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Quantitative fluorescence *in situ* hybridization for investigation of telomere length dynamics in the pituitary gland using samples from 128 autopsied patients

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ABSTRACT

In order to investigate the population dynamics of telomere status, we measured the telomere lengths of glandular cells in the adenohypophysis (AH) and pituicytes, a type of glial cell, in the neurohypophysis (NH) of 128 autopsied humans (65 men, 63 women, 0 and 102 years) using our original quantitative fluorescence *in situ* hybridization (Q-FISH) method. Telomeres in the AH shortened with aging in both men and women, but those of pituicytes did not. Pituicyte telomeres were significantly longer in women than in men. The data suggest that telomeres shorten with age in the AH, whereas those in pituicytes maintain a constant length throughout life.

Comparison of pituicyte telomere lengths among 5 generations, < 18, 18–69, 70–79, 80–89, and ≥ 90 years, revealed a tendency for telomeres to be longer in individuals in their 80 s and 90 s than in those in their 70 s. These findings lend support to the widely held notion that humans with longer telomeres may have a longer life span, and shed light on the biology of pituitary gland in terms of telomere length dynamics, as well contributing to the development of bioengineered hormone-producing cell replacement strategies and regenerative therapies.

1. Introduction

Human telomeres consist of repetitive G-rich DNA sequences and associated binding proteins located at the ends of linear eukaryotic chromosomes, apparently playing a key role in prevention of chromosomal and genomic instability (Blackburn, 2001; de Lange, 2005). Progression of telomere shortening with aging may lead to genomic instability during the initial stage of tumorigenesis (DePinho, 2000; Londono-Vallejo, 2008). Telomere biology is an interesting and new field that is evolving rapidly, but potential variations and pitfalls associated with the different methods used for measuring telomere length have been pointed out, and recently a successful 3D technique for measurement of telomere length and steric configuration has been reported, together with its prognostic implications (Aubert et al., 2012; Aubert, 2014; Knecht et al., 2012; Louis et al., 2005). Using Southern blotting (Takubo et al., 1999 and 2002) and quantitative fluorescence *in situ* hybridization (Q-FISH) (Aida et al., 2008), we have demonstrated that telomere shortening occurs with aging in almost all human organs and tissues, including the anterior lobe of the pituitary gland (Ishikawa et al., 2012). Also, using Q-FISH and our originally developed software, Tissue Telo, employing the telomere-to-centromere ratio (TCR) or normalized TCR (NTCR) (Aida et al., 2007 and 2014; Kurabayashi et al., 2008; Shiraishi et al., 2009; Takubo et al., 2010), we have attempted to confirm the telomere length distributions of two different cell types from the adenohypophysis (AH) and neurohypophysis (NH).

In the present study, we estimated NTCRs in the AH and NH of the human pituitary gland, and investigated the population dynamics of telomere status and the relationship between telomere length and aging

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in the AH and NH. Macroscopic and histopathologic studies at our institution have suggested that the pituitary is one of the organs showing the highest degree of preservation during aging (Sawabe et al., 2006), with scarcely any age-related histologic changes such as lymphocyte infiltration, fibrosis, and amyloid deposition in the AH (Lopes et al., 2012). In the NH, granular cell nests or tumorlets are more common in the elderly (Lopes et al., 2012). Age-related telomere shortening in the pituitary has never been reported, except for our previous paper (Ishikawa et al., 2012). Moreover, no reported study has documented telomere lengths in the NH or in pituicytes. The pituicyte, a type of glial cell, is the most numerous cell type in the NH, playing an important role in the secretion of neurohormones throughout life (Hatton, 1988). Therefore, it is a particularly intriguing cell type from the viewpoint of telomere dynamics in the AH and NH. Here we investigated telomere lengths at various ages in glandular cells of the AH and pituicytes of the NH.

2. Materials and methods

2.1. Subjects

We analyzed 128 pituitary glands from autopsied individuals (65 men and 63 women) aged between 0 and 102 years. The mean age was 71.5 years overall, 70.0 years for the men and 76.1 years for the women. We measured the telomere lengths of glandular cells in the AH. As a functional unit, the NH consists of the infundibulum, pituitary stalk, and posterior lobe. We used the posterior lobe as the NH and measured the telomere length of pituicytes alone in the posterior lobe.

We divided the individuals into two groups: a younger group (aged 0–60 years) and an elderly group (≥60 years).

All samples of pituitary glands were stored at −80 °C until use.

Approval was obtained from the Ethics Committees of Tokyo Metropolitan Institute of Gerontology and the Department of Pathology, Japanese Red Cross Medical Center, Tokyo, before the start of the study.

2.2. Methods

1. Tissue processing and histological assessment

Samples were fixed for 6 h in 10% buffered formalin and then subjected to standard tissue processing and paraffin embedding. Tissues were cut into sections 5 μm thick for histological observation and 3 μm thick for Q-FISH. We identified the AH and NH histologically based on expert opinion among the authors [M.F., J.A., Y.M., T.A., T.I., and K.T.]. Representative photomicrographs of HE-stained sections of the AH and pituicytes of the NH are shown in Fig. 1.

