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Age estimation in Living Egyptians using signal joint t-cell receptor excision circle rearrangement

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One of the forensic investigations' goals is to estimate the age. Age estimation contributes to identification of individuals and creates a biological profile to help in identification of the missing persons in a forensic context **(1)**.

Age can be estimated very accurately in childhood using developmental methods like dental mineralization and eruption **(2)**. **El-Bakary and his colleagues (3)** stated that Egyptian children dental age estimation by Willems and Cameriere methods was significantly correlated with real age with (98%) accuracy. Dental age estimation is more accurate in childhood compared to adulthood and the accuracy of dental age estimation methods is complicated by different sample sizes, age structures, grouping, and statistical analysis (**4)**

In adulthood, there is skeletal and dental changes. The skeletal and dental changes have some relationship with age, this relationship is influenced by many endogenous and exogenous factors **(5).**

Skeletal growth characteristics such as long-bone lengths, epiphyseal fusion, and dental eruption provide a more precise and accurate indication of age with certain degree of confidence (typically 95%) **(6).**

The current age estimation methods are less accurate and require population specific references so, new methodologies are needed to avoid these problems **(1& 7).**

The new age estimation methods are based on natural process of ageing,

which leads to alterations of tissues and organs on different biochemical and molecular levels. In a biochemical approach, many studies try to relate the racemization of aspartic acid with age **(8).** In a molecular biology approach, many studies try to relate DNA's alterations with age in

different tissues e.g. the telomere shortening and signal joint T-cell receptor rearrangement excision circles (sjTRECs) decreasing with age. The most promising nontelomere based approach for predicting age is to measure sjTRECs accumulation in T cells **(9).**

The central role of the thymus in generation of T lymphocytes is well established, as well as its age-related atrophy 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 naive T lymphocyte generation **(10).**

In order to create a broad number of T-cell receptor (TCR) molecules, each immature T lymphocyte experiences unique somatic rearrangements in its TCR loci during intra-thymic development. During this rearrangement process, the intervening DNA sequences in the TCR loci are deleted (**Figure 1**) **(7)** and circularized into episomal DNA molecules, also called signal joint TCR excision circles (sjTRECs). Such by products do not replicate during cell division and so are present in higher concentrations in the most recent thymic emigrant population, after which they are diluted out by cell division **(11).**

The number of these episomal DNA excision products (sjTREC) within the peripheral T-cell pool shows an age related reduction **(12 and 13)**.

The alteration of sjTREC levels in peripheral blood is a dynamic process and can be an index of individual identification of different age **(14)**. The size of sjTREC was 140 bp. This is remarkably robust against the negative effects of DNA degradation and can be successfully applied to aged blood samples (**15 and 16)**.

The aim of the present study is to validate the usage of a sjTREC in peripheral blood leukocytes (PBLs) as a molecular marker for Egyptian person's age estimation among different age groups.

Material and Methods

Whole blood samples were obtained from 153 unrelated healthy Egyptian individuals ranging from a few weeks to70 years old. Donors were randomly chosen among individuals who came to Kasr Alaini Hospital - Cairo University for regular checkup. The research protocol was approved by the Ethical committee - Faculty of Medicine, Cairo University. All samples were collected into Vacuette EDTA tubes and stored at −80°C, with informed consent obtained from the individual or a parent or guardian.

DNA extraction

Genomic DNA was extracted from peripheral blood using the Qiagen DNA Isolation kit (Qiagen GmbH, Hilden, Germany). Samples were dissolved in RNase-

free water and quantified spectrophotometrically at 260/280nm, RNA concentrations were assessed using the OD 260/280 ratio, and DNA integrity was studied by gel electrophoresis on a 1% agarose gel, containing ethidium bromide, the isolated DNA was stored at -20oC till use.

TaqMan qPCR

Technically, the quantification of sjTRECs is straightforward: the primers and hydrolysis (TaqMan) probe target the unique joint fragment of the circular molecule. SjTREC abundance is normalized to the total DNA content in whole blood samples which is measured by qPCR TATA box binding protein (TBP) single copy gene.

