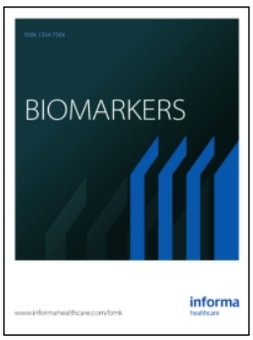

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ORIGINAL ARTICLE

Telomere length and signal joint T-cell receptor rearrangement excision circles as biomarkers for chronological age estimation

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ABSTRACT

Background: Chronological age estimation is a challenging marker in the field of forensic medicine. The current study aimed to investigate the accuracy of signal joint T-cell receptor rearrangement excision circles (sjTRECs) quantification and telomere length measurement as methods for estimating chronological age.

Methods: Telomere length was estimated in the DNA derived from the buccal cells through estimating the telomeric restriction fragment (TRF) length using Telo*TTAGGG* Telomere Length Assay while the sjTRECs quantification was carried out on DNA isolated from the blood samples using qPCR.

Results: The TRF length was shortened with increased age ($r = -0.722$, $p < 0.001$). The sjTRECs were also decreased with increased age ($r = -0.831$, $p < 0.001$). Stronger coefficient and lower standard error of the estimate was obtained when multiple regression analysis for age prediction based on the values of both methods was applied ($r = -0.876$, $p < 0.001$).

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KEYWORDS
Chronological age; telomere length; sjTRECs; quantitative real-time PCR; southern blotting

Introduction

Ageing is a natural and gradual process in human life. The chronological age, the time elapsed since birth, is different from the biological age which refers to how old a person seems influenced by genetic and environmental factors such as diseases, lifestyle and social factors independent of the passage of time alone (Freire-Aradas *et al.* 2017, Jung *et al.* 2017). Age discrimination of anonym human bodies is an important issue in the field of forensic medicine. It can provide valuable information to the medico-legal interrogator in crime inquiry as well as utility in mass catastrophe situations where age may be difficult to estimate (Ren *et al.* 2009, Alkass *et al.* 2010).

There are various methods used in the prediction of human age including analysis of bones and teeth morphological features (Meissner and Ritz-Timme 2010, Machado *et al.* 2018), chemical methods (Alkass *et al.* 2010) and molecular genetics methods (Ou *et al.* 2011, Cho *et al.* 2014, Zubakov *et al.* 2016, Márquez-Ruiz *et al.* 2018). As estimation of the chronological age of biological materials, such as bloodstains, is a pivotal important point in forensic investigations (Meissner and Ritz-Timme 2010, Machado *et al.* 2018); procedures based on forensic genetic analysis are expected to provide some advantageous information than conventional methods of age estimation which do not provide adequate morphologic or biochemical information (Ou *et al.* 2012). New DNA tests have been developed which can deduce individual age from a biological trace (Freire-Aradas *et al.* 2017).

Noteworthy, to date, the forensic use of assorted DNA analysis such as mtDNA (Elmadawy *et al.* 2013), autosomal STR (Omran *et al.* 2009), Y-STR analysis (Nagai *et al.* 2015), telomere shortening (Meissner and Ritz-Timme 2010, Ou *et al.* 2012, Alhusseini and Madboly 2016), signal-joint T-cell receptor excision circles (sjTRECs) (Yamanoi *et al.* 2018) and DNA methylation (Rom *et al.* 2017) for age prediction have been achieved (Naue *et al.* 2017).

Human telomeres contain tandem repeats of a hexameric sequence, TTAGGG, which forms a protective cap at the end of the chromosomes. The length of this repeated tandem sequence is shortened with each cell replication cycle. Accordingly, the expression of the telomerase enzyme is induced to add repeat sequences to the end of the telomeres to overcome this problem. However, the effectiveness of this process is lost with the advancement of time. Once telomere length reaches a critical limit, cell senescence occurs as a result of the arrest of cellular division (Freire-Aradas *et al.* 2017). Telomere length can therefore be used as a potential marker for age prediction (Tsuji *et al.* 2002, Ren *et al.* 2009, Srettabunjong *et al.* 2014). However,

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telomere length is affected by disease states, stress and environmental conditions (Surtees *et al.* 2011).

