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Original Article

Chronic sun exposure shortens telomeres of the epidermis

Akiya Tatsumi[1, 2], Motoji Sawabe[1] and Junko Aida[3]

1) Department of Molecular Pathology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo, Japan
2) Department of Medical Technology and Sciences, International University of Health and Welfare, Narita, Chiba, Japan
3) Research Team for Geriatric Pathology, Tokyo Metropolitan Institute of Gerontology, Itabashi, Tokyo, Japan

Abstract

The ends of human chromosome are protected by telomeres, which are composed of tandem repeats of DNA sequences. Telomeres can be damaged by a variety of environmental factors, ultimately resulting in cellular senescence and carcinogenesis. The purpose of this study was to clarify the relationship between telomere shortening in the skin and sun exposure. Using quantitative fluorescence *in situ* hybridization (Q-FISH), we examined telomere lengths (TL) in the epidermis and dermis of sun-exposed (SE) and sun non-exposed (NE) skin from the same individuals without any skin disease. There was a significant difference ($p = 0.039$) in epidermal TL between SE and NE samples, except for adolescents and young adults (AYA). Our results suggest that chronic sun exposure shortens the telomeres of the epidermis, which may explain the UV-associated acceleration of skin aging and skin disorders.

Key Words: Telomere, Skin aging, Q-FISH, Ultraviolet

Introduction

Telomeres are composed of a repetitive DNA sequence TTAGGG(n) and multiple telomere-binding proteins. Their primary role is to protect the ends of chromosomes from DNA degradation and recombination[1]. The telomere repeats shorten by 50–200 base pairs with each cell division, because of incomplete synthesis of the telomere DNA during replication. This is referred to as the "end-replication problem"[2]. Shortened telomeres are not as efficient at maintaining chromosomal integrity, and this leads to chromosomal instability[3], thus destabilizing the genome and causing cancer development[4]. Therefore, maintenance of telomere length (TL) is crucial for long-term cell survival.

In addition to replicative cell division during aging, extrinsic stresses can also accelerate telomere shortening. For instance, telomeres in the esophageal epithelium of alcoholics are shorter than those of non-alcoholics, suggesting that telomere attrition is closely related to the high frequency of esophageal carcinoma in alcoholics[5].

Likewise, telomere shortening in the skin can be accelerated by a variety of extrinsic stimuli, such as sun exposure. Actinic keratosis is a precancerous lesion that forms on damaged skin as a result of cumulative lifetime exposure to ultraviolet (UV) radiation[6]. Ikeda and colleagues have reported that epidermal tissue in surrounding actinic keratosis has shorter telomeres than epidermal tissue from normal skin[7]. Furthermore, the telomeres in normal sun-exposed (SE) epidermis are shorter than those in normal sun non-exposed (NE) epidermis. Hence, chronic exposure of the epidermis to sun has a telomere shortening effect and contributes to the aging process and cancer development. In the study by Ikeda's group, all samples were obtained from different individuals. However, as individual differences in TL are mostly present from birth[8], we considered it necessary to compare samples from the same individuals in order

Corresponding Author: Junko Aida, DDS, PhD
Research Team for Geriatric Pathology Tokyo Metropolitan Institute of Gerontology 35-2 Sakae, Itabashi, Tokyo 173-0015, Japan
Tel: +81-3-3964-3271
E-mail: aidajs@tmig.or.jp
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Table 1 Characteristics of patients from whom autopsy materials were taken.

Case no.	Age (years)	Gender	Cause of death
1	26	Male	Acute lymphoblastic leukemia
2	40	Male	Pineal tumor, Gastrointestinal bleeding
3	40	Male	Chronic myeloid leukemia
4	53	Male	Esophageal cancer, Hepatocellular carcinoma, Colorectal cancer
5	65	Female	Endometrial cancer, Ovarian cancer
6	68	Male	Esophageal cancer
7	71	Female	Occult primary tumor
8	80	Male	Floor of the mouth cancer

to eliminate the effects of such individual variation on estimates of TL changes in response to sun exposure.

Several studies have indicated that telomere shortening is evident in the peripheral areas of cancers or in precancerous lesions[7, 9]. Therefore, we considered that the use of samples of normal skin would eliminate the effect of any underlying disease or condition on TL.

Ikeda and colleagues reported that the telomeres of fibroblasts in areas of actinic keratosis were shorter than those in the normal SE and NE dermis, although there were no such differences between normal SE and NE dermis[7]. However, it has been reported that UVA radiation from the sun penetrates the dermis and shortens the telomeres of human skin fibroblasts[10].

