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Telomere length of normal leukocytes is affected by a functional polymorphism of hTERT

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Abstract

Transcriptional regulation of human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase, is essential for telomerase activity associated with telomere length. In this study, we investigated the effects of a $^{-1327}$T/C polymorphism within the hTERT promoter region on the hTERT promoter activity and leukocyte telomere length in normal individuals. The promoter activity in the $^{-1327}$T-sequence was significantly higher than that in the $^{-1327}$C-sequence ($p = 0.0004$). For leukocyte telomere length, the $^{-1327}$T-allele carriers had significantly longer than the $^{-1327}$T-allele non-carriers ($p = 0.0007$). Also, there was no age-related shortening in leukocyte telomere length in the $^{-1327}$T/T ($p = 0.6633$) and $^{-1327}$T/C subjects ($p = 0.1691$), whereas there was clear age-related telomere shortening in the $^{-1327}$C/C subjects ($p = 0.0117$). These findings suggest that the functional $^{-1327}$T/C polymorphism of hTERT is associated with leukocyte telomere length in normal individuals.
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Telomerase synthesizes telomeric repeats for addition to the end of linear chromosomes, although replication of the telomeric end is sometimes incomplete [1,2]. Thus, telomere shortening occurs after repeated cell divisions and has a key role in cellular senescence, differentiation, immortalization, and transformation [3]. A recent study showed that telomere shortening is assumed to contribute to mortality in older subjects or age-related diseases [4].

Telomere length is mainly regulated by telomerase activity associated with transcriptional activity of human telomerase reverse transcriptase (hTERT), a subunit of telomerase [5–7]. The hTERT promoter region located with the 1375 bp upstream of the transcription-starting site is rich in transcription factor binding sites [8,9]. Although the regulation of hTERT transcription has been widely studied, little is known about the genetic variations in relation to hTERT transcriptional activity.

In this study, the hTERT promoter region was sequenced for screening of genetic polymorphisms in a healthy population. A T to C transition 1327 bp upstream of the transcription-starting site of hTERT ($^{-1327}$T/C) was frequently observed (nucleotide numbering according to Horikawa et al.) [9]. Further, we investigated the association between the $^{-1327}$T/C polymorphism and (a) hTERT transcriptional activity in normal human umbilical vein endothelial cells (HUVECs), and (b) telomere length and telomerase activity in peripheral leukocytes in normal individuals.

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Materials and methods

Screening of the sequence variations within the hTERT promoter region. Written informed consent was obtained from all subjects enrolled into the study. Study subjects were genetically unrelated Japanese subjects.

We recruited 46 healthy subjects for screening of polymorphisms within the hTERT promoter region. Among these subjects, the variation(s) in the hTERT sequence (nucleotide number [9] $^{-}$1665 to $^{+}$20) for 17 subjects and the sequence variation at position $^{-}$1327 for 29 subjects were analyzed by a direct DNA sequence analysis.

Luciferase assay. A dual-luciferase reporter assay system (Promega, Madison, WI) was used according to the manufacturer's protocol. A 1.6-kb DNA fragment (nucleotide number [9] $^{-}$1623 to $^{+}$20) with the $^{-1327}$T- or $^{-1327}$C-sequence was subcloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA). Each hTERT insert was subsequently cloned into a firefly luciferase reporter plasmid pGL-3-Basic, a promoter- and enhancer-less vector (Promega), designated pGL3-$^{-1327}$T and pGL3-$^{-1327}$C. Thus, we prepared four types of firefly luciferase reporter plasmids, pGL3-$^{-1327}$T, pGL3-$^{-1327}$C, pGL3-Basic, and pGL3-Control, with the SV40 enhancer/promoter for the normalization of hTERT promoter activity and one *Renilla* luciferase reporter plasmid for standardization of transfection efficiency.

