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Title: Usefulness of telomere length in DNA from human teeth for age estimation

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Abstract Age estimation is widely used to identify individuals in forensic medicine. However, the accuracy of the most commonly used procedures is markedly reduced in adulthood, and these methods cannot be applied in practice when morphological information is limited. Molecular methods for age estimation have been extensively developed in the last few years. The fact that telomeres shorten at each round of cell division has led to the hypothesis that telomere length can be used as a tool to predict age. The present study thus aimed to assess the correlation between telomere length measured in dental DNA and age, and the effect of sex and tooth type on telomere length; a further aim was to propose a statistical regression models to estimate biological age based on telomere length. DNA was extracted from 91 tooth samples belonging to 77 individuals of both sexes and 15 to 85 years old, and was used to determine telomere length by quantitative real-time PCR. Our results suggested that telomere length was not affected by sex and was greater in molar teeth. We found a significant correlation between age and telomere length measured in DNA from teeth. However, the equation proposed to predict age was not accurate enough for forensic age estimation on its own. Age estimation based on telomere length in DNA from tooth samples may be useful as a complementary method which provides an approximate estimate of age, especially when human skeletal remains are the only forensic sample available.

Keywords Age estimation · Teeth · Telomere length · Quantitative real-time PCR

Introduction

Age estimation is currently a major challenge faced by forensic scientists. Age diagnosis not only helps to answer questions arising in criminal or civil proceedings that involve minors or adults with no valid identification documents, but also facilitates the creation of a biological profile when identification is required [1].

Several methods are available for forensic age assessment in both living and deceased persons. The most common procedures are based on the study of physiological changes in dental and skeletal structures with aging, e.g. mineralization and eruption processes in childhood and degenerative changes in adulthood. However, these morphological features can also be modified by a variety of external and internal factors apart from age. For this reason the discrepancy between biological and chronological age becomes larger with increasing age, so that age estimation is much less accurate in adults than in subadults [1]. In addition, in deceased individuals the applicability of these methods also depends on whether the type and state of preservation of the human remains make a morphological analysis possible [1].

These difficulties have led to the appearance of new approaches based on alterations caused by aging, but focused on the molecular level. These methodologies are based on changes observed at the protein level, such as aspartic acid racemization and protein glycosylation, as well as alterations at the DNA level, including mitochondrial deletions, telomere shortening, single joint T-cell receptor excision circle rearrangements, and most recently, DNA methylation markers [2–4]. Nevertheless, low accuracy, practical limitations and the lack of standardization for these methods per se and for sampling protocols are the main problems in attempts to incorporate these new methods into current forensic practice [2, 3]. More research is needed in this field to achieve greater accuracy in age prediction.

In this connection, telomere length is a promising biomarker of cellular aging since it has been found to be directly associated with the proliferative capacity of cells and the induction of replicative cell senescence, thus acting as a molecular clock [5–7]. Many studies with different methods of measurement have highlighted the inverse correlation between telomere length and age in a variety of cells and tissues [8–16]. Although some of these studies were aimed at aging research while others were specifically oriented towards forensic age estimation, only a few of them considered teeth, a valuable forensic sample [9,

14, 15]. Due to their distinctive composition as well as their location within the jaws, teeth are highly resistant to harsh conditions including postmortem DNA degradation, long after soft tissues have been lost. This makes dental tissues an excellent source of DNA of often better quality and less susceptible to contamination than DNA extracted from bones [17].

This study aimed to determine the correlation between telomere length measured in dental DNA and age, as well as the effect of sex and tooth type on telomere length. A second goal in this study was to propose a statistical regression model for estimating biological age in forensic cases for which only mineralized tissues are available.

Materials and methods

Sample collection and DNA extraction and quantification

Ninety-one healthy permanent teeth extracted from 77 patients (20 males, 53 females and 4 subjects with no sex data) aged 15 to 85 years (31.54 ± 13.17 years; males: 36.46 ± 17.28 years; females: 28.81 ± 11.35 years) were studied. Teeth were extracted at the public Oral Health Service and private dental clinics in Granada (Spain) for periodontal, orthodontic, prosthetic or surgical reasons and were free of caries, root canal treatments or restorations. The research protocol was approved by the Ethics Committee for Human Research of the University of Granada (Spain), and the study was conducted in accordance with the ethical standards laid down by the Declaration of Helsinki.