In the present study, to clarify the effects of sunlight exposure on dermal fibroblasts, we assessed TL in not only the epidermis but also the dermis from paired skin samples. For this purpose, we used a quantitative fluorescence *in situ* hybridization (Q-FISH) method, which allows measurement of TL in individual cells within formalin-fixed paraffin-embedded (FFPE) tissues[11, 12]. In our original algorithm for telomere length analysis, the centromere is used as an internal control, and more than 100 cells are analyzed per case[13]. This method has been widely proven to have accuracy and reproducibility for estimation of TL in various archival tissues[5, 7, 9]. Here, we used this method to clarify the effects of chronic sun exposure on TL in the skin by excluding any influence of individual basic differences in TL or skin disease.

Materials and Methods

Tissue specimens

Samples of normal skin were collected at autopsy from 8 individuals (6 males and 2 females; average age 55.1 years) who had died of various cancers without any skin disorders, as detailed in Table 1. We used paired SE and NE skin samples obtained from the neck and abdomen, respectively.

This study was approved by the Tokyo Metropolitan Institute of Gerontology Ethics Committee (Approval Number: 47/2014).

Histopathological assessment

FFPE samples were serially sectioned at 3 μm for hematoxylin and eosin (H&E) and Elastica van Gieson (EVG) staining and at 2 μm for Q-FISH. The staining procedures were conducted according to the established laboratory protocol, and the stained skin tissues were histologically confirmed to be normal without any autolytic or pathological changes by experienced pathologists (JA, MS). Only the stained SE skin tissues had solar elastosis, which is a histological change apparent in sun-exposed areas, and we considered that the changes evident in these samples were compatible with SE skin.

Q-FISH

FFPE sections (2 μm) were deparaffinized with xylene, dehydrated in ethanol, and air-dried. They were then pretreated with 0.2 mol/L HCl for 20 min at room temperature (RT), washed in distilled water for 10 min at RT, placed in 1 mol/L NaSCN for 30 min at 86˚C, and rinsed again in distilled water for 3 min at RT. After pepsin digestion (1 mg/mL acid H_2O) for 15 min at 38˚C, the slides were washed in distilled water for 3 min at RT followed by dehydration in an ethanol series (70%, 90%, 100%), and air-dried. The slides were also treated with 0.5 μg/ml RNase for 10 min at 37˚C to eliminate residual RNA, washed with phosphate-buffered saline for 5 min at RT, dehydrated through the above ethanol series, and air-dried. Hybridization with peptide nucleic acid (PNA) probes was then performed. A PNA telomere

probe (telo C Cy3 probe: 5'-CCCTAACCCTAACCCTAA-3'; Fasmac, Atsugi City, Kanagawa, Japan) labeled with Cy3 (final concentration 1.5 μg/ml; 0.30 μmol/L) and a PNA centromere probe (Cenp1 probe: 5'-CTTCGTTGGAAACGGGGT-3'; Fasmac) labeled with fluorescein isothiocyanate (FITC; final concentration 0.6 μg/ml; 0.12 μmol/L) were used at a concentration of 30 μg/ml (6.04 μmol/L). These probes were added to each slide, immobilized on a cover glass, and denatured for 6 min at 83°C, followed by incubation for 1 h at RT under humid environmental conditions. After incubation, the slides were washed four times for 15 min each time with 70% formamide buffer to remove any unbound probe, followed by washing with TBST (0.1 mol/L Tris, 0.15 mol/L NaCl, 0.08% Tween 20) 4 times for 5 min each time, dehydrated in serial ethanol dilutions and air-dried. The sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR, USA) for nuclear staining. The stained slides were stored in a closed box at 4°C until evaluation by microscopy.

Q-FISH imaging

Fluorescence signals in tissue sections were captured by a charge-coupled device (CCD) camera (RETIGA-2000DC, QIMAGING, Surrey, BC, Canada) fitted to an epifluorescence microscope (80i; Nikon, Tokyo, Japan) using a triple band-pass filter set for DAPI/FITC/Cy3 (Part #61010, Chroma Technology Corp, Rockingham, VT, USA) equipped with an objective lens (Plan Fluor x40/0.75, Nikon). The Image-Pro Plus software package (version 7.0, Media Cybernetics, Silver Spring, MD, USA) was used to obtain the images.

