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**Original**

# Telomeres on chromosome 21 and aging in lymphocytes and gingival fibroblasts from individuals with Down syndrome

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**Abstract: Progressive chromosome 21 loss in individuals with trisomy 21 or Down syndrome (DS) is supposedly related to their premature senescence. In addition, the telomere hypothesis of cellular aging involving telomere shortening in normal and accelerated aging *in vivo* and *in vitro* is well documented. This study investigated the integrity of two chromosome 21 regions (the 21q telomere and the 21q22.13-q22.2 region) and their relationship with aging by means of fluorescence in situ hybridization (FISH) in lymphocytes and gingival fibroblasts cells. The use of tissues from different germ layers allows detection of mosaicism. Chromosome variations in tissue from the neuroectoderm layer could explain the variable phenotype of DS. This approach is original in the literature. Lymphocyte and gingival fibroblast nuclei from 18 affected individuals aged 5-54 years were analyzed. Although not significant (*P* = 0.06), analysis from 11 tissue-matched individuals as well as the comparison between lymphocytes and fibroblasts from different subjects (*P* = 0.05) suggested that lymphocyte cells are more likely to miss 21q telomere signals. Hence, gingival fibroblasts are probably capable of more efficient cell repair, and the occurrence of mosaicism is more related to cell proliferation than to germ layer origin. Investigation of the 21q22.13-q22.2 region from six tissue-matched individuals and from different DS patients revealed no significant differences between the tissues. (J. Oral Sci. 46 , 171-177, 2004)**

Key words: telomere; Down syndrome; aging; lymphocyte; gingival fibroblast.

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## Introduction

In adult individuals with trisomy 21 or Down syndrome (DS) most of the clinical signs associated with normal aging - such as sensorial, motor and epidermic alterations - occur prematurely. These individuals show premature cognitive decline and generally develop the neuro-pathological signs of Alzheimer's disease.

An age-related unidirectional chromosome 21 loss has been observed by cytogenetic analysis in lymphocyte cultures from Down syndrome individuals (1). These losses were referred to as "occult mosaicism". In our laboratory, we confirmed these observations and verified an age-related increase in the frequency of polyploid cells

in Down syndrome (2).

In binucleated cells from 12 DS patients aged 0.01 - 8.9 years, a higher frequency of malsegregation of chromosome 21 in trisomic cells was observed than in disomic cells from 20 age-matched controls. The frequency of nondisjunction was higher than that of chromosome loss in both trisomic and disomic cells (3).

It is well known that there is progressive telomere shortening in cells from individuals with normal and accelerated aging both *in vitro* and *in vivo*. A telomere hypothesis of cellular aging has been proposed, where the loss of telomere sequences provides the signal for cells to enter into senescence, leading to the formation of dicentric chromosomes and classical breakage-fusion-bridge cycles followed by genome rearrangements and aneuploidy (4). Hence, loss of chromosome 21 telomere sequences could be involved in the origin of chromosome aneuploidy.

Significantly higher loss of telomere sequences was observed in a series of 21 Down syndrome individuals (aged 0-45 years old) compared with age-matched controls through the use of terminal restriction fragments (TRF) containing TTAGGG repeats (5).

The present research investigated the presence of chromosome 21q telomeres and of 21q22.13-q22.2 regions by FISH and their relationship with aging in two different tissues from DS individuals. Study of tissues from different germ layers allows the detection of mosaicism and could explain the variable DS phenotype.

## Methods

The sample was made up of 18 patients with Down syndrome aged 5 to 54 years. They were selected from a Dental Care Center for Exceptional Individuals supervised by the Dental School in Araçatuba, São Paulo, Universidade Estadual Paulista. Healthy young matched controls and elderly healthy controls were also investigated. We considered that as Down syndrome individuals show premature aging, we should compare them with elderly healthy controls, with ages varying from 69 to 87 years. The Research Ethics Committee of the Universidade Federal de São Paulo (UNIFESP) approved this study and all participants gave their informed consent according to the Helsinki Declaration. We performed karyotype analysis on lymphocytes from the individuals and selected only those with three distinct copies of chromosome 21, identified in 15 cells by a G-banding technique (6). Gingival cells were obtained from material arising out of dental treatment. Peripheral blood lymphocytes were cultivated for 72 h in RPMI 640 medium supplemented with 20% calf serum and stimulated with phytohemagglutinin P according to a modified technique (7). Fibroblast gingival cells were cultured in alpha-MEM with 20% calf serum according to a modified AGT technique (8).

