Limitation of Simultaneous Analysis of T-Cell Receptor and κ-Deleting Recombination Excision Circles Based on Multiplex Real-Time Polymerase Chain Reaction in Common Variable Immunodeficiency Patients

Faranaz Atschekzei  Fareed Ahmad  Torsten Witte  Roland Jacobs  Reinhold E. Schmidt

Department of Clinical Immunology and Rheumatology, Hannover Medical School, Hannover, Germany

Key Words
Common variable immunodeficiency  ·  T-cell receptor excision circles  ·  κ-Deleting recombination excision circles  ·  Quantitative real-time polymerase chain reaction

Abstract
Aim of Study: We used a triplex real-time polymerase chain reaction (PCR) to classify our common variable immunodeficiency (CVID) patients into distinct groups according to the amount of their T-cell receptor excision circles (TRECs) and κ-deleting recombination excision circles (KRECs). Materials and Methods: TREC and KREC analysis was performed using a multiplex real-time PCR assay. The T- and B-lymphocyte subsets were measured by flow cytometry. Results: The copy number of TRECs and KRECs was significantly reduced in CVID patients compared to healthy controls. The TREC copy number was inversely correlated with age in both healthy subjects and patients; however, the KREC copy number was inversely correlated with age only in CVID patients. Moreover, no association was seen between TREC/KREC copy number and clinical manifestations such as bronchiectasis, splenomegaly, granulomata, autoimmune cytopenias, organ-specific autoimmunity, enteropathy and lymphoid hyperplasia. Conclusion: TREC and KREC quantification might be a useful tool to differentiate between CVID and combined immunodeficiency, but considering the results of this study a classification of CVID patients in certain groups is hardly possible.

Introduction

Common variable immunodeficiency (CVID) comprises a group of heterogeneous diseases characterized by deficiency in antibody production. While some CVID patients only suffer from infections of the respiratory tract, many of them are afflicted with serious noninfectious complications like autoimmunity and malignancy [1], which mostly are not easy to treat and often end fatally.

Generally, CVID occurs sporadically. Only 10–20% of cases demonstrate familial accumulation [2]. Several monogenetic disorders (ICOS, CD19, CD20, CD81) re-
sulting in the CVID phenotype were identified during the past few years but the majority of the genetic mechanisms leading to CVID are still unclear [1].

The attempt to differentiate a CVID collective into homogeneous subgroups represents huge challenges. Therefore, various immunological assays are used in these patients to determine the status of their immunity such as the Freiburg classification [3]. So far, the current methods are based on lymphocyte subset determination and functional analyses by flow cytometry, and they do not provide sufficient information about lymphocyte neogenesis. Furthermore, all these methods require fresh blood and rapid handling of samples.

Recently, a real-time polymerase chain reaction (PCR) was established based on the simultaneous quantification of T-cell receptor excision circles (TRECs) and \( \kappa \)-deleting recombination excision circles (KRECs) for newborn mass screening of severe combined immunodeficiency and of B-cell immunodeficiency [4, 5]. TRECs and KRECs are circular episomes of DNA that are formed into differentiating T- and B-cell progenitors during V(D)J rearrangement in the thymus and bone marrow. It is supposed that TREC and KREC measurement could be a valuable marker for lymphocyte neogenesis [6].

Kamae et al. [7] reported the classification of CVID patients by simultaneous KREC/TREC real-time PCR. They subdivided their CVID patients (n = 40) with the aid of concurrent KREC and TREC quantification into 4 categories (A = TREC+/KREC+, B = TREC+/KREC−, C = TREC−/KREC+, D = TREC−/KREC−) and claimed an association between the clinical severity of CVID and KREC/TREC-based classification of CVID patients [7].

The main aim of the current study was to evaluate the suggested classification by Kamae et al. for our CVID patients.

