SUPPLEMENTAL DATA

METHODS

**Telomere length measurement by quantitative real-time PCR (q-PCR)**

The measurement of telomere length was performed by the same method as in the previous study (11). The method was originally developed by Cawthon and applied here without major modifications (13). Briefly, copy numbers of the telomere (T) repeat and of a single (S) copy number control gene (36B4, acidic ribosomal phosphoprotein P0) were determined by quantitative PCR (q-PCR). The result was expressed as the T/S ratio. The RTL was calculated by dividing the T/S ratio of each experimental sample by the T/S ratio of a reference DNA consisting of a mixture of DNA samples from three healthy individuals. The TERT gene amplification assay was performed by q-PCR. We used the $2^{\Delta\Delta Ct}$ method to calculate the TERT/36B4 gene copy ratio ($\Delta\Delta Ct = (Ct_{TERT_{sample}} - Ct_{36B4_{sample}}) - (Ct_{TERT_{calibrator}} - Ct_{36B4_{calibrator}})$) (14). For the $\Delta\Delta Ct$ calculation to be valid, the amplification efficiencies of the target and reference genes must be approximately equal. This can be established by determining the variation in $\Delta Ct$ ($\Delta Ct = Ct_{target} - Ct_{control}$) as a function of template dilution. If the absolute value of the slope of DNA dilution versus $\Delta Ct$ is close to zero, it implies that the amplification efficiencies of the target and housekeeping genes are similar. In our study the slope values for $\Delta Ct$ versus log DNA dilution for two runs were 0.019, 0.088 and 0.033 for telomere and TERT reactions, respectively. All samples were run in duplicate. The Master mix for telomere, TERT and 36B4 was identical except for the oligonucleotide primers. The final concentrations of reagents in the PCR were 150 nM 6-ROX, 0.2x Syber Green, 15mM Tris-HCl pH 8.0, 50 mM KCl, 2mM MgCl2, 0.2 mM each dNTP, 5 mM DTT, 1% DMSO and 1.25 U AmpliTaq Gold DNA polymerase. The final concentrations and 5’→3’ sequences of the primers for telomere, TERT and 36B4 were as follows: tel-1 270 nM, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT; tel-2 900 nM, TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA; TERT_F 300 nM : CTTGGGAACCAGGACAAAGG, TERT_Rev 300 nM : TAAAATTATCCACATGGCTCACGT;
The PCR profile for the telomere reaction was 95°C for 10 min., then 30 cycles of 95°C for 15 s and 54°C for 2 min.; for TERT: 95°C for 10 min. followed by 30 cycles of 95°C for 15 s and 60°C for 1 min.; for 36B4 95°C for 10 min. then 30 cycles of 95°C for 15 s and 58°C for 1 min. For each plate a dissociation curve was obtained to monitor any additional double stranded DNA. A dilution series of a mixture of three DNA samples derived from healthy individuals ranging from 1.25 to 40 ng was analyzed to produce a standard curve for each plate. For all the plates two runs were done to allow the obtained RTL and TERT gene amplification results to be tested for reproducibility. To evaluate the intraplate variability 8 samples were spotted twice and randomly distributed in each plate for each run. The mean intraplate coefficients of variation (CV) of telomere Ct values and 36B4 Ct values were 0.87% and 0.51%, respectively. The interplate CVs were 1.25% and 1.66%, respectively. For TERT, interplate and intraplate CVs of Ct values were 0.63% and 0.56% respectively. The slopes of the standard curves generated for each run were as follows: telomere -3.33, -3.16, 36B4 -3.25, -3.15, TERT -3.28, -3.18. The linear correlation coefficient for all curves was R²>0.98.

Flow-FISH procedure

We wished to validate the results by performing flow cytometry and TERT expression measurement in the available previously studied patients. To accomplish this we collected fresh blood samples from 14 of the sporadic and 10 of the familial cases chosen randomly from those studied previously. Additionally, we collected 21 samples from subjects with no diagnosed cancer as controls. As shown in Table 1, all three groups were matched for age and gender.