Interphase cells were hybridized with commercial DNA probes: TELVYSION 21Q TELOMERE SPECTRUM ORANGE (VYSIS, IL, USA), 45 kb, corresponding to the 21q telomere region and the LSI 21 SPECTRUM ORANGE PROBE (VYSIS, IL, USA), 200 kb, corresponding to the 21q22.13-21q22.2 chromosome region (LSI-21 probe).

The FISH procedures were performed according to modified protocols (9).

For the interphase analysis of fluorescent hybridization signals with the 21q telomere probe, we scored 100 lymphocyte nuclei per individual in DS patients, as well as in fibroblast gingival cells. Lymphocytes and fibroblast cells of 11 tissue-matched DS subjects were also analyzed. In addition, cells from young and older controls were recorded.

The scoring of signals using the LSI-21 probe was performed on 200 - 300 lymphocytes and on 200 - 300 fibroblast cells from 11 and 10 DS individuals respectively. Lymphocyte and fibroblast cells from six tissue-matched DS subjects were analyzed using this probe.

Only nuclei displaying one, two or three hybridization signals were recorded.

Scoring was done according to the following criteria: a nucleus was registered as having more than one FISH signal if the signals were of similar size and intensity and separated by a distance of more than one FISH signal diameter (3).

All cell samples were examined under an Axioplan Zeiss Imaging System equipped with appropriate filters (Fig. 1).

Linear regression analysis was used to evaluate the correlation between age and frequency of missing FISH signals using the two probes. Pearson's correlation coefficient and the non-parametric Wilcoxon test were used to compare the two cell tissues from the same individual. Statistical analysis was done using Excel and the SPSS software package and references (10).

## Results

Table 1 shows the frequency of missing FISH signals with the two probes in patients and controls.

No significant differences were observed using linear regression analysis ($P > 0.05$). However, there was a slight tendency for increased missing signals with increasing age in the regression line (Fig. 2).

Eleven tissue-matched patients using the 21q telomere probe were compared (Table 1) and did not show any

significant difference ($P = 0.06$). The comparison between lymphocytes from 17 subjects and fibroblasts from 12 DS patients (data not shown) showed no significant difference ($P = 0.05$).

The comparison of lymphocytes and fibroblast nuclei signals from six tissue-matched individuals using the LSI-21 probe showed no statistically significant difference (Table 1). Lymphocytes from 10 DS patients and fibroblasts from 11 subjects did not show any significant difference (data not shown).

No significant differences were observed using linear regression analysis for young and elderly control groups ($P > 0.05$).

Pearson's correlation coefficients between the two tissues were r = 0.162 and r = -0.06 for the 21q telomere and LSI-21 probes respectively, showing a low correlation. Pearson's correlation coefficients between the two tissues for the control group were r = 0.73 for probe 21qtel and r = 0.72 for the LSI-21probe.

## Discussion

Fluorescence *in situ* hybridization (FISH) with chromosome-specific probes allows analysis of chromosomes or chromosome regions in interphase nuclei. This investigation makes it possible to screen a much larger population of cells from various tissues for aneuploidy and mosaicism (1).