Signal joint T-cell receptor rearrangement excision circles (sjTRECs) are small circular extrachromosomal DNA by-products found in the T-lymphocyte as a result of the rearrangements of the T-cell receptors gene segments encoding the variable parts of T cell receptor (TCR) α and β chains during the intrathymic development (Kong *et al.* 1999, Lorenzi *et al.* 2008, Freire-Aradas *et al.* 2017). The sjTRECs are linearly declined in human peripheral blood with increasing age (Geenen *et al.* 2003, Lorenzi *et al.* 2008, Zubakov *et al.* 2010). So, molecular analysis of sjTRECsis one of the innovative tools for human age determination from biological samples and deemed as a promising tool for forensic implementation (Lorenzi *et al.* 2008, Ou *et al.* 2012). The sjTRECs estimation can be influenced by immune system disturbance (Zubakov *et al.* 2010).

Developments in the field of forensic genetics in Egypt have grown rapidly. Until recently, the data about age prediction in Egyptians using molecular genetics methods was limited. Therefore, Egyptian forensic DNA data advancement is essentially recommended. The current study aims to assess the use of sjTRECs and telomeric restriction fragments (TRF) length polymorphism from biological materials as biomarkers for chronological age estimation in Egyptians.

Clinical significance

- Chronological age prediction based on biological material is a powerful tool in forensic investigations.
- This study confirms that TRF length estimation & sjTRECs quantification can be used as biomarkers for age estimation.
- This study suggests sjTRECs quantification to be more preferable than TRF length estimation in fields where age prediction is required.

Materials and methods

Samples collection and DNA extraction

A total of 124 unrelated healthy Egyptian volunteers of both sex (68 men and 56 women) were included in this study. Age ranged from a few days up to78-years-old. Inclusion criteria were: Egyptian, healthy donors and unrelated (to exclude the effects of ethnicity and inheritance on telomere length). Exclusion criteria were: Individuals with a history of chronic diseases, active smokers and obese. Written informed consents were obtained from patients. This study was approved by the Benha University Research Ethics Committee & Ethical Committee, Graduate School of Medicine, Gifu University. This study adhered to the tenants of the Declaration of Helsinki.

Whole blood samples as well as buccal swabs (2 swabs from each volunteer) were collected from all subjects. Blood samples were collected into EDTA tubes and buccal swabs were collected into clean sterile 15 ml falcon tubes. Genomic DNA was isolated from the collected blood and buccal swab samples using the Qiagen DNA isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was then quantified and evaluated using a Nanodrop spectrophotometer and gel electrophoresis. The extracted DNA was stored at −80 °C.

Telomere length estimation

Telomeric restriction fragment (TRF) length was analyzed by Southern blotting using the Telo*TTAGGG* Telomere Length Assay (Roche Diagnosis GmbH, Germany) Figure 1. The isolated DNA from buccal swabs of each volunteer was concentrated by ethanol precipitation method and resolved in 17 μL of milliQ water to obtain a concentration of 1–2 μg. DNA was then digested with *HinfI* and *RsaI* restriction enzymes for 2 h at 37 °C. The reaction was stopped by adding 5 μL of gel electrophoresis loading buffer (5×) and the reaction vials were quickly spun down. The DNA fragments were separated using a 0.8% agarose gel electrophoresis. Running on the gel was at 5 V/cm in 1× TAE buffer until the bromophenol blue tracking dye is separated about 10 cm from the starting wells (total run time ∼3.5 h). Separated DNA fragments were then transferred into a nylon membrane by Southern blotting and hybridized to a digoxigenin (DIG)-labelled probe specific for telomeric repeats. The membrane was further washed twice and then incubated in the detection buffer. Finally, the hybridized telomeric fragments were visualized by a highly sensitive chemiluminescence substrate. The membrane was exposed to LI-COR C-DiGit Blot Scanner and the membrane image was managed by Image Studio Software Version 3.1 for the C-DiGit® Blot Scanner. Mean TRF length has been defined according to the following formula: $\mathrm{TRF} = \frac{\sum(\mathrm{OD}i)}{\sum(\mathrm{OD}i/Li)}$ Where OD_i is the chemiluminescent signal and L_i is the length of the TRF at position *i*. This analysis was achieved using the Telomeric 1.2 software (Available at http://bioinformatics.fccc.edu). Linear regression between mean TRF lengths and ages was carried out using the Graphpad Prism 6 software (GraphPrism Software, La Jolla, California, USA).