2. FISH using tissue sections

The slides were processed by the Q-FISH method, as reported previously (Aida et al., 2007 and 2008). Tissue sections were hybridized with PNA probes for the telomere (telo C-Cy3 probe: 5′-CCCTAACCCT AACCCTAA-3′; catalogue number F1002, Fasmac, Atsugi, Japan) and the centromere (Cenp1-FITC probe: 5′- CTTCGTTGGAAACGGGGT-3′; custom-made, Fasmac) and the nuclei were stained with DAPI (Molecular Probes, Eugene, OR). TCRs were analyzed in the glandular cells of the AH and pituicytes of the NH separately.

3. Image analysis of telomeres (Fig. 2)

Q-FISH digital images were captured by a CCD camera (ORCAER-1394, Hamamatsu Photonics KK, Hamamatsu, Japan) mounted on an epifluorescence microscope (80i, Nikon, Tokyo, Japan) equipped with a triple band-pass filter set for DAPI/FITC/Cy3 (Part#61010, Chroma Technology Corp., Rockingham, VT, USA) and a x40 objective lens (Plan Fluor 40 x /0.75, Nikon, Tokyo, Japan).

Microscope control and image acquisition were performed using the Image-Pro Plus software package (version 5.0, Media Cybernetics Co. Ltd., Silver Spring, MD, USA). The captured images were analyzed with our own original tissue analysis software, 'TissueTelo Ver. 3.1′, which can calculate the TCR in individual nuclei automatically, as reported previously (Aida et al., 2007, 2008 and 2014; Kurabayashi et al., 2008; Shiraishi et al., 2009; Takubo et al., 2010).

4. TCR normalized by cell block

As a control for variations in sample preparation, we also performed Q-FISH on a cell block-section of a cultured cell line, TIG-1 (Ohashi et al., 1980), that had been subcultured (34 PDL; population doubling level) and had a telomere length of 8.64 kbp measured by Southern blot analysis, placed on the same slides with the pituitary gland sections. Each TCR for the target cells was divided by the mean TCR for the cell block on the same slide to give the NTCR (Aida et al., 2008).

5. Statistical analyses

The NTCRs of the two cell types were compared, both within individual cases and between cases, using Student's *t*-test and paired *t*-test. Correlations were analyzed in terms of Pearson's correlation coefficient and single regression analysis. In all comparisons, differences at $p < 0.05$ were considered to be significant. All calculations were performed using GraphPad Prism 5 for Windows version 5.01 (GraphPad Software, Inc., USA).

3. Results

3.1. Relationship of NTCR to age in the 128 cases (Fig. 3)

(1) AH

Telomeres in the AH showed significant shortening with increased age in both men (M) and women (W) (M: $y = -0.0102X + 2.2800$: $p = 0.0001$, W: $y = -0.0053X + 2.0570$: $p = 0.0294$). The annual telomere length reduction rate in men overall (NTCR: 0.0102) was significantly greater than in women overall (0.0053). The telomere lengths of men and women intersected at 45.6 years of age.

(2) NH

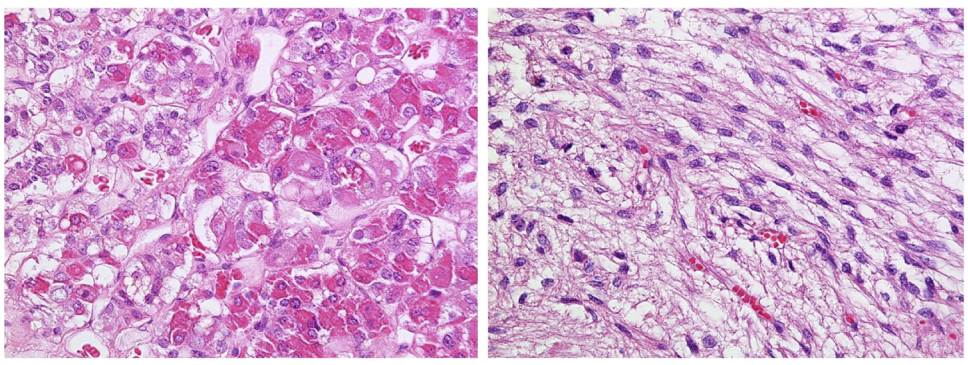

Fig. 1. Representative histology of the pituitary glands from an 89-year-old man (left, adenohypophysis) and an 88-year-old woman (right, neurohypophysis). Inflammatory cell accumulation and fibrosis are not evident in these photographs, even though these individuals were very old.
Left: Three cell types – chromophobic, acidophilic, and basophilic cells – are recognizable in the adenohypophysis in an HE-stained section. Acidophilic cells are most numerous. When measuring telomere lengths, we did not distinguish among these three cell types, but avoided inclusion of endothelial cells.
Right: Many pituicytes surrounded by the neuropil. The pituicytes have elongated nuclei and unipolar or bipolar processes. We measured the telomere lengths of these cells and were careful to avoid endothelial cells.