FISH analysis of interphase nuclei in tissues from

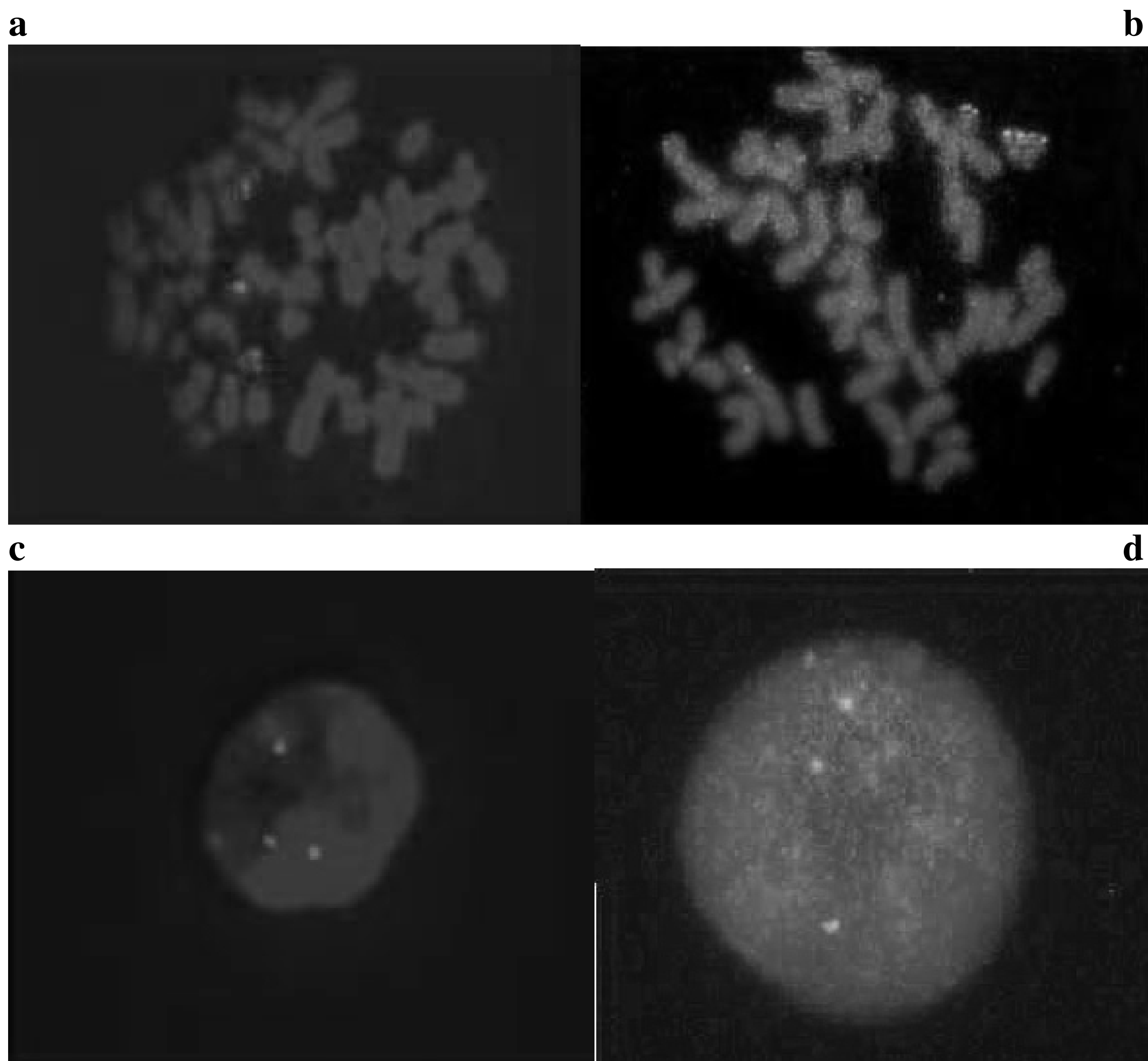



Fig. 1 Metaphase and interphase nuclei with three FISH fluorescent signals in the chromosomal regions 21q22.13-q22.2 (a; c) and 21q telomere (b; d) from Down syndrome individuals.

