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TECHNICAL NOTE

Detection and quantification of the age-related sjTREC decline in human peripheral blood

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Abstract The decline of signal joint T-cell receptor rearrangement excision circles (sjTRECs) in human peripheral blood has been demonstrated to be age-related, which can be a potential marker for individual age determination. However, little is known about the quantitative relationship between the levels of sjTREC and age. The aim of the present study was to investigate the levels of sjTREC in peripheral blood leukocytes (PBLs) among different age groups in Chinese population, so as to clarify whether it could serve as a suitable marker for biological age estimation in forensic practice. sjTREC levels were measured by real-time quantitative PCR analysis in peripheral blood samples from individuals of known age (n=248). The quantification results showed that sjTREC declined in human PBLs in an age-dependent manner (r=−0.8177, P<0.01). The formula for age estimation based on peripheral sjTREC decline was Y=−24.921x−39.932± 10.47 (Y age, year; X log sjTREC/TBP; 10.47: standard error). Furthermore, there was no difference between males and females with regard to sjTREC levels. These results suggest that assessment of sjTREC in PBLs might be a valuable additional tool in age determination, especially in cases where traditional morphologic information is absent or inefficient in forensic practice.

Keywords Real-time quantitative PCR (RQ-PCR) · Signal joint T-cell receptor excision DNA circles (sjTRECs) · Age · Sex · Chinese population

Introduction

In forensic practice, when forensic samples such as blood samples cannot provide enough morphologic information, useful information on age diagnostics can be made with the assistance of methods based on molecular biology. A variety of biological indicators, such as the telomere DNA restriction fragment (TRF) length [1, 2], and mitochondrial DNA (mtDNA) 4,977 bp fragment deletion degree [3], have been indicated to be closely related to individual age and aging, which may be effective markers for the forensic age determination. However, most of these studies are still in the early stage due to obstacles including the interferences of the environmental, genetic and disease effects, the differences between the groups of people and organs, and lack of precise quantitative relationship between single biological indicator and age. In view of this, the Study Group on Forensic Age Diagnostics recommended the implementation of three morphologically independent features in each single case to obtain age estimates as precisely as possible [4]. Therefore, the pursuit of newer age-related markers will be

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complementary to the current techniques for age estimation, thus improving the accuracy of age determination.

The central role of the thymus in the generation of a diversified population of peripheral T lymphocytes is well-established, as well as its age-associated involution at a rate of approximately 3% per year until middle age and at a rate of 1% per year thereafter, which leads to a reduction in this organ's function of naïve T lymphocyte generation output [5]. For a long time, measurement of thymic output is indirect, based mainly on the phenotypic markers on naïve T cells. Recently, measurement of thymic output has been achieved by the analysis of the amount of T cell receptor rearrangement excision circles (TRECs) within T cell population [6]. TRECs are by-products of the rearrangements of gene segments encoding the variable parts of T cell receptor (TCR) α and β chains in the thymus, which give rises to TCR diversity generation. Since these extra-chromosomal DNA by-products do not replicate during cell divisions, they indicate the extract number of the recent thymic naïve T cell emigrant population. Signal joint TRECs (sjTRECs) are the most appropriate TRECs resulting from δRec-ψJα rearrangement (*TCRD* locus deletion) that occurs late during thymopoiesis, and largely accepted as a surrogate marker for thymic function [7]. Previous studies have clearly shown a progressive decline in peripheral sjTREC levels with age [8, 9], echoing the age-related thymic atrophy. However, very little is known about the quantitative relationship between human sjTRECs and donor age.

Real-time quantitative polymerase chain reaction (RQ-PCR) is widely used in forensic research for its accuracy and sensitivity [10, 11]. Three oligo nucleotides are used in RQ-PCR analysis: a forward primer, a reverse primer, and a so-called *Taqman* probe. All of them are specific for the target and are able to bind to it. In order to determine the relationship between the levels of sjTREC and age, we adopted a sensitive back-to-back RQ-PCR method to detect sjTREC in peripheral blood samples, from a large series (n=248) of individuals ranging from new born (cord blood) to 78 years old, so as to see if this relationship could be used to develop a formula to predict the age of an individual within a narrow range suitable for the forensic practice.

Materials and methods

Samples

Whole blood samples were obtained from 248 unrelated healthy individuals ranging from 0 (cold blood) to 78 years old of Chinese Han people, an ethnic group living in the southern China (Table S1). Donors were randomly chosen among individuals whose ancestors had lived in the region for at least three generations. The research protocol was approved by the Human Subjects Committee at the Department of Forensic Biology of Zhongshan School of Medicine, Sun Yat-sen University. All samples were collected into Vacuette EDTA K3 tubes and stored at −80°C, with informed consent from the individual or a parent or guardian.

