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METHODS MANUSCRIPT

# An improved method for detecting telomere size differences in T-lymphocyte interphases from older people with Down syndrome with and without mild cognitive impairment

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## Abstract

Telomere size (quantified by fluorescence intensity and physical lengths) in short-term T-lymphocyte cultures from adults with Down syndrome (DS) with and without mild cognitive impairment (MCI-DS) or dementia was compared. For these studies, dementia status was determined based on longitudinal assessments employing a battery of cognitive and functional assessments developed to distinguish adult-onset impairment from preexisting developmental disability. In the course of our studies using a MetaSystems Image Analyzer in combination with ISIS software and a Zeiss Axioskop 2, we found that Fluorescein isothiocyanate (FITC) telomere fluorescence referenced to chromosome 2-identified FITC probe fluorescence as a nontelomere standard (telomere/cen2 ratio) showed great promise as a biomarker of early decline associated with Alzheimer's disease (AD) in this high-risk population. We have now obtained a cen (2) CY3 probe that can clearly be distinguished from the blue–green FITC interphase telomere probe, providing a clear distinction between telomere and centromere fluorescence in both interphase and metaphase. We used FITC/CY3 light intensity ratios to compare telomere length in interphases in adults with DS with and without MCI-DS or dementia. Five age-matched female and five age-matched male pairs ($n = 10$) all showed clear evidence of telomere shortening associated with clinical progression of AD ($P < 0.002 - P < 0.000001$), with distributions of mean values for cases and controls showing no overlap. We also examined the time needed for microscopy using interphase versus metaphase fluorescence preparations. With interphase preparations, examination time was reduced by an order of magnitude compared with metaphase preparations, indicating that the methods employed herein have considerable practical promise for translation into broad diagnostic practice.

*Keywords:* Down syndrome; Aging; PNA techniques; cen(2) CY3 and FITC probes

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## Introduction

Down syndrome (DS; Trisomy 21) is the most prevalent chromosomal cause of intellectual disability, with an incidence rate of approximately one in every 690–730 newborns [10]. It is caused by the presence of a third copy of chromosome 21, either in whole (~97% of cases), in part (partial trisomy), or as mosaicism. In previous generations, survival of newborns with DS into middle age was unusual [1, 10]. It produces a phenotype that includes a distinctive appearance and established risks for a broad range of health-related concerns. This includes a dramatically increased risk for Alzheimer's disease (AD), the present focus.

Many studies have documented an increased risk for Alzheimer's disease (AD) among middle aged and older adults with DS [1], and consensus has developed regarding the significance of this association, both directly for this rapidly growing population and for understanding AD pathogenesis more generally. While the devastating consequences of end-stage AD are clearly recognizable, even for individuals with quite severe lifelong cognitive and functional impairments associated with Down syndrome, those impairments complicate early diagnosis. Given that treatments, once developed, will be most effective prior to advanced stage neurological damage, methods are urgently needed to recognize early-stage AD within this extremely vulnerable population.

Within the neuro-typical population developing AD, early clinical declines are characterized as mild cognitive impairment (MCI) [2], and while no consensus standards for MCI have been developed for the population with DS, our group has developed methods for operationally distinguishing "MCI-DS" from both aging, *per se*, and frank dementia [3, 4]. In addition to classifying MCI-DS clinically, we have examined telomere length in short-term T-lymphocytes as a biomarker of dementia status in a series of studies employing a variety of measurement methods, consistently demonstrating telomere shortening associated with cognitive decline [5–9]. While these methods included measures of fluorescence light intensity during interphase using an FITC probe, sensitivity/specificity were improved when we were able to use a cellular-based internal standard, either an FITC probe for chromosome 2 centromeres or a physical measurement of chromosome length excluding telomeric regions. While these methods maximized correspondence with clinical status, they required measurements during metaphase and were extremely labor intensive.

Nevertheless, the high correspondence between these measures of telomere length and clinical status of adults with DS showed considerable promise for this biomarker of AD clinical progression. Therefore, we sought to improve upon efficiency without sacrificing precision. This was accomplished by obtaining a cen (2) CY3 probe that could serve as an internal standard distinct in color from the FITC telomere probe, allowing quantification of telomere fluorescence (reflecting length) in interphase preparations, as shown in Fig. 1. We hypothesized that this method would retain the sensitivity and specificity found in our previous studies employing cellular-based standards while providing substantial savings in microscopy time.

