Trends in TRECs values according to age and gender in Chinese children, and their clinical application

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Abstract

T cell receptor excision circles (TRECs) are small circularized DNA elements produced during rearrangement of T cell receptor (TCR) genes. Because TRECS are fairly stable, do not replicate during mitosis, and are not diluted during division of naïve T cells1, they are suitable for assessing the number of newly formed T cells 2. In this study, we detected TRECs in 475 healthy Chinese children aged 0–18 years in different clinical settings. We found a strong correlation between TRECs levels and peripheral CD4 naïve T cell numbers, but not between TRECs levels and effector or memory CD4 and CD8 T cell numbers. TRECs levels fell significantly compared with normal controls in patients with severe combined immunodeficiencies (SCID) (n=7), wiskott-aldrich syndrome (WAS) (n=22), or activated PI3Kδ syndrome (APDS) (n=5). TRECs levels in those with signal transducer and activator of transcription 1 (STAT1) deficiency (n=8) decreased or did not change significantly, a finding consistent with that for CD4 naïve T cells. We also measured TRECs levels in seven PIDs after hematopoietic stem cell transplantation (HSCT) (WAS=5; chronic granulomatous disease (CGD)=2), and found the complications after HSCT may reduce TRECs levels by interfering with production of naïve T cells. In conclusion, we established reference values for TRECs, which can be used to screen for primary immunodeficiency diseases (PIDs) during early life and track immune reconstitution after HSCT.

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Abstract

T cell receptor excision circles (TRECs) are small circularized DNA elements produced during rearrangement of T cell receptor (TCR) genes. Because TRECS are fairly stable, do not replicate during mitosis, and are not diluted during division of naïve T cells[1], they are suitable for assessing the number of newly formed T cells[2]. In this study, we detected TRECs in 475 healthy Chinese children aged 0–18 years in different clinical settings. We found a strong correlation between TRECs levels and peripheral CD4 naïve T cell numbers, but not between TRECs levels and effector or memory CD4 and CD8 T cell numbers. TRECs levels fell significantly compared with normal controls in patients with severe combined immunodeficiencies (SCID) (n=7), wiskott-aldrich syndrome (WAS) (n=22), or activated PI3Kδ syndrome (APDS) (n=5). TRECs levels in those with signal transducer and activator of transcription 1 (STAT1) deficiency (n=8) decreased or did not change significantly, a finding consistent with that for CD4 naïve T cells. We also measured TRECs levels in seven PIDs after hematopoietic stem cell transplantation (HSCT) (WAS=5; chronic granulomatous disease (CGD)=2), and found the complications after HSCT may reduce TRECs levels by interfering with production of naïve T cells. In conclusion, we established reference values for TRECs, which can be used to screen for primary immunodeficiency diseases (PIDs) during early life and track immune reconstitution after HSCT.

Key words: TRECs; reference values; PIDs; HSCT

Introduction

The thymus provides a suitable microenvironment for maturation of T cells; therefore, thymic output may reflect thymus function. T cell receptor excision circles (TRECs) are circular DNA segments generated in T cells during sequential rearrangement of the variable V, D, and J segments of TCR genes. About 70% of all newly produced T cells are TREC-positive. These circularized DNA elements cannot replicate or be stored in the cells[3]. Thus, quantitation of TRECs is an excellent surrogate marker of the number of naïve T cells that have emigrated recently from the thymus[4].

Since measurement of TRECs can be done quickly by real-time quantitative PCR (RT-qPCR), TRECs levels have been used to assess thymic output under healthy and disease conditions; they are especially useful for diagnosis and management of T cell-related disorders[5]. In recent years, TRECs have been used to screen newborns for SCID[6]. Low TRECs levels have been detected in preterm newborns and low birth weight (BW) babies. Newborns with Down's syndrome and ataxia telangiectasia have low TRECs levels[7,8].

As TRECs are a marker of T cell reconstitution, levels should predict occurrence of GVHD after HSCT. Indeed, patients with low TRECs levels after HSCT are more likely to suffer GVHD[9,10]. Assessing trends in TRECs levels according to age is used as a forensic investigation tool to estimate age[11]. TRECs levels could be used to distinguish between benign and malignant diseases; indeed, studies show that levels in patients with acute lymphocytic leukemia and acute myeloid leukemia are lower than those in healthy persons[12]. In addition, TRECs analysis has been applied to autoimmune diseases. TRECs levels in patients with systemic lupus erythematosus fall as disease activity increases[13]. Levels are also low in patients with autoimmune thyroiditis. However, levels in those with autoimmune type 1 diabetes are higher than those in healthy controls[14].

