Telomere Shortening in Down Syndrome Patients— When Does It Start?

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Down syndrome (DS) is one of the most common aneuploidy. In general population, its prevalence is 1:600–1:800 live births. It is caused by a trisomy of chromosome 21. DS is phenotypically manifested by premature aging, upward slant to the eyes, epicanthus, flattened face, and poor muscle tone. In addition to physical changes, this syndrome is characterized by early onset of diseases specific to old age, such as Alzheimer's disease, vision and hearing problems, and precocious menopause. Since DS symptoms include premature aging, the shortening of telomeres might be one of the markers of cellular aging. Consequently, the aim of the study was to determine the length of the telomeres in leukocytes from the blood of juvenile patients with DS ($n=68$) compared to an age-matched control group ($n=56$) and also to determine the diagnostic or predictive value for this parameter. We show that, for the first time, in juveniles, the average relative telomere length in studied subjects is significantly longer than in the control group (50.46 vs. 40.56, respectively arbitrary units [AU]; $p=0.0026$). The results provide interesting basis for further research to determine the causes and consequences of telomere maintaining and the dynamics of this process in patients with DS.

Introduction

AMONG NUMEROUS FUNCTIONS of telomeres, they limit the number of cell divisions, thus preventing the accumulation of mutations that might lead to a malignant transformation. Telomere loss (also observed in Down syndrome [DS]) could provide the signal for cells to enter senescence, leading to the formation of dicentric chromosomes and classical breakage–fusion–bridge cycles, followed by genome rearrangements and aneuploidy. Consequently, this might lead to further impairments and faster dementia, observed in DS and in the course of many aging-associated disorders (Honig *et al.*, 2012). A key concern to aging adults with DS is the increased risk of Alzheimer's disease (AD). The profile and sequence of cognitive impairments in adults with DS are similar to those seen with AD in the general population. Memory processes are affected early in the course of DS dementia (Krinsky-McHale and Silverman, 2013). Severe cognitive deterioration, such as acquired apraxia and agnosia, has been reported in 28% of individuals with DS at the age of 30 years, with a higher prevalence of these impairments in the subsequent years (Head *et al.*,

2012). The earliest manifestations of dementia in DS may involve changes in personality and behavior. The diagnosis of this mental disorder in DS can be challenging against the background of preexisting intellectual impairment. Standardized criteria for the diagnosis of dementia in DS include both informant-based and direct measures (O'Caoimh and Clune, 2013). The severity of preexisting cognitive impairment may also be a predictor of the rate of cognitive deterioration in DS (Temple *et al.*, 2001). However, determining whether it is a cause or a consequence is not clear yet. We still do not know what factors, when and how, induce increased aging and telomere loss in DS patients. This might be an individual issue resulting from the mother's metabolic profile (Révész *et al.*, 2014), initial telomere at birth, or environmental factors (Nakamura *et al.*, 2014).

Advanced maternal age is a well-documented risk factor for chromosome 21 nondisjunction in humans, but the understanding of this association at the genetic level is still limited. In particular, the state of maternal genetic age is unclear. Most studies focus on maternal and parental age and maternal telomere length (TL) in the context of DS birth risk (Ghosh *et al.*, 2010; Eisenberg, 2014). Since replicative

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senescence could partially account for aging of the immune system in DS patients and in elderly individuals, TL assessment was proposed as a marker of aging and dementia status of those patients (Vaziri *et al.*, 1993). However, even if telomere shortening is known to be associated with DS, the exact mechanism and association remain unclear. It is still not known which factors can trigger or accelerate this process and when they are turned on.

Materials and Methods

Studied patients and controls

The study group comprised 68 DS children (mean age 4.5 ± 3.1 years; age ranged from 2 to 21 years; 28 boys, 40 girls). All patients were recruited from the Centre for Orthodontic Mini-implants at the Department and Clinic of Maxillofacial Orthopedics and Orthodontics, Poznan University of Medical Sciences in Poznan, Poland. The control group comprised 56 healthy age-matched children (mean age 4.5 ± 3.4 years; 32 boys, 24 girls; age ranged from 2 to 17 years). Both patients and control participants were Caucasians. The study protocol was approved by the ethics committee of the Poznan University of Medical Sciences (97/11), and written consent was provided by the parents of participating minor individuals.

