
KREC = kappa-deleting recombination excision circles
IL = interleukin

[16,17]. We evaluated TREC and KREC copy numbers from the original Guthrie cards as well as from peripheral blood of several SCID patients and compared their findings with those of healthy controls. Our objective was to determine if these assays are able to identify T and B cell lymphopenia as well as dysfunctional T and B cells.

METHODS

PATIENTS

The estimated number of births in Israel is 170,000 per year. Our study group consisted of seven patients, all terms, born in Israel during a 1 year study period (2010–2011) and later diagnosed with SCID in three tertiary hospitals. TRECs were analyzed in their peripheral blood samples upon diagnosis and in their original neonatal Guthrie cards with dried blood spots obtained immediately after birth. To conform to the diagnostic demands of neonatal screening, 15 Guthrie cards with dried blood spots from 15 healthy newborns were similarly analyzed. In addition, 24 Guthrie cards with dried blood spots (generously provided by Dr. Francis K. Lee, from the Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention, Atlanta, GA, USA) obtained from cord blood (that had higher and lower TREC copy numbers but were within the normal range for newborns) were used as normal controls, and those obtained from cord blood with T cell depletion (thus representing SCID-like newborns with undetectable or minimal TREC copy numbers) were used as SCID-like controls. Identifying numbers were removed from all Guthrie cards. Disks 3 mm in diameter were punched out of the dried spots. DNA was extracted by using the QIAamp DNA Micro kit (QIAGEN) and then re-suspended in 20 µl H2O. RNase P was used as the DNA content detector. The study was approved by the Israel Ministry of Health, the institutional review board was aware of this study, and informed consent was obtained from the parents of the patients.

IMMUNOLOGICAL WORKUP

Cell surface markers of peripheral blood mononuclear cells were determined by immunofluorescent staining and flow cytometry (Epics V, Coulter Electronics, Hialeah, FL) with antibodies purchased from Coulter Diagnostics. Lymphocyte proliferation in response to phytohemagglutinin was determined by tritiated thymidine incorporation, as previously described [18]. For the lymphocyte proliferation studies, the cells were harvested 3 days after stimulation and samples were counted in a liquid scintillation counter. All assays were performed in triplicate, and a stimulation index was calculated as the ratio between stimulated and unstimulated lymphocyte responses and compared with the stimulation index obtained from normal controls. Serum concentrations of immunoglobulins were measured by nephelometry.

Table 1. Clinical and immunological findings of seven patients diagnosed with severe combined immunodeficiency

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
SCID phenotype	T-B-NK+	T+B-NK+	T-B+NK+	T-B-NK+	T-B-NK+	T-B-NK+	T-B-NK+
SCID genotype	Unknown	*RAG2*	22Q11 deletion	*RAG2*	*DCLRE1C*	*RAG2*	*RAG2*
Clinical presentation	FTT, infection	Rash, infection, Omenn	Dysmorphism, cardiac defect, infection, DiGeorge	Rash, infection	Stomatitis, infection	Rash, pneumonia Omenn	PCP pneumonia
Age at symptom onset (mo)	6	2	0	1	7	2	4
Age at diagnosis (mo)	12	4	8	1	7	3	9
Lymphocyte count/mm³	100	10,686	1690	1400	1560	4200	180
CD3/mm³	56	2871	50	324	450	1300	53
CD3+CD4+/mm³	10	2351	55	294	168	400	36
CD3+CD8+/mm³	46	855	7	14	95	800	28
CD4+/CD8+	0.2	2.7	8	21	1.8		
CD19+/mm³	2	0	1244	0	13	0	15
CD3-CD56+/mm³	60	3800	80	700	956	1000	130
IgM (mg/dl)	103	110	80	7	119	UD	37
IgG (mg/dl)	887	UD	1540	151	183	UD	150
IgA (mg/dl)	136	UD	109	UD	UD	UD	20
PHA mitogenic response*	6.8%	6.5%	10%	0%	0%	0%	0%
TRECs/0.5 µg DNA at time of diagnosis†	Undetectable	Undetectable	Undetectable	Not done	Undetectable	Undetectable	Undetectable
TRECs/3 mm punch from Guthrie cards‡	Undetectable	Undetectable	18	Undetectable	Undetectable	Undetectable	Undetectable
Outcome	W/A 6 mo after HSCT	W/A 12 mo after HSCT	Died of EBV-related lymphoma at age 14 mo	W/A 23 mo after HSCT, GVHD	W/A 2 mo after HSCT	Died 1 wk after HSCT from respiratory failure	W/A 12 mo after HSCT