Several studies have examined thymic function in healthy children and adults[15,16]. However, in China, we have no reference values for TRECs in different pediatric age groups. Therefore, it is difficult to determine a cut-off value for TRECs in a clinical setting. Also, it is not completely clear which diseases impact TRECs values. Here, we examined trends in TRECs levels in 475 healthy children (aged 0–18 years) according to

age; the cohort included premature newborns and low BW newborns. Combined with analysis of lymphocyte subsets, we demonstrated a strong correlation between TRECs levels and CD4 naïve T cell numbers. To test the significance of these findings in a clinical setting, we evaluated TRECs levels in patients with different PIDs and patients with PIDs treated with HSCT.

Methods

Healthy volunteers

A total of 410 healthy children (227 males, 183 females) aged 0–18 years were recruited. All were healthy volunteers from Chongqing, China. We defined "healthy" as follows: normal nutritional status, no evidence of infectious disease, no medication, no contact with persons with infectious disease, no immunizations for at least the last 4 weeks, no blood transfusions, no history of atopic disorders, and no evidence of hematological or immunological disorders. All participants underwent regular blood tests, liver and renal function tests, serological tests for hepatitis B virus, and real-time PCR for EBV and cytomegalovirus. Blood samples were obtained from September 2014 to June 2015. DNA was extracted from peripheral blood.

Dry blood spots sample collection

Whole blood samples (n=65) were dropped onto *Whatman 903 paper* in accordance with the established routine neonatal screening process. Three 3.2 mm discs were punched per sample and stored at 4*C until they were processed. DNA was extracted according to a standard protocol (QIAGEN).

Patients

The volunteers recruited to the study were aged from 0–18 years; all were from nonconsanguineous families in mainland China, and included premature newborns and low BW newborns. Those with PIDs were admitted to the Children's Hospital of Chongqing Medical University and diagnosed according to clinical features, laboratory inspection, and genetic analysis. WAS or CGD patients accepted for transplantation were followed up at Children's Hospital of Chongqing Medical University. Clinical data were collected during hospitalization. Informed consent was obtained from participants or their guardians.

Quantification of TRECs

Genomic DNA was isolated from peripheral blood samples according to standard protocols (QIAGEN). The sequences of the primers used for PCR were as follows: TRECs, 5'-CACATCCCTTTCAACCATGCT-3' and 5'-GCCAGCTGCAGGGTTTA GG-3'; probe, 5'-FAM-ACACCTCTG GTTTTTGTAAAGGTGCCCACT-3'-TAMRA; and T cell receptor alpha constant sequences, 5'-AGGAATCCTTGTCTCTGAAAAA TGC-3' and 5'-TTCCTTTTAGTTTCTTGGCCTATGC-3'; probe, 5'-HEX-TGAAGA GAGGACCCTGTTAC-CGCC A-3'-TAMRA. TRECs were measured by real-time quantitative PCR, as described previously. Briefly, 5 μl of DNA was amplified in 25 μl of PCR solution containing primers and probes specific for TRECs. Standards were prepared according to protocols published by Sottini et al[17]. All DNA samples were run in triplicate alongside no-template controls.

Lymphocyte subset analysis

All the healthy volunteers were taking part in the Public Welfare Scientific Research Project of China (grant no. 201402012)[18]. The BD Multi-test IMK Kit (cat. 340503) was used to analyze lymphocyte subsets (B cells, CD3 T cells, CD4 T cells, CD8 T cells, and CD32CD56/CD16 natural killer cells). The following mAbs were used to detect T cell subsets: anti-human CD3 (PerCP-Cy5.5), anti-CD4 (FITC; fluorescein isothiocyanate), anti-CD8 (BV510), anti-CD27 (APC; allophycocyanin), anti-CD45RA (PE-Cy7), anti-TCR (PE; phycoerythrin), and anti-TCRgd (BV421) (all from BD Biosciences). Subsets were analyzed as described previously.

