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Investigating the Variation of TREC/KREC in Combined Immunodeficiencies

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ABSTRACT

T-cell receptor excision circles (TREC)/Kappa-deleting recombination excision circles (KREC) assay has been recently recognized for detecting patients with primary (T- and/or B-cell) immunodeficiency (PID). We aimed to investigate the alterations of these biomarkers in some combined immunodeficiency patients compared to the healthy controls in different age groups.

TREC and KREC were assessed in a total of 82 PID patients, most of them with exact genetic diagnosis (3 months to 42 years); using quantitative real-time-polymerase chain reaction (PCR). Patients had a final diagnosis of common variable immunodeficiency (n=23), ataxia-telangiectasia (AT) (n=17), hyper-IgE syndrome (HIES) (7 with *DOCK8* deficiency, 4 with signal transducer and activator of transcription 3 (STAT3) deficiency, and 8 children with unknown genetic defects), Wiskott-Aldrich syndrome (WAS) (n=20), purine nucleoside phosphorylase (*PNP*)deficiency(n=1), dedicator of cytokinesis2 (*DOC*K2) deficiency (n=1), recombinase activating gene1 (*RAG1*) deficiency (n=1).

Very low to zero amounts of TREC and/or KREC were detected in 14 out of 23 cases of common variable immunodeficiency (CVID), 14 out of 17 cases of AT, 8 out of 20 cases of WAS, 6 out of 7 cases of *DOC*K*8*-deficiency patients, 4 out of 8 cases of HIES with unknown genetic defects and all patients with defects in *DOCK2*, *PNP*, and *RAG1*. *STAT3*-deficient patients were normal for both biomarkers. All patients showed a significant difference in both markers compared to age-matched healthy controls.

Our findings highlight that apart from severe types of T/B cell defects, this assay can also be used for early diagnosis the patients with late-onset of disease and even PIDs without a positive family history.

Keywords: Neonatal screening; Primary immunodeficiency disorders; Real-time polymerase chain reaction

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INTRODUCTION

T-cell receptor excision circles (TREC) and Kappa-deleting recombination excision circles (KREC) are episomal DNA circles generated during the rearrangement processes of T cell receptor (TCR) and B cell receptor (BCR) in the thymus and bone marrow (BM), respectively. None of these biomarkers are divided during lymphocyte proliferation. In addition, the function of the thymus and bone marrow is decreased over the lifespan. Therefore, TREC and KREC copies decrease with advancing age.[1] The simultaneous assessment of TREC and KREC during the newborn period has been recently raised for early diagnosis of diseases with genetic defects in proteins responsible for T and B cell developments in thymus and bone marrow respectively.[1,2]

Primary immunodeficiency disorders (PIDs) comprise a group of heterogeneous genetic disorders, affecting cellular and humoral immunity.[1,3-5] There is evidence that the worldwide prevalence of severe forms of PID is higher than the previous estimation of1 per 50,000 to 100,000 live births. Nevertheless reports from some countries with a high percentage of consanguinity and those with unidentified early deaths suggest that the prevalence of PID might be higher than that reported previously.[7] Fortunately, there are some molecular assessments like TREC/KREC testing which are more readily available for diagnosis of T and B cell defects early on before severe and recurrent infection and development of morbidity. It can be easily scheduled in national newborn screening programs to early detect suspected patients and follow them for more evaluations. Luckily, simultaneous detection of TRECs and KRECs has been developed and can help us to screen PIDs patients regardless of genetic defects.[2,3]

Combined immunodeficiency (CID) patients consist of a large group of PID patients and they suffer from recurrent or chronic infections due to severe T cell defects.[3] Severe combined immunodeficiency (SCID) which is the most profound form of CID with both T and B cell defects, is fatal in the first months of life without effective treatment.[1,8] Patients with a family history of PID are commonly diagnosed by prenatal genetic tests; while others are identified following the incidence of clinical manifestations and/or the report of abnormal laboratory findings.[1,2] Therefore, TREC/KREC assay is a beneficial method in reducing treatment costs and increasing survival.[9]

