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Change in the telomere length distribution with age in the Japanese population

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Abstract Telomeres play a role in cellular aging and they may also contribute to the genetic basis of human aging and longevity. A gradual loss of the telomeric repeat sequences has been reported in adult tissue specimens. This study determined the percentage of telomere restriction fragment in various molecular-sized regions in addition to measuring the average telomere length. Mean telomere restriction fragment (TRF) length was determined by Southern blot analysis using a longer telomeric repeat probe with higher sensitivity. A significant decrease in longer telomere fragments and a quick increase in the shortest fragments were observed, especially in male subjects. There was a tendency that the age-adjusted telomere length was longer in females than that observed in males, while males lose the telomeric sequence faster than females. These data indicated that the percentage of longer telomeres fragments decreased, while the shortest fragments increased quickly with age. In addition, the longest telomere fragments decreased and the short fragments increased with a relatively stable frequency with age. There was also a significant difference in the longest telomere fragment percentage between males and female in their 40s and 50s, whereas no difference was observed in the mean TRF length. Interestingly, the changing rate of the longest and the shortest range group of TRF percentage associated with aging seemed quite different between before and after 50-year old with a gender-related contrast. This contrast implies a drastic change around the age of 50 of unknown factors that affect telomere attrition.

Keywords Telomere · Age · Japanese · Gender

Introduction

Telomeres are specialized DNA-protein complexes located at the ends of the linear chromosomes of eukaryotes. They are made up of a large number of tandem repeats of the sequence TTAGGG [1, 2] and act to preserve genome integrity and stability by preventing the recognition of chromosomal ends as double-stranded DNA breaks. Previous studies have demonstrated that the replication of normal somatic cells is finite and that, after a critical number of cell divisions, the cells reach a state termed replicative senescence. At this point, further cell division cannot occur, because DNA replication during mitosis is incomplete resulting in a loss of 50–200 terminal bp per cell division in telomeres [3]. Telomerase is an RNA-dependent DNA polymerase that is capable of synthesizing terminal telomere repeats and extending the length of the telomeres, thus compensating for the telomere loss occurring with cell division [4]. Telomerase has been reported to be active in some exceptional cases, i.e., in cancer cells and in stem cells which can divide without limit.

Telomeres undergo attrition in their length with each cycle of somatic cells [4], and thus the history of somatic cells is a major determinant of telomere length. In humans, the telomere length of replicating somatic cells is inversely related to the age of an individual [5, 6], although it is highly variable among subjects of the same age [6, 7]. Another determinant in this phenomena is

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heredity [4, 8]. The differences in the length of the terminal restriction fragment (TRF) within subpopulations of somatic cells are far smaller than the differences among persons of the same age [9, 10]. These observations suggest that the telomere length could thus predict the functional status of tissues more reliably than chronological age and they might also be useful in diagnosing age-related diseases associated with a restricted replicative capacity. Due to the wide inter-individual variations in the telomere length, it is difficult to define a general borderline between physiological and pathophysiological telomere attrition, and thus it is very important to specifically define an internal control.

Age-related telomere attrition has been demonstrated in a variety of human tissues by using TRF analysis [11, 12]. Recently, several studies have shown that the loss of a few hundred base pairs from short telomeres could be important to cellular aging but may not be detectable by traditional mean TRF analysis [13, 14]. Friedrich et al. [15] analyzed TRF in peripheral blood leukocytes, skin, and synovial tissue from elderly patients and found that TRF measurements in easily accessible tissues, such as the blood, could serve as a surrogate parameter for the relative telomere length in other tissues. Another report also suggested that peripheral blood leukocytes are an excellent source for investigating telomere shortening [16]. Therefore, the present study, investigated the telomere changes associated with aging in blood cells in normal adults by determining the percentages of different lengths of telomeres, based on molecular size markers. This helped to answer the following questions concerning telomere length: (1) How are telomeric sequences lost in normal peripheral blood cells? (2) Do males and females demonstrate different telomere lengths and shortening rates, and if so, how?

