Twin studies - investigations of the telomerase system and potential telomere-mitochondria interplay

Ph.D. Thesis

Dóra Melicher

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
András Falus, Member of Hungarian Academy of Sciences

Official reviewers:
János Aradi, Ph.D.
Gábor L. Petheő, M.D., Ph.D.

Head of the Final Examination Committee:
Zsuzsa Schaff, Member of Hungarian Academy of Sciences

Members of the Final Examination Committee:
András Hrabák, Ph.D.
József Prechl, M.D., Ph.D.

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1. Introduction

Telomeres, which contain hexanucleotid repeats (TTAGGG), are protective heterochromatic structures formed by DNA tandem repeats bound by specialized protein complexes, that cap the end of linear chromosomes and play a key role in maintaining genome stability. Most somatic tissues lack telomerase activity and show progressive telomere shortening coupled to cell division that can lead to loss of chromosomal stability and changed epigenetic signature. Telomere length can be modified by genetic and epigenetic factors, and dysfunction and defects in the stability of TL contribute to the pathobiology of several human disease manifestations. Telomerase is a ribonucleoprotein enzyme complex that maintains telomere length by telomere synthesis. The human telomerase complex consists of two main components, a catalytic reverse transcriptase protein subunit (hTERT), and a telomerase RNA component (hTERC or hTR). Telomerase plays a crucial role in the pathology of aging and cancer through maintaining genome integrity, controlling cell proliferation, and regulating tissue homeostasis.

Telomerase is repressed in most human somatic cells, but lymphocytes are unique in the sense that they retain the ability to upregulate telomerase upon activation, which allows the intense clonal expansion and proliferation required to generate effector cells and long lived memory cells while maintaining telomere length. Capacity for cellular replication is vital for lymphocyte function, and telomerase activity is commonly referred to as a marker of the proliferative activity of cells.

Mitochondria are essential cell organelles, generating most of the ATP, fundamental for cellular functions, and have an integral role in both cell growth, differentiation, replication and death. The aging process is characterized by increases of mitochondrial reactive oxygen species production, accumulating mutations in mitochondrial DNA, dysfunction of mitochondrial biogenesis and an overall decline in mitochondrial function. Mitochondrial DNA copy number, the number of circular mtDNA per cell, provides an indirect way for measuring mitochondrial biogenesis, the process that could compensate for the increasing energy demand and compromised mitochondrial function. Lately, key links have been revealed between telomere shortening and mitochondrial dysfunction, two mechanisms that are both considered to play central role in the aging process. Studies mostly on cell lines demonstrated that mitochondrial stress could contribute to telomere attrition and vice versa. Common mechanisms and complicated telomere-mitochondria interplay was also proposed, however the relationship is still not completely understood.

Monozygotic and dizygotic twins are uniquely valuable subjects for studies aiming to control over genetic background and early environmental influences. Both intra- and interpair comparisons between twins are equally powerful tools, studying twins can make new contributions in revealing and understanding genetic as well as epigenetic mechanisms.

2. Objectives

Both telomerase activity, hTERT protein level, telomere length and mitochondrial DNA copy number are important markers in reflecting the replicative capacity of cells. We aimed to contribute to the deeper understanding of the telomerase system and telomere-mitochondria interplay by simultaneously investigating these four variables in a genetically controlled study design, and being the first research group to study them in monozygotic twins.

We alongside aimed to help in deciphering the possible contribution of genes and environment by comparing the degree of phenotypic similarities of monozygotic and dizygotic twins. Applying the unique research potential of twin studies, we approach the issue of possible genetic contribution, by calculating intraclass correlation coefficients for mtDNAcn and TL among MZ and DZ twins in estimating co-twin similarity. Studying possible associations of mtDNAcn and TL firstly in twins, also belonged to our main objectives.

We further detail our specific objectives below:

Analysis of the distribution of T lymphocyte subgroups in peripheral blood mononuclear cells of monozygotic twins of different age and gender by applying multi-parametric flow cytometry.

- Comparison and assessment of co-twin similarities of T-cell subgroup distribution in monozygotic twins.

Measurement of the catalytic reverse transcriptase protein subunit of telomerase (hTERT) in T lymphocyte subsets.

- Analysis and comparison of hTERT protein level in T lymphocytes, helper-, cytotoxic-, and regulatory T-cells.
- Comparison and assessment of co-twin similarities of hTERT protein level of different T-cell subgroups of MZ twins.

Detection of telomerase activity in PBMCs, utilizing the Telomeric Repeat Amplification Protocol (TRAP).

- Comparison and assessment of co-twin similarities of telomerase activity of MZ twins.

