

DNA Collection and extraction

DNA was extracted using the method described in Freeman *et al.*¹. Briefly, Buccal mucosa cells were collected by swabbing the inside of subjects' mouths. Swabs were placed in protective buffer containing 100mM NaCl, 10mM Tris-HCL, pH 8.0; 10mM EDTA, pH 8.0, 0.2mg/ml Proteinase K and 0.5% w/v SDS (Sigma Aldrich, Dorset, UK). Tubes were labeled with barcodes and shipped to the laboratory at room temperature for extraction. To Extract DNA, swabs were spun at 300g for 4 min to collect cells and the resulting supernatant was incubated at 65°C for 2 hours to promote proteinase K activity. The supernatant was decanted into fresh tubes and treated with 600µl of an organic deproteinization reagent (Yeast reagent 3 (Autogen Bioclear Ltd., Wiltshire, UK) diluted with equal volume of 100% Ethanol (Sigma Aldrich)). The mixture was vigorously shaken by hand for 30 seconds and debris was removed by pelleting at 5000g for 25 min. The DNA containing supernatant was then transferred to fresh tubes, 1.2ml Isopropyl alcohol was added and samples were mixed gently for 1 min before pelleting the DNA at 500g for 25 min. The isopropyl alcohol was then decanted and DNA pellets were vigorously re-suspended in ethanol (70% v/v) for 10 min prior to final pelleting at 5000g for 10 min. After removal of ethanol, the remaining DNA pellets were allowed to dry before re-suspending at 37°C in 1ml 10mM Tris-HCL, 0.1mM EDTA, pH 0.8.

Measurement of mean relative telomere length

The method designed by Cawthon² determines mean telomere length across all chromosomes for all cells sampled. The method involves two quantitative PCR reactions for each subject; one for a single-copy gene (S) and the other in the telomeric repeat region (T). Given each of these reactions is compared to a standard reference sample before the (T)/(S) ratio is calculated, the ratio will equal 1 when the experimental sample is identical to the reference sample. Thus the ratio of one individual to another will correspond to the relative telomere lengths of their DNA. All DNA samples were run in triplicate for telomere and single-copy reactions at both age 5 and age 10. i.e., 12 reactions per study subject.

For the single-copy reaction (36B4), 10µl PCR reactions were set-up using a QIAgility robotic pipettor (Qiagen, MD, USA) to ensure maximum pipetting accuracy and contained the following reactants: 30 ng gDNA, 5 µl 2x SYBR Q-PCR ROX Mix (Qiagen), 0.5pmol forward primer (36B4u; Sigma, MO, USA), 0.5 pmol reverse primer (36B4d;Sigma), 0.5 µl 1M DTT (Sigma), 0.1 µl DMSO (Sigma), 1.6 µl dH₂O. Primer sequences were 36B4u: CAG CAA GTG GGA AGG TGT AAT CC; 36B4d, CCC ATT CTA TCA TCA ACG GGT ACA A. Thermalcycling was performed on an AB9700 TaqMan real-time PCR machine (Applied Biosystems, CA., USA), with an initial incubation step at 95°C for 15 minutes, followed by 30 cycles of 95°C for 15 s and 58°C for 1 min.

For the telomere reaction, 10µl PCR reactions were set-up using a QIAgility robotic pipettor (Qiagen, MD, USA) to ensure maximum pipetting accuracy and contained the following reactants: 30 ng gDNA, 5µl 2x SYBR Q-PCR ROX Mix (Qiagen), 0.9 pmol

forward primer (Tel1; Sigma), 0.9 pmol reverse primer (Tel2; Sigma), 0.5 μ l 1M DTT (Sigma), 0.1 μ l DMSO (Sigma), 0.6 μ l dH₂O. Primer sequences were: tel 1, GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG T; tel 2, TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA. Thermalcycling was performed on the AB9700 TaqMan real-time light cycler (Applied Biosystems), with an initial incubation step at 95°C for 15 followed by 30 cycles of 95°C for 15 s and 54°C for 2 min.

Primer concentrations were optimized for each batch in accordance with AB9700 Taqman guidelines (Applied Biosystems). Each plate contained a 1.68-fold serial dilution of a reference sample used as a standard curve, ranging from 40 to 1.06 ng/ μ l DNA. The reference sample DNA consisted of a pool of DNA from eight separate individuals (who were not part of the 236 individuals tested for telomere length in this study). As reaction conditions varied between telomere and single-copy Q-PCRs, they were performed in separate plates. Sample grid position was preserved between plates to minimize experimental error due to positional effects.

Data acquisition and ratio relative calculation

Relative quantities of samples for each of the reactions were calculated as follows: firstly, reaction efficiencies (E) were calculated from the standard curve slope using $E = 10^{(1/-\text{slope})}$, where at 100% efficiency $E = 2$. Next, relative quantities (RQs) were calculated using $RQ = E^{\Delta Ct}$ where ΔCt is the difference between the average triplicate Ct value of the within-plate mean Ct, and the Ct value of the individual sample. Sample RQ values were calculated for each reaction separately (T and S). Finally, the telomere length relative to the amount of single-copy transcript was calculated using the ratio

RQ(T)/RQ(S). The coefficient of variation (CV) for the triplicate samples was .97% for the telomere (T) and .49% for the single-copy gene (S).

The correlation between MZ twins was $r = .22$ for age-5 and $r = .13$ for age-10 TL, suggesting little resemblance between them. Behavioral genetic studies of adults have reported conflicting heritability estimates ranging from 36%-82%³⁻⁶. To our knowledge there are no studies of the heritability of TL in childhood.

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