Materials and methods

Subjects

All subjects completed an in-person interview that ascertained information about factors that may be related to blood cell/peripheral blood mononuclear cell (PBMC) telomere length. Among 231 subjects ranging in age from 20 to 68 years, 192 usable blood samples (taken using 10 ml Vacutainer tubes containing EDTA/heparinized syringes) were drawn and stratified into 10-year age groups. The groups were very similar with respect to smoking status, family income, the levels of physical activity, gender make-up and socio-economic status. This research was performed following approval by the Conjoint Health Research Ethics Board of Kyushu University.

Telomere detection

Telomere detection was performed as previously described [7, 14, 17]. Briefly, blood cell DNA was extracted from samples using PureGene DNA Extraction Kits (Gentra Systems, Minneapolis, MN) and the quality was assessed by agarose gel electrophoresis. Aliquots of DNA (1 ug) were digested at 37°C with 3U MspI for 2 h. The digests (20 ul) were loaded onto a 0.8% agarose gel (Bio-Rad, Hercules, CA) and resolved by electrophoresis at 100 V for 30 min. After electrophoresis, the gels were examined by ethidium bromide staining and photographed with a P/N Polaroid film, and then samples were denatured, neutralized, and transferred by Southern blotting to a positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany). The transfer was done overnight at room temperature using 20× SSC. The DNA was cross-linked to the nylon membrane using a UV Stratalinker (TM 2400; Stratagene, La Jolla, CA). The blotted DNA fragments were hybridized to a 500 bp long (TTAGGG)n digoxigenin (dig)-labeled probe specific for telomeric repeats. Hybridization was carried out at 42°C overnight in a Techne-hybridiser HB-1D, and the samples were then washed as recommended by the manufacturer. The membrane was then incubated with anti-digoxigenin-AP-specific antibody. The telomere probe was visualized by CSPD (provided with the kit). The membrane was then exposed to Fuji XR film with an intensifying screen. The smears of the autoradiogram were captured on an Image Master, and the telomere length was then assessed quantitatively.

Terminal restriction fragment (TRF) analysis

Cherif and coworkers showed that a loss of a few hundred base pairs from short telomeres could play an important role in cellular aging but they may not be detected by traditional mean TRF analysis [13, 14]. In this study, we therefore compared the telomere length using a telomere percentage analysis with four intervals of length as defined by a molecular weight standard. This method has previously been used to determine the change in the telomeres in rats [14], however, the present study is the first to use it to determine the change in the telomere length in human beings. In brief, the intensity (photo-stimulated luminescence: PSL) was quantified as follows: each telomeric sample was divided into grid squares as follows according to the molecular size ranges: 23.1–9.4, 9.4–6.6, 6.6–4.4, and 4.4–2.3 kb. The percent of PSL in each molecular weight range was measured (%PSL = intensity of a defined region-background × 100/total lane intensity-background). The mean TRF was estimated using the formula($\sum$(ODi-background)/$\sum$(ODi-background/Li) [18], where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

Statistical analysis

The normality of the data was examined using the Kolmogorov–Smirnov test, and homogeneity of variance using the Levene Median test. The difference in the mean TRF length and a telomere percentage analysis with age and gender were analyzed using a two-way ANOVA test followed by all pairwise multiple comparison procedures using Tukey's post-hoc test. If the normality and variance of the data were not acceptable in the first test, then logarithmic or square-root transformations were used to normalize the data for fitting to the two-way ANOVA. We found no significant difference in interaction between age and gender, and other factors, such as age on mean TRF or telomere loss were assessed using line regression models. The data are shown using the mean ± SE bars. The criterion for significance is $P < 0.05$. All analyses were carried out using a Sigma Statistical Analysis Soft ware (Sigma 2.03, 2001; Sigma, St. Louis, MO).