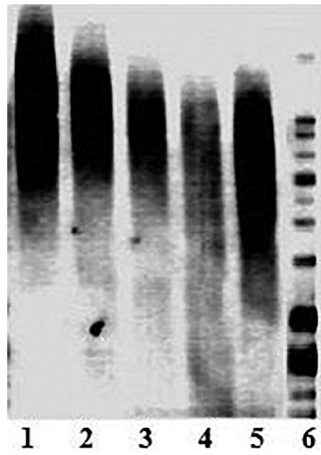

Figure 1. Example of scanned blotting of TRF in Egyptians. Lane1: control; lanes 2–5 are samples with mean TRF 11.08, 8.6, 5.96 and 6.7 Kb and lane 6 is a molecular weight marker ranging from 1.1:21.2 Kb.

Taq man qPCR assay for sjTRECs quantification

For sjTRECs amplification and quantification, real-time PCR, using the specific primers and probes was done in ABI7900 qPCR system (Applied Biosystem, USA) using TaqMan® Universal Master Mix II (Applied Biosystems, USA) according to manufacturer's instructions. The amplification profile was as follows: initial denaturation at 95 °C for 10 min followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. Taqman TM Hydrolysis technology was used in a 20 μL reaction mixture containing 250 nM of each primer set, 250 nM, Taqman hydrolysis probe labelled with carboxyfluorescein (FAM) at the 5′ end and with N,N,N,N-tetramethylrhodamine (TAMRA) at the 3′ end. The final reaction volume was 20 μl containing 9 μl of extracted DNA, 1 ml Taqman® assay (20x) and 10 μL TaqMan® Universal Master Mix II (2x).

The sequence of primers and probe for sjTRECs (ACCESSIONAE000521) were:

5′-CCATGCTGACACCTCTGGTT-3′ (P1: forward primer),
5′-TCGTGAGAACGGTGAATGAAG-3′ (P2: reverse primer) and
5′-FAM-CACGGTGATGCATAGGCACCTGC-TAMRA-3′ (Taqman probe) (Hazenberg *et al.* 2001). Primers and probe for the internal reference gene (human TATA box binding protein (TBP); accession NG00816) were 5′-TTAGCTGGCTCTGAGT ATGAATAAC-3′ (forward primer),
5′-AACCAATAAAACCTACTCC TCCCTTAA-3′ (reverse primer) and 5′-FAM-CAGTCCAGACTG GCAGCAAGAAAAT-TAMRA-3′ (Taqman probe). Fluorescence of FAM liberated from the probe by TaqMan was measured to determine the amplification threshold cycle (Ct). The normalized sjTRECs amount in each sample was calculated as a difference between Ct values of TBP and sjTRECs assays (dCt) (Zubakov *et al.* 2010). Linear regression analysis was performed using GraphPad Prism 6 software (GraphPrism Software, La Jolla, California, USA) to determine the association between dCt and donor age.

Statistical Analysis

The collected data were organized and tabulated. SPSS version 20 software (SPSS Inc, ILL Company, USA) was used for the univariate, bivariate and stratified analyses of the data. Correlations among variables were studied by using the Pearson coefficient and Multivariate linear regression models. Comparison of the correlation coefficient of the two variables was done using the transformation Z test. Differences were considered significant at $p \leq 0.05$.