Statistical analysis

Data were analyzed using GraphPad prism software and the IBM SPSS Statistics package (version 20). Independent-samples t-tests were used to compare the means of two groups (parametric data). Pearson's

and partial correlation coefficients were calculated for parametric and nonparametric data, respectively. A P-value < 0.05 was considered statistically significant.

Results

Trends in TRECs levels in healthy children according to age and gender

We hypothesized that understanding trends in TRECs levels in healthy children according to age will help us to identify disease at the early stages; therefore, we quantified TRECs levels in 475 healthy children, including premature newborns and low weight newborns. In accordance with a previously published paper[18], the children were categorized into seven different age groups (0–1 months (m), 1 m–6 m, 6 m–1 years (y), 1–4 y, 4–8 y, 8–12 y, and 12–18 y) (Table 1). TRECs levels fell significantly with increasing age (r -0.5179; p<0.0001) (Fig. 1), which is consistent with the findings of Ou et al[19]. TRECs declined rapidly from 1 m to 4 y and from 12 y to 18 y. There were no obvious changes between newborns and babies aged 1–6 m. Females tended to have higher levels of TRECs than males before the age of 1 year; however, the difference was not statistically significant. After the age of 1 year, TRECs levels in females were similar to those in males (Fig. 2).

We also analyzed TRECs levels according to gestation age (GA) (Fig. 3A). TRECs levels increased with the increasing GA. Levels in preterm infants were significantly lower than in full term babies (Fig. 3B), which is consistent with the findings of de Felipe et al[20]. Here, we found that full term newborns with low BW had lower TRECs levels than those of normal BW (Fig. 3C). However, the TRECs levels in preterm infants with low BW were comparable with those of normal BW infants (Fig. 3D).

TRECs levels correlate significantly with CD4 naïve T cell numbers

To examine the correlation between T cell subsets and TRECs levels, we analyzed the absolute numbers of different T cell subsets in the 410 healthy children. There was a correlation between TRECs levels and the numbers of CD3 T cells (r 0.220, p<0.0001) (Fig. 4A) and CD4 T cells (r 0.318, p<0.0001) (Fig. 4B). We also found a significant correlation between TRECs levels and CD4+CD45RA+CD27+ (CD4 naïve) T cell numbers (r 0.305, p<0.0001) (Fig. 4C). However, there was no correlation between TRECs levels and CD8+/CD8+CD45RA+CD27+ (CD8 naïve) T cell numbers (p>0.1) (Fig. 4D/E), or between TRECs levels and CD4+/CD8+ central memory T cell or CD4/CD8 effector memory T cell numbers (Fig. 4F–H).

Because CD4 naïve T cell numbers showed a strong correlation with TRECs levels, we next analyzed the correlation between TRECs levels and CD4 naïve T cell numbers in different age groups (Fig. 5). We found significant correlations between TRECs numbers and CD4 naïve T cell numbers in the 1–4 y (r 0.243, p 0.023) (Fig. 5D) and 4–8 y (r 0.409, p<0.0001) age groups (Fig. 5E).

TRECs levels in children with PIDs and secondary immunodeficiency

To confirm the specificity and sensitivity of our method, we analyzed TRECs levels in patients with SCID and X linked agammaglobulinemia (XLA). TRECs levels in patients with SCID of the IL2rg, RAG1, JAK3, and Lig4 mutation genotypes were 0, whereas those in patients with XLA were normal (Table 1), which is consistent with previous publications[21].

Next, we determined the significance of TRECs levels in PIDs (Table 2). We identified greater reductions in TRECs levels in patients with classical WAS, whereas those in X linked thrombocytopenia (XLT) patients were only slightly decreased or within the normal ranges (Fig. 6). These data suggest that TRECs levels could be used to identify classical WAS or XLT early, thereby enabling appropriate selection of therapeutics. However, the data may mean that classical WAS might be caused by a thymic output defect[22]. Gain-of-function (GOF) mutations in PIK3CD cause APDS. Therefore, we asked whether there was a clear reduction in TRECs levels in APDS, consistent with other combined immunodeficiency (CIDs). We found that TRECs levels in most patients with STAT1 mutations were normal, although some did have lower levels (Fig. 6). We also analyzed the lymphocyte subsets from STAT1 patients (Table 3) and found that changes in TRECs levels were consistent with changes in CD4 naïve T cell numbers in STAT1 patients (Fig. 7).