In addition, this assay has been illuminated as a high throughput method with high sensitivity and the ability to utilize DNA extracted from a minimum volume of peripheral blood samples collected onto the filter papers.[9,10] Although this method has been approved for neonatal screening of PID, some researchers are trying to use it for following up the treatments including hematopoietic stem cell transplantation (HSCT), enzyme replacement, and gene therapy in infected patients with HIV.[1] There is some evidence on using the TREC and or KREC assay for identifying the patients with defects in the genes involved in lymphocyte development and differentiation such as recombination activating genes 1/2 (*RAG-1 /2*), adenosine deaminase (*ADA*), and interleukin-2 receptor (*IL-2R*).[2,3] However, the ability to detect the patients with mutations in the genes influencing the function of immune cells like Zeta-chain-associated protein kinase 70 (*ZAP-70*) has been published previously.[11]

In the present study, we aimed to investigate the levels of TREC and KREC in patients with different types of PID and compared them with age-matched healthy controls (HCs). Moreover, we analyzed the possible correlation between these two biomarkers and the age in every type of PID.

PATIENTS AND METHODS

Samples

This study was conducted on 82 PID patients selected based on a clinical diagnosis of combined immunodeficiency who were referred to Immunology, Asthma, and Allergy Research Institute (IAARI), Tehran University of Medical Sciences (TUMS) from across the country between 2011 and 2018. They were referred to the IAARI for a definite diagnosis, treatment, and follow-up. Patients were clinically diagnosed based on the European Society for Immunodeficiencies (ESID) criteria (www.esid.org). This study was approved by the ethics committee of Tehran University of Medical Sciences (IR.TUMS.IAARI.REC.1395.382).Written informed consent was obtained from the guardians of the patient and healthy controls.

As shown in Table 1, 82 patients with a definitive

diagnosis of CID and genetic analysis of some patients were included: common variable immunodeficiency (CVID; n=23; age range 3 to 42 years), ataxia-telangiectasia (AT; n=17; age range 3 to 24 years), hyper-IgE syndrome (HIES; 7 patients with dedicator of cytokinesis-8 (*DOCK8*) deficiency, 4 cases with signal transducer and activator of transcription-3 (*STAT3*) deficiency and 8 children with unknown genetic defects; age range 8 months to 25 years), Wiskott-Aldrich syndrome (WAS; n=20; age range 3 months to 14 years), purine nucleoside phosphorylase deficiency (*PNP* deficiency; n=1; age: 20 months), *DOCK2* deficiency (n=1; age: 7 months) and recombination activating genes-1 deficiency (*RAG1* deficiency; n=1; age: 14 years). To compare our findings with the healthy controls, we used the results of our previous report on evaluating TREC and KREC in 251 healthy individuals (age range 0-60 years).[12] For this purpose, we compared each patient group with its age-matched healthy controls. Demographic, laboratory, and immunological data were obtained from IAARI's PID registry. For lymphocyte count, we used the normal range of CD3 and CD19 among the Iranian population in different age ranges which were in accordance with the textbook.[13,14] As previously described, samples were collected on GE 903 Guthrie cards (GE Healthcare Life Sciences Corp, Marlborough, Massachusetts, USA), dried at room temperature, and kept at -25°C for further evaluation. Dot blood samples on Guthrie cards from the heel stick of infants under the age of one year and venous blood samples of more than one-year-old participants were used. EDTA-blood samples of PID patients were obtained from IAARI's blood bank. All blood samples were transferred on Guthrie cards for punching and performing the test. The informed consent forms for entering the study were signed by all participants or their guardians.

Quantitative Triplex Real-time PCR

TREC/KREC/ACTB triplex real-time quantitative PCR (RT-qPCR) assay was carried out in a final volume of 30 microliters. As previously described with slight technical modifications,[2] DNA was extracted from each 3.2 mm punched dried blood spot (DBS) sample and RT-qPCR reactions were performed by hydrolysis probe chemistry on an ABI 7500 Real-time PCR system (Applied Biosystems, California, USA) according to the manufacture instructions (ImmunoIVD, Stockholm, Sweden). To ensure the efficacy of DNA extraction from Guthrie cards as well as the RT-qPCR process, actin-β (ACTB) amplification was applied. Samples with ACTB≥1000 copies per 3.2 mm punched DBS were considered as high-quality samples. Finally, TREC and KREC copy numbers were measured per punch of Guthrie cards. TREC/KREC assay was done at the laboratory of ImmunoIVD Company in Stockholm, Sweden. According to our previous study,[15] TREC<11 copies/3.2 mm DBS and KREC<6 copies/3.2 mm DBS were considered as the appropriate cut-offs for detecting T and/or B cell lymphopenia. For interpreting the findings of this study, we also used the reference ranges of these two biomarkers in different age groups of Iranians as described in our previous study.[12]