Telomere analysis

Digitized images of the slides were examined using our original software Tissue Telo ver.3.1, which had been developed for TL analysis at Tokyo Metropolitan Institute of Gerontology. Using this software, nuclear areas were manually identified as combined images with DAPI (blue), FITC (green), and Cy3 (red). Telomere and centromere signals were recognized as pixels with the brightest intensity (top 5%) of each nuclear area outlined by hand. Pixels showing the lowest intensity (bottom 20%) were regarded as autofluorescence signals and compensated for in the nuclear areas. The signals extracted based on these thresholds provide more accurate and consistent results[14].

As most cell nuclei are cut in tissue sections, the telomere signal from any given nucleus does not reflect that from the nucleus as a whole. Therefore, the telomere signal was measured with reference to the centromere signal[14]. This is because centromere length is almost the same among individuals, and the telomere and centromere are considered to be distributed evenly in the nucleus. Therefore, the telomere to centromere intensity ratio (TCR) was calculated using Tissue Telo ver.3.1 and applied as the relative TL of each cell.

Selection of target cells

In Q-FISH images, we selected basal cells and fibroblasts as cell types representative of the epidermis and dermis, respectively. We analyzed manually over 100 cells for each single cell type in each skin sample. Cells located in the lower part and in contact with the basal membrane of the epidermis were counted as basal cells. Spindle-shaped cells within the dermis were recognized as fibroblasts; any spindle cells around a cavity-like space were excluded because they were considered likely to be endothelial cells.

Normalization of TL

To avoid any variation in the staining conditions, a cell block containing TIG-1 cells was used as a control to normalize the TL[14, 15]. The TIG-1 cell strain had been established at Tokyo Metropolitan Institute of Gerontology. The TL of the cells at a population doubling level (PDL) of 34 was 8.6 kbp, as determined by Southern blotting analysis. A slice of the cell block was placed on a slide for Q-FISH, and then stained and analyzed at the same time as the tissue sections. As with basal cells and fibroblasts, over 100 TIG-1 cells were counted in each case. The ratio of the TCR of target cells to the TCR of TIG-1 cells – the normalized TCR (NTCR) – was then calculated. We recognized the mean NTCR as the representative TL for each case.

Effect of aging on skin TL

Adolescent and young adults (AYAs), defined as individuals between ages 15 and 39, are thought to have a biological behavior for cancerization different to that of patients over 40 years of age[16]. Therefore, we compared TLs among cell groups in the older group.

Statistical analysis

The paired skin samples in each case were analyzed using unpaired *t*-test because the numbers of cells analyzed were different. To compare the TL between two groups, paired *t*-test was used. The correlation between TL and subject age was then analyzed using Pearson's correlation coefficient. All statistical analyses were performed with EZR, which is based on R commander

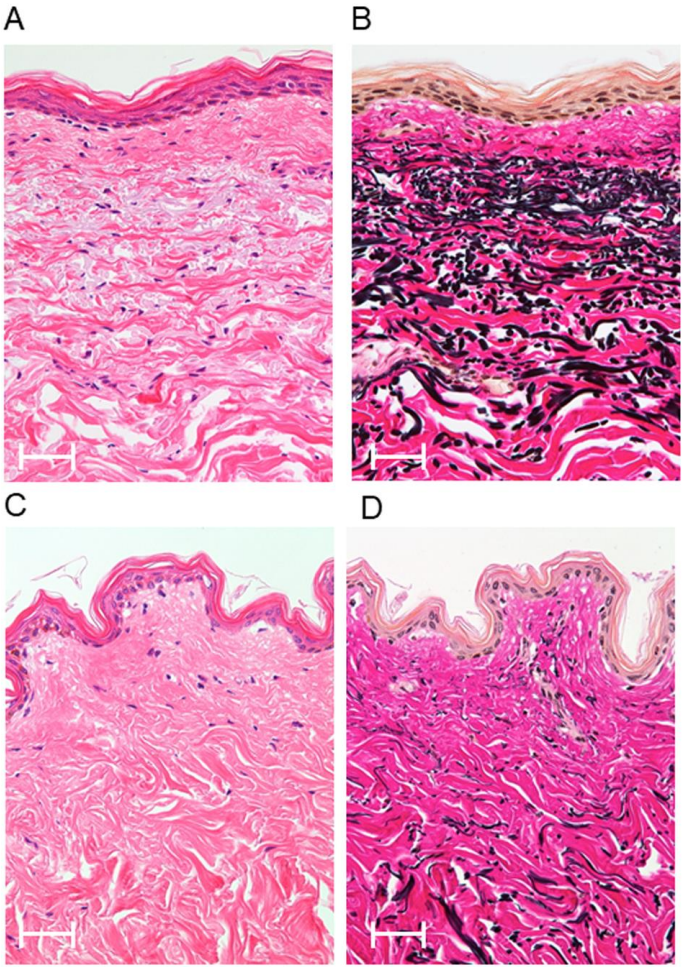

Figure 1

Samples of SE (Fig. 1A) and NE (Fig. 1C) skin stained with hematoxylin and eosin (H&E). All were samples of normal skin tissue without any autolytic or pathological changes. Samples of SE (Fig. 1B) and NE (Fig. 1D) skin from the same sources subjected to Elastica van Gieson (EVG) staining. The dark purple regions are recognizable as elastic fiber areas. The SE skin shows solar elastosis, involving accumulation of abnormal elastin as a result of chronic sun exposure. All images are from case no. 7. Scale bars are 50 μm (x200).