HUVECs (passage number, 3) were purchased from TAKARA (Tokyo, Japan). Early passage HUVECs (passage number, 5–7) were used to avoid the influence of any transformation by subculture on this assay. Transfection with luciferase plasmid into HUVECs was performed using FuGene 6 Transfection Reagent (Roche, Nutley, NJ). Luminescence was measured in each transfectant 24 h and 48 h after transfection. The value corresponding to the transcriptional activity of hTERT promoter for pGL3-$^{-1327}$T or -$^{-1327}$C was calculated using the formula: relative luciferase activity (%) = [(pGL3-$^{-1327}$T or -$^{-1327}$C) − (pGL3-Basic)]/[(pGL3-Control) − (pGL3-Basic)] × 100.

Assay for telomere length. To measure telomere length of leukocyte DNA, as assessed by mean length of terminal restriction fragments (TRF), we used Southern hybridization of telomeric DNA [10] and real-time kinetics quantitative polymerase chain reaction (PCR) [11], and correlation of results by these two methods was previously confirmed [11]. After the confirmation of correlation between these two different methods for measuring telomeres of our samples, we calculated telomere length. Study subjects were 133 males over 40 years of age because the rate of telomere shortening decreases after 40 years of age and is higher in males [12,13]. Genotyping of the $^{-1327}$T/C polymorphism was performed using Megabase 1000 (General Electric, Fairfield, CT), according to the manufacturer's protocol for the single nucleotide primer extension-based method.

Telomerase activity. Telomerase activity in leukocyte from healthy subjects was measured using the method for real-time quantitative PCR telomeric repeat amplification protocol (TRAP) assay, as described previously [14], and telomerase activity in each genotype of the $^{-1327}$T/C polymorphism was analyzed by the values of threshold cycle of telomeric repeat amplification in the real-time quantitative PCR TRAP assay. Nine study subjects were selected to match in age among three genotypes of the $^{-1327}$T/C polymorphism.

Statistics. Mean values of the two groups in this study were compared by Student's t test. Mean values of the three groups in this study were compared by ANOVA. Single regression analysis was used to detect a correlation coefficient (r) in TRF length assay. Statistical analyses were performed using StatView (ver 5.0, for Macintosh, SAS, Cary, NC). A p value less than 0.05 was considered to be statistically significant.

Results

We analyzed the sequence of the hTERT promoter region to screen for genetic variations in 17 subjects, and 2 subjects were showed to be heterozygous for a T to C transition at 1327 bp upstream of the transcription-starting site [9]. This $^{-1327}$T/C transition has been reported (rs 2735940) in the database of single nucleotide polymorphism (http://www.ncbi.nlm.nih.gov/SNP/index.html), although there is no report of epidemiologic or experimental data for this substitution. To examine whether this T/C substitution is polymorphism or not, i.e., this substitution is present more than 1% among population, the genotype distribution of the $^{-1327}$T/C substitution was analyzed in an expanded population of 46 subjects. As a result, the genotype distribution was 15.2% for the $^{-1327}$T/T genotype, 39.0% for the $^{-1327}$T/C genotype, and 45.8% for the $^{-1327}$C/C genotype, suggesting that this T/C substitution is a polymorphism.

To investigate the effects of the $^{-1327}$T/C polymorphism on hTERT transcriptional activity, we performed an experimental study using a dual-luciferase reporter assay system. The mean value of relative luciferase activity representative of hTERT promoter activity in HUVECs transfected with pGL3-$^{-1327}$T was significantly higher than that in HUVECs transfected with pGL3-$^{-1327}$C at 24 h or 48 h after the transfection: 4.592 ± 0.285 (%, mean ± SD) for the pGL3-$^{-1327}$T and 3.711 ± 0.686 for the pGL3-$^{-1327}$C after 24 h of the transfection ($p = 0.0026$), and 6.368 ± 1.017 for the pGL3-$^{-1327}$T and 4.842 ± 0.203 for the pGL3-$^{-1327}$C after 48 h of the transfection ($p = 0.0004$) (data were obtained from three independent experiments performed in triplicate). The results are indicative of the relationship between the $^{-1327}$T-sequence and higher hTERT transcriptional activity.