Results

In this study, the mean TRF length obtained from buccal cells showed an inclination to become shorter with age. The correlation between average TRF and age of the individuals included was obtained as a straight line by regression analysis with correlation coefficient ($r = -0.722$, $R^2 = 0.522$), ($p < 0.001$). The regression curve can be used roughly to calculate the age of an unknown individual. The TRF length would be significantly used for the determination of unknown age via the equation: Age in year $= -8.210X + 76.05 \pm 15.7$ where X is the telomere length and ±15.7 is the standard error in years. In order to evaluate the precision of these results, the actual ages of the subjects were compared with the estimated ages and the mean prediction error (ME) was 12.5 years.

For further clarification of the changes in telomere length with age, included individuals were divided into seven different age groups for each decade of life. Group 1(18 individuals; few days to 10 years), group 2 (16 individuals; 11–20 years), group 3 (16 individuals; 21–30 years), group 4 (18 individuals; 31–40 years), group 5 (16 individuals; 41–50 years), group 6 (18 individuals; 51–60 years) and group 7 (22 individuals over 60 years). The results showed also a highly significant ($p < 0.001$) decrease in mean TRF length with increased age as illustrated in Figure 2.

Regarding sjTRECs amplification and quantification done for the collected blood samples, our results revealed that the amount of sjTRECs was reduced as the donor age increased. The amount of sjTRECs in each sample was normalized and calculated as a difference between Ct values of TBP normalizer, which was stable in all ages and sjTRECs (dCt). We applied the linear regression between donor age and the normalized sjTRECs ($dCt_{TBP} - dCt_{sjTRECs}$) which illustrated a significant decrease in the level of sjTRECs with increasing age of the donor ($r = -0.831$, $R^2 = 0.690$) ($p < 0.001$). The unknown donor age can be predicted from the formula: Age $= -4.994X - 8.715 \pm 12.6$ where X is the dCt. The standard error of the estimate was ±12.6 years and the mean prediction error (ME) was 9.9 years. The mean dCt ($dCt_{TBP} - dCt_{sjTRECs}$) when the donor age was divided into age groups is shown in Figure 3.

Comparing the correlation coefficients of both methods using the fisher`s Z transformation test revealed no significant difference between the two coefficients ($p = 0.135$).

Multiple regression analysis for age prediction based on the values of both methods was applied with correlation coefficient ($r = -0.876$, $R^2 = 0.768$) ($p < 0.001$). Age prediction can be applied via the equation: Age = (−3.966 TRF) + (−3.73 dCT) + 22.103 ± 11.03 with stronger coefficient, lower standard error of the estimate (±11.03) and lower mean prediction error (8.4 years). Statistical analysis of the correlation coefficient revealed: (1) the relation between TRF and age ($r = -0.722$) and multiple regression analysis ($r = -0.876$) revealed a significant difference between the two coefficients ($p = 0.017$), (2) the relation between the amount of sjTRECs and age ($r = -0.831$) and multiple regression analysis ($r = -0.876$) revealed no significant difference between the two coefficients ($p = 0.372$).

Discussion

Enforcement of gene expression and DNA profiling are novel techniques in the scope of age identification in forensic medicine especially the trace analysis of biological samples of low quality and quantity (Tsuji *et al.* 2002, Hewakapuge *et al.* 2008, Alhusseini and Madboly 2016, Zubakov *et al.* 2016). In this study, we elucidated the significance of two