DNA extraction

Genomic DNA was extracted from 300 μl of whole blood using the Wizard Genomic DNA extraction kit® (Promega) according to the manufacturer's instructions and stored at −20°C until use. Sample purity and quantity was determined by a Gene Quant pro RNA/DNA calculator (Amersham Pharmacia, USA).

Primers and *Taqman* probes

In order to easily visualize the circled extra-chromosomal DNA by-products, we adopted a sensitive back-to-back RQ-PCR method to detect sjTREC in peripheral blood samples [12]. As shown in Fig. S1, the forward and reverse sjTREC PCR primers were designed to be located to in the *TCRD* locus, with their 3′ ends, respectively, close to δRec and ψJα locus. In this position, the primers did not allow amplification of target sequence when *TCRD* locus remained part of genomic DNA, but could generate a PCR fragment solely when *TCRD* gene was deleted and a TREC containing a unique signal joint (sj) sequence was formed. The sequence of primers and probe for sjTREC (ACCESSION AE000521) were 5′-CCATGCTGACACCTCTGGTT-3′ (P1: forward primer), 5′-TCGTGAGAACGGTGAATGAAG-3′ (P2: reverse primer), and 5′-FAM-CACGGTGATGCATAGG-CACCTGC-TAMRA-3′ (*Taqman* probe) [12]. In addition, primers and probe for the internal reference gene, human TATA box binding protein (TBP; accession NG00816), were 5′-TTAGCTGGCTCTGAGTATGAATAAC-3′ (forward primer), 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′ (reverse primer), and 5′- FAM-CAGTCCAGACTGG CAG-CAAGAAAA T-TAMRA-3′ (*Taqman* probe).

Real-time quantitative PCR

The contents of sjTREC and TBP in each sample were determined by RQ-PCR using standard curve method. Plasmid constructs encoding the sjTREC sequence or TBP were used as standards and diluted over the range 10^6–10^1. Standard curves for sjTREC and TBP were run respectively in each experiment together with extracted DNA samples, positive and negative controls. RQ-PCR was performed using an ABI Prism® 7500 Sequence Detector System and data analyzed using SDS 2.0 software. *Taqman*™ Hydro-

lysis technology was used in a 20 μl reaction mixture containing 250 nM of each primer (forward and reverse primer), 250 nM *Taqman* hydrolysis probe and 10 μl Premix Ex Taq™ (TaKaRa). Each reaction contained 0.2 μg template DNA. Thermal cycling conditions were 95°C for 5 min then 95°C for 15 s, 60°C for 15 s, and 72°C for 32 s for 40 cycles. sjTREC and internal reference gene TBP were triplicately measured in each experimental samples. The absolute amounts of sjTREC and TBP were calculated from the sample cycle threshold (*C*t) and the standard curve regression equation, respectively, and then the normalized value of sjTREC level was determined as log sjTREC/TBP. The efficiencies of the PCR reactions were calculated by using the formula $E=[10^{(-1/S)}]-1$, with *S* being the slope of the standard curve.

Statistics

The normality of the data was examined using the Kolmogorov–Smirnov test, and homogeneity of variance using the Levene median test. Results (log sjTREC/TBP) were expressed as minimum, maximum, and mean± standard deviation. Linear regression equation was obtained for estimating the age of donors by peripheral sjTREC levels. One-way ANOVA test was used to test for differences between groups, followed by all pairwise multiple comparison procedures using Tukey's post hoc test. Gender difference was assessed using the unpaired student *t* test. All statistical analysis was performed using SPSS© 16th edition software. Results were considered significant when $p<0.05$.

Results

Primer specificity

The quantification results confirmed that DNA could be correctly extracted from all blood samples, and the quantities obtained were sufficient for all the analyses to be done. The sjTREC/TBP amplification specifically yielded 134/112 bp of good quality for all samples tested (Fig. 1). No sizing discrepancy was found between the PCR product and the expected result from direct sequencing analysis. Negative controls, water and common animal DNA (dog, cow, and mouse DNA), were not detected with this primer pair (data not shown).