Statistical Analyses

Data were statistically analyzed using Excel 2013 (Microsoft office, USA) and SPSS statistical software version 21.0 (Microsoft Corporation, Chicago, Illinois, USA). Curves were plotted by GraphPad Prism software version 5 (GraphPad Software, San Diego, California, USA). First, the normality of quantitative variables was investigated by the Kolmogorov-Smirnov test. Given the non-parametric distribution of the data, the Mann-Whitney test was utilized for meaningful comparisons between two groups. Descriptive statistics are shown as median as well as lower (Q1) and upper (Q3) quartiles. Spearman correlation was used for non-parametric data. The p values of less than 0.05 were considered as statistically significant levels.

RESULTS

Different TREC and KREC Levels in CID Patients

Hyper-IgE Syndrome (HIES)

As shown in Table 1, we included nineteen cases with HIES: seven *DOCK8*-deficient patients, four cases with *STAT3* deficiency, and eight cases with unknown genetic defects. Among *DOCK8* deficiency patients, only one patient (P1) showed low or undetectable levels of both TREC and KREC; another child (P3) had the normal copies of both biomarkers, and the remaining cases indicated only low or undetectable TREC copies (P2, 4-7). The results were compared with our previously published data on distributing TREC and KREC in different age groups in Iranian healthy individuals.[12] All *STAT3*-deficient patients

Table 1. The results of T-cell receptor excision circles (TREC) and Kappa-deleting recombination excision circles (KREC) and data of immunological investigations from included patients

	Diagnosis	Mutated gene	Patients	age of sampling (year) (min-max)	Genetic analysis	Gender (male/female) (n/n)	The median (Q1-Q3) of TREC (copies/3.2mm DBS)	The median (Q1-Q3) of KREC (copies/3.2mm DBS)	Overall *p* (compared to HCs)
CID	HIES (19 n)	*DOCK8* (7n)	P 2, 4-7	5.5-23	P4: Exon 1-48 Del P5: Exon 1-44 Del	1/4	0 (0-4)	41 (50-435)	TREC (0.009), KREC (0.008)
			P1	13	-	Female	0	2	
			P3	4	Exon 25-26 Del	Male	92	163	
		STAT3 (4n)	P 8-11	1.5-16	P8 & P9: Exon13:c.1144 C>T (p.Arg382Trp) P10: Exon17:c.1594 A>C (p.Lys 531 Gln)	2/2	200 (56-453)	380 (186-630)	
		Unknown (8n)	P 12-19	0.67-25	-	3/5	49 (1-216)	109 (42-287)	
	CVID(23 n)	-	P 20-28	3-24	-	6/3	141 (39-257)	103 (20-294)	TREC (0.009), KREC (0.005)
			P 29-40	9-44		10/2	2 (0-3)	18 (13-36)	
								1 (0-4)	
			P 41,42	3.65-7		1/1	173 (172-174)	0-1	
	WAS (20 n)	*WASP*	P 43-54	0.25-14	P45: c.121 C>T (p.Arg41Ter) P46: c.631 C>T (p.Arg211Ter) P47: c.1074dupa (p.Pro359fsTer135) P50: c.208 G>A (p.Gly70Arg)	Male	30 (16-214)	188 (29-418)	TREC (0.0001), KREC (0.02)
			P 55-62	0.58-14	P55: c.631 C>T (p.Arg211Ter) P56: c.755 G>A (p.Trp252Ter) P57: c.631 C>T (p.Arg211Ter) P58: c.91 G>A (p.Glu31Lys) P59: c.1390 G>T (p.Glu464Ter) P62:c.1001del G (p.Gly334fsTer111)		4 (1-8)		
	AT (17 n)	-	P 63, 64	3.5-9	-	1/1	21 (14-29)	24 (10-38)	TREC (0.0001), KREC (0.0001)
			P 65-78	4.5-15	-	3/1	0	9 (8-31)	
						5/5		1 (0-2)	
			P 79	15	-	Male	11	7	

	PNP deficiency (1 n)	*PNP*	P 80	1.66	-	Male	0	11	
	DOCK2 deficiency (1 n)	*DOCK2*	P 81	0.58	-	Female	0	35	
	RAG1 deficiency (1 n)	*RAG1*	P 82	14	c.2275 C>T (p.Arg759Cys)	Male	0	0	