Each sample was labeled with a random number, and the date of extraction, the tooth number according to the FDI numbering system, and the subject's date of birth and sex were recorded. Chronological age for each case was calculated from the date of birth to the date of tooth extraction. Each extracted tooth was washed and decontaminated before being pulverized under liquid nitrogen in a cryogenic laboratory mill (6750 Freezer/Mill®, SPEX® SamplePrep, Stanmore, UK). Then DNA was extracted from 500 mg of dental powder using a standard organic method of proteinase K, EDTA and SDS digestion, followed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction, and Amicon® Ultra-0.5 Ultracel-30 membrane (Merck Millipore, Darmstadt, Germany) purification and concentration. Double-stranded DNA concentrations were determined with the Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™, Carlsbad, CA, USA) and a fluorescence

microplate reader (GloMax® Multi Microplate Multimode Reader, Promega, Madison, WI, USA), with excitation at 490 nm and emission at 510–570 nm.

Telomere length measurement

Telomere length was determined by quantitative real-time PCR (qPCR) according to Cawthon's method [18], with some modifications. Each PCR well contained 5 ng of dried DNA and 9 µL of a mix of QuantiFast SYBR Green PCR kit (Qiagen, Hilden, Germany), nuclease-free water and the primer pair specific for the plate. All analyses were processed with an Applied Biosystems 7900 HT Fast Real-Time apparatus (Applied Biosystems, Grand Island, NY, USA). The thermal cycle conditions began with a 50°C UNG activation step for 2 min and incubation at 95°C for 5 min, followed by 30 cycles of 95°C for 15 s and 54°C for 2 min for telomere PCR, or 40 cycles of 95°C for 15 s and 58°C for 1 min for the RPLP0 PCR protocol. For each measurement the mean of the two closest threshold cycle (Ct) values out of three was used. The results were recorded as average relative telomere length, represented by the ratio of relative telomere repeat copy number (T) to single-copy gene copy number (RPLP0, S) [18]. To obtain the relative values, the T/S ratio at the third standard curve point was used as the reference T/S ratio.

Statistical analysis

Statistical analysis was done with SPSS software, version 23.0 (IBM Corporation, Armonk, NY, USA). Descriptive statistics were recorded and expressed as the mean ± standard deviation of the mean. To compare mean values for quantitative variables between sexes, the nonparametric Mann-Whitney U test for two independent samples was used. The Kruskall-Wallis test, a nonparametric test for more than one independent sample, was used to compare quantitative variables between age groups and types of teeth. Statistical dependence analysis between variables was carried out with the Pearson correlation coefficient. Regression analysis was used to obtain a linear regression equation to predict age from the value of relative telomere length. A value of p less than 0.05 was considered statistically significant.

Results

The quantity of DNA was determined for all teeth and mean DNA concentration for the overall sample, expressed in ng/µL, was calculated (Table 1). A significant negative correlation between DNA concentration and age was seen ($R^2 = 0.63$, $p < 0.001$), indicating lower DNA concentrations with increasing age. Differences in DNA concentration among types of teeth (incisors, canines, premolars and molars) were also studied (Table 1). Statistically significant differences were obtained between mean DNA concentration in molars and the concentrations calculated for incisors, canines and premolars. No significant differences between sexes were observed when mean DNA concentration values were compared between males (38.30 ± 21.66 ng/µL, $n = 20$) and females (46.78 ± 11.66 ng/µL, $n = 53$).

Table 1 Age, DNA concentration and relative T/S ratio values according to types of teeth

Tooth type	*n*	Age (years)	DNA concentration (ng/µL)	Relative T/S ratio
		Mean ± SD (Min - Max)	Mean ± SD	Mean ± SD
Incisors	11	56.17 ± 7.94 (50.00-73.83)	16.14 ±10.01	0.70 ± 0.28
Canines	5	57.13 ± 7.13 (50.00-64.83)	24.99 ± 14.38	0.51 ± 0.10
Premolars	6	57.95 ± 14.24 (43.67-85.00)	21.46 ± 12.91	0.54 ± 0.03
Molars	69	28.39 ± 11.10 (15.50-85.00)	47.34 ± 12.13*	1.02 ± 0.24**
Total	91	35.28 ± 16.25 (15.50-85.00)	40.63 ± 16.93	0.92 ± 0.29

n: number of cases; SD: standard deviation; Min: minimum; Max: maximum
* Significant differences between molars and incisors ($p < 0.001$), canines ($p < 0.01$), and premolars ($p < 0.001$)
** Significant differences between molars and incisors ($p < 0.001$), canines ($p < 0.001$), and premolars ($p < 0.001$)