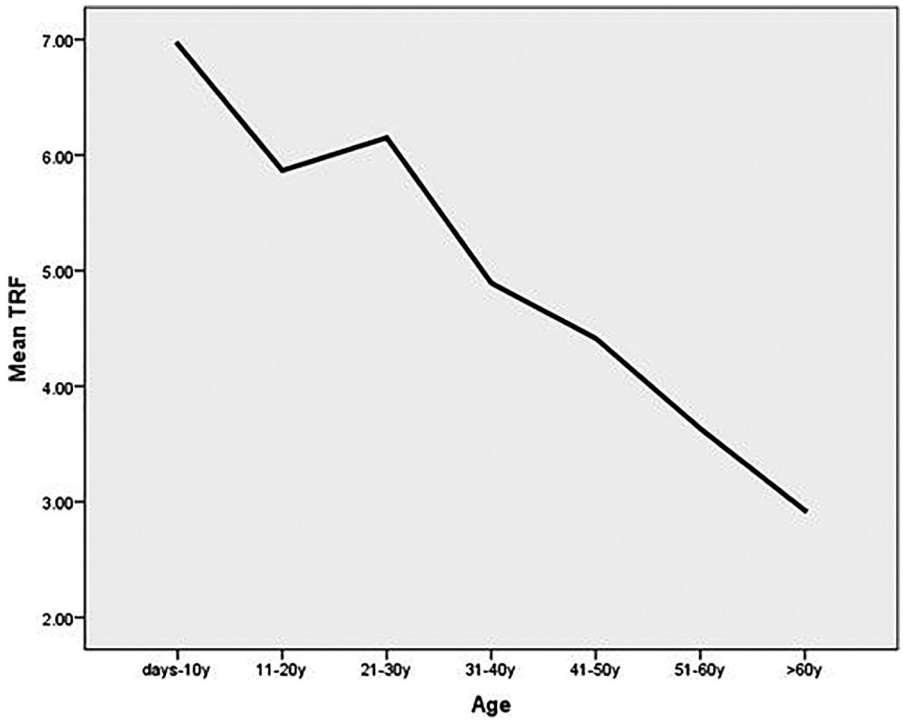

Figure 2. Mean TRF length in the age groups.

different biomarkers (sjTRECs and telomere length) from various biological samples (buccal swabs and peripheral blood samples) collected from Egyptian healthy individuals from a wide age span.

Telomere shortening with advancing age can be used as evidence for age prediction of unknown individuals (Hewakapuge *et al.* 2008, Karlsson *et al.* 2008). In line with this assertion, the mean TRF length was measured to give the average correlation between the different telomere length of each chromosome and the age of the donors. The present study has proved the hypothesis of attrition of telomere length with age as mean TL significantly shortened with aging ($r = -0.722$), ($p < 0.001$) and also showed a highly significant ($p < 0.001$) decrease in mean TL among the different age groups. These findings support the data obtained by previous reports done on age prediction in Japanese (Tsuji *et al.* 2002) and Tibetan Chinese (Ren *et al.* 2009) populations from blood samples. Using the Southern blotting technique as a method for telomere length estimation, they reported telomere length shortening with advanced age. TRF was used as a method for age estimation using the equations: Age $= 0.0095X + 148.9 \pm 7.037$ with correlation coefficient $r = -0.8320$ ($R^2 = 0.6922$) and age $= -16.539X + 236.287 \pm 9.832$ with correlation coefficient $r = -0.913$ and p-value ($p < 0.001$), respectively. Takasaki *et al.*, using dental pulp DNA from Japanese subjects, concluded that TRF would be used for age estimation with the equation age $= -0.0119X + 168.0 \pm 7.52$ (Takasaki *et al.* 2003).

Quantitative PCR is another reliable technique used for the determination of relative telomere length; it was firstly described by Cawthon (Cawthon 2002) and has been used in studies in Sweden ($r = -0.233$, $p < 0.01$) (Nordfjall *et al.* 2005) and the Netherlands ($r = -0.987$, $p < 0.01$) (Zubakov *et al.* 2010). Moreover, it has been carried out to study the age estimation in Egyptians by Alhusseini and Madboly (2016). Their results showed that the relative telomere length was significantly decreased with advancing age with a correlation coefficient $r = -0.903$ ($p < 0.001$).

Conversely, studies done by Karlsson *et al.* (2008), Hewakapuge *et al.* (2008) and Zubakov *et al.* (2016) observed a low correlation of age with telomere repeat length as R^2 value was significantly low ($R^2 = 0.09$, 0.037 and 0.14, respectively). They suggested that the use of telomere attrition as a method for age prediction is not suitable for application in forensic cases as there are wide variations between individuals which limited thoroughness (Karlsson *et al.* 2008, Meissner and Ritz-Timme 2010, Zubakov *et al.* 2016). The variation of r values attributed to the difference between each study regarding hereditary telomere length, the health of the individual, size and type of sample, laboratory conditions, oxidative distress and antioxidant activity in human cells (Hewakapuge *et al.* 2008, Karlsson *et al.* 2008).