Absolute telomere length measurement, using quantitative polymerase chain reaction standard curve method.

- Comparison and assessment of co-twin similarities of telomere length of MZ and DZ twins.

Absolute mitochondrial DNA copy number measurement, using quantitative polymerase chain reaction standard curve method.

- Comparison and assessment of co-twin similarities of mtDNA copy number of MZ and DZ twins.

Assessment of co-twin similarities of mitochondrial DNA copy number, telomere length, hTERT protein level and telomerase activity in PBMCs of MZ twins, calculation of intraclass correlation coefficients and assessment of within-pair similarity measures with increasing age.

Bivariate association analysis and regression model of mtDNA copy number, telomere length and clinical data of MZ and DZ twins.

Co-twin similarity assessment and comparison of MZ and DZ twins in mtDNA copy number and telomere length.

3. Methods

Subjects and study design

32 subjects, comprising of 16 monozygotic twin pairs were included in the monozygotic twin study consisting of combined telomerase activity measurements, flow cytometric detection of hTERT protein level and analysis of the distribution of various T-cell subpopulations, mtDNA copy number and telomere length measurements. The mean age of participants was 46.0 ± 12.88 (years ± SD), with an age-range of 21 – 69 years. Eleven pairs of the twin subjects were female and five pairs were male.

142 subjects, comprising of 96 monozygotic- (48 complete pairs) and 46 dizygotic twins (23 complete pairs) were analysed in the mtDNA copy number and telomere length measurement study. The mean age of participants was 50.54 ± 15.43, with an age-range of 20 – 75 years. 50 pairs of the twin subjects were female and 21 pairs were male. The study participants were recruited from the members of the Hungarian Twin Registry. The subjects - one twin pair at a time - attended a full-night polysomnography in the frame of an ongoing clinical study, attending the Sleep Laboratory of, the Department of Pulmonology, Semmelweis University, Budapest, Hungary. The following morning, within an hour after awakening peripheral blood was drawn.

All volunteers gave their written informed consent.

Flow cytometry

The identification of lymphocyte subpopulations and the detection of intracellular hTERT protein level, in subpopulations of lymphocytes was carried out using the method of concurrent staining of cell surface antigen, multi-parametric flow cytometry was applied for the analysis.

T lymphocytes were defined on the basis of their CD3 expression. Helper T-cells were identified by CD3+/CD4+ double positivity, while cytotoxic T-cells were described by the co-expression of CD3 and CD8. Regulatory T lymphocytes were characterized by the intracellular presence of Foxp3 transcription factor in CD3+/CD4+ cells. Propidium iodide uptake assay was used for the determination of the viability of PBMCs before staining.

The assay procedure for the detection of hTERT protein level was carried out as follows: $5x10^5$ PBMC suspensions were incubated with fluorochrome-conjugated antibodies for exofacial staining in the dark for 15 min, at room temperature. During washing steps the supernatant of PBS diluted cell suspension was removed by centrifugation, allowing a minor volume of liquid (ca. 100 μL) to remain. Cell pellet was fixed by 4% paraformaldehyde solution for 10 min, at room temperature. After washing in PBS, the cells were permeabilized by 0.1% saponin solution for subsequent staining for intracellular Foxp3 and hTERT protein. Intracellular staining lasted for 15 min. Unbound

antibodies were removed by washing with 0.1% saponin solutions. All antibodies were carefully titrated in earlier experiments.

Measurements were carried out using a FACSCalibur flow cytometer (Becton Dickinson San Jose, CA, USA) on the day of the staining, collecting $1x10^5$ cells/tube. CellQuest-Pro software (Becton Dickinson San Jose, CA, USA) was used for the acquisition and analysis. Lymphocyte subpopulations were determined inside the lymphocyte gate, on the basis of their exofacial protein pattern. The relative amounts of intracellular hTERT protein were analyzed by the comparison of geometric mean channel values of fluorescence.

Measurement of telomerase activity with TRAP-ELISA assay

For the detection of telomerase activity, the Photometric Enzyme Immunoassay for Quantitative Determination of Telomerase Activity, utilizing the Telomeric Repeat Amplification Protocol (TRAP) was applied. To determine telomerase activity $2x10^5$ cells were used for the assay. The cells were lysed in CHAPS lysis buffer, and the TRAP assay was performed using the TeloTAGGG Telomerase PCR ELISA^{PLUS} kit (Roche Diagnostics GmbH) following the manufacturer's instruction. Briefly, the PCR based assay was carried out in a reaction mixture (biotinylated telomerase substrate