Results

Age-related shortening rate of telomeres

The mean TRF length shortened by 77 bp/year with aging in all subjects (Fig. 1a). The mean TRF was greater than 5 kb in all age ranges observed in this study. This is compatible with previous reports [5, 19]. To determine whether the telomere attrition rate is constant with aging, the rates of subjects 20–40 and 50–60 were compared (Fig. 1b). The average of attrition rate was –76 bp/year up to the age of 50, and decreased to –68 bp/year after age 50 (Fig. 1b). In addition, the gender-related difference in the telomere attrition rate was analyzed. The attrition rate was 103 bp/year in males and 54 bp/year in females (Fig. 1c). To identify estrogen-associated telomeric change, pre- (<50 years old) and post-menopausal (>50 year old) telomere attrition rates of females were analyzed. In males, the attrition rate was 125 bp/year prior to age of 50 and was reduced to to 84 bp/year beyond that age. On the contrary, in females, the attrition rate was 46 bp/year before age 50, and rose to 210 bp/year after 50 (Fig. 2b). Thus, an unexpected tendency was observed that the telomere attrition rate after 50 years old became faster in females than in males.

Telomere length percentage analysis

The changes in the telomere length percentage profile associated with aging were compared between males and females. The telomere percentage profile revealed that the longest telomere column (23.1–9.4 kb) reduced linearly in

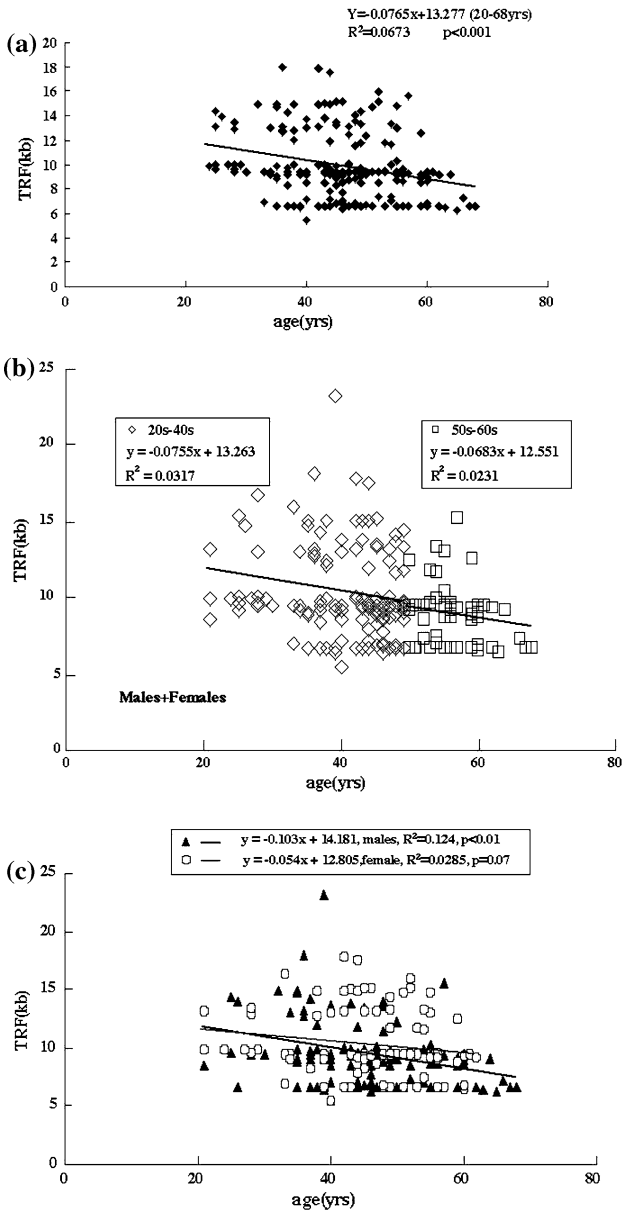

Fig. 1 Mean telomere restriction fragment length (TRF) and age. (**a**) Distribution of mean telomere restriction fragment length with age ranging 20–68 years old. (**b**) Attrition rate of mean TRF of 20–40s and that of 50–60s. (**c**) The attrition rate of the mean TRF of males and females