According to this study, the mean TRF length in Egyptians is generally shorter than the other populations (Tsuji *et al.* 2002, Ren *et al.* 2009). This may be attributed to the difference in ethnicity, environmental conditions as UV exposure, lifestyle, smoking, oxidative distress and antioxidant activity in human cells (Hunt *et al.* 2008, Karlsson *et al.* 2008, Ren *et al.* 2009, Surtees *et al.* 2011).

During T cell receptors (TCRs) gene rearrangement, sjTRECs are formed as consequences of split out of unutilised part in a ring pattern. They do not replicate during cell divisions and are highly accepted as a marker for thymic function (Kong *et al.* 1999, Cho *et al.* 2014). It is well known that

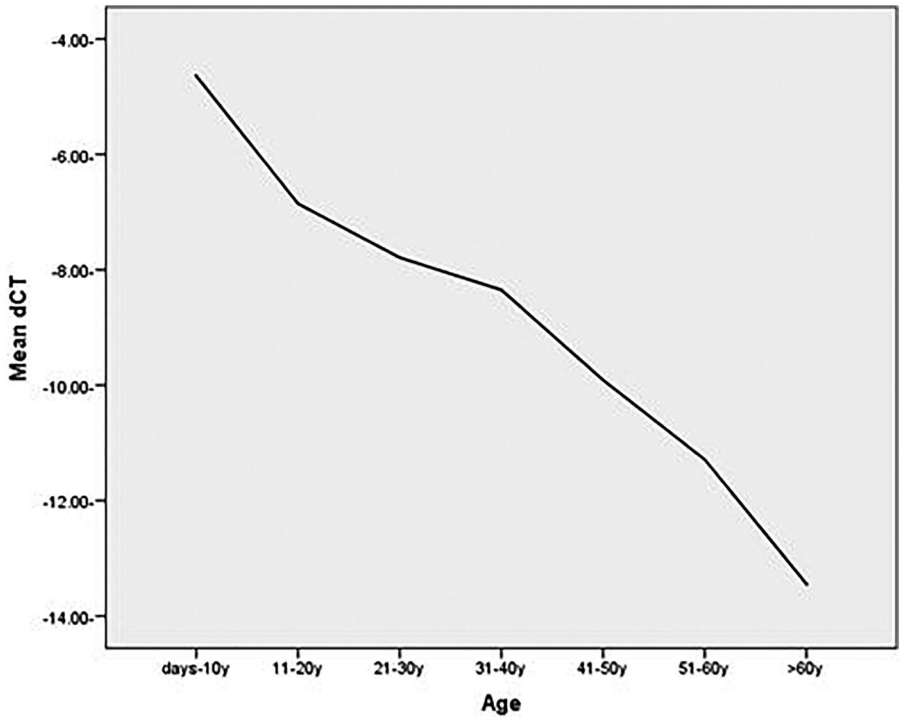

Figure 3. Mean dCt ($dCt_{TBP} - dCt_{sjTRECs}$) in the age groups.

the thymus, as a part of the immune system, deteriorates with age progression and expectedly declines sjTRECs in peripheral blood (Pido-Lopez *et al.* 2001, Cho *et al.* 2014). In this framework, the correlation between the donor age and sjTRECs obtained in our study, with $R^2 = 0.690$, was in the range of correlation reported in the previously published studies diversified from $R^2 = 0.667$ (Ou *et al.* 2011) to 0.834 (Zubakov *et al.* 2010). Previous studies found that sjTRECs levels declined in an age-dependent manner in blood samples in Chinese ($R^2 = 0.759$) (Ou *et al.* 2012), Korean ($R^2 = 0.648$) (Cho *et al.* 2014), Dutch ($R^2 = 0.835$) (Zubakov *et al.* 2010) and lower correlation in the Netherlands ($R^2 = 0.546$) (Zubakov *et al.* 2016).