IgA = immunoglobulin A, PHA = phytohemagglutinin, SCID = severe combined immunodeficiency, FTT = failure to thrive, TREC = T cell receptor excision circle, HSCT = hematopoietic stem cell transplantation, GVHD = graft versus host disease, RAG = recombination activating gene, PCP = *Pneumocystis jiroveci* pneumonia, EBV = Epstein-Barr virus, W/A = well and alive, mo = months, wk = weeks

*Percentage, CPM patient/CPM control
†Normal TREC levels › 400 copies/0.5 µg DNA
‡Cutoff levels ›10 copies/3 mm punched disk

QUANTIFICATION OF TREC AND KREC

The amount of TREC was determined by RQPCR. Reactions in the peripheral blood were evaluated using 0.25 µg genomic DNA extracted from the patient's PBMC. Reactions on the Guthrie cards were determined using 5 µl of extracted DNA. PCR reactions contained TaqMan universal PCR master mix (Applied Biosystems, USA), specific primers (900 nM) and FAM-TAMRA probes (250 nM), as previously described [18]. RQPCR was carried out in an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). The standard curve was constructed by using serial dilutions of a known TREC plasmid (generously provided by Dr. Daniel Douek, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD). Each dilution was performed in triplicate. In order to calculate the amount of signal and coding joint of KRECs for each patient, the obtained cycle threshold was compared to the cycle threshold of the TREC copies in the different

PBMC = peripheral blood mononuclear cells

standard serial dilutions, as described above. Amplification of RNase P (TaqMan assay, Applied Biosystems) served as a quality control to verify similar amounts of genomic DNA that were used in the assays for both the TREC and KREC analyses. The findings in the age-matched normal individuals were used as control values (> 400 TREC copies in 40 samples in which immunodeficiency was ruled out). Each experiment was performed in duplicate, and the threshold for each cycle was positioned at the same level.

RESULTS

PATIENTS' DESCRIPTION AND OUTCOME

Seven term infants were included in this study [Table 1]. They all presented with features suggestive of immunodeficiency during infancy. None of their mothers had undergone prenatal genetic testing. Their mean age at the time of their presenting symptoms was 3.1 ± 2.4 months, but the diagnosis of SCID was made only after a mean of 4.1 ± 2.9 months. Two
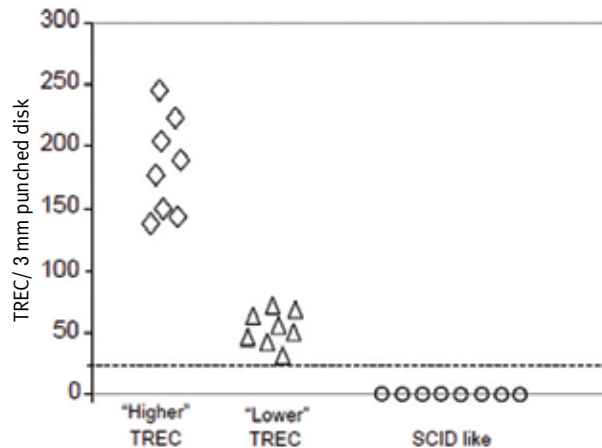
of the patients had normal lymphocyte counts (10,686 and 4200/mm^3) and they both exhibited a typical Omenn phenotype, including diffuse erythroderma and lymphadenopathy. Another patient had facial dysmorphism and associated abnormalities, and she was diagnosed as having a complete DiGeorge phenotype. She was the only SCID patient with normal levels of B lymphocytes. Genetic diagnosis could not be made in one patient: although he showed a typical T-B-NK+ SCID, some of the known genes associated with this phenotype (*RAG1*, *RAG2* and *DCLRE1C*) were normal. Five of the six patients who underwent HSCT survived, and the seventh succumbed to Epstein-Barr virus-related lymphoma before HSCT could be performed.