Table 1 Frequency of missing FISH signals for the 21qtel and LSI 21 probes

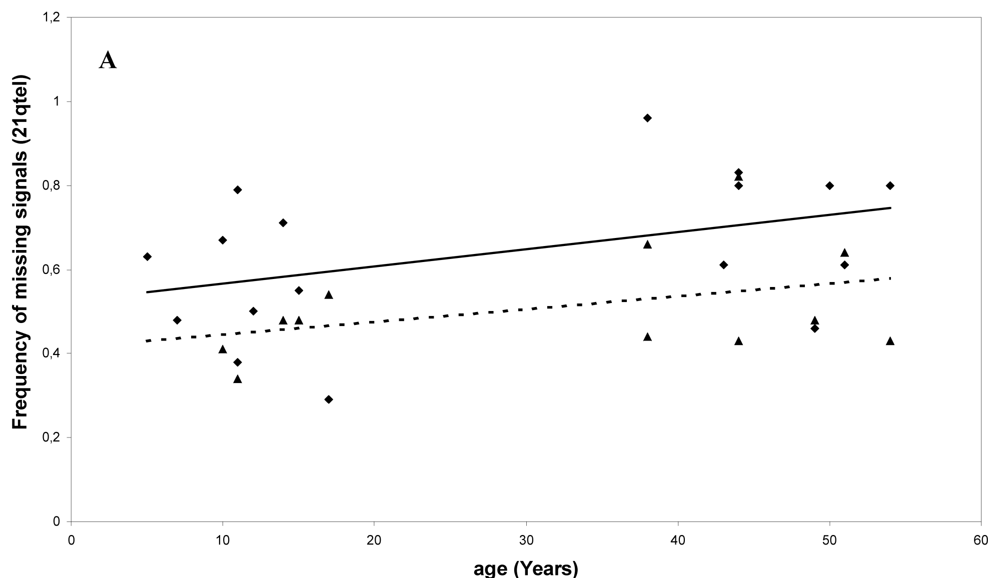
| Patient (Down syndrome) | Age | 21qtel probe Interphase lymphocytes | 21qtel probe Interphase fibroblasts | LSI 21 probe Interphase lymphocytes | LSI 21 probe Interphase fibroblasts |
|---|---|---|---|---|---|
| DS03 | 10 | 0.67 | 0.41 | | |
| DS05 | 11 | 0.38 | 0.34 | 0.125 | 0.1 |
| DS07 | 14 | 0.71 | 0.48 | 0.085 | 0.135 |
| DS08 | 15 | 0.55 | 0.48 | | |
| DS09 | 17 | 0.29 | 0.54 | 0.144 | 0.165 |
| DS11 | 38 | 0.96 | 0.44 | | |
| DS13 | 44 | 0.83 | 0.82 | 0.147 | 0.120 |
| DS14 | 44 | 0.8 | 0.43 | 0.110 | 0.089 |
| DS15 | 49 | 0.46 | 0.48 | 0.113 | 0.225 |
| DS17 | 51 | 0.61 | 0.64 | | |
| DS18 | 54 | 0.8 | 0.43 | | |
| Mean ± SD | | 0.64 ± 0.18 | 0.51 ± 0.13 | 0.12 ± 0.02 | 0.14 ± 0.05 |
| Wilcoxon test: | | $P \geq 0.06$ ($\alpha = 0.05$) | | $P \geq 0.26$ ($\alpha = 0.05$) | |
| | | | r = 0.162 | | r = -0.06 |
| Controls | | | | | |
| | 08 | 0.14 | 0.07 | 0.03 | 0.07 |
| | 11 | 0.19 | 0.12 | 0.02 | 0.09 |
| | 14 | 0.17 | 0.15 | | |
| | 15 | 0.11 | 0.09 | 0.03 | 0.10 |
| | 17 | 0.12 | 0.12 | | |
| | 69 | 0.15 | 0.11 | 0.05 | 0.08 |
| | 71 | 0.20 | 0.16 | | |
| | 72 | 0.28 | 0.17 | 0.01 | 0.11 |
| | 75 | 0.14 | 0.10 | | |
| | 83 | 0.24 | 0.13 | | |
| | 87 | 0.12 | 0.11 | 0.02 | 0.05 |
| Mean + SD | | 0.169 ± 0.05 | 0.13 ± 0.003 | 0.03 ± 0.001 | 0.083 ± 0.002 |

different germ layers, such as lymphocytes from mesoderm and gingival fibroblast cells from neuroectoderm, allows the evaluation of mosaicism in brain-related tissue as a second tissue.

The use of fibroblast cultures from material obtained during routine dental treatment is an easy way to obtain a second tissue sample for mosaicism investigation and presents many practical advantages.

Many different technical and cellular factors including DNA probe length, overlapping signals, cross-hybridization, cells in the G2 cell cycle stage or strong counterstaining (11) could influence the visualization and scoring of the fluorescence signals, leading to false negative or false positive results, but these factors would influence the results of FISH randomly, being tissue- and age-independent.

As expected, the frequency of missing FISH signals using probe LSI-21 showed greater similarity and less variable results between lymphocyte and fibroblast nuclei, probably as a result of greater DNA probe length.