TaqMan qPCR include amplification of 50ng DNA extracted from the whole in a 25µl reaction mixture containing 700nM of each primer, 150nM of hydrolysis probe and 12.5µl 2X TaqManR Universal PCR Master Mix (Applied Biosystems) using ABI Prism 7300 detector (Applied Biosystems). PCR runs started with incubation at 50oC for 2min, then at 95oC for 10min followed by 45 cycles of denaturation at 95oC for 15sec and annealing/elongation at 60oC for 30sec. The oligo sequences used in the assays were as follows:

- For sjTREC quantification according to **Hazenberg and his colleagues (17)**:

1.Forward primer 5'-CCATGCTGACACCTCTGGTT-3'.

2.Reverse primer 5'-TCGTGAGAACGGTGAATGAAG-3'.

3.Hydrolysis probe 5'-(FAM) CACGGTGATGCATAGGCACCTGC-3' (TAMRA).

- For the TBP gene amplification:

1.Forward primer 5'-TTAGCTGGCTCTGAGTATGAATAAC-3'.

2.Reverse primer 5'-AACCAATAAAACCTACTCCTCCCTTAA-3'.

3.Hydrolysis probe 5'-(FAM) GAGTCCAGACTGGCAGCAAGAAAAT-3' (TAMRA).

The normalized value of sjTREC of each sample was calculated as a difference between Ct value of TBP and sjTREC assays (dCt) **(15).** According to (R) the efficiencies (E) of the PCR reaction was calculated by using the formula E= (10(-1/s) -1 where S being the slope of the standard curve **(18)**.

Statistics

Normality of the data was examined using the Kolmogorov-Smirnov test, and homogeneity of variance using the Levene median test. Linear regression equation was obtained for estimating the age of donors by sjTREC levels in blood samples, and gender difference was assessed using the unpaired student t test, Results were expressed as minimum, maximum, and mean } standard deviation. Linear regression equation was obtained for estimating the age of donors by peripheral

sjTREC levels. All statistical analysis was performed using SPSS- 16th edition software. Results were considered significant when p=0.05.

Results

A linear range was observed for both the sjTREC and TBP amplicons, and their efficiencies were very close to each other (0.94060.021 for sjTREC and 1.03760.092 for TBP), which is a prerequisite to the use of direct dCT method for accurate normalized sjTREC quantification **(19)**.

Normalized sjTREC Quantification in blood Samples

The sjTREC quantification by qPCR analysis was successfully performed in all 153 collected blood samples. In contradistinction to the TBP normalizer, which was stable across all ages, sjTREC contents reduced as the donor age increased **(Figure 3)**.

The sjTREC levels (dCtTBP -sjTREC) were normalized in a cohort of 153 blood samples attained from healthy subjects ranging from few weeks -70 years old, 63 were males and 90 were female (**Table 1**). sjTREC contents declined progressively in these collected samples with increasing donor age through a life span (**Table 2**).

Correlation between Normalized sjTREC Quantification and Individual Age

Considering the donor age as the dependent variable and sjTREC levels (dCt values) as the predictor, we adopted linear regression to assess the correlation between sjTREC level and individual age. Linear regression equation was also

obtained for age estimation, with its accuracy evaluated using R^2. A straight line was obtained by regression analysis between individual age and dCt, R^2 being 0.870 with confident interval (95%) (**Table 2 and Figure 2**). Normalized sjTREC level of each sample could be included in the following formula to predict **scheuer** the donor age: Age= -30.671 + (-5.998Y): (Y is dCtTBP-sjTREC; standard error of the estimate ± 7.35 years).

Assessment of Gender Difference in sjTREC Quantification

Normalized sjTREC contents in male and female samples were compared to find out whether there is gender-specific change as regards sjTREC level in human blood samples. No differences were found between males and females (**Table 3**).