## Materials and methods

Samples of up to 10 ml of the whole blood were collected in a green-topped tube (Becton-Dickinson and Co., Franklin Lakes, NJ, USA, containing 60 USP units of sodium heparin, Cat # 367671) and delivered to our laboratory at room temperature on the same day. All individuals included in the present analyses had full trisomy 21. Samples were coded using a unique participant ID along with the date and general demographics. Institutional Review Board and Institutional Biohazard Committee approval was obtained for all studies and informed consent was obtained from participants or their legally authorized representative prior to sample collection or assessments of cognition, health or functional abilities. Dementia status of each participant was established simultaneously with blood sampling and prior to telomere measurements developed expressly for this purpose and described elsewhere [9]. All cases used in this study were carefully screened for any other diseases that might shorten telomeres, including malignancies. All samples and slides were coded and masked as to clinical status and demographics to avoid any influence of investigator bias on results.

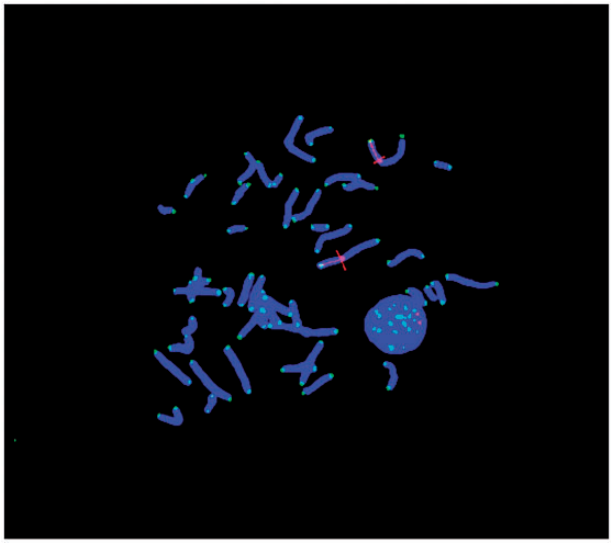

**Figure 1:** PNA CY3 probe on chromosome 2 and in an interphase. A metaphase and an interphase nucleus with telomeres labeled with an FITC telomere probe and with chromosome 2 centromeres hybridized with a CY3 probe. Telomeres appear as blue–green, against the blue background of chromosomes counterstained with DAPI. The chromosome 2 centromeres appear as red. All cases were screened for any other diseases that may shorten telomeres including malignancies.

Short-term cultured T-lymphocytes from each participant were hybridized with an FITC-labeled peptide nucleic acid (PNA) probe, as previously described, to label telomeres [5–13]. In addition, a centromere CY3 PNA probe [a gift for investigational use from Agilent (Santa Clara, CA, USA)/DAKO, Glostrup, Denmark], was used as a nontelomere fluorescent internal standard in interphase. All conditions were at room temperature unless otherwise noted. Briefly, coded slides were air-dried for 48 h. Slides were immersed in 2X saline sodium citrate (2X SSC) buffer (Oncor, Gaithersburg, MD, USA, Cat # 32-804850) for 5 min, then in 2X SSC buffer at 37 °C for 30 min. The dehydration step was achieved by immersion in a cold ethanol series (70%, 85%, and 100%) for 2 min each. Slides were left in a vertical position until dried and then treated with a solution of 20 μl of 1 part PNA cen(2) CY3 probe + 9 parts PNA telomere FITC probe (Agilent, Santa Clara, CA, USA, Telomere FISH Kit, Cat # K5325) that was prepared by a brief vortex and microcentrifuge. The slides were cover-slipped and sealed with rubber-cement (Elmer's, Westerville, OH, USA, Cat # E904) and placed in a preheated slide incubator (Boekel Scientific, Feasterville, PA, USA, slide moat model #24000), adjusted to 80 °C for 5 min. Slides were maintained at relative humidity of 32% in the dark for 4 h for maximum hybridization. The glue and coverslips were carefully removed with slides placed in kit rinse solution for 1 min.