In addition, we analyzed TRECs levels in patients with secondary immunodeficiencies such as primary nephrotic syndrome (NS) after prednisone treatment. We found that TRECs levels were normal in NS patients with or without immunosuppressive treatment (Table 4 and Fig. 8).

TRECs levels in PID patients after HSCT

We followed up TRECs levels in five patients with classical WAS and two with CGD who underwent HSCT (Fig. 9). The clinical features of these patients are summarized in Table 5. Patients P2 and P5 characterized by respiratory tract infections, eczema, and thrombocytopenia. P2 developed GVHD in the liver, eyes, and CNS after HSCT, and P5 suffered multiple organs disorders. P6 on set with systemic tuberculosis after BCG vaccination and suffered liver and kidney damage after HSCT. P2, P5, and P6 had low levels of TRECs up until 4 weeks after HSCT. The other four patients showed elevated TRECs levels in the following 4 weeks, which returned to the normal range at around 1 year post-HSCT.

Discussion

TRECs are thought to be the most reliable tools for tracking recent thymic output. Indeed, such measurements have been used in multiple clinical settings, including diagnosis of SCID, T cell development-associated immune deficiencies, T cell reconstitution after HSCT, aging, and autoimmune diseases.

In this study, we quantified TRECs levels in 475 children aged 0–18 years, including premature newborns and low BW newborns. To the best of our knowledge, this is the largest study designed to determine TRECs reference levels in a healthy Chinese pediatric population. We found that TRECs levels tended to fall with age, mainly due to division of peripheral cells and reduced thymic activity due to age-associated thymic involution[23]. TRECs levels fell rapidly between 1 month and 1 year-of-age, reflecting increased rates of thymopoiesis. A second large decrease occurred from 12–18 years-of-age, which might be due to changes in hormone levels. We also found that TRECs levels were lower in premature newborns and low BW newborns. TRECs levels tended to increase with GA. These data suggest that during newborn screening, the GA and its reciprocal BW should be included in the screening strategies[24]. Ward et al found a 9.8% increase in TRECs levels per week of gestation[25]; however, our study identified no linear correlation between GA and TRECs levels, although this may be due to an insufficient number of subjects in this case.

Gender-related differences in TRECs levels were very conflicting[26,27]. We found that TRECs levels in females were similar to those in males. Sex hormones such as prolactin may regulate development of CD4 T cells[28]. Indeed, the number of mature CD4 T cells in estrogen-treated mice increases. Also, testosterone may induce apoptosis of thymocytes[29]. In addition, production of cytokines is affected by sex hormones such as IL7 and IL15, which may drive development of T cells[30].

We found a weak correlation between TRECs levels and numbers of CD4 naïve T cells, which is inconsistent with the consensus that TRECs levels are closely related to CD4 naïve T cell numbers. In the large number of healthy young participants, we found a correlation between TRECs levels and the absolute number of peripheral CD4 naïve T cells only in the 1–4 y and 4–8 y age groups; no study has found this before. This finding may be due to the fact that we examined CD4 naïve T cell numbers in peripheral blood; thus the numbers may be affected by factors other than thymic output (i.e., proliferation, death, and redistribution of peripheral CD4 naïve T cells)[31]. The CD4 naïve T cells have a short lifespan and soon undergo apoptosis, thereby contributing to T cell homeostasis in which newly generated thymic emigrants make up for the loss of peripheral cells[32]. Activated of CD4 naïve T cells are regulated by some nutritional factors[33]; therefore, measurement of TRECs levels and naïve T cell numbers should be combined with an assessment of thymus function.

WAS, characterized by eczema, thrombo-cytopenia, and immunodeficiency, has three phenotypes: classical WAS, XLT, and X-linked neutropenia (XLN)[34]. Treatment differs according to the phenotype and associated complications. Those with classical WAS need urgent curative treatment, such as a stem cell transplant or gene therapy[35]. For those with XLT, symptomatic treatment is the major therapy of choice, although splenectomy is sometimes recommended as it effectively stops the tendency to bleed[36]. XLT patients have

a better prognosis than those with WAS[37]. Thus, WAS may be a good model to test whether TRECs are a good biomarker and predictor of disease severity. Our data revealed that TRECs levels in patients with classical WAS (n=14) were significantly suppressed, whereas those in patients with XLT (n=8) were mildly suppressed or normal. There was a strong correlation between TRECs levels and CD4 naïve cell numbers in those with classic WAS, which is consistent with the findings of a previous study[36].

