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Telomere length in Hutchinson-Gilford Progeria Syndrome

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ABSTRACT

Hutchinson-Gilford Progeria Syndrome (HGPS) is a rare premature aging disorder caused by mutations in the gene *LMNA*, which encodes the nuclear matrix protein lamin A. Previous research has shown that the average telomere length in fibroblasts from HGPS patients is shorter than in age-matched controls. How mutations in lamin A lead to shortened telomere lengths is not known nor is the contribution of individual chromosome ends to the low average length understood. To measure the telomere length of individual chromosomes, we used Quantitative Fluorescence in situ Hybridization (Q-FISH). In agreement with previous studies, we found that the average telomere length in HPGS fibroblasts is greatly reduced; however, the telomere length at chromosome ends was variable. In contrast, the telomere length in hematopoietic cells which typically do not express lamin A, was within the normal range for three out of four HGPS patient samples. Our results suggest that mutant lamin A decreases telomere length via a direct effect and that expression of mutant *LMNA* is necessary for telomere loss in HGPS.

1. Introduction

Hutchinson-Gilford Progeria Syndrome (HPGS) is a segmental premature aging disease which manifests in the first 2 years of life (Pollex and Hegele, 2004). Symptoms include postnatal growth restriction, loss of hair and subcutaneous fat, decreased joint mobility, and atherosclerosis (Hennekam, 2006). Patients die at a mean age of 12.6 years from progressive atherosclerosis of the coronary and cerebrovascular arteries leading to heart attacks and strokes (Baker et al., 1981; Hennekam, 2006). Cognitive development is normal and no increase in cancer incidence has been observed (Hennekam, 2006). In about 90% of cases, HGPS is caused by a C → T mutation at nucleotide 1824 in exon 11 of the lamin A/C gene (*LMNA*) which activates a cryptic splice site in the mRNA (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). This results in the translation of a protein with a 50 amino acid deletion near the C-terminus (Eriksson et al., 2003). The deletion includes a cleavage recognition site that is required for complete processing to the mature form of lamin A (Eriksson et al., 2003).

The two classes of nuclear lamin proteins, A-type and B-type, are involved in many important nuclear functions including DNA replication, transcription, chromatin organization, nuclear shape and nuclear position in the cell (Zastrow et al., 2004). The two predominant A-type lamins, A and C, are expressed by alternative splicing of the *LMNA* mRNA (Zastrow et al., 2004). Lamin A and C are expressed in all differentiated tissues except for some hematopoietic lineages including CD20-positive B lymphocytes, CD3-positive T lymphocytes as well as neuroendocrine cells (Broers et al., 1997; Jansen et al., 1997). Lamin A interacts with many components of the nucleus including other lamins, lamin associated protein 2α (LAP2α), and actin as well as DNA and histones and potentially serves as a scaffolding network for multiprotein complexes (Zastrow et al., 2004).

HGPS resembles aging at the cellular level as well. The mutant protein, often referred to as 'progerin', remains farnesylated at the C-terminus due to the lack of protein processing (Eriksson et al., 2003). This likely causes most of the cellular defects including lobulation of the nuclear membrane, diminished replication potential, slow DNA damage response and abnormal chromatin organization (Goldman et al., 2004; Liu et al., 2005). It has also observed that telomere length is shorter in HGPS fibroblasts when compared to age-matched controls (Allsopp et al., 1992).

Telomeres are repetitive G-rich DNA sequences and associated binding proteins found at the ends of linear eukaryotic chromosomes. They are key in preventing genomic instability (Blackburn, 2001; de Lange, 2005). The telomere binding proteins aid in forming a protective structure which 'caps' chromosome ends and prevent their processing as double strand breaks (de Lange, 2005). Telomeres shorten with each cell division, *in vivo* and *in vitro*, due

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to the 'end replication problem' as well as sporadic losses following damage or replication errors (Lansdorp, 2005). Consequently, telomeres from elderly donors are much shorter that those from young donors. When telomeres reach a critical length, cells either stop dividing (senesce) or undergo apoptosis (Stewart and Weinberg, 2006). Limitations in the replicative potential of cells imposed by telomere shortening may restrict the proliferation of abnormal cells; however, progressive telomere attrition also contributes to the loss of cells and tissue function with age (Aubert and Lansdorp, 2008).

In order to better understand the role of telomeres in HGPS, we examined the telomere length in cells from HGPS patients using two approaches. First, to determine how telomere lengths of individual chromosomes vary in fibroblasts of HGPS patients, we quantified telomere lengths using quantitative fluorescence in situ hybridization (Q-FISH) (Poon and Lansdorp, 2001). We show that telomere length is significantly shorter in HGPS cells; however no particular chromosome had consistently short or long telomeres. Second, to determine a causative role for lamin A in telomere shortening in HGPS patients, we used flow-FISH (Baerlocher et al., 2006) to examine telomere length in hematopoietic cells, which do not express lamin A (Baerlocher et al., 2006; Broers et al., 1997). We show that telomere length is in the normal range for three out of four HGPS patients examined. These results suggest that mutant lamin A is directly involved in the generation of short telomeres.