designed for the application of statistical functions frequently used in biostatistics[17]. Differences at $p < 0.05$ were considered significant and all tests were two-tailed.

Results

Figures 1A and 1C show H&E staining in SE and NE skin. Samples from 8 cases were adopted for Q-FISH because they were collected as paired skin tissues from individuals who had no skin diseases including inflammation. In all cases, SE skin had more abundant elastin fibers in the dermis than NE skin, and these histological changes were especially remarkable in SE skin from case no. 7. The accumulation of elastic fibers was identified more accurately by EVG staining (Figs. 1B and 1D).

Typical Q-FISH images of skin tissue and TIG-1 cells are shown in Figures 2A and 2B. Basal cells had brighter signals than the other epidermal layers and were thought to represent the epidermis. Fibroblasts appeared to be far brighter than the epidermis. Signals for the telomere (red) and centromere (green) were visible in each nucleus. The distribution of TCR in a representative case is shown in Figure 2C.

Comparison of TL among various cell groups in each case

Figure 3 shows a box plot of TL in SE and NE skin in individual cases. For the epidermis, in one (case no. 5) out of eight cases, the telomeres in SE epidermis were significantly shorter than those in NE epidermis ($p =$

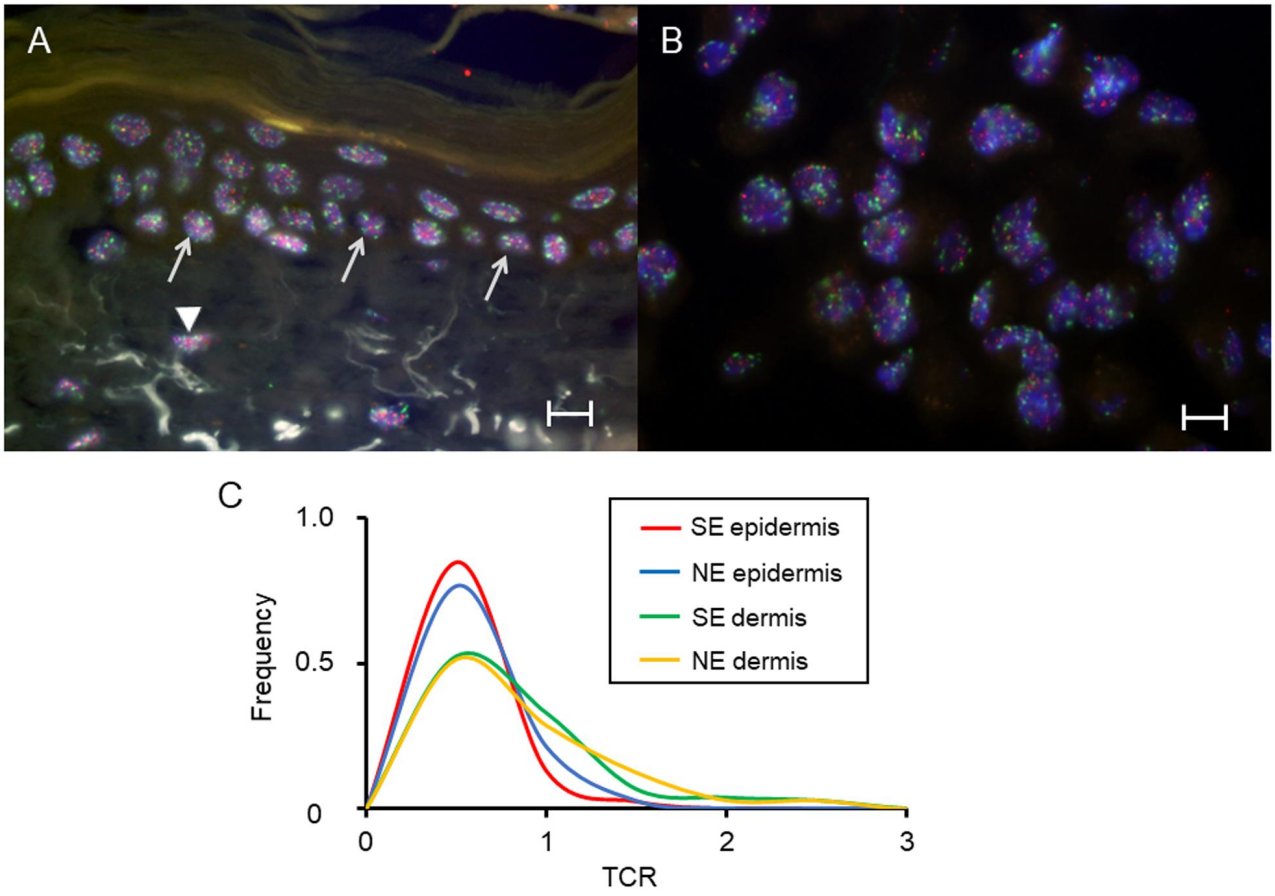

Figure 2

Representative Q-FISH images of skin tissues (A) and TIG-1 cells (B) labeled for the telomere (red), centromere (green), and with DAPI (blue). Basal cells and fibroblasts were analyzed as representative cells of the epidermis and dermis, respectively. Arrows indicate basal cells and arrowhead indicates fibroblasts. Scale bars are 10 μm. (C) Representative distribution of TCR in SE and NE skin. Each image and the distribution of TCR are from case no. 4.