GOF mutations in the PI3K genes PIK3CD (p110δ) and PIK3R1 (p85α) cause a combined immunodeficiency syndrome called APDS[38]. TRECs levels and CD4 naïve T cell numbers were suppressed significantly in those with APDS[39]. The PI3K-AKT pathway is crucial for transition of intermediate single positive thymocytes to double positive thymocytes[40]. T cell development in those with PIK3CD GOF mutations is skewed, as evidenced by a reduction in CD4 and CD8 thymocyte numbers in an APDS mouse model[41]. APDS mice show severe lymphopenia in the periphery, along with increased senescence of effector T cells and total T cells[42]. Signal transducer and activator of transcription 1 (STAT1) is a transcription factor that mediates cellular responses to interferons (IFNs), as well as other cytokines and growth factors. Mutation of STAT1 is associated with chronic mucocutaneous candidiasis or Mendelian susceptibility to mycobacterial disease, with or without autoimmune disease[43]. TRECs levels in patients with STAT1 mutation are normal in the publications before44,45; however, we found that TRECs levels were consistent with the levels of CD4 naïve T cells, which were normal or mildly decreased. A previous study shows that only 28% of patients with STAT1 mutations have low CD4 T cell numbers[46]. We found a similar result here[47]. Lower CD4 T cell counts are associated with a higher mortality in STAT1 patients; therefore, patients with low TRECs levels probably require HSCT as soon as possible. The proportion of CD3/CD4 T cells and TRECs levels in NS patients were normal[48,49]. Overall, the data suggest that TRECs levels correlate with CD4 naïve T cell numbers; therefore, they may be a good biomarker for multiple PIDs.

TRECs levels may also predict occurrence of GVHD and associated complications. In the first few months after HSCT, peripheral expansion of implanted cells is very important[50]. We found that TRECs levels started to rise within 4 weeks of HSCT, and achieved normal levels by 1 year post-HSCT. Consistent with the findings of Weinberg et al[51], we found that GVHD might be associated with low TRECs levels. P2 developed GVHD in multiple organs, and his TRECs levels remained low. P5 and P6 had low TRECs levels after HSCT and developed multiple organ disorders. The major factors that affect TRECs levels are the number of HLA matches, the type of graft, TRECs levels before HSCT, and the proposal conditioning regimen[52]. However, more cases are needed to identify a correlation between TRECs levels and complications after HSCT.

Conclusions

The present study used RT-qPCR to analyze TRECs levels in 475 healthy children of different ages in China. This is the largest population of healthy children to be analyzed in China. We established reference values for TRECs and quantified trends in TRECs levels according to age and sex. We provide solid data showing a correlation between CD4 naïve T cell counts and TRECs levels according to age. Postnatal factors such as nutrition and hormone levels induce rapid changes in the phenotype of CD4 naïve T cells. We also showed that TRECs levels are of clinical utility by quantifying them in patients with WAS, APDS, or STAT1 mutations, in patients post-HSCT, and in patients with secondary immunodeficiency disease. Although the methods used to measure and detect TRECs differ between laboratories (which will lead to differences in TRECs levels in the same individual), it is still meaningful to establish a TRECs reference value within a consistent environment. Our institution is the main PID diagnosis and treatment center in China; therefore, such a reference value system will enable prompt screening of PIDs in a timely manner.

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Ethics approval

Informed consent was obtained from all individual participants included in the study. This study was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the ethics committee of Chongqing Medical University.

Authorship Contributions

Q.Z and ZX.D designed the study and wrote the manuscript; Q.Z, RX.D and YN.L performed the experiments and analyzed the data; Q.Z, YP.W, XM.C, Z.S, LN.Z, Y.D and XM.T followed the patients; all the authors reviewed the manuscript before it was submitted.

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Consent to participate

Written informed consent was obtained from individual or guardian participants.

Consent to publication

Written informed consent was obtained from individual or guardian participants.

Availability of data and material

The data that support the findings of this study are available from the corresponding authors, XD.Z upon reasonable request.

Disclosure of Conflicts of Interest

The authors declare no conflict of interest.

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