CID: Combined immunodeficiency; HIES: Hyper-IgE syndrome; CVID: Common variable immunodeficiency; WAS: Wiskott-Aldrich syndrome; AT: Ataxia-telangiectasia syndrome; *PNP*: Purine nucleoside phosphorylase; *DOCK2*: Dedicator of cytokinesis-2; *DOCK8*: Dedicator of cytokinesis-8; *STAT3*: signal transducer and activator of transcription-3; WASP: Wiskott-Aldrich syndrome protein; RAG1: recombination activating genes-1; TREC: T-cell receptor excision circle; KREC: Kappa-deleting recombination excision circle; DBS: Dried blood spot; unknown: no genetic defect was detected.

showed normal levels of both biomarkers. Four out of eight HIES patients with unknown genetic defects had low TREC copies; while the remaining patients showed normal levels of both markers (Figure 1A). Overall, the level of TREC copies in HIES patients was lower than that in HCs (p=0.009); while an increased level of KREC was observed in these patients compared to HCs (p=0.008) (Figure 2A and B). A negative correlation was found between TREC and the age of HIES patients which was not significant (R: -0.50; p=0.055); while a significant inverse association was observed between KREC copy numbers and the age of patients (R: -0.52; p=0.041).

Common Variable Immunodeficiency (CVID)

Twenty-three cases with CVID were entered in this study; twelve cases indicated low TREC levels, six of which had abnormal KREC levels; two cases showed undetectable KREC and normal levels of TREC, and finally, nine patients had normal levels of both TREC and KREC (Figure 1B). Moreover, both TREC and KREC copy numbers in CVID patients were significantly lower than those in HCs (p=0.009 and p=0.005, respectively) (Figure 2C and D). A significantly negative correlation was observed between TREC copies and the age of CVID patients (R: -0.62; p=0.002). An inverse association was also found between KREC and the age of patients which was not statistically significant (R: -0.103; p=0.64).

Wiskott-aldrich Syndrome (WAS)

As shown in Figure 1C, twenty cases with the diagnosis of WAS were investigated in the current study. Twelve patients fell within the normal region of both TREC and KREC, and the remaining eight cases showed low TREC copy numbers, two of which had borderline KREC. TREC levels in WAS patients were lower than those in HCs (p=0.0001). While the copy numbers of KREC in patients were higher than those in HCs (p=0.02) (Figure 2E and F). A negative relationship was detected between TREC and KREC copies and the age of WAS patients which were not statistically significant (R: -0.20; p=0.50 and R:-0.24; p=0.31, respectively).

Ataxia-telangiectasia (AT)

Seventeen cases with AT were entered in this study. One patient showed the borderline results of both TRECs and KRECs and two cases indicated normal levels of both markers. Although fourteen patients had undetectable TREC, twelve of which showed low or zero KREC copies (Figure 1D). A very considerable difference was found between the AT patients and HCs in terms of the levels of both biomarkers (p=0.0001 for both) (Figure 2**Error! Reference source not found.**G and H). An inverse association was found between both biomarkers and the age of AT patients which was not significant (R: -0.104; p=0.66 and R: -0.22; p=0.41, respectively).

Other CID Cases

One patient with *DOCK2* deficiency indicated undetectable TREC copy numbers (16) but a normal level of KREC (Table 1). The *PNP*-deficient patient showed zero copies of TREC; while KREC copies fell above the related cut-off (Table 1**Table 1**). Herein, we measured the copies of both biomarkers in a *RAG1*-deficient patient with late-onset of disease at 14 years of age; this case demonstrated zero copies of both markers. Figure 3 shows the number of TREC and KREC copy numbers obtained in different CID patients altogether in one graph to easily compare the expression of these biomarkers in the corresponding diseases.