0.021) (Fig. 3A). In four other cases there was also a tendency for SE epidermis to have shorter telomeres than NE epidermis, but this was not significant (case no. 3: $p = 0.076$, case no. 4: $p = 0.094$, case no. 6: $p = 0.178$, case no. 7: $p = 0.274$).

For fibroblasts in the dermis, five cases had longer telomeres in NE than in SE, but three cases had shorter telomeres in NE. However, there were no significant differences between TL in the SE and NE dermis in each case (Fig. 3B). The measured values of TL are shown in Table 2.

Comparison of TL in SE and NE skin

Figure 4 shows the box plot distribution of TL in the cases overall. As shown in Figure 4A, there was no significant difference in TL between SE and NE in both the epidermis and dermis ($p = 0.154$, $p = 0.448$).

Comparison of TL in the epidermis and dermis

Telomeres in the epidermis were significantly shorter than those in the dermis ($p \leq 0.0001$) in both SE and NE skin (Fig. 4).

Effect of aging on skin TL

Regression analysis was performed to determine the correlation between TL and age; the regression equation obtained was (SE epidermis: $y = -0.0028x + 0.9825$, $r = -0.488$, $R^2 = 0.238$, $p = 0.220$; SE dermis: $y = -0.0004x + 1.3986$, $r = -0.054$, $R^2 = 0.003$, $p = 0.899$; NE epidermis: $y = -0.0002x + 0.9030$, $r = -0.034$, $R^2 = 0.001$, $p = 0.936$; NE dermis: $y = 0.0012x + 1.3501$, $r = 0.176$, $R^2 = 0.031$, $p = 0.676$). There was no significant difference between the two groups (Fig. 5).

Although we tried to compare groups classified by age, i.e. the AYA group and the older group, the number of the former cases was too small (n=1) to be statistically

