



A novel multiplex qPCR method for assessing the comparative lengths of telomeres

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Abstract

Background: The comparative length of telomeres is considered to be related to diseases such as cancer, aging, and cardiovascular diseases. qPCR is currently one of the main methods for detecting telomere length. However, due to the unique sequence of telomeres (highly repetitive six-base sequence), it is difficult to design primers and probes to expand and detect telomere and to put internal reference gene and telomere into the same tube for detection to reduce the possible inter-pore errors and improve amplification efficiency. Besides, the stability and accuracy of the test results are greatly affected by the difference between reference genes and telomere copy number.

Methods: In this study, the single-copy genes were replaced with high-copy genes (300 copies) as the internal control to reduce the copy number difference of the internal genes and telomere. In addition, a multiplex qPCR system was constructed to detect the telomeres and an internal reference gene product. We also detected the lengths of telomeres in the genomic DNA in immortalized cells (293T and HeLa) from different generations of cells.

Results: We detected the comparative telomere lengths of 1500 random Chinese volunteers of different ages with the multiplex qPCR method; the result shows that the comparative length of telomeres is negatively related to age. In addition, we compared our qPCR detection method with a terminal restriction fragmentation (TRF) method. Both of them were highly consistent, indicating that the qPCR method was reliable.

Conclusions: In conclusion, we developed a stable, convenient, and accurate comparative telomere length detection method.

KEYWORDS

multiplex quantitative PCR, the comparative length of telomere, TRF analysis

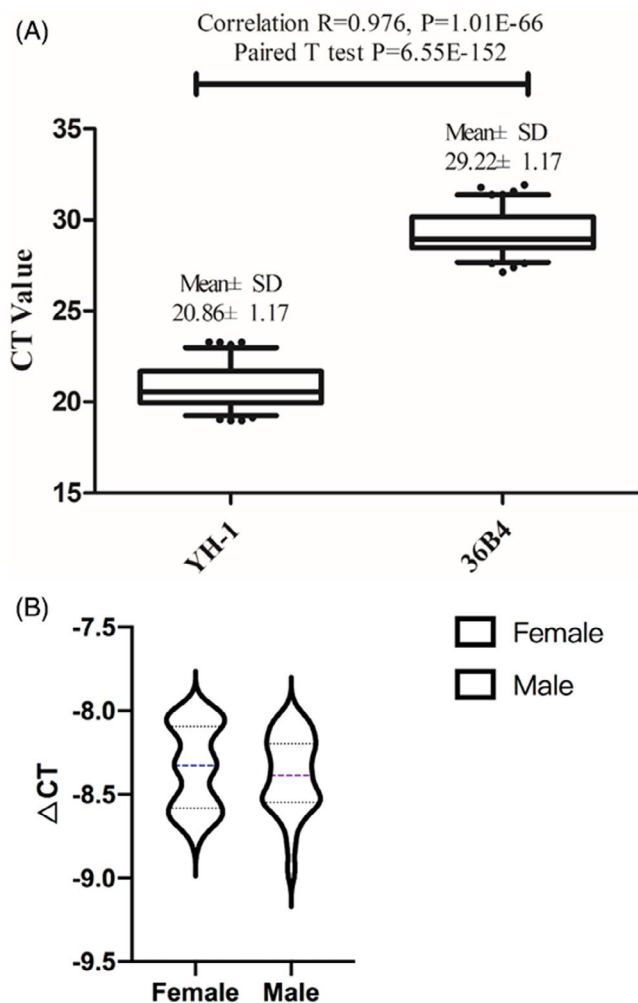


FIGURE 2 A, Stability of YH-1 and 36B4 based on cycle threshold values. Figure 2A shows the stability of YH-1 gene and 36B4 gene. The mean Ct value of YH-1 gene was 20.86 ± 1.17 , and that of 36B4 gene was 29.22 ± 1.17 . The expression levels of the two genes changed similarly in the population. B, Stability of YH-1 internal reference gene in sex. Figure 2B shows the expression level of YH-1 gene in different genders. The YH-1 gene was expressed at similar levels in male and female samples