DNA isolation

DNA was extracted from peripheral blood leukocytes using a minicolumn-based DNA isolation kit (A&A Biotechnology, Gdynia, Poland) and stored at -20°C , as previously described (Rubiś *et al.*, 2012). One hundred microliters of peripheral blood were used for isolation of DNA. High-concentration samples of genomic DNA were prepared in decimal concentrations (quantitative polymerase chain reaction [qPCR] standards) to cover all possible measurements range. One hundred microliters of peripheral blood were used for isolation of DNA, and 10 ng of genomic DNA were taken for qPCR.

Real-time PCR

TL was assessed using two pairs of primers, that is, telomere specific and single-copy (*albumin*) gene specific (Cawthon, 2009; O'Callaghan and Fenech, 2011). The specificity of primers has been affirmed by O'Callaghan and Fenech (2011) in conditions providing efficiency close to 100% (2.5 mM MgCl_2 ; 0.5 μM primers) (Table 1). Initial denaturation and polymerase activation (hot start) were

performed at 95°C for 10 min, followed by two cycles of $94^{\circ}\text{C}/15\text{ s}$ and $49^{\circ}\text{C}/15\text{ s}$ without fluorescence acquisition. The signal was detected during another 40 cycles of $94^{\circ}\text{C}/10\text{ s}$, $66^{\circ}\text{C}/10\text{ s}$, and $72^{\circ}\text{C}/10\text{ s}$. Melting analysis ($65\text{--}95^{\circ}\text{C}$ range, 0.2°C resolution) at the end of the reaction was performed to verify the specificity of the product and indicated a T_m of 81.7. The efficiency of the reaction was no lower than 97.8% and not higher than 102%. Importantly, this result was repeatable for all the samples that were analyzed (in serial dilutions). Similarly, reaction conditions for *albumin* were as follows: denaturation at $95^{\circ}\text{C}/10\text{ min}$ —hot start, followed by 45 cycles of $94^{\circ}\text{C}/10\text{ s}$, $61^{\circ}\text{C}/10\text{ s}$, and $72^{\circ}\text{C}/10\text{ s}$. The concentration of primers was 0.5 μM and magnesium chloride was 2.5 mM. The T_m of the product (analysis performed as above) was 80.7 and the efficiency was 99.6%. The TL was assessed using a qPCR system (LC 2.0; Roche) and SybrGreen kit (Roche Diagnostics). Blind quality control samples were included into each qPCR. All samples were run in triplicates (additionally, one sample was repeatedly included in all the studied tests). The results were verified by analysis of four samples using the Southern blot assay, which revealed that the qPCR method is reproducible.

Southern blot analysis

To verify the results obtained by TL real-time PCR, a part of the samples was analyzed by the reference method, Southern blot, as previously described (Kimura *et al.*, 2010). We used a standard Southern blot (TeloTAGGG Telomere Length Assay Kit; Roche Diagnostics) to evaluate the TL assessing the terminal restriction fragment (TRF) according to the manufacturer's protocol. Briefly, 1.5 μg of DNA were digested with restriction enzymes (*HinfI* and *RsaI*), and then, DNA fragments were separated by gel electrophoresis and transferred to a nylon membrane. Subsequently, DIG-labeled probe specific for telomeric sequence was applied and detected using anti-DIG antibody conjugated with HRP. Chemiluminescent visualization of telomeric DNA was conducted with the ChemiDoc XRS system (BioRad) and

TABLE 1. THE COMPOSITION OF THE qPCR MIXTURE FOR AMPLIFICATION OF TELOMERIC REPEATS AND ALBUMIN GENE FRAGMENTS

Reagents	Volume (μl)	Final concentration
Sybr Green reaction mix	1	1 $\times$
Primers F/R	1	0.5 μM each
MgCl_2	0.8	2.5 mM
H_2O	6.2	
DNA	1 (10 ng)	1 ng/ μL
Final volume	10	

F, forward; qPCR, quantitative polymerase chain reaction; R, reverse.