males (Fig. 3a). A significant difference in the percentage of the longest telomeres (23.1–9.4 kb) was observed between males and females in their 40s ($P = 0.031$) and 50s ($P = 0.027$) (Fig. 3c). The second longest column (9.4–6.6 kb) was significantly higher during the 20s than in any other later age range in both sexes (Fig. 3a, b). This implies that younger adults experience faster telomere attrition. Previous studies identified at least two phases of telomere shortening, one at age 4 and one in early adult hood [16], and the latter phase is similar to the present

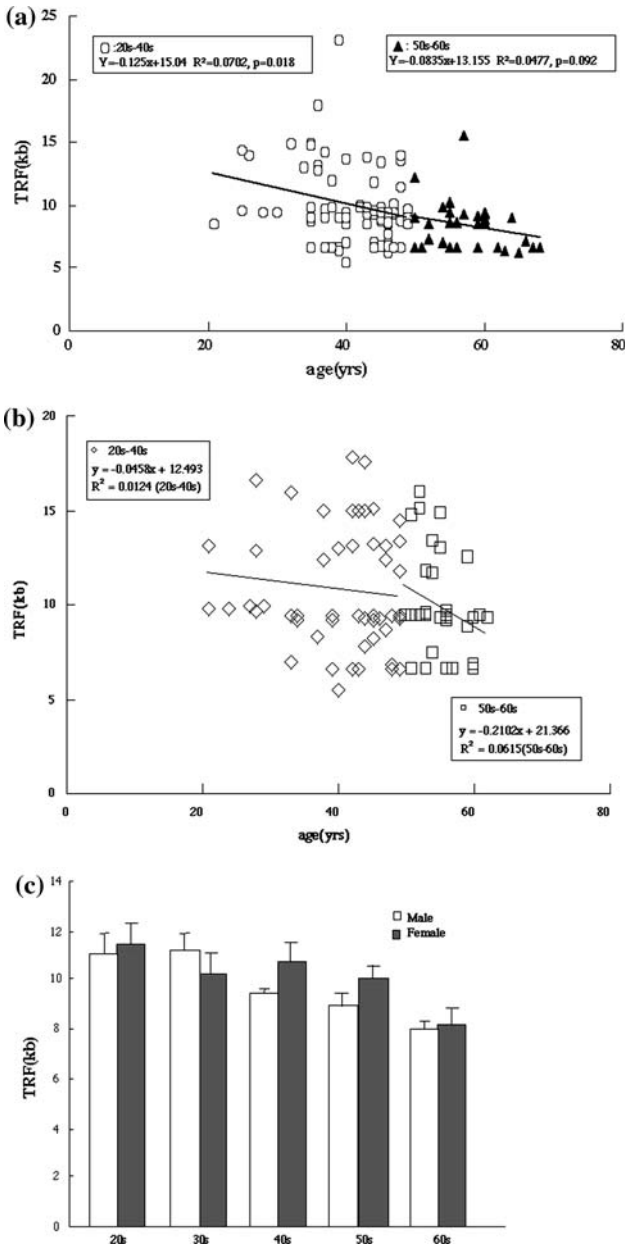

Fig. 2 Distribution of the mean TRF of 20–40s and 50–60s. The distribution of mean TRF in males (**a**) and females (**b**). The mean TRF of every decade from 20s to 60s in males and females (**c**)

result. The shortest telomere column (4.4–2.3 kb) increased with age in both sexes (Fig. 3a, b, d). There seemed to be a notch occurring in the 50s, and this may imply that an increasing rate of change in the shortest column at around age 50.