Linearity, amplification efficiency, and sensitivity of the RQ-PCR assay

Serial tenfold dilution of plasmid constructs encoding the sjTREC sequence or TBP were used as standards and amplified in triplicate RQ-PCR reaction. Figure 2a and b present the standard curves plotting copy numbers of plasmid versus the logged *C*t value. A linear range over six orders of magnitude was observed for both the sjTREC and TBP amplicons. The slope of the standard curve indicates the efficiency of the PCR reaction. The optimal efficiency of 100% corresponds to a slope of −3.32, but in practice, efficiencies between 90% and 110% are considered acceptable. Calculation of the efficiency for each one of the amplification reactions resulted in *E*sjTREC=92% and *E*TBP=98%. Therefore, amplification of the sjTREC and TBP were employed for further analysis. Moreover, the lowest amount of sjTREC and TBP that could be reliably quantified both corresponded to ten copies per reaction.

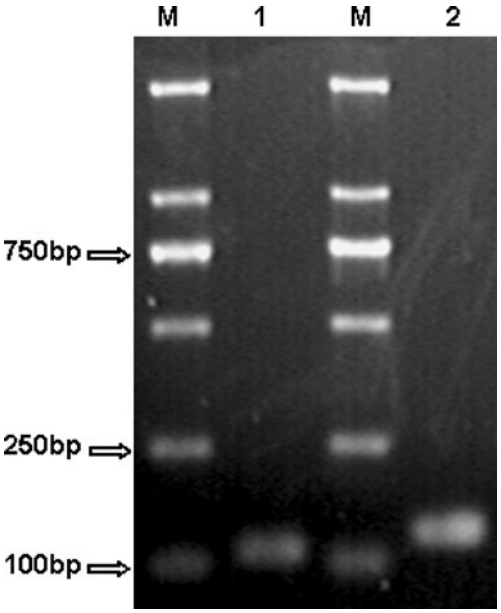

Fig. 1 Specificity of the primer pairs. PCR products with primer pairs using 200 ng DNA from human PBLs were as follows: *M* DL2000 marker (Takara), *1* amplified with TBP primers, *2* amplified with sjTREC primers

sjTREC/TBP quantification in collected whole blood samples from 248 healthy subjects

The sjTREC/TBP quantification was successfully performed in all blood samples tested by RQ-PCR analysis. An example of amplification curves obtained for DNA from blood samples of 2.5-, 31-, and 78-year-old individuals was illustrated in Fig. 2c and d, and the quantification results of these three samples were listed in Table S2. The level of sjTREC/TBP was detected as low as 0.2 μg of genomic DNA with the Gene Quant pro RNA/DNA calculator (Amersham Pharmacia, USA).

The sjTREC levels (log sjTREC/TBP) were assessed in a cohort of 248 healthy subjects ranging from new