Next, we measured leukocyte TRF length to test the hypothesis that the $^{-1327}$T/C polymorphism affects telomere length, closely related to the final stages of the telomere system. This speculation was also raised by previous reports that an inter-individual variation in leukocyte telomere length was genetically determined [15,16]. The TRF length in normal leukocytes was significantly different among the three genotypes: 7.80 ± 1.23 (kb, mean ± SD) for the $^{-1327}$C/C genotype ($n = 67$), 8.47 ± 1.04 for the $^{-1327}$T/C genotype ($n = 52$), and 8.53 ± 0.96 for the $^{-1327}$T/T genotype ($n = 14$) ($p = 0.0031$; Fig. 1). When analyzing the telomere length between the subjects without or with the $^{-1327}$T-allele, we obtained the results which showed that the genotypes with $^{-1327}$T/T and $^{-1327}$T/C were significantly longer than that in the $^{-1327}$C/C genotype: 7.80 ± 1.23 (kb, mean ± SD) for the $^{-1327}$C/C genotype ($n = 67$), 8.47 ± 1.04 for the $^{-1327}$T/C and $^{-1327}$T/T genotypes ($n = 66$) ($p = 0.0007$). Mean age was not significantly different between groups: 53.4 ± 5.0 (years, mean ± SD) for the $^{-1327}$C/C, 52.7 ± 4.4 for the $^{-1327}$T/C, and 51.9 ± 4.4 for the $^{-1327}$T/T ($p = 0.5200$). Also, there was no age-related shortening in TRF length in the $^{-1327}$T/T ($r = 0.128$, $p = 0.6633$) and $^{-1327}$T/C subjects ($r = -0.194$, $p = 0.1691$), whereas there was clear age-related telomere shortening in the $^{-1327}$C/C subjects ($r = -0.306$, $p = 0.0117$; Fig. 1). These observations suggest that the $^{-1327}$T/C polymorphism is strongly associated with telomere length in peripheral leukocytes in normal individuals.

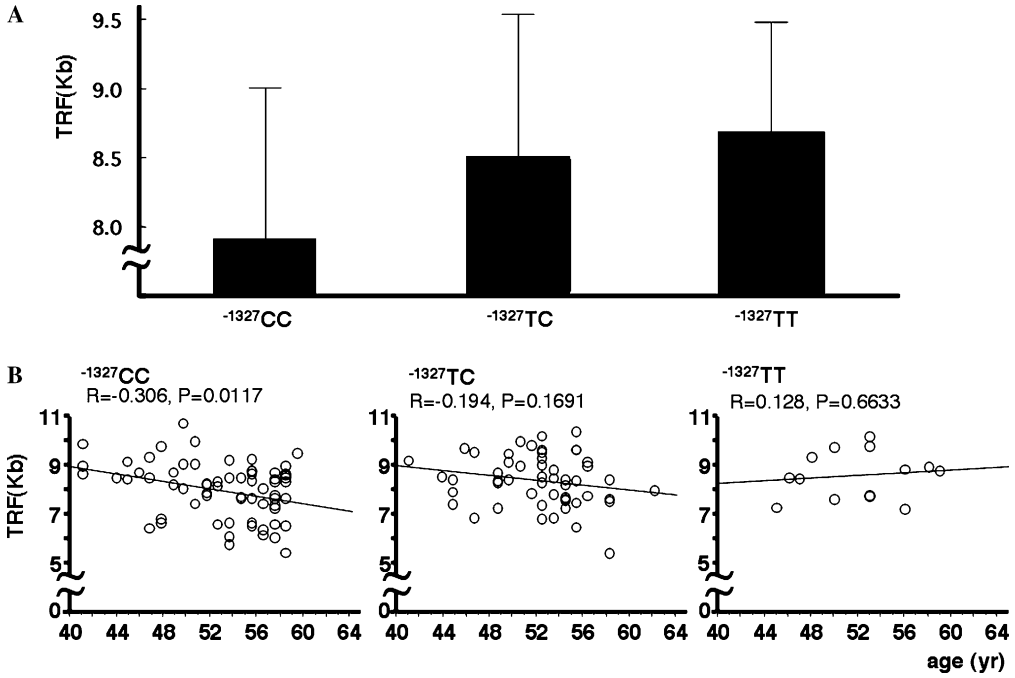

Fig. 1. Relationship between leukocyte telomere length and the $^{-1327}$T/C polymorphism. (A) Bars show mean TRF length (i.e., telomere length) (mean ± SD) in each genotype. (B) Plot of leukocyte TRF length against age and regression line are shown separately for the $^{-1327}$CC, $^{-1327}$TC, and $^{-1327}$TT genotypes.