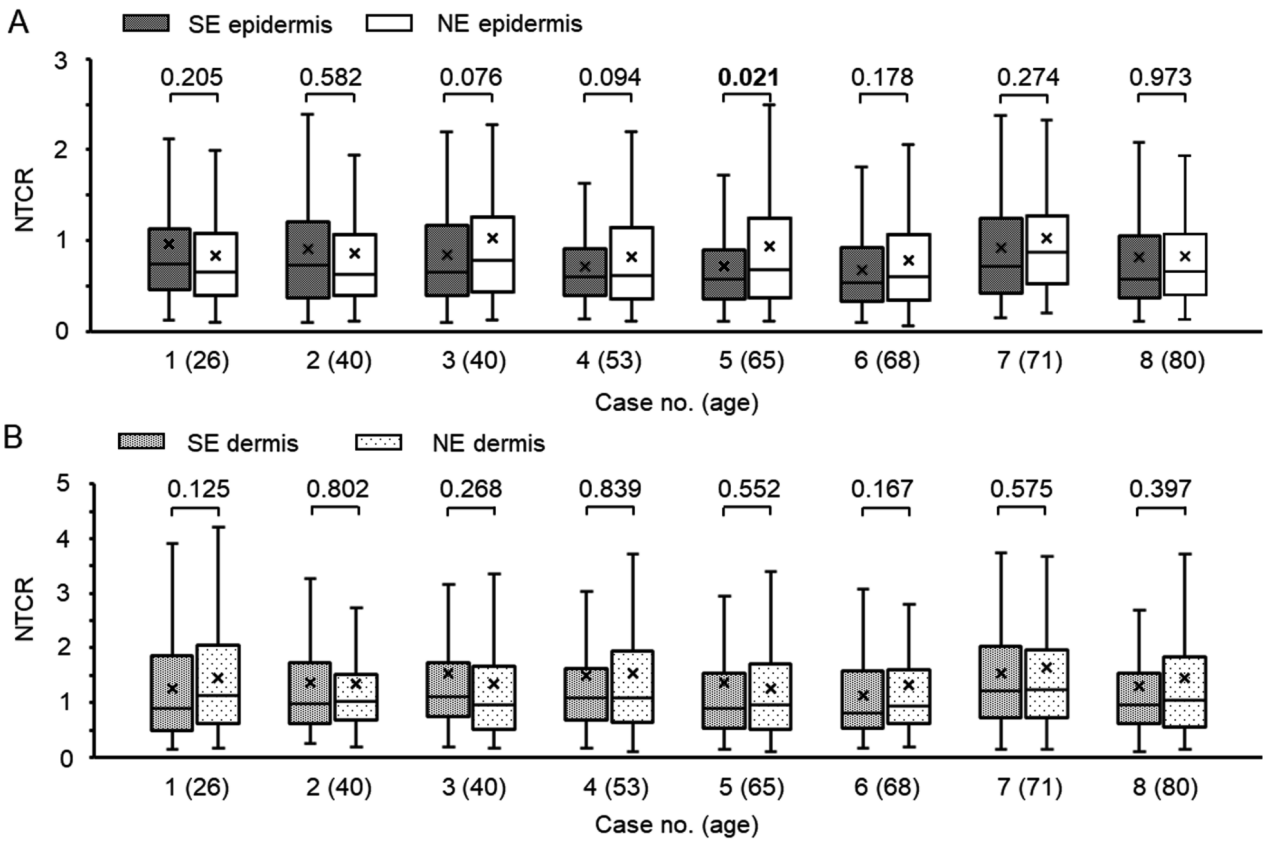

Figure 3

Comparison of TL in SE and NE skin from each case. Box plots of the NTCR in the epidermis (3A) and dermis (3B) are shown for each case. Center bars and cross marks represent the median and the mean levels, respectively. The boxes show the 25th and 75th percentiles of the data, and the whisker ends correspond to the 1st and 99th percentiles. As a value representing TL, the mean value was used for statistical analysis, and *p*-values were calculated by unpaired *t*-test. Differences were considered statistically significant at $p < 0.05$. Significant *p*-values are shown in bold. In one (case no. 5) out of eight cases, telomeres in SE epidermis were significantly shorter than those in NE epidermis ($p = 0.021$). In the epidermis of other cases, and in the dermis, there was no significant difference between SE and NE skin.

Table 2 Relative telomere lengths in each pair of skin samples.

Case no.	Epidermal TL				*p*-value*	Dermal TL				*p*-value*
	SE		NE			SE		NE		
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
1	0.964	0.436	0.834	0.315	0.205	1.251	0.479	1.447	0.509	0.125
2	0.917	0.739	0.868	0.732	0.582	1.374	1.090	1.337	1.147	0.802
3	0.854	0.292	1.035	0.415	0.076	1.528	0.577	1.340	0.571	0.268
4	0.720	0.204	0.835	0.256	0.094	1.488	0.651	1.529	0.615	0.839
5	0.716	0.264	0.945	0.419	**0.021**	1.377	0.889	1.256	0.551	0.552
6	0.681	0.276	0.779	0.331	0.178	1.132	0.515	1.329	0.672	0.167
7	0.923	0.524	1.025	0.479	0.274	1.547	0.505	1.646	0.809	0.575
8	0.830	0.322	0.827	0.287	0.973	1.306	0.590	1.448	0.626	0.397

*: unpaired t-test between SE and NE mean NTCR. NTCR: normalized telomere to centromere ratio. Significant *p*-value (<0.05) is shown in bold font.
Abbreviations: TL, telomere lengths; SE, sun exposed; NE, sun non-exposed; SD, standard deviation