SYBR green dye. All PCRs were carried out on a 7500 Real-Time PCR System, and amplification was conducted as follows: Stage 1: 1.5 min at 95°C, Stage 2: 30 cycles (telomere and internal gene reaction) of 15 sec at 95°C, Stage 3: 1 min at 50°C, Stage 4: 45 sec at 72°C, and fluorescence signals were collected at 50°C. The reactions were carried out in a 96-well plate. Software v2.3 was used for analysis. The telomere length for each sample was determined using the telomere to high-copy gene ratio (T/H ratio) by calculating the Δ Ct [$\text{Ct}(\text{telomere})/\text{Ct}(\text{high-copy gene})$]. The T/H ratio for each sample (x) was normalized to the mean T/S ratio (test sample/standard sample) of the reference sample [$2^{-(\Delta\text{Ct}_x - 2\Delta\text{Ct}_{\text{ref}})} = 2^{-\Delta\Delta\text{Ct}}$], which was also used for the standard curve as a reference sample and as a validation sample. In every run, two reference samples were included to validate each reaction. The experiment was considered reliable if the T/H ratio of the control sample ranged within the 95% variation interval (0.95–1.05).

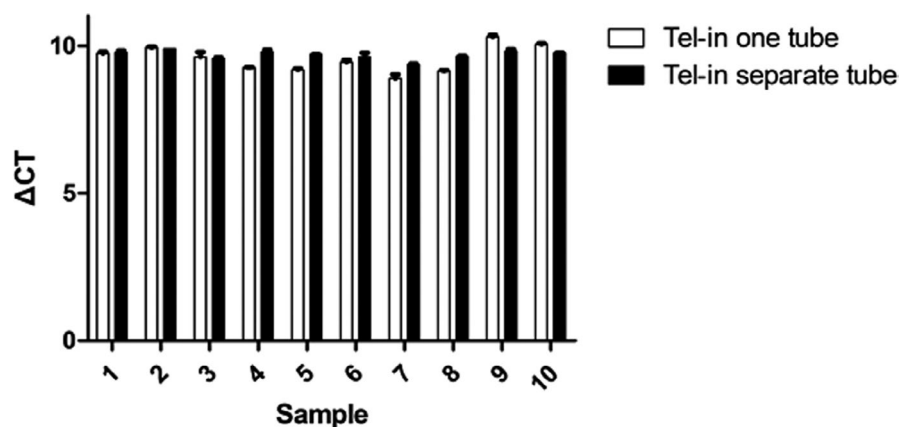
2.2 | Sample collection and preparation

A total of 1,500 random peripheral blood samples were collected from the Central Hospital of Songjiang District, Shanghai. Samples were collected under informed consent from the volunteers. The samples are divided into 6 different age-groups (20–30, 31–40, 41–50, 51–60, and above 60 years of age). The gender ratio of the total sample is basically the same, but the difference is obvious in the age range: take 50 years old as the limit, the sample age ≥ 50 , the number of samples is large, while the sample age < 50 , the number of samples is small.

2.3 | Cell lines

Three cell lines, 293T, HeLa, and HUVEC cells, were used in this study. The genomic DNA extraction kit (DP304) produced by Tiangen Biochemical Technology Co., Ltd. (Beijing, China) was used for cell line genome DNA extraction. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific Inc., Waltham, MA) supplemented

FIGURE 3 Differences in the amplification efficiency of telomeres. Figure 3 shows the amplification of telomeres and reference genes in the same tube and in different tubes. Under two different amplification conditions, telomere expression levels in the same sample did not change significantly