Analysis of the longest and the shortest column of telomere length percentage before and after age 50

Based on the changes in the telomere length with age between the male and female subjects, these findings suggest that there is a relatively stable change in females in comparison to that in males. The telomere percentage length profile showed a significant gender-related difference in the longest telomeres (23.1–9.4 kb) (Fig. 3c), thus indicating that telomere attrition between individuals in their 40s and 60s thus occurs faster in females than in males. In addition, the same tendency was apparently observed but no statistical significance was detected in the mean TRF length (Fig. 2c). A lower percentage in the longest telomeres was observed in males than that in females in the 40s ($28.67 \pm 1.3\%$ vs. $33.65 \pm 1.9\%$, $P = 0.031$) and 50s ($26.89 \pm 1.9\%$ vs. $31.88 \pm 1.6\%$, $P = 0.027$) and there was no difference seen for those in 20s, 30s and 60s (Fig. 3c). In contrast, there was tendency of a greater frequency of the shortest telomere of 4.4–2.3 kb in males than that in females in 40s and 50s ($18.35 \pm 1.9\%$ vs. $20.12 \pm 1.2\%$; $16.74 \pm 1.4\%$ vs. $19.54 \pm 1.7\%$, respectively) in Fig. 3d. The difference in the long- and short-telomere percentage change associated with aging in males and females between before and after age of 50 can clearly be seen in Fig. 4. The attrition of the longest column (23.1–9.4 kb) before age 50 was observed in males but not in females, and after the age of 50 it became slower in males but faster in females. On the other hand the shortest column (4.4–2.3 kb) increased more gradually after 50 years of age than before in males, while it increased more quickly in females.

Discussion

Newborn TRF is mainly determined genetically and the variation of TRF among population reflects its genetic variation even in adults [4, 6–8]. TRF attrition is thought to be mainly caused by incomplete DNA replication at chromosome ends and DNA erosion by hyper-oxidation [3, 20]. Therefore, cumulative accelerated cell division of leukocytes in inflammatory conditions and continuous oxidative stress with aging are currently regarded as the main causative factors for telomere-shortening with aging. In this study, we tried to detect the age-related TRF change as a net value of age-acceleration by various kinds of factors including those mentioned above in 'normal' population. A wide variation of TRF in the population may conceal minor changes in TRF. We have also tried to perform a telomere percentage analysis to detect the concealed change of the telomeric length. This is the first study to observe the process of the telomere shortening using telomeres percentage analysis in normal adults. The present study indicates that the length of telomeres decreased with age in normal peripheral blood cells in 192 normal adults, consistent with previous observations [7, 16]. A review of the literature indicates that there has been no previous analysis of the changing telomere size in humans. So far,