P1-TS, optimized anchor-primer P2 nucleotides and Taq DNA polymerase) with the use of an internal standard (IS) and negative control (heat inactivation of telomerase-associated RNA) for each sample. After 20 min of incubation at 25 °C to allow the telomerase mediated extension of the TS primer, the reaction was performed in thermocycler (GeneAmp PCR System 9700, ABI) after the following steps: the reaction mixture was subjected to 94 °C for 5 min after which, 30 PCR cycles followed at 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 90 sec. After the cycling process, the samples were incubated at 72 °C for 10 minutes. To quantitate the cellular telomerase activity, the PCR product was denatured and then hybridized with a digoxigenin-labeled telomeric repeat-specific probe. The PCR product was visualized using peroxidase-conjugated antibodies against digoxigenin and measured at 450 nm, respectively at 690 nm using an ELISA reader (Labsystems Multiscan MS, Thermo).

Relative telomerase activity was calculated following the manufacturer's instructions: in the first step the absorbance readings of the negative samples were subtracted from the absorbance readings of the samples. When the difference in absorbance was higher than 0.2 A450nm-A690nm units, samples were considered telomerase positive.

Measurement of telomere length and mtDNA copy number

DNA was extracted from the previously isolated PBMCs, using the QIAamp DNA Blood Mini Kit (Qiagen Inc., Valencia, CA). Each DNA sample was quantified and qualified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Absolute telomere length (TL) and absolute mitochondrial DNA copy number (mtDNAcn) were measured using quantitative polymerase chain reaction (qPCR). First the mtDNAcn was calculated based on the ratio between the amount of mtDNA (Cytochrome b [MT-CYB]) and that of a single-copy gene (albumin [ALB]). TL was measured by calculating the number of telomere repeats relative to that of a single-copy gene (ALB) used as a quantitative control, relative to a reference sample. All qPCR reactions were performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA) with TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems, Foster City, CA). Three qPCRs were performed to quantitate copy numbers for telomeres, mitochondrial genomes (Cytochrome b [MT-CYB]), and the albumin gene [ALB] as a single-copy standard.

The primer sequences for telomeres (O'Callaghan et al., 2008) and primers and TaqMan probe for MT-CYB (Hakonen et al., 2007) had been previously reported. The primers and probe of the ALB gene, and also probe for

telomere were designed by using Primer3 program (Untergasser et al., 2012). The probe had a fluorophore at 5'-end, 6-carboxyfluorescein and a quencher at 3'-end, tetramethylrhodamine. Primer concentrations were raised to 100 nM and cycling conditions were as follows: 10 min at 95°C, followed by 50 cycles of 95°C for 15 sec, 60°C for 1 min. Each sample was run in triplicate using 20 ng of DNA and accepted only in case the standard deviation of the Ct values were <1Ct (CV>5%).

Amplification of telomeres, MT-CYB and ALB were performed in separate runs, using the same reference samples in the same well positions. For the standard curve method, 6-point serial-dilution of cloned amplicons with no template control was applied and the same calibrator sample was used in all runs to allow comparison of results across runs. Data were analyzed by StepOne Software v2.3 (Applied Biosystems, Foster City, CA). All standard curve met the criteria that the correlation coefficient (R2) was greater than 0.95, and the PCR efficiency was in the range of 90-100%.

Statistical analysis

Statistical analysis was carried out by applying linear mixed effects regression models using the lme4 package of the software. Statistical significance level was set at $p<0.05$. Continuous variables were expressed as mean ± standard deviation (SD) unless otherwise specified. Logarithmically transformed data were used in

case of mtDNAcn and TL in order to approximate a normal distribution.

Intraclass correlation coefficients (ICCs), estimating the intra-individual stability of a variable across conditions, were computed for the twin pairs to estimate the level of co-twin similarity. ICCs were calculated using the residual variance proportion of a baseline and age and sex corrected mixed effects model. Confidence intervals were derived using a family based bootstrap with 1000 draws.

To assess the bivariate associations between mtDNAcn, TL and available predictors, standard coefficients from a full information maximum likelihood estimated regression were applied that also controlled for age and sex. The interpretation of the models is analogous to Pearson's correlations but account for the lack of independence between twin pairs and incidental missing data on the predictors. P values were calculated using cluster corrected standard errors. To test the relationship between mtDNAcn and TL, the aforementioned strategy was used, and both standardized and unstandardized coefficients were presented. Additionally, regression model controlling for all significant predictors based on the bivariate analysis was used. The analyses were conducted using Mplus 8. Statistics data was assessed using GraphPad PRISM v6.05 (GraphPad SoftwareInc., California, USA).