TREC AND KREC COPY NUMBERS

TREC and KREC copy numbers in PBMC were analyzed in the SCID patients at the time of diagnosis. All patients had undetectable TREC, while KREC were detectable only in the T-B+NK+ SCID patient [# 3 in Table 1]. The originally stored Guthrie cards of all seven patients were checked to determine whether both T and B cell deficiencies could have been diagnosed by screening TREC and KREC copy numbers immediately after birth. The 16 normal controls (those with higher and lower TREC copy numbers within the normal range for newborns) and the 8 SCID-like controls were first analyzed to test the accuracy of extracting TREC and KREC copy numbers from Guthrie cards [Figure 1]. The normal controls had copy numbers > 30/3 mm punched disk (range 142–212 copies, mean 183.4, for the higher group and 36–69 copies, mean 53.03, for the lower group). This is considered the diagnostic cutoff score for a neonatal screening program for SCID or T cell lymphopenia [7]. As expected, the TREC levels in the SCID-like controls were undetectable [Figure 1]. Next, we analyzed TREC of dried blood spots from the stored Guthrie cards of 15 normal control neonates: they had on average TREC copy numbers of 85.5/3 mm punched disk (range 35–212 copies). None had TREC below the cutoff range value for diagnosing SCID. In contrast, all seven SCID patients had values beyond the test range for TREC in their originally stored Guthrie cards: six had altogether undetectable levels and one had remarkably low levels [Figure 2].
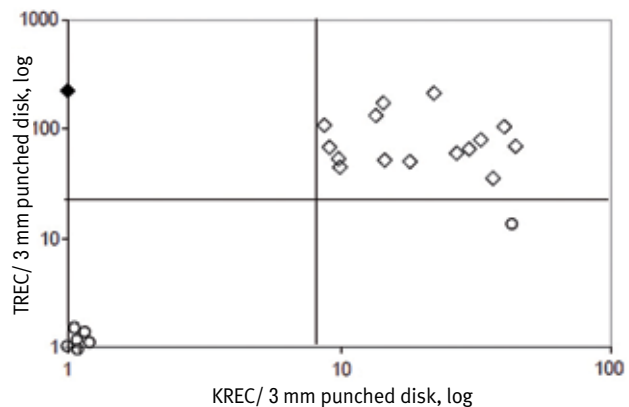
We also analyzed KREC copy numbers in the dried blood spots of the 15 normal control neonates using their stored Guthrie cards (the copies ranged from 8 to 56). Based on those results, we considered < 8 copies/3 mm punched disk as the diagnostic cutoff score for B cell lymphopenia. A simulated Guthrie card of a typical patient with no detectable B cells due to X-linked agammaglobulinemia demonstrated undetectable KREC levels as well. The lack of KREC could be verified on their original stored Guthrie cards of all six patients in whom

HSCT = hematopoietic stem cell transplantation

Figure 1. TREC copy numbers in dried cord blood spot samples from the control Guthrie cards, including samples with higher and lower TREC copy numbers and samples with T cell depletion (SCID-like patients). Copy numbers are presented per 3 mm punched disks. Diagnostic cutoff scores for T cell lymphopenia is provided

◇ normal range for newborns – "Higher" TREC
△ normal range for newborns – "Lower" TREC
○ T cell depletion (SCID-like patients)

Figure 2. TREC and KREC copy numbers (per 3 mm punched disks) in dried blood spot samples from stored Guthrie cards of 15 normal control neonates (open diamond), seven SCID patients (open circle) and one simulated Guthrie card of a typical patient with no B cells (black diamond). Diagnostic cutoff scores for T and B cell lymphopenia are provided. The results parallel the patients' immunophenotypes

the lymphocyte immunophenotype revealed significantly reduced or undetectable levels of B cells. Taken together, all patients with confirmed mutations in the genes involved in T and B cell development (*RAG* and *DCLRE1C* genes) demonstrated values beyond detectable ranges for both TREC and KREC, paralleling their T and B immunophenotype. In the

typical case of T-B+ SCID [patient #3 in Table 1], KREC copy numbers were normal in her original stored Guthrie card, similar to the result obtained from her peripheral blood upon diagnosis (KREC copy number of 184).

DISCUSSION

TREC, a DNA biomarker used as a surrogate for the number and function of circulating T cells, has successfully proved its efficacy for SCID screening in the USA [7]. Here, we demonstrated that this screening assay is also valid in Israel, a country whose population comprises some ethnic groups in which consanguineous marriages are traditional. TREC were undetectable or they were significantly lower in all the SCID patients tested by us than in healthy neonates. These data were derived from the DNA obtained from both their originally stored Guthrie cards and from peripheral blood samples that were obtained as part of the diagnostic workup. Uniquely, we also demonstrate that KREC assay can identify absence of B cells in newborns, hence assisting and strengthening the diagnosis of SCID, which in some cases is associated with B cell defects. Moreover, KREC might be beneficial in early identification of patients with inherited B cell deficiencies, such as X-linked and autosomal recessive agammaglobulinemia. Thus, simultaneous measurement of TREC and KREC copy numbers in Guthrie card samples is a useful approach for identifying patients with T and/or B cell deficiency.