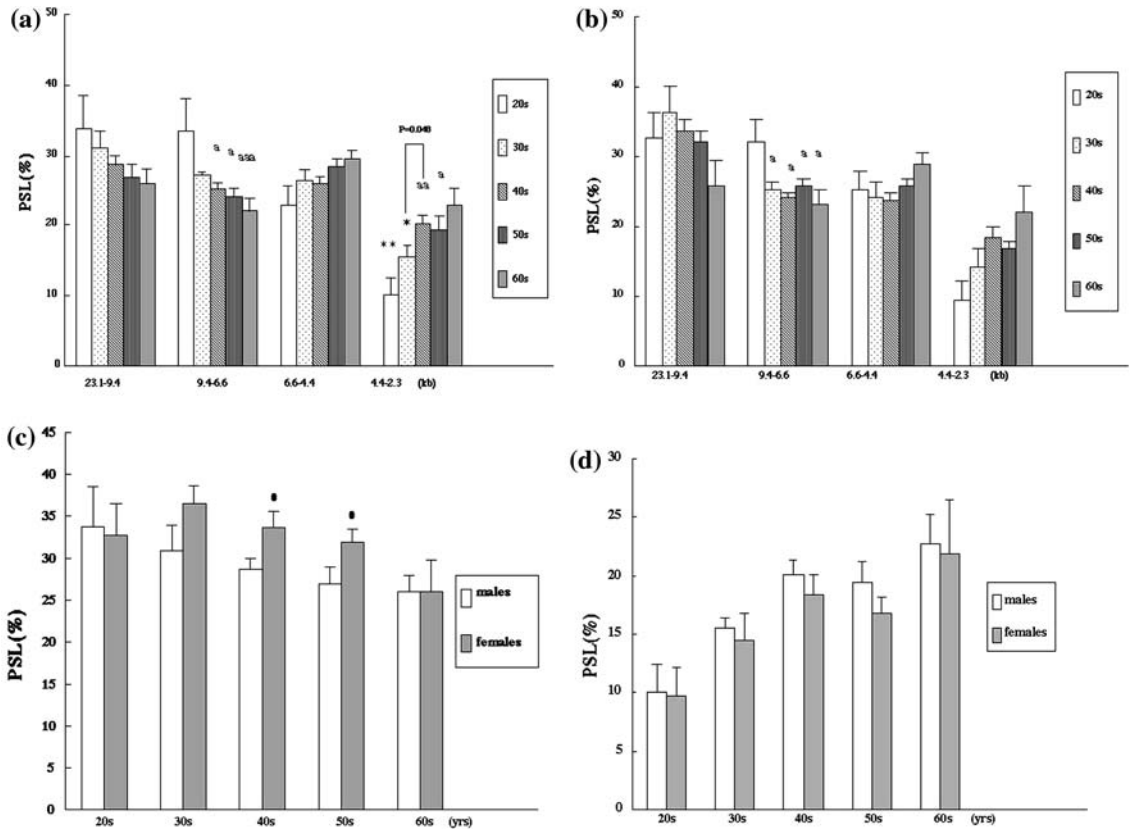

Fig. 3 Telomere fragment length percentage profile. Long (9.4–23.1 kb) (**b**, **d**) and short (2.3–4.4 kb) (**a**, **c**) telomere fragments in age ranges between 20s and 60s in males and females (**c**, **d**). The vertical bars are the mean ± SE ^{a} $P < 0.05$, ^{aa} $P < 0.01$, ^{aaa} $P < 0.001$ compared to 20s. * $P < 0.05$, ** $P < 0.01$ compared to 60s. # $P < 0.05$ in comparison to males

only a limited number of studies have confirmed that very short telomeres are associated with telomere dysfunction, while also limiting cellular survival in mice [13] and rat [14]. From the telomere percentage profile in the present study, the telomere attrition rate seems to be namely reflected in the longer ($\gtrsim$10 kb) and shorter ($\lessapprox$5 kb) telomere fragments. In addition, this analysis allows the detection of significant differences in the telomere percentage profile between individuals in their 30s and 40s that could not be detected by the TRF analysis. This indicates that the telomere length percentage analysis was useful and sensitive for detecting detailed telomere-size changes. A previous study found that in many human organs or tissues, a rapid shortening of the telomeres occurs in the young and only a slower reduction occurs after age 40s [21]. In addition, the above observation regarding older subjects is similar to our present results. It is possible that the rapid loss of telomeric DNA is caused by a high rate of proliferation. Blood cells are constantly replaced by new cells, which are generated by proliferation and differentiation of hematopoietic progenitors, and cell divisions are required to sustain hematopoiesis throughout life. This would be initiated from a partially differentiated pool of progenitors that already have undergone significant telomeric shortening [16].

Gender-related differences in telomere were also observed with aging. TRF attrition with aging was observed in a normal Japanese population, but the correlation was mild. This mild correlation seemed to mainly be related to the lower correlation among females (Fig. 1c). Previous data have shown that the variability in the mean TRF length among individuals may arise from the telomere length at birth, the rate of loss thereafter, or both [5, 6, 22, 23], and may also be affected by estrogen [24]. This process may also be regulated by a competing set of positive and negative factors throughout life [5], such as age, gender and race.