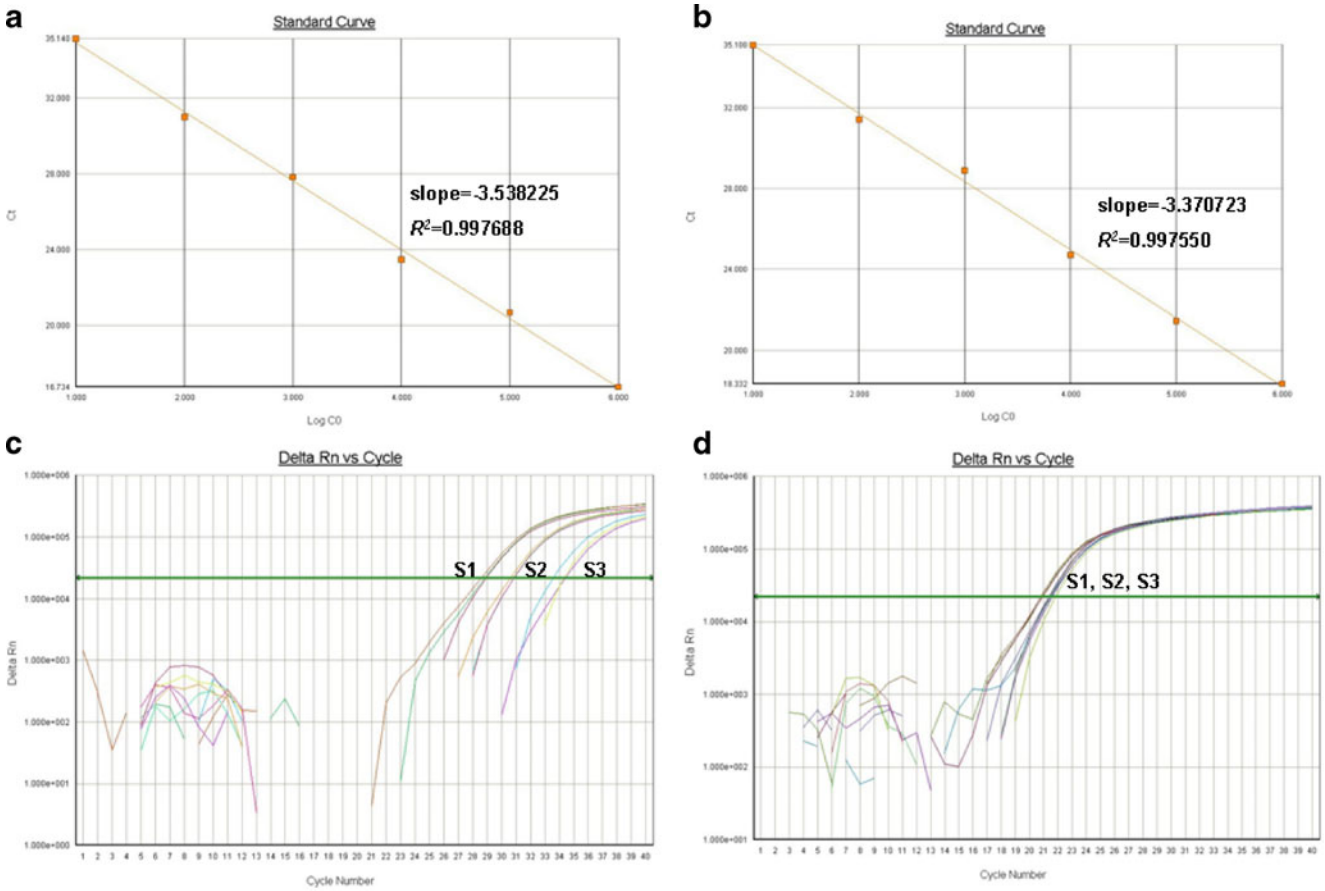

Fig. 2 Standard curves of sjTREC (**a**) and TBP (**b**) containing standard plasmids diluted over the range 10^6–10^1 copies, and the example of sjTREC (**c**) and TBP (**d**) amplification curves obtained for blood samples from 2.5 (*S1*)-, 31 (*S2*)-, and 78 (*S3*)-year-old individuals

born (cord blood) to 78 years old. As shown in Table S3, the numbers of sjTRECs remained at a high level in PBLs for the first 1–14 year, with a relatively constant decline rate. Between 10–14 and 15–19 age group, a sharp drop was observed, resulting from the progressive enrichment of the thymic stroma in adipose cells (thymic adipose involution), followed by a gradual decline thereafter. However, another remarkable decrease in sjTREC levels could be seen between 50–54 and 55–59 age group. Over a life span, a total decline of ~2 logs was found, as Zhang et al. [8] had reported. It should be noted that even people over the age of 70 had significant numbers of sjTREC in their PBLs.

Since apparent decline in sjTREC levels were seen around the age of 14 and 54, respectively, the samples were therefore divided into three main groups: minors (1–14 years, n=63), adults (15–54 years, n=167), and old (≥55 years, n=18). Among these three age groups, a highly significant difference was obtained in sjTREC levels (P<0.001; Table 1). These data indicated that there was significant alteration with age in sjTREC levels, which could serve as an index of individual identification in different age classes.

Regression analysis between sjTREC levels and donor age

A straight line was obtained by regression analysis between the sjTREC levels and age, the correlation coefficient being r=−0.8177 (Fig. 3). The detected sjTREC level of each sample could be included in the following formula to roughly calculate the age of the subject: Y=−24.921 x−39.932±10.47 (Y age, year; X log sjTREC/TBP; 10.47 standard error). In addition, estimated ages were within 10 years of actual ages in 65.3% of all subjects.

Comparisons of male and female sjTREC levels

To find out whether there is gender-specific change with regard to sjTREC level in human peripheral blood, we analyzed the sjTREC levels in male and female samples. In all 5-year groups, no differences were found between males and females (data not showed).