SCID is considered a rare disease, although its true incidence is not known. In Israel, it is expected that the incidence of SCID is higher than in other western countries due to the relatively high rate of consanguinity in both the Jewish [19] and the Arab populations [20]. This trend will probably be similar to the higher frequency of other inherited disorders, such as congenital hypothyroidism [21], glucose-6-phosphate dehydrogenase deficiency [22] or phenylketonuria [16] that have been reported in Israel. Given that there are effective treatment strategies available for these children, every effort should be made to enable the earliest possible diagnosis of these diseases, initiate appropriate treatment, and enhance outcome in terms of complications and overall survival, as already shown in other reports [4,5,14]. There was a delay of more than 4 months between the appearance of the first symptoms to final diagnosis in our group of patients. Importantly, when SCID was diagnosed antenatally or at birth because of a diagnosis of SCID in a previous sibling, there was 90% survival, compared to only 39.6% when diagnosis of the first presenting person in the family was late [4]. Similarly, there was an infant mortality rate of 42% for 138 neonates who were not tested at birth compared to 15% for 20 neonates who were tested at birth [5].

A recent study of 5766 newborns and some known cases of SCID and of whole-blood specimens selectively depleted of naive T cells confirmed the utility of TREC measurement [23].

Another report showed that the TREC assay can diagnose not only lymphopenic SCID patients but also SCID patients with residual T cells, including maternally engrafted cells or leaky T cells (e.g., Omenn phenotype) [24]. In agreement with these reports, two of our patients who displayed normal amounts of circulating T cells had no detectable TREC. These cells were dysfunctional, self-reactive oligoclonal T cells that characterized the Omenn phenotype; therefore, it was unlikely that TREC would be present.

Wisconsin was the first state in the USA to formally implement TREC for neonatal detection of SCID, and their screening program has been the longest (44 months) recorded in the U.S. [13]. They reported a total of 71,000 infants who were screened, 8 of whom demonstrated T cell lymphopenia from various causes. The Wisconsin program achieved a false-positive rate of only 0.03% in term infants and 0.14% in premature infants [13]. As of May 2011, six states and one U.S. territory were performing newborn screening for SCID using the TREC assay. Those programs identified 14 cases of classic SCID as well as 40 cases of T cell lymphopenia that were not related to SCID from a total of 961,925 newborns. All 14 cases were referred to an immunologist and received appropriate therapy. These data suggest that infants with low or absent TRECs can be identified and referred for confirmatory testing and prompt intervention [25], as also seen in our study.

Universal implementation of newborn screening for SCID will invariably raise some challenges. Many other new or known conditions with T cell lymphopenia – not necessarily SCID – that need medical attention will be detected. Indeed, a case of complete DiGeorge syndrome was also identified by our screening program, in addition to the typical SCID patients. The major challenge will be to develop the appropriate protocol for following and treating these patients. The benefit of an SCID screening program is to improve the health status of affected individuals by enabling early diagnosis and initiation of optimal treatment. However, we are aware that drawbacks of such a screening program include false-positive results (causing additional costs, parental stress and anxiety) and false-negative results (potentially causing a delay in diagnosis in missed cases). Screening programs always raise concerns about privacy and autonomy and balancing health care expenses. Several studies reported the economic benefits of performing early versus late transplantation in SCID patients [6]. In early transplantations, the baseline clinical status is better and the complications are fewer, therefore expensive treatment is not necessary. Moreover, patients who had their transplant early in life had usually been outpatients for most of their transplant processes [25].

We confirm that quantifying TREC levels is a sensitive and specific screening test for SCID also in Israel. We also demonstrated the feasibility of a sensitive and specific screening test for B cell lymphopenia by means of quantifying KREC

levels. It is expected that SCID and other forms of T cell lymphopenia screening will become more accepted worldwide. The Israeli medical infrastructure is especially amenable to neonatal genetic screening for treatable conditions, such as SCID, for the early diagnosis, prompt initiation of treatment and expectation of cure of such patients.

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Capsule

Fgf9 from dermal γδ T cells induces hair follicle neogenesis after wounding

Understanding molecular mechanisms for regeneration of hair follicles provides new opportunities for developing treatments for hair loss and other skin disorders. Gay et al. show that fibroblast growth factor 9 (Fgf9), initially secreted by γδ T cells, modulates hair follicle regeneration after wounding the skin of adult mice. Reducing Fgf9 expression decreases this wound-induced hair neogenesis (WIHN). Conversely, overexpression of Fgf9 results in a two- to threefold increase in the number of neogenic hair follicles. The authors found that Fgf9 from γδ T cells triggers Wnt expression and subsequent Wnt activation in wound fibroblasts. Through a unique feedback mechanism, activated fibroblasts then express Fgf9, thus amplifying Wnt activity throughout the wound dermis during a crucial phase of skin regeneration. Notably, humans lack a robust population of resident dermal γδ T cells, potentially explaining their inability to regenerate hair after wounding. These findings highlight the essential relationship between the immune system and tissue regeneration. The importance of Fgf9 in hair follicle regeneration suggests that it could be used therapeutically in humans.

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