**Subjects and Methods**

DNA was extracted from peripheral white blood, obtained from 134 adult patients with CVID (median age 46, range 19–84 years; female/male ratio 81/50, European ethnicity) who come regularly to our clinic for treatment and 50 healthy controls (median age 42, range 22–69; female/male ratio 81/50, European ethnicity) who come regularly to our clinic for treatment and 50 healthy controls (median age 42, range 22–69; female/male ratio 81/50, European ethnicity) according to the manufacturer’s protocol (QIAamp DNA Blood Midi Kit, Qiagen). The clinical diagnosis of CVID was based on the ESID criteria for CVID (www.esid.org):

- Increased susceptibility to infections
- Marked decrease in IgG and marked decrease in IgA with or without low IgM levels (measured at least twice; <2 SD of the normal levels for their age)
- Poor antibody response to vaccines

All subjects gave their permission to the study by written consent, according to the Ethics Committee procedure at our Institution.

A multiplex real-time PCR assay was used to quantify T- and B-lymphocyte mobilization by the detection of TRECs and KRECs. The study protocol and reagents for triplex real-time PCR were provided by the Jeffrey Modell Diagnostic and Research Centre for Primary Immunodeficiency at the Municipal Hospital St. Georg, Leipzig, Germany. The primer was described previously by Sottini et al. [8]. Absolute copy numbers of TRECs, KRECs and the housekeeping gene ACTB (copies/µg DNA) were obtained by extrapolation from standard regression lines, which were determined by each gene during quantitative real-time PCR reaction using an ABI PRISM® 7700 device. Suitable cutoff scores were established using statistical methods. The statistical evaluation of the data was performed using the R statistics program and was supported by the Institute of Biometrics MHH.

T- and B-lymphocyte subsets were measured by flow cytometry.

**Results**

**KREC and TREC Copy Numbers Related to Age in Patient and Control Groups**

In order to clarify whether this is a useful approach, we studied TRECs and KRECs in a case-control study using triplex real-time PCR. We found that the TREC copy number decreases with age both in healthy controls (p = 2.25e–6, cor. = −0.6, Pearson’s product-moment correlation, fig. 1a) and CVID patients (Pearson’s product-moment correlation, p = 3.93e–7, cor. = −0.4, fig. 1b). The copy number of KRECs remained unchanged in the control group (Pearson’s product-moment correlation, p = 0.69, cor. = −0.05, fig. 1c) with increasing age but declined in CVID patients with age (Pearson’s product-moment correlation p = 2.55e–3, cor. = −0.26, fig. 1d).

**Comparison of KREC/TREC Copy Numbers between Patients and Controls**

The copy numbers of TRECs and KRECs were significantly reduced in CVID patients compared to healthy controls (fig. 2a, b).

However, the age of blood donors was different for the case and control groups (Welch’s t test, mean of patients’ age = 47, mean of controls’ age = 42, p = 0.08). We conducted a logistic regression analysis to adjust for differences in age. The reduction of KREC and TREC copy numbers remained a significant parameter in the multivariate statistical models through the group comparisons (TRECs: OR 0.70, 95% CI 0.597–0.823, p < 0.0001; KRECs: OR 0.91, 95% CI 0.859–0.967, p = 0.0023; table 1).
All variables included in this model (KRECs; TRECs; age) had a significant impact on the state of health or sickness. On this basis, the CVID patients included into this study were divided into 2 age groups, according to the median age of 45 years. Then, the cutoff values were determined for TRECs and KRECs in each age group.

It is notable that the cutoff value for elderly CVID patients was lower (cutoff for KRECs 6.2 copies/μg DNA; cutoff for TRECs 1.6 copies/μg DNA) than that for young CVID patients (cutoff for KRECs 8.8 copies/μg DNA, cutoff for TRECs 5.2 copies/μg DNA). By using these cutoffs we could distinguish between low and normal KREC and TREC values.

The association between KREC/TREC copy number and immune status of CVID patients was investigated, and we found a direct correlation between TREC copy number and the absolute number of CD4+ T lymphocytes (p = 9.30e–3; cor. = 0.5) in CVID patients younger
than 45 years but not in the older patients (p = 0.68; cor. = 0.08; data not shown). The statistical analysis of the data showed a significant positive correlation between KREC copy number and the absolute number of CD19+ B lymphocytes (p = 1.60e –4 ; cor. = 0.6) in young patients but not in older CVID patients (data not shown).