2. Materials and methods

2.1. Cell lines and patient samples

The HGPS cell lines AG03513, AG06297 and AG11498 were obtained from the NIA Aging Cell Repository (Coriell Cell Repository, Camden, NJ). Cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 15% fetal calf serum, 200 μM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 atmosphere incubator. At the time experiments were preformed, the HGPS cell lines were at the following population doublings: AG03513 – PD 17, AG06297 – PD 35 and AG11498 – PD 7. The six healthy control fibroblast cell lines that were used in the telomere length studies have been previously reported in (Martens et al., 1998). Blood samples were drawn from patients diagnosed with classical HGPS after informed consent from patients and their parents (Merideth et al., 2008). Blood was drawn in Heparin or EDTA tubes and shipped at room temperature. Upon arrival blood samples were frozen until analysis. Controls for Flow-FISH were 400 healthy persons ranging from birth to 100 years of ages (Yamaguchi et al., 2005).

2.2. Quantitative fluorescence in situ hybridization

Q-FISH was preformed as previously described (Poon and Lansdorp, 2001). Briefly, metaphase cells were harvested, fixed with methanol–acetic acid then dropped onto slides. Slides were fixed with formaldehyde, treated with pepsin, and dehydrated with ethanol. The hybridization mix containing the Cy3-labeled $(CCCTAA)_3$ peptide nucleic acid telomere probe was added to each slide which was then denatured at 80 °C for 2 min prior to incubation at room temperature for 1 h. Slides were washed, counterstained with DAPI then mounted using DABCO. Images were acquired and analyzed as described.

2.3. RNA extraction and RT-PCR

RNA was extracted from BJ neonatal human foreskin fibroblasts, and T cells and granulocytes from control samples using the RNAeasy kit (Qiagen). A reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay was performed with the isolated RNA to make complementary DNA (cDNA). The cDNA was amplified with either primers for *LMNA* (forward: CAAGGCATCTGCCAGCGG and reverse: TTTCTTTGGCTTCAAGCCCC) or *β-actin* (forward: AGAGATGGCCACGGCTGCTTC and reverse: GCATTTGCGGTGGACGATGGAG). PCR products were visualized on a 1.5% agarose gel by ethidium bromide staining.

2.4. Flow-FISH

Details of the Flow-FISH method are as previously described (Baerlocher et al., 2006). Briefly, red blood cells from samples were lysed with NH_4Cl. Leukocytes were denatured in formamide at 87 °C, hybridized with a fluorescein-conjugated $(CCCTAA)_3$ peptide nucleic acid probe, and counterstained with LDS751 DNA dye. Analysis of fluorescence was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The cell types analyzed included total leukocytes, granulocytes, total lymphocytes, CD45RA-positive/CD20-negative naive T cells, CD45RA-negative memory T cells, CD20-positive B cells, and CD57-positive NK/NKT cells. Bovine thymocytes were used as an internal standard in every sample. Cellquest Pro software (BD Biosciences, CA, USA) was used to quantify the flow cytometry results; median telomere lengths were calculated using an automated Microsoft Excel calculator.

2.5. Statistical analysis

HGPS samples were compared to control samples using an independent *t*-test, assuming unequal variance. A *p*-value of <0.005 was considered significant.

3. Results

3.1. Telomere length in HGPS fibroblasts

To investigate the nature of telomere shortening in HGPS, Q-FISH was performed on metaphase chromosome spreads of three HGPS primary fibroblast cell lines derived from biopsies of three different patients (Fig. 1). The fibroblast cell lines from HGPS patients were grown using standard culture conditions. Cells from early passage cultures were arrested in metaphase and used for telomere length analysis. At least twelve metaphases were analyzed for each cell line. No significant chromosomal abnormalities such as fusions, translocations or aneuploidy were detected in the metaphases from HGPS patients (data not shown).

The samples AG11498 and AG06297 have the common 1824 C → T mutation; however, the mutation status of AG03513 is unknown although the patient had classical HGPS symptoms. Telomere length in AG06297 and AG03513 were significantly shorter than the control samples and AG11498 was around the same length as the control samples and is short for the age of the patient based on data from Allsopp et al., 1992 (Table 1) (Martens et al., 2000). While AG11498 was not shorter than control cells it should be noted that the average age of the control cell lines was 50 years which likely accounts for this. Telomere lengths were variable between different chromosome ends as well as between samples (Figs. 2 and 3). Signal free ends, representing telomeres that are too short to detect by Q-FISH, were observed in the HGPS samples but not in the normal fibroblast metaphases (Table 1) (Fig. 1, white asterisks).

We found no evidence for consistent biased shortening of any particular chromosome end between samples, although all samples exhibited some extremely short chromosomes including chromosome 18q in AG06297 (Fig. 2 and Supplementary Figs. 1 and 2). The telomeres of chromosome 17p, which have been previously reported to be among the shortest (Britt-Compton et al., 2006; Martens et al., 1998), were below the mean telomere length in all HGPS samples however in AG11498 it was not statistically different from the mean telomere length for that sample (Fig. 3). In addition, the telomeres of chromosome 4q, often one of the longest telomeres and always above the mean in normal individuals (Martens et al., 1998), was only significantly different than the mean telomere length in AG03513.

In the control fibroblast samples, the average telomere length of chromosome 18, which is gene poor, were above the total mean telomere length. The average telomere length of chromosome 19, which is gene-rich, was always found to be below the mean telomere length in the control samples. In the HGPS samples, the telomere length of chromosomes 18 and 19 was more similar to each other than they were in control samples. In some cases, such as chromosome 18q in sample AG06297, chromosomes were found to be on the opposite side of the mean from usual (Figs. 2 and 3). In summary, chromosome-specific differences in telomere length observed in cells from normal control individuals were not preserved in all HGPS cell lines. This suggests a much more random chromatin arrangement in the HGPS cells.