A slower attrition of the mean TRF in females was observed and supported by the further observation that longer telomeres (>9.4 kb) remained stable until age 50 in females.

There are conflicting observations concerning differences in the telomere length between males and females. Some reports [25, 26] have found some minor variations, but no significant mean TRF length difference between males and females, while others have shown that the TRF

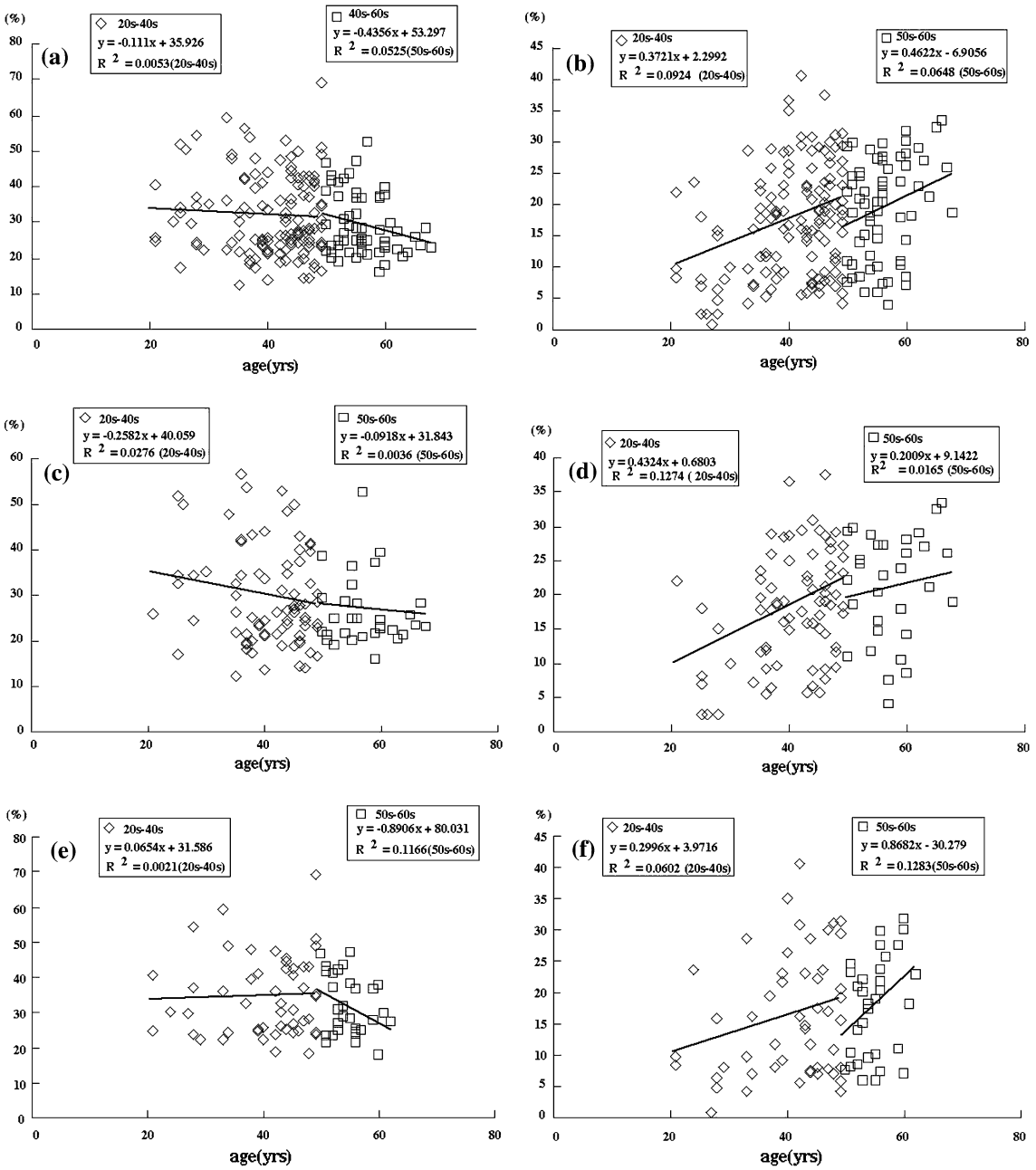

Fig. 4 Distribution of percentage of long-telomere fragments and short-telomere fragments with age. The longest range (9.4–23.1 kb) is described in (**a**), (**c**), and (**e**). The shortest range (2.3–4.4 kb) in (**b**), (**d**) and (**f**). (**a**) and (**b**) are for males + females. (**c**) and (**d**) are for males. (**e**) and (**f**) are for females. Note that the differences between (**c**) and (**e**), and between (**d**) and (**f**) to see the gender-related change of telomere-length distribution after age of 50

length in blood cells is longer in females than in males after adjusting for age [8, 22]. This latter observation is consistent with the findings of this study.

Using the percentage method, a significantly higher percentage of the longest telomere fragments was observed in females than in males. In addition, there was a propensity of a lower percentage of the shortest telomeres in females compared to males in both their 40s and 50s, with difference observed in the 60s, after menopause, after adjusting for age. This is consistent with the findings of a previous study which reported that estrogen can stimulate telomerase through an estrogen response element existing on the catalytic subunit of the enzyme [24], while also reducing oxidative stress [20].

In addition, this study demonstrated an average rate of decline in TRF length of 77 bp per year in all subjects

including males and females, which is similar to the rate of 67 bp per year reported in a previous report including Japanese males and females [21]. These data also showed telomeric shortening with a slower pace in males and with a faster pace in females over age 50. A previous study showed that telomeric loss did not occur at a consistent speed [16].