The telomere length was measured by FISH and flow cytometry as previously described (15). Briefly, lymphocytes were isolated by ficoll separation (Ficoll-Paque PLUS, GE Healthcare) within 24 hours after blood collection and cryopreserved at 2x10⁷ cells per mL in freezing medium (45% FBS, 45% RPMI, 10% DMSO) in liquid nitrogen. Frozen cells were rapidly thawed, washed once in RPMI+20%PBS, counted (Coulter Counter, Beckman, USA) and resuspended in two 15 ml tubes (control and sample tube)
at 4x10^6 cells in 5 mL of RPMI medium with 20% FBS. The lymphocyte suspension was centrifuged at 500xg for 10 minutes at room temperature (RT) and the supernatant discarded. The cell pellet was resuspended in 600 ul of hybridization solution and in 600 ul of fluorescein-PNA probe (CCCTAA), for the control and sample tubes, respectively (Telomere PNA kit/FITC, Dako, USA, Cat. No. K5327). The cell suspension was transferred to flow tubes (in duplicates), incubated at 82.5°C in a water bath for 10 minutes, vortexed and hybridized at RT overnight in the dark. The following day the samples were washed twice as follows: 1 ml of Wash Solution (dilution 1:10 with water, Telomere PNA kit/FITC, Dako, USA, Cat. No. K5327) was added to each tube, the cell suspension was incubated at 40.5°C in a water bath and centrifuged at 500xg for 10 minutes. Finally, the cell pellet was resuspended in 1 ml of filtered PBS and analyzed by flow cytometry.

Bovine thymocytes (used as internal control for flow cytometric analysis) were obtained from calf thymus and prepared as previously described (15).

**Flow cytometric analysis**

Flow cytometric analysis of all samples and controls was performed in the clinical flow cytometry laboratory at the OSU Medical Center using a FC500 flow cytometer and CXP software version 2.2 as previously described (15). To minimize instrument related variations the same instrument was utilized for all experiments. The instrument was started daily according to the standard operation procedure that includes demonstration of acceptable quality control data points for calibration of fluidics, compensation and linearity. Further quality control for Quantum™ FITC-% MESF (premix) beads (Bangs Laboratories, Inc. catalog Code 555pB) was monitored with each sample to assess variation of mean fluorescence channel intensity (MFI) from sample to sample. Immediately following the last wash post hybridization all patient samples and bovine thymocytes with and without probes were delivered to the flow cytometry laboratory. All tubes were spiked with a 10 mL of FITC Quantum TM beads and immediately analyzed. Events consistent with lymphocyte single nuclei were selected using side scatter characteristics. The intensity of the FITC signal was measured on a linear scale using FL1 channel. All
samples were run in duplicates and a minimum of 25,000 events acquired per sample. With the exception of bovine thymocytes with and without probes the flow operator was blinded to the identity of the sample. Following the acquisition of events the identity of each individual sample was revealed and X-mean for each sample was derived by subtracting the MFI value for samples with no probe (MFI_{NP}) from the corresponding (paired) samples with probe (MFI_{P}). To account for unavoidable experiment to experiment differences in permeabilization, hybridization and washes a duplicate sample of bovine thymocytes (isolated and cryopreserved at -130° C prior to this study) was included in each experiment. Bovine thymocytes were processed in an identical manner to the patient samples including pairs of labeled and unlabeled cells. The X-mean for thymocytes was calculated by subtracting the MFI of processed cells without probe (TNP) from the MFI of thymocytes with probe (TP). The derived thymocyte X-mean was then used as a denominator to express final results for patient telomere length for each experiment. Utilization of the patient/thymocyte ratio assured uniformity between all samples that were processed, stained and analyzed in different batches. Moreover, to further assure reproducibility of the flow method to measure the average length of telomeres several patient and control samples with different telomere lengths were randomly, blindly and repetitively incorporated into several experiments. These samples demonstrated essentially identical results when run on different days establishing good precision of this method.

**TERT gene expression**

2.5 ml of blood was collected into PAX gene tubes (PreAnalytiX GmbH, Germany) and after 2 h of incubation at RT the samples were stored at -70°C. RNA extraction was performed according to the manufacturer’s protocol and 500 nanograms were utilized to retrotranscribe into cDNA by the High Capacity Reverse Transcriptase kit (Applied Biosystems). The Taqman TERT gene expression assay (Applied Biosystems, Cat. No. Hs0097265_m1) and Taqman GAPDH gene expression (Applied Biosystems, Cat. No. 4352934E) were used in the amplification reaction performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The conditions for PCR reaction were as
follows: 95°C for 10 minutes to activate the polymerase followed by 40 cycles of denaturation (95°C, 15 seconds) and annealing (60°C, 1 minute). The formula $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{(TERT)} - Ct_{(GAPDH)}$ was employed to calculate the relative $TERT$ mRNA level.