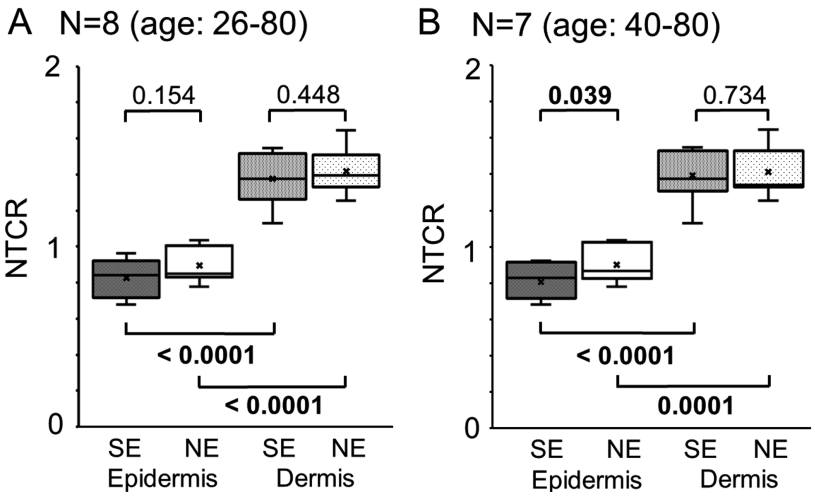

Figure 4

Comparison of NTCR between SE and NE skin. Box plot description is the same as that for Figure 3, and the calculated *p*-value is shown. (A) A box plot of the mean NTCR values for the samples overall is shown (n = 8; mean: 55.4 years; standard deviation: 17.5). When 8 paired samples were analyzed, there was no significant difference in TL between SE and NE skin (epidermis: $p = 0.154$; dermis: $p = 0.448$). In both SE and NE skin, telomeres in the epidermis were significantly shorter than those in the dermis ($p < 0.0001$). (B) After excluding one subject (case no. 1) in the adolescent and young adult (AYA) group, the remaining older cases were analyzed statistically and the data are shown as a box plot (n = 7; mean: 59.6 years; standard deviation: 14.4). Telomeres in the SE epidermis were significantly shorter than those in the NE epidermis ($p = 0.039$), whereas there was no difference in TL between SE and NE dermis ($p = 0.734$). In both SE and NE skin, telomeres in the epidermis were significantly shorter than those in the dermis ($p \leq 0.0001$).

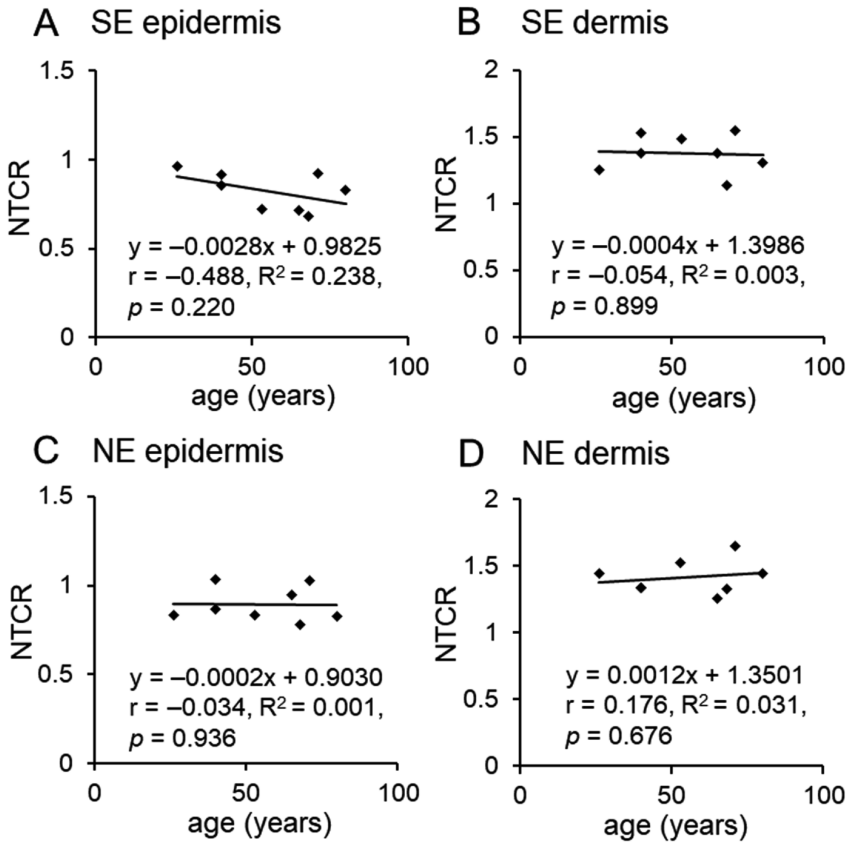

Figure 5

Scatter plot of TL and age for the SE epidermis (A), SE dermis (B), NE epidermis (C), and NE dermis (D). Each regression equation, correlation coefficient and calculated *p*-value are shown. In all groups, there was no significant correlation between TL and age.

reliable. Therefore, we compared skin TLs between SE and NE in the older group (aged 40 years and over). In the 7 cases in the older group, the telomeres in SE epidermis were significantly shorter than those in NE epidermis ($p = 0.039$) (Fig. 4B). By contrast, there were no significant differences in the TLs of fibroblasts ($p = 0.734$).