4. Results

Investigating the telomerase system and telomere-mitochondria interplay, we carried out the first study, whereby four variables were simultaneously assessed, applying twin study design. We pursued co-twin similarity measurements of hTERT protein level, telomerase activity, telomere length and mitochondrial DNA copy number in 16 pairs of monozygotic twins, applying multi-parametric flow cytometry, TRAP-ELISA assay and qPCR standard curve method on peripheral blood mononuclear cell samples. Analyzing co-twin similarity of lymphocyte subsets, we were the first to demonstrate very high intraclass correlation coefficients of hTERT protein level in T lymphocytes (0.891) and in both helper T-cells (0.896), regulatory T-cells (0.885) and cytotoxic T-cells (0.798). In combined assessment of PBMCs, high intraclass correlation was calculated for telomere length (0.815), considerable for mtDNA copy number (0.524) and again, exceptionally high ICC was detected in hTERT protein level (0.946), while in contrast, telomerase activity showed no co-twin similarity (ICC=0). Besides, we provided novel information in characterizing the relative amount of hTERT protein in different lymphocyte subpopulations, whereby we found that regulatory T-cells contained significantly higher level of hTERT protein (27.01 ± 4.42, [mean ± SE])

compared to T lymphocytes (14.28 ± 1.34), cytotoxic- or (15.20 ± 1.74) helper T-cells (14.11 ± 1.34).

To further investigate the association of telomere length and mtDNA copy number, we substantially increased the sample size to a total of 142 healthy subjects, comprising of 96 monozygotic and 46 dizygotic twins, whereby significant positive association ($r=0.28$, $p<0.01$) was confirmed in age and sex corrected analysis. Following bivariate estimates and correction with significant predictors, the independent positive associations were further verified. In addition, we assessed age and sex adjusted co-twin similarities of telomere length and mtDNA copy number of the 142 monozygotic and dizygotic twins. In the case of telomere length strong intraclass correlation values were measured for both MZ (ICC=0.794) and DZ twins (ICC=0.785). ICC values for mtDNA copy number were also strong, with MZ twins (ICC=0.758) presenting slightly higher results compared to DZ twins (ICC= 0.641).

5. Conclusions

Both telomerase activity, hTERT protein level, telomere length and mitochondrial DNA copy number are important in reflecting the replicative capacity of cells. Aiming to contribute to the deeper understanding of the telomerase system and potential telomere-mitochondria interplay, we simultaneously defined and compared mitochondrial DNA copy number, telomere length, telomerase activity and level of the catalytic reverse transcriptase protein subunit of telomerase enzyme, hTERT in genetically matched subjects. Using PBMCs of 32 monozygotic twins, we applied qPCR standard curve method, TRAP-ELISA assay and multi-parametric flow cytometry. To estimate the level of co-twin similarity, we pursued intraclass correlation coefficient measurements.

Analyzing lymphocyte subgroups of identical twins, we were the first to show very high ICC for hTERT protein levels in T lymphocytes and both in helper-, cytotoxic-, and regulatory T-cell subgroups, while analyzing the similarity of the distribution of T lymphocyte subpopulations, high ICC was detected in regulatory- and cytotoxic T-cells. Assessing the results of four simultaneously measured parameters, high intraclass correlation was calculated for telomere length, considerable for mtDNA copy number and again, exceptionally high ICC was detected in hTERT protein

level, while in contrast, telomerase activity showed no co-twin similarity.

We presented novel information by firstly characterizing the relative amount of hTERT protein in different lymphocyte subpopulations, whereby we showed that regulatory T-cells contained significantly higher level of hTERT protein compared to other T lymphocytes, cytotoxic- or helper T-cells, in light of which, further investigations could help provide more insights into the regulation of the activation of regulatory T-cells. Multi-parametric flow cytometry could be the bridge between proteomics and cytomics, since it can characterize the functional and morphological properties of single cells at the same time.

To investigate the potential association of telomere status and mitochondrial function, we were the first to examine the relationship between mtDNA copy number and telomere length in MZ and DZ twins, involving a total of 142 subjects into our study. We confirmed significant positive association between the two variables, verified by bivariate and regression analysis. Assessing co-twin similarities of MZ and DZ twin subjects, we found that twins were similar in their intraclass correlation measures irrespective of zygosity, suggesting a possibly more important role of common (shared) environmental factors compared to non-shared (unique) environmental and to a smaller degree also individual genetic influences.

The results of our pilot studies should inspire additional investigations with considerably increased sample sizes and by involving twins discordant for certain disease phenotypes, while longitudinal studies should also be encouraged. In conclusion, we propose that further studies should investigate the potential of using the combination of telomere length and mtDNA copy number as possible biomarkers in health, and in specific disease conditions in the future.

6. Bibliography of own publications

6.1. Publications related to the current Ph.D. thesis

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