After gene expression levels were analyzed, relative telomere length, expressed as relative T/S ratio, was calculated for all teeth. Relative T/S ratio in all samples ranged from 0.38 to 1.49 (0.92 ± 0.29, Table 1). The regression analysis showed a significant negative correlation between relative T/S ratio and age ($R^2 = 0.35$, $p < 0.001$), indicating that telomere length shortened with age (Figure 1), and that this trend was stronger in males ($R^2 = 0.37$, $p < 0.01$) than in females ($R^2 = 0.17$, $p < 0.01$). The relationship between

relative T/S ratio and tooth type was also studied. Statistically significant differences were obtained between mean relative T/S ratio in molars and the means obtained in incisors, canines and premolars (Table 1). No statistically significant differences between sexes were found in mean relative T/S ratio (0.94 ± 0.32 in males vs. 1.00 ± 0.24 in females).

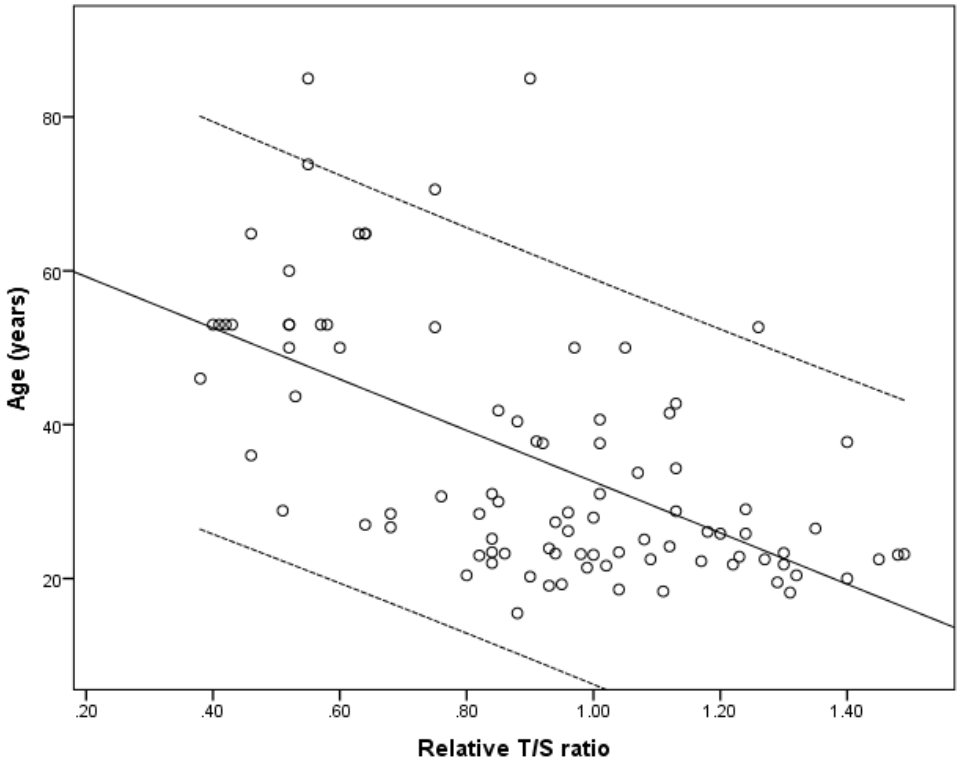

Fig. 1 Correlation between relative telomere length (expressed as relative T/S ratio) and age. The solid straight line represents the linear regression, and the dotted straight lines show the 95% confidence intervals for the predicted individual value

The formula derived to estimate the subject's age was $y = 65.89 - 33.30x$ (where y represents age in years and x represents the relative T/S ratio). The standard error of age estimation for this study sample was ± 13.18 years. To evaluate the accuracy of this equation, the actual ages of the subjects in the study were compared to the predicted ages (Figure 2). The mean prediction error was ± 9.85 years. Estimated ages were within ±10

years of actual ages in 61.04 % of all subjects. The differences (in years) between actual and estimated ages by age group for all teeth are shown in Table 2.