Discussion

Molecular human age detection is a newly emerging field with relevance to forensic implication **(20).** The individual age from birth time to testing time, as one important identification factor, can currently be estimated from DNA information **(16)**.

Unlike odontological or skeletal approaches for age estimation **(21)** or some biochemical methods **(22)**, DNA-based approach using blood sample does not require the availability of samples such as bones or teeth and requires small DNA amounts (0.2 μ g DNA template or less) **(23)** thus expands the availability of

molecular age estimation for practical applications among living child or adult persons **(15).** Moreover, the living persons will not expose to x-rays hazards.

So, molecular age estimation would be useful in aging living individual such as illegal immigrates, comatose patients after car accidents and child prostitution.

Our results showed that sjTREC levels ranged from (-17.3 to -1.9) among 153 participants, males formed 41% of them. There was no statistical significant difference in sjTREC levels between males and females. We were concordant with studies done by **Ou and his colleagues (14 and 16).** We weren't in agreement with **Zubakov and his colleagues (15) and Pido-Lopez and his colleagues (24)**, as they observed a small but statistically significant gender effect on sjTREC quantification.

There was a highly significant negative regression correlation between sjTREC levels and individual age (R^2 = 0.87, standard error of the estimate ±7.35 years). We suggested that assessment of sjTREC in peripheral blood might be a valuable tool in age estimation. Similarly, **Zubakov and his colleagues (15)** found that sjTREC levels declined in an age dependent manner in blood samples with (R^2 = 0.835, standard error of the estimate ± 8.9 years), **Ou and his colleagues (16)** found that sjTREC levels declined in an age dependent manner in blood samples with (R^2=0.759) and **Cho and his colleagues (25)** declared the linear negative

regression curve between sjTREC levels and age with (R^2 = 0.807, standard error of the estimate ± 8.49 years).

However, our results were slightly lower than a study reported by **Lorenzi and his colleagues (23).** This may be due to the difference in sample size, type and the method

employed in the study to measure the sjTREC levels and endogenous control in samples **(16).** Another possible explanation is that sjTREC in peripheral blood might vary widely depending on genetic, medical and environmental factors, which needs further investigations.

A person's bio-geographic ancestry didn't influence this approach using sjTREC levels for age estimation. This age-related DNA maker approach is suitable for age estimation from blood samples among Dutch **(15),** Chinese **(16) and** Koreans **(25).** We found that using sjTREC levels for age estimation is suitable for Egyptians too.

Unfortunately, age-estimation using sjTREC levels method is restricted to blood samples and body parts containing blood and is not possible for other body parts or fluids, such as semen or saliva, as they do not contain T cells in quantities required for sjTREC detection (**15**). Many infections and environmental stressors can affect the thymus gland causing thymic involution .

Ferrando-Martı´nez and his colleagues (26) suggested that an inhomogeneity in thymic function in elderly individuals can compromise the accuracy of using sjTREC levels for age estimation. For this reason our sample was healthy middle age Egyptian individuals. Future research into pathological conditions involving the immune system and its effect on molecular markers may allow further improvement of accuracy in individual age estimation from biological materials.

We concluded that, within the forensic context, our approach is expected to provide investigative leads in criminal cases by allowing an accurate age estimation of unknown individuals from minute blood. Furthermore, our method is relevant for several other practical applications and clinical applications where age records were lost.

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Table (1): Personal data among study groups.

Age group (year)	Male	Female	Total
0-14	18	21	39
15-29	9	21	30
30-44	12	24	36
45-59	12	18	30
60-	12	6	18
Total	63	90	153

Table (2): sjTREC levels (dCt values) for five age groups.

Age group	No	Minimum	Maximum	Mean±SE	One-way ANOVA		Confidence level (95%)	Confident interval	
0-14	39	-10.30	-1.90	-6.88±2.0	F value	P value	0.505	Lower	-11.04
15-29	30	-10.90	-7.50	-9.4100 ±0.90871	280	0.000**		Average	-10.538
30-44	36	-12.30	-9.10	-10.7917±0.84291				Upper	-10.033
45-59	30	-15.90	-10.70	-13.0800±1.45777					
> 60	18	-17.30	-12.70	-15.6050±1.44111					

** = highly significant, (No) is the number of the cases

Table (3): Comparison of sjTREC levels among male and female blood samples.