In accordance with our data, there was no significant difference between the coefficients of both methods ($p = 0.135$)suggesting that both methods can be used significantly as biomarkers for chronological age estimation. Remarkably, multiple regression analysis for age prediction based on the values of both methods showed a stronger coefficient ($r = -0.876$, $R^2 = 0.768$) ($p < 0.001$) and lower standard error of the estimate (±11.03).

Results brought about from our study also showed a significant difference ($p = 0.017$) between the correlation coefficient of the relation between TRF and age ($r = -0.722$), and the correlation coefficient of the multiple regression analysis ($r = -0.876$). No significant difference ($p = 0.372$) was observed between the correlation coefficient of the relation between the amount of sjTRECs and age ($r = -0.831$) and the correlation coefficient of the multiple regression analysis ($r = -0.876$). Overall, the data we present here provide enhanced evidence that human age estimation from blood using sjTRECs quantification is feasible at reasonably high accuracy and superior to Telomere length estimation.

Conclusion

Based on the accuracy of estimated age, amount of DNA used, simplicity of the method, and time consumed, this study suggests sjTRECs quantification using real-time PCR to be more preferable than Telomere length estimation using Southern blotting in the estimation of age. However, a wide-range anthropological examination is recommended to be legitimized before the establishment of these methods officially in forensic practice.

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Ethical approval

This study was approved by the Benha University Research Ethics Committee & Ethical Committee, Graduate School of Medicine, Gifu University. The study adhered to the tenants of the declaration of Helsinki. The samples used in this assay were obtained from Egyptian volunteers after written consents were obtained from them, or their legally authorized representative.

Disclosure statement

The authors declare that they have no conflict of interest. The authors confirm that this manuscript has not been published elsewhere and is not under consideration by any other journal. All authors have approved the manuscript.

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Data availability statement

All relevant raw data will be freely available to any scientist wishing to use them for non-commercial purposes without breaching participant confidentiality.

References

Alhusseini, N.F. and Madboly, A.G., 2016. Telomere length measurement by quantitative real-time PCR: a molecular marker for human age prediction. *American journal of biochemistry and biotechnology*, 12 (1), 64–71.

Alkass, K., *et al.*, 2010. Age estimation in forensic sciences: application of combined aspartic acid racemization and radiocarbon analysis. *Molecular & cellular proteomics*, 9 (5), 1022–1030.

Cawthon, R.M., 2002. Telomere measurement by quantitative PCR. *Nucleic acids research*, 30 (10), e47.

Cho, S., *et al.*, 2014. Age estimation via quantification of signal-joint T cell receptor excision circles in Koreans. *Legal medicine*, 16 (3), 135–138.

Elmadawy, M.A., *et al.*, 2013. Investigation of mtDNA control region sequences in an Egyptian population sample. *Legal medicine*, 15 (6), 338–341.

Freire-Aradas, A., Phillips, C., and Lareu, M.V., 2017. Forensic individual age estimation with DNA: from initial approaches to methylation tests. *Forensic science review*, 29 (2), 121–144.

Geenen, V., *et al.*, 2003. Quantification of T cell receptor rearrangement excision circles to estimate thymic function: an important new tool for endocrine-immune physiology. *Journal of Endocrinology*, 176, 305–311.

Hazenberg, M.D., *et al.*, 2001. T cell receptor excision circles as markers for recent thymic emigrants: basic aspects, technical approach, and guidelines for interpretation. *Journal of molecular medicine*, 79 (11), 631–640.

Hewakapuge, S., *et al.*, 2008. Investigation of telomere lengths measurement by quantitative real-time PCR to predict age. *Legal medicine*, 10 (5), 236–242.

Hunt, S.C., *et al.*, 2008. Leukocyte telomeres are longer in African Americans than in whites: the National Heart, Lung, and Blood Institute Family Heart Study and the Bogalusa Heart Study. *Aging cell*, 7 (4), 451–458.

Jung, S., Shin, K., and Lee, H.Y., 2017. DNA methylation-based age prediction from various tissues and body fluids. *BMB reports*, 50 (11), 546–553.