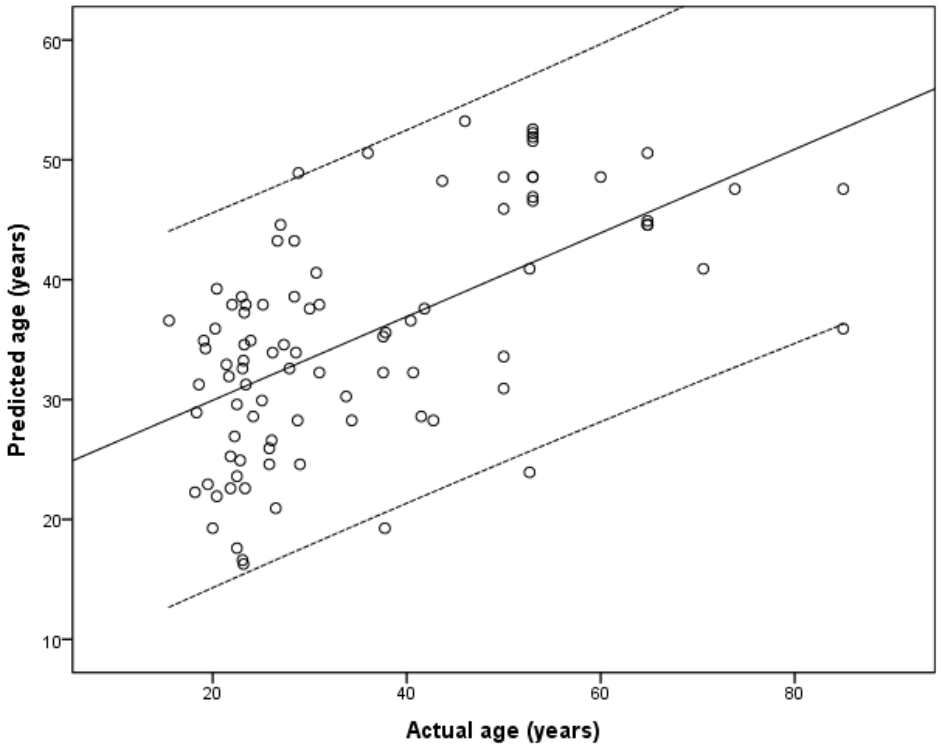

Fig. 2 Predicted versus actual age according to individual relative telomere length. The solid straight line represents the linear regression, and the dotted straight lines show the 95% confidence intervals for the predicted individual value

Table 2 Number of cases and percentages according to differences between actual and estimated ages by age group[a]

Age group[a]		Difference (years)		
	n	±0-5	±6-10	>±10
Group 1 (%)	56	23 (41.07)	15 (26.78)	18 (32.14)
Group 2 (%)	26	14 (53.85)	4 (15.38)	8 (30.77)
Group 3 (%)	9			9 (100.0)
Total (%)	91	37 (40.66)	19 (20.88)	35 (38.46)

n: number of cases

[a] Group 1: 15–35 years; Group 2: 36–55 years; Group 3: ≥56 years

Discussion

In the present study a modification of Cawthon's qPCR method [18] was used to measure telomere length in DNA extracted from teeth. It is important to note the intrinsic value of teeth as a preferable source of DNA for forensic analysis compared to bone [17]. However, the quality and quantity of DNA extracted from teeth are influenced not only by external conditions such as temperature, degree of humidity, pH and post-mortem time interval [19, 20], but also by individual factors such as the subject's age and sex, and tooth type and health status [21]. Our samples were stored in a controlled environment to protect them from post-extraction DNA decay, and the same decontamination protocol was used in all samples. With regard to DNA extraction, complete grinding and organic extraction with phenol/chloroform have previously yielded successful results in DNA analysis from mineralized tissues [17]. Among factors pertaining to the type of tooth, the higher DNA concentrations in molars (Table 1), as observed by other authors [21, 22], might be explained by the larger pulp volume and root surface, given that pulp and the cellular cementum are the best sources of nuclear DNA from teeth [17]. Also, the higher DNA concentrations observed at younger ages can be attributed mainly to decreased pulp volume and cellularity with increasing age [17]. On the other hand, we did not find significant differences in dental DNA concentrations between sexes, in accordance with an earlier study [22].

Although terminal restriction fragment (TRF) length analysis by Southern blotting [23] has long been considered the gold standard method, qPCR assay was used here to determine telomere length in our samples. The qPCR method requires small amounts of DNA and is less time-consuming than other methods. Other advantages include its high throughput, simplicity, robustness and reproducibility. It has been used in other recent studies to estimate age based on telomere shortening [24, 25]. Moreover, the RPLP0 gene was shown to be stable and suitable for use as a quantitative control with our samples.

Our results with the qPCR method showed a significant correlation between relative telomere length and age, indicating that telomere length decreased with age (Figure 1). However, some younger individuals had shorter telomeres than some older individuals. The shortest relative telomere length (0.38) was found in a 46-year-old male, while the longest relative telomere length (1.49) was observed in a 23-year-old male (Figure 1).