Telomere length is mainly regulated by telomerase activity that is generally associated with hTERT transcriptional activity. However, several reports showed that transcriptional activity of hTERT did not always correlate directly with telomerase activity and the presence of post-translational modification [17]. Thus, we analyzed the relationship between the $^{-1327}$T/C polymorphism and telomerase activity in leukocyte from healthy subjects, and mean age in each genotype group was 36.0 ± 11.0 (y, mean ± SD) for the $^{-1327}$C/C genotype ($n = 4$), 36.0 ± 7.1 for the $^{-1327}$T/C genotype ($n = 2$), and 36.0 ± 12.2 for the $^{-1327}$T/T genotype ($n = 3$). Telomerase activity was examined using the threshold cycle values (C_t) of telomeric repeat amplification in the real-time quantitative PCR TRAP assay, thus higher C_t indicating lower telomerase activity. Telomerase activity in the subjects with the $^{-1327}$T-allele was higher than that in the subjects without the $^{-1327}$T-allele: 29.9 ± 5.6 (C_t, mean ± SD) for the $^{-1327}$C/C genotype, 28.0 ± 4.2 for the $^{-1327}$T/C genotype, and 21.8 ± 4.0 for the $^{-1327}$T/T genotype, and this difference was marginally significant ($p = 0.0713$). Observation suggests that the $^{-1327}$T-allele is associated with higher telomerase activity in leukocyte.

Discussion

The present study demonstrates for the first time that the $^{-1327}$T/C polymorphism within the hTERT promoter region has functional roles: the $^{-1327}$T sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. The relationship of the $^{-1327}$T/C polymorphism to telomere shortening, telomere length, and telomerase activity was found in normal peripheral leukocytes. Leukocyte telomere shortening has been highlighted as a critical marker in the research of cell senescence and cancer, thus, our observations show an impact in the fields.

Transcriptional regulation of hTERT has a key role in telomerase activity and telomere shortening; therefore, we focused on the hTERT promoter region in this study. In our promoter assay, we found approximately a 25% higher promoter activity in the $^{-1327}$T-sequence compared the $^{-1327}$C-sequence. Although the finding with such a modest effect, the data were so strong statistically significant. This significance was caused by the small range of the standard deviations, and possible reasons of the very little inter-assay are as follows; we used a dual-luciferase assay system for standardization of transfection efficiency and early passage HUVECs (passage number, 5–7) to avoid the influence of any transformation by long-term culture on this assay. Particularly, long-term culture of HUVECs showed cell senescence [18]. Although HUVECs have slight activity of telomerase [19], telomerase activity in senescent HUVECs is not fully understood. These suggest that long-term culture of HUVECs is not adapted to evaluate hTERT promoter assay. Thus, we used the present assay system that telomerase promoter with promoter gene works under transient condition using early passage HUVECs although it is important to examine the promoter assay under permanent condition in HUVEC. As a result of careful assay design, we found the relationship of the $^{-1327}$T/C polymorphism on hTERT transcription activity in HUVECs.

We measured leukocyte DNA TRF length, but not that of endothelial cells, because telomere length in both leukocytes and endothelial calls is inversely correlated with age (average decline 30–40 bp/year in normal leukocytes) [12,13,20–22], and leukocyte DNA was available for this study. Also, endothelial cells and leukocytes are exposed to the same hemodynamic stress, thus the rate of turnover is considered to correlate between these cells [21]. The $^{-1327}$T-sequence was strongly associated with longer telomere length. We postulated that $^{-1327}$T-sequence with higher hTERT transcriptional activity is associated with more effective extension of the telomeric end during cell division, and our results reveal a possible causative role of the $^{-1327}$T/C polymorphism in inter-individual variations in leukocyte telomere length.

In conclusion, we report a potential role of the $^{-1327}$T/C polymorphism within the hTERT promoter region in the hTERT promoter activity and leukocyte telomere shortening among normal individuals.

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