Discussion

In this study, we assessed the differences in TL between SE and NE skin. The important findings were as follows: (1) In five of the seven individuals over 40 years of age, SE epidermis had shorter telomeres than NE epidermis. In the samples from patients aged 40 years or more, there was an overall tendency for the SE epidermis to have significantly shorter telomeres than the NE epidermis; (2) There was no significant difference in TL between SE and NE dermis; (3) telomeres in the epidermis were significantly shorter than those in the dermis.

Our present results were obtained using normal skin from the same individuals, and were consistent with those reported by Ikeda and his colleagues[7]. We collected paired samples of SE and NE skin from the same individuals, since inter-individual differences in TL were thought to be present[8]. Furthermore, epidermis from around cancer or precancerous lesions was expected to have shorter telomeres than normal tissues[7, 9]. Therefore, it was considered necessary to obtain normal skin samples from the same individuals to confirm whether sun exposure indeed affected TL in the skin.

When all paired samples were analyzed statistically, no significant difference in TL between SE and NE skin was found. This was probably because of the small number of samples collected at autopsy. Considering that cell damage due to sun exposure might accumulate with age, and that the prevalence of skin cancer in AYAs is low[16], we analyzed 7 samples, excluding case no. 1 (26 years of age). This revealed that telomeres in SE epidermis were significantly shorter than those in NE epidermis. This result appears to confirm that chronic sun exposure shortened telomeres in the skin, thus supporting the study of Ikeda and colleagues[7]. Fouquerel and colleagues have reported that UV radiation from the sun leads to the formation of DNA photoproducts including pyrimidine dimers in telomere repeat sequences[18]. If these photoproducts are not removed, they can damage the telomere. In addition, telomeres are hypersensitive to DNA damage induced by UV radiation[19], and in fact TL shortening was certainly evident in SE epidermis. These findings confirm the need for protection against excessive UV radiation to prevent excessive telomere attrition. However, as the present study was limited by a small sample size, future analysis of more samples to reveal the effect of UV radiation on telomeres in more detail will be needed.

In the present study, we observed solar elastosis in the SE specimens. However, there were no significant differences in TL between SE and NE dermal fibroblasts. The UV radiation in sunlight includes UVA and UVB; UVB has a shorter wavelength and higher energy than UVA[20]. Epidermal cells are affected by both types of UV, but dermal fibroblasts are affected only by UVA. Similarly to the present study, Ikeda and colleagues have reported that TLs in dermal fibroblasts of normal skin showed no significant difference between SE and NE[7]. Therefore, telomeres of dermal fibroblasts appear less vulnerable to UV than those of the epidermis.

In all samples, telomeres in the epidermis were shorter than those in the dermis. This is in agreement with a study by Sugimoto et al., who analyzed 100 specimens of epidermis and 60 specimens of dermis using Southern blotting[21]. The difference in TL between epidermis and dermis probably reflects the fact that the turnover of epidermal cell is higher than that of dermal cells[22]. As telomeres become shorter with every cell division, the fast turnover of epidermal cells may accelerate telomere shortening. Moreover, various extrinsic stimuli may damage epidermal cells, and the epidermis could potentially have high telomerase activity, which is linked to stabilization of TL[23]. However, we did not measure telomerase activity because this is not possible using FFPE autopsy samples.

It is well established that telomeres in various tissues shorten with age[8, 13]. However, no age-dependent telomere attrition in the skin was found in our present study. This was probably because our samples were obtained only from adults, and the number of samples was small. These represent the main limitations of the study, and it will be necessary to investigate larger numbers of subjects, especially children and AYAs, to clarify the correlation between TL and age.

In conclusion, we have demonstrated telomere shortening in the epidermis due to chronic sun exposure. These findings provide information on telomere dynamics that might be applicable to prevention and treatment of UV-related skin disorders.

Conflict of interest

The authors have no potential conflicts of interest to disclose in relation to this study.

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