The association found between telomere length and age was consistent with previous results [8–12, 16, 26] obtained with the TRF or the qPCR method in a variety of human cells and tissues (Table 3). However, higher correlations were found with the TRF method, an observation also evident when teeth samples were studied. Takasaki et al. [15] reported a correlation of 0.562 between age and average TRF length when DNA was obtained from dental pulp, whereas we obtained a lower correlation between relative telomere length and age when the entire tooth was analyzed with the qPCR method. It should be recalled that each method uses different methodologies and generates different telomere parameters. The qPCR assay renders relative telomere length values, whereas the TRF assay provides absolute telomere length in kilobases – a value that includes subtelomeric repeats and thus masks the real length of telomeric repeats [24, 25]. In addition, differences between the results from different studies might be due to variations in laboratory technique [27], methods and protocols [24, 27, 28], or to pre-analytical factors such as the method of DNA extraction [29] or residual PCR inhibitors [24]. Other factors that can significantly influence telomere length include differences in ethnicity [30, 31] and sample size, tissue types [13], and genetic, environmental or lifestyle factors [32–34]. On the other hand, Kumei et al. [15], who used dot-blot hybridization, found telomere length to be strongly dependent on age ($R^2 = 0.825$) (not shown in Table 3). However, their results were obtained from only four dental pulp samples from females, and they did not propose an equation for age estimation.

Table 3 Comparison of different studies of telomere length for age estimation

Study	Sample size	Tissue	R^2	SE	Method
Tsuji et al. (2002)	60	Blood	0.69	±7.04	TRF
Takasaki et al. (2003)	100	Dental pulp	0.56	±7.52	TRF
Karlsson et al. (2008)	96	Blood	0.3	±22	qPCR
Hewakapuge et al. (2008)	167	Buccal cells	0.04	ND	qPCR
Ren et al. (2009)	105	Blood	0.83	±9.83	TRF
Srettabunjong et al. (2014)	100	Blood	0.39	±9.60	TRF
Zubakov et al. (2016)	305	Blood	0.14	±15.36	qPCR
Present study	91	Teeth	0.35	±13.18	qPCR

R^2: correlation with age; SE: standard error in years; ND: not determined; TRF: terminal restriction fragment length analysis; qPCR: quantitative real-time polymerase chain reaction method

We used a formula to predict the actual age of each individual in the present study from the relative telomere length; however, the estimated standard error of the age estimates was too high (approximately ± 13 years) for forensic purposes. It is worth noting that our results are in the same range of deviation as reported for the most common morphological methods used to estimate dental age [35]. This result may reflect large differences in relative telomere length between individuals of the same age, as others have suggested [10, 11, 16]. For example, the relative telomere length of 11 individuals aged 23 years ranged from 0.82 to 1.49 (1.06 ± 0.25), illustrating a clear between-individual variation in our sample.

Although no significant differences were found in relative telomere length between sexes, as others reported [16, 26], a stronger correlation between telomere length and age was observed in men than in women, in consonance with published data [11, 16]. However, previous studies reported a higher rate of telomere attrition in males than in females as well as longer telomeres in females [12, 36]. Our results should therefore be considered with caution due to the relatively small sample size for males.

To our knowledge, this is the first study that compares relative telomere length among different types of teeth. Our results showed significantly higher relative telomere length in molars. Although higher DNA concentrations were also observed in this tooth

type, there was no significant correlation between DNA concentration and relative telomere length which might explain this finding. Therefore, the fact that molars in our sample belonged mostly to the youngest age group may account for our results. Nevertheless, large-scale studies will be needed to establish possible differences in telomere length among types of teeth more accurately, given the importance of this parameter when different teeth from the same individual are available for study.

In conclusion, molecular biology methods are undoubtedly becoming more widespread as a tool to predict age for forensic purposes, and may be especially useful for age estimation when morphological information is limited. Our results suggest a significant correlation between age and relative telomere length measured in tooth samples by qPCR. In addition, relative telomere length was not affected by sex and was significantly higher in molars. Unfortunately, the large standard error of estimates obtained with the equation we developed to predict age makes this method unsuitable for forensic age estimation on its own, although it could be used as a complementary method to assign an age interval – information that can be crucial for a final identification, especially when human skeletal remains are the only forensic sample available. The method for age estimation reported here could be complemented with the study of emerging age-predicting biomarkers in teeth samples, such as DNA methylation analysis, in order to obtain more accurate models to predict age for forensic purposes from human skeletal remains.

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Compliance with ethical standards

The research protocol was approved by the Ethics Committee for Human Research of the University of Granada (Spain), and the study was conducted in accordance with the ethical standards laid down by the Declaration of Helsinki.

Conflict of interest

The authors declare that they have no conflict of interest.

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