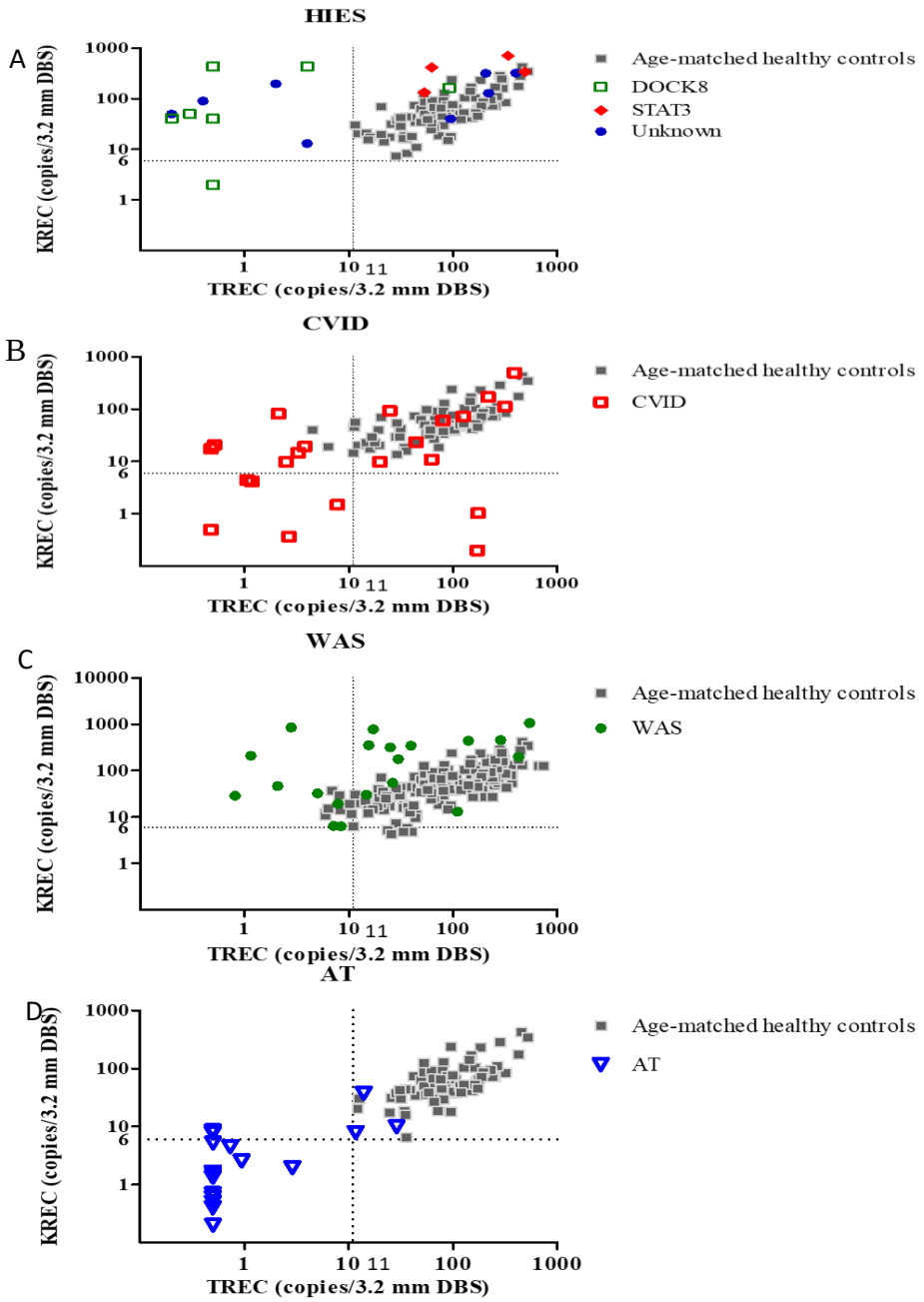

Figure 1. T-cell receptor excision circles (TREC) and Kappa-deleting recombination excision circles (KREC) copy numbers from patients with (A) hyper-IgE syndrome (HIES; n=19) and age-matched healthy controls (n=101; age range 1-25 years), (B) common variable immunodeficiency (CVID; n=23) and age-matched healthy controls (n=100; age range 3-42 years), (C) Wiskott-Aldrich syndrome (WAS; n=20) and age-matched healthy controls (n=168; age range 0-30 years), (D) ataxia-telangiectasia syndrome (AT; n=17) and age-matched healthy controls (n=75; age range 2-20 years). Dotted lines show cut-offs for TREC (<11) and KREC <6) numbers (given as copies per 3.2 mm DBS).