Sex	No	Mean dCt ± St deviation	T-test	P value
Female	90	-10.2732 ± 3.16673	1.242	0.216
Male	63	-10.9175 ± 3.14297		

(No) is the number of the cases

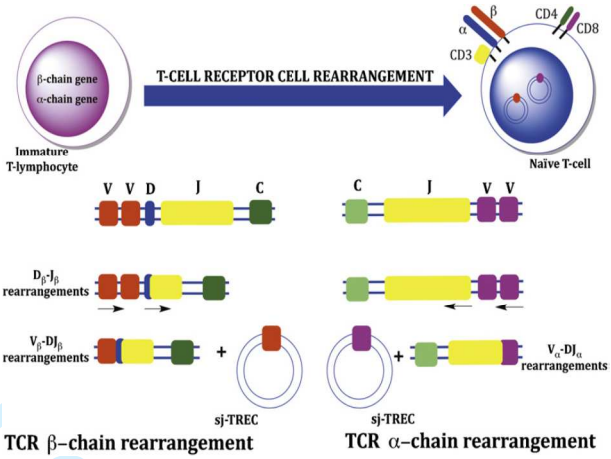

Figure (1): Rearrangement of T-cell receptor (TCR) and formation of sjTREC **(Zapico and Ubelaker, 2013).**

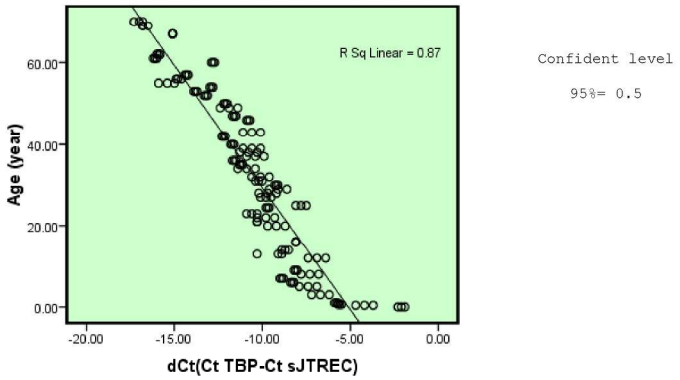

Figure (2): Correlation between SjTREC levels in 153 blood samples and individual's age (aged few weeks–70 years old).

215x279mm (300 x 300 DPI)

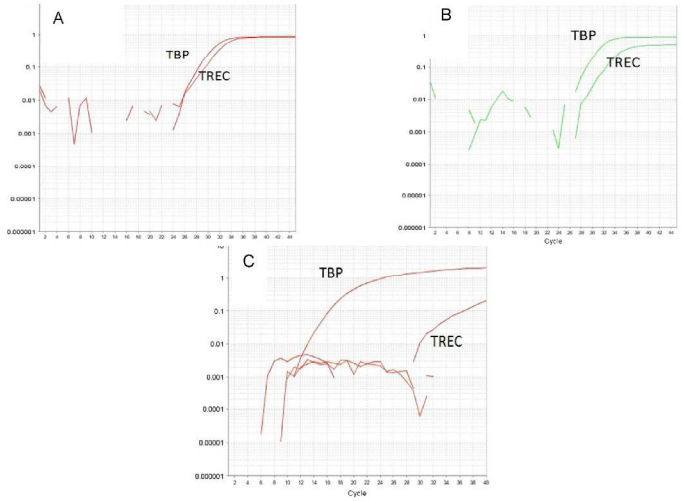

Figure (3): Typical illustrations of sjTREC and TBP amplification curves. Panel A to C was obtained for blood samples from 2-, 30- and 60- years-old individuals, respectively.

215x279mm (300 x 300 DPI)