Karlsson, A.O., *et al.*, 2008. Estimating human age in forensic samples by analysis of telomere repeats. *Forensic science international: genetics supplement series*, 1 (1), 569–571.

Kong, F.K., *et al.*, 1999. T cell receptor gene deletion circles identify recent thymic emigrants in the peripheral T cell pool. *Proceedings of the National Academy of Sciences of the United States of America*, 96 (4), 1536–1540.

Lorenzi, A.R., *et al.*, 2008. Determination of thymic function directly from peripheral blood: a validated modification to an established method. *Journal of immunological methods*, 339 (2), 185–194.

Machado, M.A., *et al.*, 2018. Effectiveness of three age estimation methods based on dental and skeletal development in a sample of young Brazilians. *Archives of oral biology*, 85, 166–171.

Márquez-Ruiz, A.B., González-Herrera, L., and Valenzuela, A., 2018. Usefulness of telomere length in DNA from human teeth for age estimation. *International journal of legal medicine*, 132 (2), 353–359.

Meissner, C. and Ritz-Timme, S., 2010. Molecular pathology and age estimation. *Forensic science international*, 203 (1–3), 34–43.

Nagai, A., *et al.*, 2015. Genetic polymorphisms at 17 Y-STR loci in an Egyptian population. *Forensic science international: Genetics supplement series*, 5, e436–e437.

Naue, J., *et al.*, 2017. Chronological age prediction based on DNA methylation: massive parallel sequencing and random forest regression. *Forensic science international: genetics*, 31, 19–28.

Nordfjall, K., *et al.*, 2005. Telomere length and heredity: indications of paternal inheritance. *Proceedings of the national academy of sciences*, 102 (45), 16374–16378.

Omran, G.A., Rutty, G.N., and Jobling, M.A., 2009. Genetic variation of 15 autosomal STR loci in Upper (Southern) Egyptians. *Forensic science international. Genetics*, 3 (2), e39–e44.

Ou, X., *et al.*, 2011. Detection and quantification of the age-related sjTREC decline in human peripheral blood. *International journal of legal medicine*, 125 (4), 603–608.

Ou, X.L., *et al.*, 2012. Predicting human age with bloodstains by sjTREC quantification. *PLoS one*, 7 (8), e42412.

Pido-Lopez, J., Imami, N., and Aspinall, R., 2001. Both age and gender affect thymic output: more recent thymic migrants in females than males as they age. *Clinical and experimental immunology*, 125 (3), 409–413.

Ren, F., *et al.*, 2009. Estimation of human age according to telomere shortening in peripheral blood leukocytes of Tibetan. *American journal of forensic medicine and pathology*, 30, 252–255.

Rom, S., *et al.*, 2017. DNA methylation-based age prediction from saliva: high age predictability by combination of 7 CpG markers. *Forensic science international: genetics*, 29, 118–125.

Srettabunjong, S., *et al.*, 2014. The study on telomere length for age estimation in a Thai population. *American journal of forensic medicine and pathology*, 35, 148–153.

Surtees, P.G., *et al.*, 2011. Life stress, emotional health, and mean telomere length in the European Prospective Investigation into Cancer (EPIC)-Norfolk population study . *Journals of gerontology, series A: biological sciences and medical sciences*, 66 (11), 1152–1162.

Takasaki, T., *et al.*, 2003. Age estimation in dental pulp DNA based on human telomere shortening. *International journal of legal medicine*, 117 (4), 232–234.

Tsuji, A., *et al.*, 2002. Estimating age of humans based on telomere shortening. *Forensic science international*, 126 (3), 197–199.

Yamanoi, E., *et al.*, 2018. sjTREC quantification using SYBR quantitative PCR for age estimation of bloodstains in a Japanese population. *Legal medicine*, 32, 71–74.

Zubakov, D., *et al.*, 2010. Estimating human age from T-cell DNA rearrangements. *Current biology*, 20 (22), R970–R971.

Zubakov, D., *et al.*, 2016. Human age estimation from blood using mRNA, DNA methylation, DNA rearrangement, and telomere length. *Forensic science international. Genetics*, 24, 33–43.