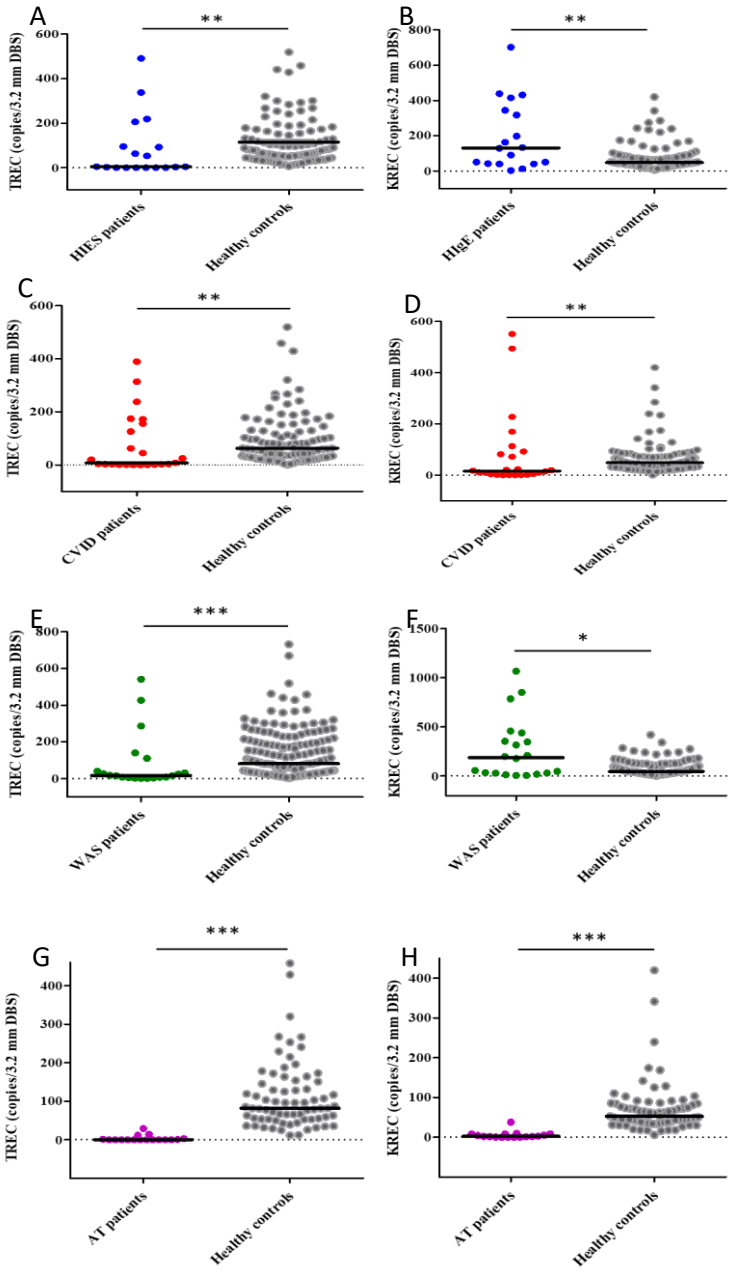

Figure 2. Comparison of T-cell receptor excision circles (TREC) and Kappa-deleting recombination excision circles (KREC) copy numbers between 4 different types of CID and age-matched healthy controls (HCs). (A) and (B) The comparison of TREC and KREC, respectively, between patients with the hyper-IgE syndrome (HIES; n=19) and age-matched healthy controls (n=101; age range 1-25 years), (C), and (D) the comparison of TREC and KREC, respectively, between patients with common variable immunodeficiency (CVID; n=23) and age-matched healthy controls (n=100; age range 3-42 years), (E) and (F) the comparison of TREC and KREC, respectively, between patients with Wiskott-Aldrich syndrome (WAS; n=20) and age-matched healthy controls (n=168; age range 0-30 years), (G) and (H) the comparison of TREC and KREC, respectively, between patients with ataxia-telangiectasia syndrome (AT; n=17) and age-matched healthy controls (n=75; age range 2-20 years). Data have been shown as median (black lines). Mann-Whitney test was done for comparison between two groups *(*p<0.05, **p<0.01, ***p<0.001).*