Table 1 sjTREC levels (log sjTREC/TBP) for three age groups (0–14, 15–54, and ≥55 years)

Group	Age (year)	*n*	M±SE	One-way ANOVA		Post hoc multiple comparisons
				F value	*P* value	*P* value
Group 1	0–14	63	−2.12±0.31	192.608	0.000<0.001	0.000<0.001 (vs. group 2)
						0.000<0.001 (vs. group 3)
Group 2	15–54	167	−3.02±0.39			0.000<0.001 (vs. group 1)
						0.000<0.001 (vs. group 3)
Group 3	55	18	−3.76±0.37			0.000<0.001 (vs. group 1)
						0.000<0.001 (vs. group 2)

Discussion

TREC measurements have been presented in a number of different ways. For example, a TREC standard enables the reporting of the absolute number of TREC molecules per unit number of cells or TREC molecules per microgram of DNA within PBMC or T lymphocytes [8, 13, 14]. In an alternative approach, TREC DNA has been reported to be relative to genomic DNA within a particular cellular subset (PBMC, T lymphocytes, naïve T lymphocytes) [15] and yet others have sorted individual T cell subsets and measured TREC within that subset [16] Un-unified methods and units used to measure sjTRECs affect the interpretation and comparison of sjTREC data. In 2008, Lorenzi et al. [17] described and evaluated a method which directly quantified sjTREC from PBLs in whole blood, removing the necessity for separating PBMC and other T cell subsets. Moreover, it requires small quantities of whole blood (0.2 μg DNA template or less). The assay is validated and has been tested in a large number of healthy control children and adults.

In the present study, we adopted the modified sjTREC assay reported by Lorenzi et al. [17] to quantify sjTREC in PBLs samples from Chinese population, with TBP used as internal reference gene. The quantification was based on the amplification of a plasmid reference standard containing sjTREC or TBP template. For each sample, a precise number of sjTREC and TBP copies can be deduced. Through this methodology, sjTREC levels (log sjTREC/TBP) were determined in 248 subjects ranging from 0 to 78 years old. As shown in Fig. 3, we demonstrated that sjTREC level declined in an age-dependent manner in peripheral blood, with a correlation coeficient of $r=-0.8177$, which is slightly lower than the $r=-0.86$ reported recently by Lorenzi et al. [17]. Moreover, alteration of sjTREC levels among three age groups (0–14, 15–55, and >55 years) was highly significant. These findings suggested that the alteration of sjTREC levels in peripheral blood is a dynamic process and can be an index of individual identification of different age. In addition, a correlation with gender was not found in our study, although such a correlation was previously reported [9].

Several studies were devoted to assess the accuracy of age estimation using the morphologic methods and proposed some applicable techniques. However, in the forensic practice, there is often no morphologic information. As a pilot study, it underlines the significance of peripheral sjTREC level as an age indicator and the standard error (SE) of estimation was found to be 10.47 years, which was commensurate with that of TRF length (SE=9.832 years) reported by Ren et al. [1]. The variation is gross. One possible explanation for this is that sample size in the present study might be too small. Another is that sjTREC in

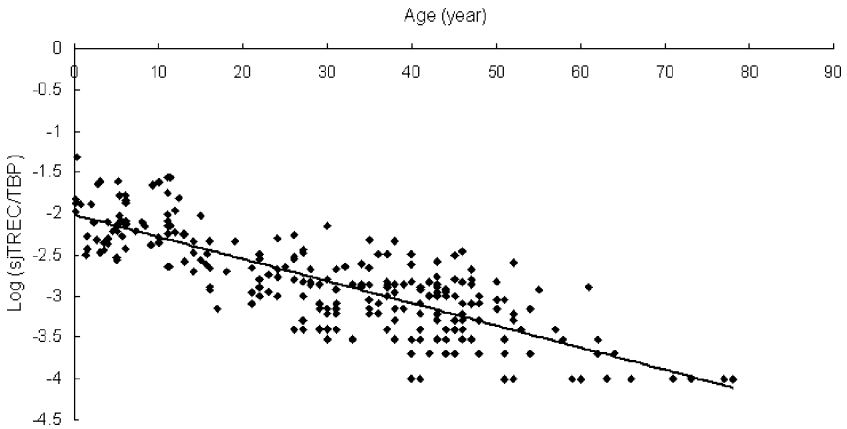

Fig. 3 sjTREC levels (Log sjTREC/TBP) in 248 donors (aged 0–78 years old) declined with age. The *straight line* was obtained by regression analysis, the correlation coefficient being $r=-0.8177$ ($R^2=0.6686$)

peripheral blood might vary widely depending on genetic and environmental factors, which needs further investigations. Therefore, our method can give a rough estimation of age of a subject in forensic examination, and requires improvements by combining with other morphologically independent age indicators' measurements, such as TRF length and mtDNA 4,977 bp fragment deletion.

In summary, although a practical estimation of age depends on many factors such as environmental and genetic factors, the present study provide a novel additional solution to the problem of age estimation based on age-dependent change in peripheral sjTREC level. However, before the application of this method in forensic practice, it still requires large-scale investigation and statistical analysis of population data.

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