FIG. 1. Comparative analysis of telomere length (TL) assessment using qPCR [AU] and Southern blot analysis [kbp] in a subgroup of DS patients. C, control sample; 1–3, study samples. AU, arbitrary units; DS, down syndrome; qPCR, quantitative polymerase chain reaction.

analyzed using ImageJ to evaluate the mean length of TRF, which refers to the telomere length for each studied sample (Fig. 1). The difference between results obtained with the use of both methods might probably result from the fact that the subtelomeric region is also measured with the Southern blot.

Statistical analysis

Statistical analysis of the results was performed using the Shapiro–Wilk test, Mann–Whitney test, and Student's *t*-test calculated through GraphPad Prism 5. A *p* value of <0.05 was considered statistically significant, $**p < 0.01$, $*p < 0.05$. The error for TL assessment calculated on the basis of qPCR standard curves (serial decimal dilutions of studied samples) was 0.00144 and for *albumin* was 0.00451 (Roche Light-Cycler 2.0 software). Outliers were assessed based on the fit points method, which minimized the differences between replicates, and the range of *C_p* values for individual samples in different runs/replicates did not exceed 0.3.

Results

The relative telomere length (RTL) assessment, performed with the use of qPCR, revealed that the average TL in blood leukocytes from DS patients was significantly longer than in the control subjects (50.46 vs. 40.56, respectively [AU]; $p = 0.0026$; Fig. 2). For normalization of quantitative expression data, a reference single-copy gene *albumin* was used.

Further detailed analysis indicated that there was no significant difference between male and female subjects in the DS group ($p = 0.6492$) (Fig. 3A). Similarly, in the control group, there was no significant difference ($p = 0.0641$) in the TL between male (44.20 [AU]) and female subjects (35.71 [AU]) (Fig. 3B).

The comparative analysis of RTL in evaluated females from both groups revealed that in the studied group the telomere repeats number was 50.127 [AU], whereas in control subjects, it was 35.709 [AU]; $p = 0.0023$ (Fig. 4A). In a similar comparison of TL between males from the DS and control groups, no significant differences were shown ($p = 0.1527$) (Fig. 4B).

FIG. 2. Analysis of relative telomere length (RTL) [AU] in Down syndrome patients (D) and control subjects (C). The assessment was performed with the use of an optimized qPCR. $**p = 0.0026$.

FIG. 3. RTL [AU] according to gender in the DS (A) and control (B) groups. F, female; M, male.

Comparative analysis of TL in DS patients relative to age revealed a trend demonstrating telomere shortening with age, but in the range of studied patients' age, no significant difference was revealed (Fig. 5).

Discussion

Telomeres in DS

DS with its severe symptoms remains untreatable, and unfortunately, we still cannot prevent it or identify the molecular basis of the detailed mechanism of the disease. It is well known that the most common risk factor for DS is maternal age. Interestingly, it was reported that older paternal age at conception (PAC) is also associated with a longer leukocyte telomere length (LTL) in offsprings (Aviv and Susser, 2013). A variety of studies showed that LTL is on an average longer in the offspring of older fathers. It is well established that TL shortens with age in most proliferating tissues. As indicated by Kimura *et al.* (2008), the mean TL in sperm does not decrease. In fact, it increases with the donor's age. The most common explanation for longer TL is the high activity of telomerase in the testes. However, how it affects the TL in offsprings is unclear, especially since they get only half of their chromosomes from sperm (Eisenberg *et al.*, 2012). In the studies performed by Lim *et al.* (2002), 143 mothers who were older than 30 years were enrolled. For this group, there was a

FIG. 4. RTL [AU] in female (A) and male (B) subjects from the studied and control groups. $**p=0.0023$.

statistically significant correlation between maternal age and mean TRF length of the newborns, with older mothers giving birth to newborns with longer telomeres. This might explain why DS children might initially (in childhood) have longer telomeres.

FIG. 5. Distribution of RTL [AU] in blood leukocytes according to the age in DS patients. The study group comprised 68 DS patients (mean age 4.5 ± 3.1 years, age ranged from 2 to 21 years).