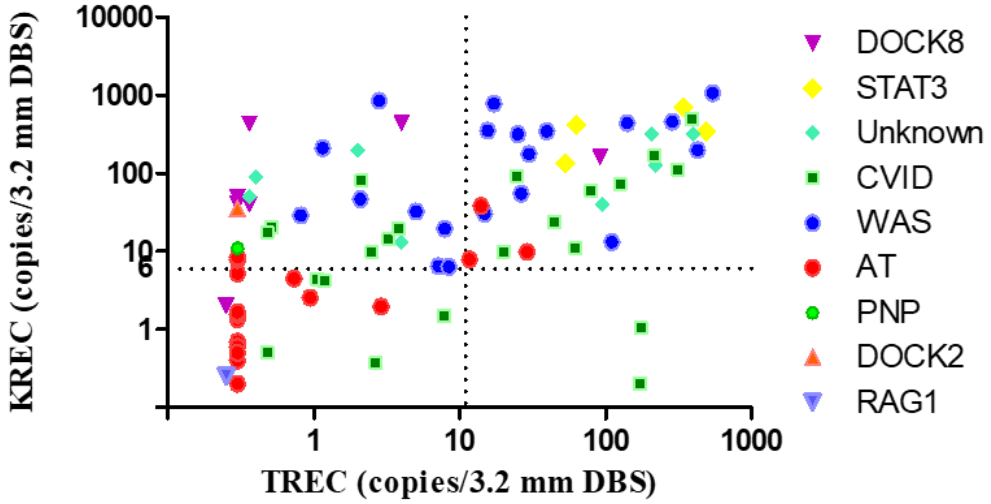

Figure 3. T-cell receptor excision circles (TREC) and Kappa-deleting recombination excision circles (KREC) copy numbers from patients with hyper-IgE syndrome (HIES; n=19) (*DOCK8*: purple triangles; *STAT3*: yellow diamonds; unknown: green diamond), common variable immunodeficiency (CVID; n=23) (green squares), Wiskott-Aldrich syndrome (WAS; n=20) (blue circles), ataxia-telangiectasia syndrome (AT; n=17) (red circles), Purine nucleoside phosphorylase (PNP; n=1) (green circles), Dedicator of cytokinesis-2 (*DOCK2*; n=1) (orange triangle), recombination activating genes-1 (*RAG1*; n=1) (blue triangle). Dotted lines show cut-offs for TREC (<11) and KREC<6) numbers (given as copies per 3.2 mm DBS).

DISCUSSION

The important role of TREC and KREC assay for evaluating the patients with T cell and B cell defects especially SCID has been shown in numerous reports [10,17]; but there are a few reports on evaluating these biomarkers in patients with the other forms of combined immunodeficiency.[18-20] In the present study, we investigated the alterations of both markers in PID patients referred to a referral center for PID patients in Iran compared with HCs. Due to TRECs and KRECs (partially) levels decreasing with age, every group measurement was compared with the corresponding TREC/KREC intervals from healthy age-matched controls before the analysis.

HIES is induced by mutations in the genes involved in modulating the immune system such as *STAT3* and *DOCK8*.[4] *DOCK8* mutation leads to a decreased thymus output and a low number of peripheral naïve T cells which can be found using TREC assay.[21] In this study, we evaluated 19 HIES patients. One out of seven HIES patients with a definitive diagnosis of *DOCK8* deficiency (P1), showed low levels of both TREC and KREC, another child (P3) had the normal copies of both biomarkers, and the remaining five cases indicated only low or undetectable TREC copies (P2, 4-7). Although *DOCK8* has been considered as a vital transcription factor downstream of several surface receptors on T and B cells, the role of this molecule on the gene recombination of TCR and BCR has remained unclear. As expected, all four *STAT3*-deficient patients had high copies of both markers. Even if *STAT3* is a critical transcription factor in T cell signaling pathways,[21] but Satoh and et al, could not find any correlation between the impaired expression of *STAT3* in thymic epithelia and T cell immigrants in stat3$^{-/-}$ transgenic mouse models.[22] Four out of eight HIES patients with unknown genetic defects showed only abnormal results in TREC while the rest had normal values of both markers. The median level of TREC in HIES patients was lower than that calculated in HCs while KREC copies were higher than HCs. Both markers in patients indicated a reduction during aging.

Although CVID is caused by B-cell dysfunction and characterized by hypogammaglobulinemia, defective T-cell function and development can also be a vital effective factor.[4,18,23] Research studies have shown decreased levels of both TREC and KREC copy numbers in CVID patients in comparison with HCs.[18] One study investigated low TREC copies in both CD4

and CD8 T-cell subpopulations in CVID patients.[24] Reversely, De Vera and et al, could find higher levels of TREC in fifteen CVID patients age range from nineteen to sixty-five years old than HCs. In the current study, 6 out of 23 CVID cases showed a low level of both TREC and KREC, six patients indicated low TREC copies but normal level of KREC, and two cases had undetectable KREC despite normal TREC. The remaining patients fell within the normal region of the TREC/KREC graph. All HCs showed normal levels of TREC and KREC copy numbers. Collectively, the levels of both TREC and KREC in CVID patients were lower than those in HCs. A negative correlation was found between both biomarkers and the age of patients which were in agreement with the previous reports.[18] As proposed by Chinen and et al, TREC and KREC can be used to classify CVID patients without a definitive molecular diagnosis for considering the most appropriate therapeutic option for them.[25]