Slides were removed from the rinse solution and placed in the kit pre-heated wash solution for 5 min, with washing temperature maintained at ± 3 °C from a target of 65 °C. Next the slides were immersed in a series of alcohol baths, as above. The slides were air-dried vertically in a darkened biohazard chemical fume hood for ½ h. Afterwards, 20 μl of 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Abbott Molecular, Des Plaines, IL, USA), mounting solution was pipetted onto each slide then covered with a 24 × 50 mm coverslip, with the top and bottom edges sealed with rubber cement. The covered slides were wrapped in aluminum foil and placed in a 20°C freezer for 1–2 days. Twenty (20) interphase cells per sample were randomly selected and then digitized for analysis using a Zeiss Axioskop 2 plus fluorescence microscope (Oberkochen, GER) installed with Metasystem software Platform and ISIS version 5.2 (Newton, MA, USA). Interphase cells should be clearly visible at low power (×10) using a DAPI filter, providing easily visible fluorescent signals within the interphase nuclei. Images were recorded using a high-power oil immersion objective (100×) for visualization of the fluorescent signal and photography. A 100-watt mercury lamp light source was used. Suitable excitation/emission filters were chosen to visualize the DAPI (360 nm/460 nm), FITC (490 nm/525 nm), and CY3 (358 nm/461 nm). Effort was taken to expose the sample to as little of excitation light as possible to minimize fluorescent fading. For each cell within each sample, total FITC and CY3 fluorescence was recorded and a ratio was calculated (FITC/CY3). These 20 values per individual provided the data that were related to clinical status.

A second set of 20 metaphase cells per sample were examined for a subset of eight individuals (pairs 3, 4, 8, and 9 in Table 1) to provide a comparison of evaluation time. Methods of evaluation for these metaphase preparations have been described previously (the only change being the use of the CY3 rather than a FITC centromere probe as an internal standard). Here, total time to collect data for 20 cells per case was recorded. Note that analysis of multiple cells per sample is a necessity for any procedure employing fluorescence markers of the type used here. Individual cells and nuclei vary in the position and orientation within the cytoplasm's three-dimensional space, and the specifics of this positioning invariably affects observed fluorescence for any individual cell. This contributes to the substantial within-sample variability, as indicated in Table 1, and is best controlled by representative sampling of multiple cells within each sample.

**Table 1:** Mean ratio of FITC telomere fluorescence/CY3 cen (2) fluorescence for 20 interphase cells (T-lymphocytes) for individual adults with DS with and without MCI-DS or dementia

| Pair | Case No. | Sex | Age* | Status | FITC/CY3*** | SD | P |
|---|---|---|---|---|---|---|---|
| 1 | gDs 0178 | F | 60.5 | Control | 3.42 | 3.16 | 0.000085 |
| | nDs 1707 | F | 52.3 | MCI-DS | 0.30 | 0.30 | |
| 2 | nDs 0752 | F | 61.1 | Control | 3.09 | 2.47 | 0.0009 |
| | nDs 0738 | F | 57.1 | MCI-DS | **0.83** | 0.81 | |
| 3 | eDs 1721 | F | 49.4 | Control | 4.82 | 4.41 | 0.00025 |
| | eDs 1882 | F | 48.5 | MCI-DS | 0.76 | 0.83 | |
| 4 | eDs 0725 | F | 62.1 | Control | 6.78 | 2.23 | <0.000001 |
| | eDs 0621 | F | 64.2 | Dementia | 0.81 | 0.65 | |
| 5 | nDs 0871 | F | 61.6 | Control | 2.08 | 1.17 | 0.0001 |
| | nDs1912 | F | 55.9 | MCI-DS | 0.41 | 0.40 | |
| 6 | gDs 1523 | M | 58.3 | Control | 2.20 | 1.52 | 0.000063 |
| | nDs 1621 | M | 53.8 | MCI-DS | 0.63 | 0.27 | |
| 7 | nDs 0033 | M | 77.9 | Control | 3.14 | 2.02 | 0.0000005 |
| | gDs 0792 | M | 63.8 | MCI-DS | 0.67 | 0.45 | |
| 8 | nDs 1863 | M | 60.7 | Control | 3.76 | 4.29 | 0.002 |
| | nDs 0930 | M | 58.9 | MCI-DS | 0.51 | 0.54 | |
| 9 | nDs 1812 | M | 60.9 | Control | **1.61** | 0.71 | <0.000001 |
| | nDs 1511 | M | 59.5 | MCI-DS | 0.43 | 0.44 | |
| 10 | nDS 1619 | M | 59.3 | Control | 1.93 | 1.45 | 0.0002 |
| | nDs 1826 | M | 57.7 | MCI-DS | 0.56 | 0.24 | |