Telomeres in leukocytes

Recent studies have shown that longer LTL is associated with reduced atherosclerotic risk in adults and increased survival in the elderly (Aviv and Susser, 2013). In most studies, it was revealed that there was a difference in the average length of telomeres between DS patients (shorter) and control subjects (longer), but the subjects were usually adults. There is no doubt that, as indicated in numerous studies (Vaziri *et al.*, 1993; Zigman, 2013), the rate of telomere loss is indeed greater in DS patients and is even three times faster than in controls.

As demonstrated by Brando *et al.* (2004), telomeres of DS patients shorten by 47% within 40 years. In addition, it was shown that the males lost telomeric DNA at a rate slightly faster than that of females (50 ± 4.2 vs. 40 ± 3.6 bp/year, respectively), but this difference did not reach statistical significance. Thus, it was suggested that telomere shortening might be a biological marker of DS patients' dementia status (Jenkins *et al.*, 2010, 2012). Interestingly, telomeric length in fibroblasts from DS fetuses was shown to be significantly shorter when compared with their control counterparts (Gimeno *et al.*, 2014). This is especially surprising since (i) telomerase should be still active in the fetus and should not let for a decrease in TL and (ii) it would implicate early and vast telomere attrition. This might also suggest that the TL is associated with additional factors.

As we show in juvenile patients, the TL was significantly longer in DS patients than in control subjects. We can hypothesize on several mechanisms that might cause such results. DS is directly related to slowing of cellular metabolism caused by hypothyroidism, which certainly decreases the rate of telomere loss, at least, in children (Purdy *et al.*, 2014). DS patients also showed increased estradiol levels, which might induce the telomerase activity and also affect telomere shortening (Zhao *et al.*, 2011; Zhou *et al.*, 2013). Surprisingly, an increased telomerase activity was reported in young (average age 4.2) DS patients (Abdel-Salam *et al.*, 2013), but not associated with increased TL as shown in older (age ranging from 42 to 80) DS patients (Jenkins *et al.*, 2012). Observation of a high telomerase activity accompanied by telomere loss is regarded as one of the cytogenetic parameters that represent a state of genetic instability (Jenkins *et al.*, 2008). Longer telomeres in our study could be the result of better physical condition of young DS patients compared to adults, which is closely related to the genetic stability. Moreover, DS patients were shown to reveal a higher cortisol level known to inhibit the telomerase activity (Choi *et al.*, 2008). Hormonal disorders in DS are complex, and it seems that the changes in TL are the results of these dysfunctions. The hormonal profile might also be responsible for the differences in TL in a gender-dependent manner. Our study demonstrated that female DS patients had longer telomeres than control subjects ($p=0.0026$).

DS patients often reveal immunological system disorders and immunodeficiency, which are associated with heavy infections and autoimmune diseases (e.g., celiac) or acute leukemia (Kusters *et al.*, 2009). It results in a lowered proliferation of lymphocytes, which might have a protective action on TL. Consequently, TL assessment in leukocytes might reflect dementia and malignancy status and help to

reveal the pathomechanism of DS (Sukeník-Halevy *et al.*, 2011).

Summary and conclusions

There are several limitations to the TL study that must be considered. Variability between individuals in TL measured in peripheral blood leukocytes, especially from DS patients, may be a consequence of major amount of factors that influence blood cell composition. This may be affected by age, sex, hormones, lifestyle, addictions, obesity, and many others. As an individual marker, it is then probably not strong enough. However, when combined with other markers, it might be a significant factor indicating the rate of aging or dementia status. Noteworthy, the TL assessment still requires a golden standard of measurement method since those used so far did not always give comparable results (Gardner *et al.*, 2014). However, there is no doubt that further studies should be conducted to assess this factor that might be indeed recognized as an indicator of DS subjects aging or general condition assessment.

To summarize, there are still a lot of things we do not know about telomere metabolism in DS. However, since their shortening with age is a known fact, they might be used as a marker of senescence and symptoms severity predictive marker. Since there are contradictory data concerning the moment when telomere shortening starts, this mechanism should be carefully studied. It would be also advisable for patients with DS to avoid unhealthy lifestyle habits and characteristics, such as obesity, cigarette smoking, chronic stress, and alcohol intake, which lead to marked telomere shortening.

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Disclosure Statement

No competing financial interests exist.

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