WAS is an X-linked disorder caused by mutations in the Wiskott-Aldrich syndrome protein (WASP) gene, leading to thrombocytopenia, autoimmunity, and frequent infections.[26,27] There are controversial reports on the expression levels of TREC and KREC in WAS patients. One study on Swedish and German populations reported low TREC or KREC copies in four out of eleven WAS patients[26] and another report described a normal level of TREC in a 19-year old WAS patient.[11] Although animal studies on WASP knockout (KO) mice revealed reduced TREC level,[27] some other human studies are not likely to be certain about the influence of WASP expression on TCR gene rearrangement in the thymus.[28] Herein, eight out of twenty patients (P55-62) had a low level of TREC, while two of them showed the borderline results of KREC. The median level of TREC in WAS cases was lower than that in HCs, while it was higher than controls for KREC which were in agreement with the evidence reporting the normal number of B cells in WAS patients despite B cell dysfunction.[29] Decreased levels of both biomarkers with advancing age were shown.

AT is caused by mutations in the ataxia-telangiectasia (*ATM*) gene which has a critical role on V(D)J recombination and immunoglobulin class switch rearrangement (CSR).[30] AT patients suffer from immunodeficiency complications due to decreased numbers of T and B cells.[31] In the present study, from all 17 AT patients, only two cases (P63 and P64) had normal values of TREC and KREC and the remaining had low or undetectable copy numbers. Amazingly, there are some reports on the beneficial effects of newborn screening for SCID to accidentally identify the other types of PIDs including AT.[2,20,32] Moreover, a considerable correlation between low levels of TREC and KREC and diminished CD3 T and CD20 B lymphocytes in about sixty percent of AT patients has been reported.[33] Herein, the level of both TREC and KREC in AT patients was lower than those in HCs. In addition, the correlation between both parameters and age in AT patients was negative.

DOCK2 deficiency is known as a rare genetic disorder of CID with early-onset of disease. T, variable B, and NK cell abnormalities have been indicated in this disorder.[16,34] In the case of TREC, our finding was following the study published by Dobbs et al. who showed a decreased TREC copy numbers based on their regional cut-off values, in two of five *DOCK2*-deficient patients.[34] Based on immunophenotyping results, our patient (P81) was classified as T (-) B (-) NK (-) PID while KREC levels were unexpectedly normal.

PNP deficiency is another rare form of CID that is initially identified by hypouricemia, decreased serum level of Inosine as well as T and/or B lymphopenia. *PNP* deficiency decreases the sensitivity of thymocytes to apoptosis during negative selection in the thymus.[19] Herein, a *PNP*-deficient patient (P80) showed undetectable TREC despite relatively normal KREC. We compared KREC copies in this patient with those in HCs and observed that the KREC level was below the lower limit of the related reference range for KREC despite the normal count of B lymphocytes in the patient. A study performed on nine DBS samples from *PNP*-deficient patients showed low/undetectable TREC and/or KREC copies only in two patients.[19] Another study revealed low or absent levels of coding and signal joints TREC and KREC in two sisters (1 and 2.5 years) with *PNP* deficiency which was in agreement with low T and B cell numbers (only in older cases), respectively.[35] According to the previous reports, our late-onset *RAG1*-deficient patient showed undetectable levels of both TREC and KREC.[2,15,36]

In the current study, insufficient numbers of studied samples was a limitation of our research that may affect the results. Other limitations of our study were the hard conditions for transmitting these kits to Iran and the inability to obtain some data of included people.

In conclusion, a considerable discrepancy was found in TREC/KREC copy numbers between the CID patients and HCs. Compared to the results of our previous report on age-sex matched healthy controls, we observed a lower TREC, but higher KREC copy numbers in HIES and WAS patients, lower biomarkers in CVID and AT patients. Regarding the variation in T and B cell numbers and functions in CID patients and the low incidence of different types of CIDs, it is necessary to evaluate more cases to find out the threshold level of TREC and KREC in each disease uniquely. Despite limitations in screening some types of PIDs such as *DOCK8* deficiency, this method would be a powerful way to identify some types of PID with late- or early onset of disease in newborn screening. Reduced levels of TREC and KREC versus normal or increased numbers of T or B cells in some patients illustrate it as a more precise method for diagnosing the patients.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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