*Note*: Bolded values indicate the highest MCI-DS value and the lowest control value. Age*—represents the age of subject at specific time blood was drawn. Status**—controls have not developed indications of clinical AD progression; MCI-DS indicated some decline in cognition with qualitative parallels to MCI in adults without intellectual disability; Dementia indicates clear declines in cognition and functional abilities. Mean ratio***—indicates the FITC Intensity value × $10^4$ measured in fluorescence intensity units/CY3 Intensity value × $10^4$ measured in fluorescence intensity.

## Results and discussion

Several approaches were employed to determine the strength of association between telomere length and clinical status. First, we treated matched pairs of individuals as individual replications of a series of individual comparisons using a series of independent *t*-tests. All showed that telomere length was shorter for the pair member developing AD-associated decline in clinical status (Table 1). Second, the mean ratio value for each individual provided the data for an overall comparison of the two groups (with decline in clinical status versus without). A *t*-test for dependent samples confirmed that this difference was highly significant [$t(9) = 5.76$, $P < 0.0003$] replicating our previously reported findings of high correspondence between telomere length and clinical status. Third, acknowledging the imperfect matching of pairs with and without indications of AD clinical progression, an analysis of covariance was conducted with Group and Sex as independent variables and Age as a covariate. Again, the effect of group was highly significant and no other effect approached significance, $F(1,16) = 25.5$, $P < 0.0002$, and verify the usefulness of the CY3 probe as an internal reference for quantification of telomere length.

The magnitude of the overall group difference, with no overlap between the two within-group distributions, represents especially strong evidence of the utility of telomere length as a biomarker of early AD progression within this population and perhaps others. (Note the bolded values in Table 1 indicating the highest MCI-DS value and the lowest control value.)

Having confirmed that the exceptionally high correspondence between telomere length and clinical status was maintained with the shift to analysis of cells at interphase, an additional analysis focused on the savings in time required for microscopy.

Measurements of 20 interphase cells compared with 20 metaphase cells in a subsample of four pairs (#s 3, 4, 8, and 9) revealed an enormous and highly significant difference, $F(1,3) = 5{,}169.2$, $P < 0.00001$. Examination time for metaphase preparations ranged from 5 to 5.5 h while corresponding times for interphase preparations ranged from 32 to 40 min, an order of magnitude difference.

Results suggest the usefulness of adding the CY3 PNA probe as a nontelomere fluorescence reference in studies of telomere length in this and perhaps other populations. As indicated in Table 1, however, this was a small-scale study and considerable variability was observed among controls. It would be desirable to identify the source of this variability and to see these findings replicated in a considerably larger sample prior to translation into practice. We plan to address this concern in future work and are anxious to see if other labs can obtain the same or similar results.

If these findings continue to hold up to replication, translation into practice would improve diagnostic accuracy for

individuals with DS suspected of developing AD, and would contribute to improved planning to address support needs. Further, if these methods prove to provide a valid biomarker for early AD progression in other populations, they could improve the accuracy of differential diagnosis and guide treatment selection.

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**Authors' contributions: E.C.**

J.—project design and analysis of data and conclusions drawn;
L.Y.—laboratory studies, fluorescent microscopy, data maintenance and analysis of data;
E.M.—laboratory studies, fluorescent microscopy, data maintenance and analysis of data;
S.K.-M.—recruitment, psychological testing and analysis of data;
W.Z.—psychological testing and analysis;
N.S.—epidemiology and statistical analysis;
W.S.—Program Project Director, psychological design and analysis and interpretation of data and conclusions drawn.

*Conflict of interest statement*. None declared.

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