regression - LSI21 probe

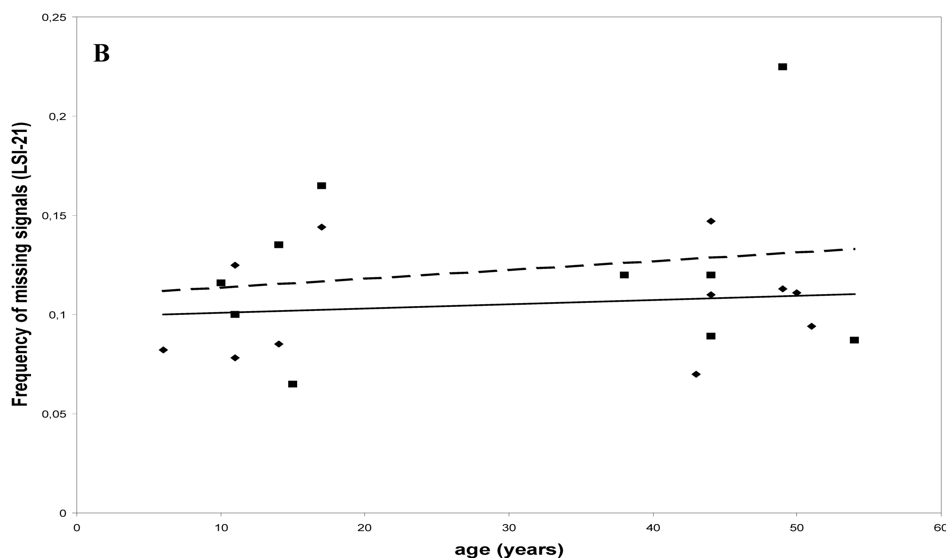



Fig. 2 Distribution of frequencies of missing signals by individual age.

A Relationship between age and frequency of missing FISH signals using 21q telomere probe in lymphocyte and fibroblasts nuclei. Solid line: lymphocytes regression (y = 0.525+ 0.004 x); dot line: fibroblasts regression (y = 0.416+ 0.0030 x). ◆: lymphocytes, ■: fibroblasts

B Relationship between age and frequency of missing FISH signals using LSI 21 probe in interphase lymphocytes and interphase fibroblasts. Solid line: lymphocytes regression (y = 0.0988 - 0.0002 x); dashed line: fibroblasts regression (y = 0.1091 + 0.0004 x).
◆: lymphocytes, ■: fibroblasts

A slight, but not significant increase in missing FISH signals with increasing age was observed with probe 21qtel.

The Wilcoxon analysis for comparison of 11 tissue-matched DS patients in relation to 21q telomere signals revealed no significant differences at $P > 0.06$ (Table 1). The comparison between the two tissues from different individuals did not show significant differences at $P = 0.05$. Thus, we suggest that lymphocytes are more likely to miss FISH 21q telomere signals than gingival cells. We propose that these differences could be a consequence of the telomere's role in controlling the number of cell

divisions in tissues with different proliferation rates.

Some studies have demonstrated that proliferation of gingival fibroblasts does not appear to be age-related, probably because their microenvironment is continually moistened by saliva (12). Many growth factors have been identified in human saliva, such as basic fibroblast growth factor, insulin growth factor and epidermal growth factor, which are involved in cell proliferation, cell repair, migration, differentiation and anti-apoptosis activity (13-16). On the other hand, an age-related decrease of basic fibroblast growth factor in human saliva has been identified (17).

A reduction in the frequency of aneuploidy in cortical explants cultured in medium containing fibroblast growth factor 2 has been observed (18), indicating that the frequency of aneuploid cells could be experimentally reduced by growth conditions.

An investigation of chromosome telomere integrity in multiple tissues from subjects at different ages detected significant differences between a 20-week fetus and a 72-year-old male (19). The 72-year-old male showed the shortest telomeres and the most variation in telomere size for all tissues, but the greatest differences were observed in blood cells.

Therefore, we suggest that blood cells are more likely to lose 21q telomere sequences than gingival cells probably as a result of their higher proliferation rate, and that the occurrence of mosaicism is more related to cell proliferation than to germ layer origin.

On the other hand, our results did not show any differences in the levels of chromosome 21 aneuploidy or mosaicism between lymphocytes and gingival fibroblasts.

Key points of this present work were therefore summarized as follows; 1) The frequency of FISH signals was investigated using 21q telomere and 21q22.13-q22.2 probes in two different germ layer tissues – lymphocytes and gingival cells – from individuals with Down syndrome. 2) Comparison between lymphocytes and gingival fibroblasts from different subjects as well as between 11 tissue-matched Down syndrome individuals using the 21q telomere probe suggested that lymphocyte cells are more likely to have missing FISH signals. 3) Analysis of six tissue-matched DS patients with the 21q22.13-q22.2 probe as well as comparison between FISH signals in the two tissues from different individuals revealed no significant differences.

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