The difference between males and females was observed in the telomere attrition rate after age 50. At an earlier age, telomere attrition is faster in males than in females, but after 50, the tendency was reversed. The female longer telomere fragments are reduced faster after the age of 50. This observation may reflect accelerated cellular aging in the postmenopausal period. Slower telomere regression in female before 50 could be an effect of estrogen, and estrogen reduction in the postmenopausal period would then result in faster shortening of the telomeres. This possibility can be applied to the male increased telomere attrition because of the serum estrogen level is lower in males than in females prior to age 50. The reduced male telomere attrition after 50, however, cannot be explained by changes in estrogen-related telomere maintenance. The explanation for the reduced of telomere-shortening rate in males after 50 is unknown. One possible hypothesis is that androgen facilitates telomere attrition and a serum androgen reduction after age of 50 yields slower telomere attrition in male. However, there has been no report confirming the telomere-shortening effect of androgen, so far. Further investigation is necessary to confirm this hypothesis. Furthermore, the observed accelerated telomere attrition in females compared to males after age 50 cannot be explained by either estrogen- or androgen-related telomeric regulation. The process of telomere attrition with aging after age 50 may be affected by unknown factors irrespective of the hormonal environment.

In conclusion, this study revealed that telomere shortening occurred in an age- and gender-related manner based on the percentage of various telomere fragment sizes in blood cells. In males, there was a decrease in the longer telomere fragments and an increase in the shortest segments with age, especially in healthy adults from 20s to 40s. Beyond age 50, the rate of these changes was altered in a gender-related manner. In females, there was also a significant decrease with age in longer telomere fragments before age 50 and this was significantly accelerated after 50. The variation in the telomere attrition rates between males and females beyond age of 50 cannot be simply explained by estrogen-related effects but by other unknown factor(s).

Further investigation is therefore necessary to elucidate the relationship between the longevity and telomere length distribution, and its gender-related alterations.

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