Moreover, no association was observed between TREC/KREC copy numbers and clinical manifestations such as opportunistic infections, autoimmune diseases and malignancies.

**Discussion**

In this study we have shown that the KREC level decreases with age in CVID patients, while it remained unchanged in healthy controls. The number of TRECs is assumed to decrease in elderly subjects due to peripheral cell division and reduced thymic activity because of age-associated thymic involution. Indeed, the number of TRECs in this study was significantly lower in CVID patients compared to the healthy controls and decreased with age indicating T-cell impairment in CVID patients.

Our results do not confirm the data of Kamae et al. [7]. Our measured TREC/KREC values scattered widely and were lower than those in the stated study. This result can be explained by the fact that KREC and TREC levels decline with increasing age in patients and the median age of our patients was significantly higher than in the study mentioned (median age 15 years, range 2–52 years) [7]. Hence, a breakdown into these 4 categories as described by Kamae et al. for their limited CVID patients (n = 40) was unascertainable. Even, after division of our CVID patients into 2 age groups and statistical determination of cutoff values for each age group, a classification of patients based on TREC/KREC quantification was not possible.

This method might work in a limited cohort with much younger patients, but in the current study we could not confirm its suitability for the classification of CVID patients in distinct groups. Also our contrasting results may be based on the fact that with increasing case numbers of such a heterogeneous disease the classification into homogeneous groups might not be possible.

Meanwhile, our further next-generation sequencing analysis (data evaluation is already in progress) revealed that some of the CVID patients, particularly those with too low or undetectable KRECs, have deficiencies such as CTLA4, LRBA, or mutations in TACI and IGLL1 genes. One of the CVID patients with undetectable KRECs and TRECs has shown compound heterozygous RAG1 mutations and was immediately rediagnosed to combined immunodeficiency (data not shown). These new findings indicate that patients with too low or undetectable KREC and/or TREC levels might have a serious form of B- or T-cell defects, resulting from homozygous or compound heterozygous mutations in key genes of the adaptive immune system. Therefore, TREC and KREC measurement might be an early step toward a better diagnosis.

In the light of these results we conclude that KREC/TREC quantification might be used in individual cases to distinguish between CVID and combined immunodeficiency, but in general a classification of CVID patients into distinct groups is not possible using this method. A TREC and KREC measurement in individual instances could serve as an indicator for T-cell abnormalities and for terminal B-cell defects, which need further investigations.

**Acknowledgments**

This study was supported by DZIF TTU 07.801 (German Centre for Infection Research). The statistical analysis was supported by the institute for Biometry MHH.

**Disclosure Statement**

The authors have no other relevant affiliations or financial participation with any organization or entity with financial interest.

---

Table 1. Logistic regression model

<table>
<thead>
<tr>
<th>Variable</th>
<th>d.f.</th>
<th>Parameter estimate</th>
<th>Standard error</th>
<th>Wald χ²</th>
<th>p &gt;χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>1</td>
<td>5.079</td>
<td>1.0503</td>
<td>23.3849</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>KREC</td>
<td>1</td>
<td>-0.0923</td>
<td>0.0302</td>
<td>9.3292</td>
<td>0.0023</td>
</tr>
<tr>
<td>TREC</td>
<td>1</td>
<td>-0.3552</td>
<td>0.0819</td>
<td>18.8024</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>1</td>
<td>-0.0426</td>
<td>0.0159</td>
<td>7.1624</td>
<td>0.0074</td>
</tr>
</tbody>
</table>

This table shows the coefficients (parameter estimate), their standard errors, the Wald χ² statistic, and associated p values. The coefficients for TRECs, KRECs and age are statistically significant.
References


2 Yong PF, Thaventhiran JE, Grimbacher B: 'A rose is a rose is a rose’, but CVID is not CVID common variable immune deficiency (CVID): what do we know in 2011? Adv Immunol 2011;111:47–107.