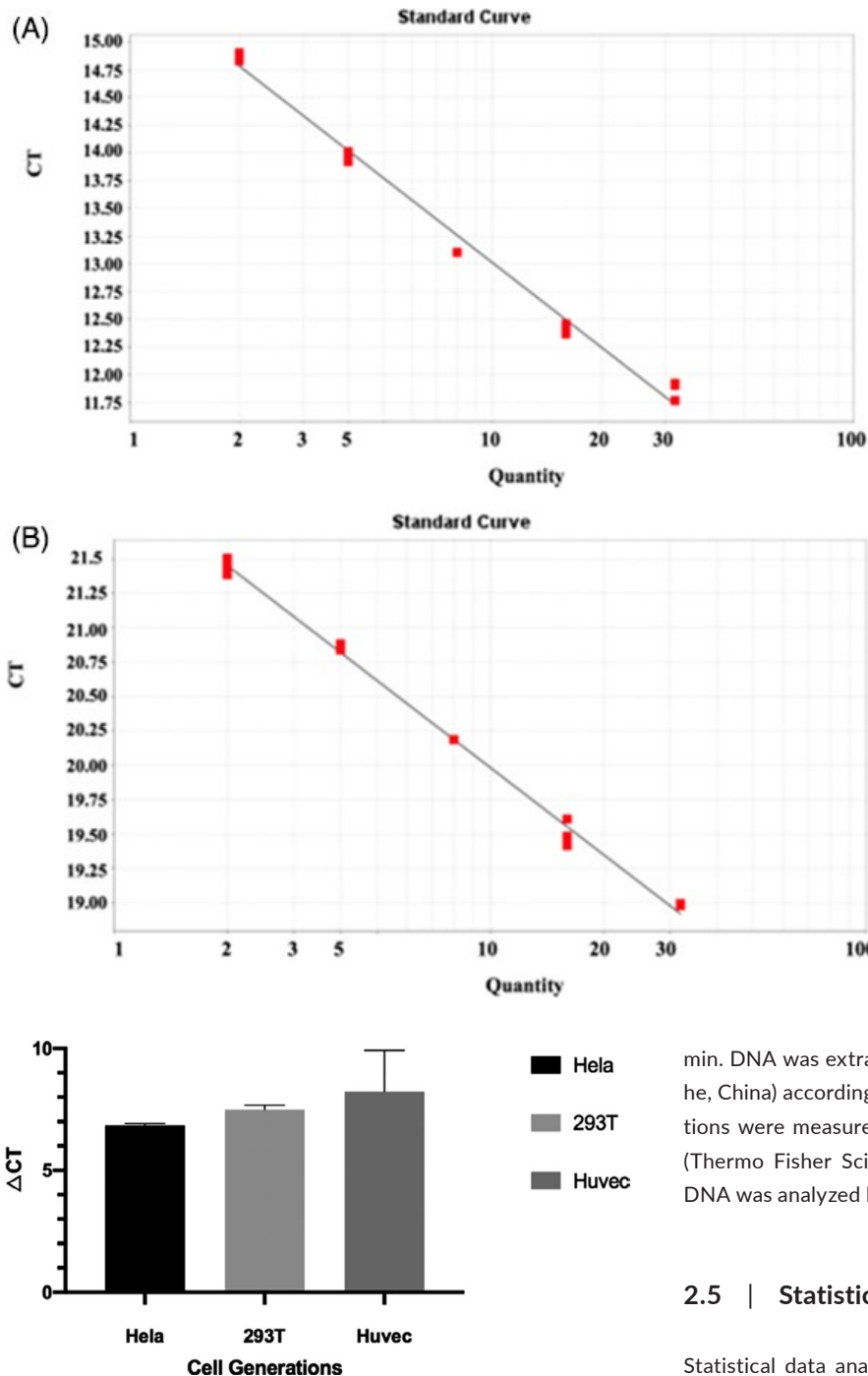


FIGURE 5 Comparison of telomere lengths in different cell lines. Figure 5 shows the telomere length variation of immortalized 293T and HeLa cells and normally differentiated HUVEC cells under different cell algebras

with 100 U/mL penicillin, 100 g/mL streptomycin, and 10% fetal bovine serum (Thermo Fisher Scientific Inc., Waltham, MA). All cell lines were maintained at 37°C of 5% CO₂.

2.4 | DNA extractions

200 ul of fresh blood was taken and added to the red blood cell lysate. After 1 min, the DNA was centrifuged for 2 mins at 10,000 rpm/

FIGURE 4 A, The standard amplification curve of telomeres. Figure 4A shows the standard curve of telomere amplification. The amplification efficiency of telomeres was consistent at different concentrations. B, The standard amplification curve of YH-1. Figure 4B shows the standard curve of YH-1 gene amplification. The amplification efficiency of YH-1 was consistent at different concentrations

min. DNA was extracted using a commercial DNA extraction kit (Yihe, China) according to the protocol of the vendor. DNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and the quality of DNA was analyzed by agarose gel electrophoresis.

2.5 | Statistical analysis

Statistical data analysis was performed using GraphPad Prism v5 (GraphPad Software Inc., CA, USA).

3 | RESULTS

3.1 | High copy genes were selected as internal genes

Compared to previous single genes such as 36B4, Figure 1 showed high-copy number genes bridge the gap with the telomere copy number, improving the stability of detection. We detected the lengths of telomeres in fifteen blood samples at DNA template concentrations of 40 ng and 80 ng. Our data showed that the high-copy number gene was approximately 300 bp and the amplification products were around 200 bp.

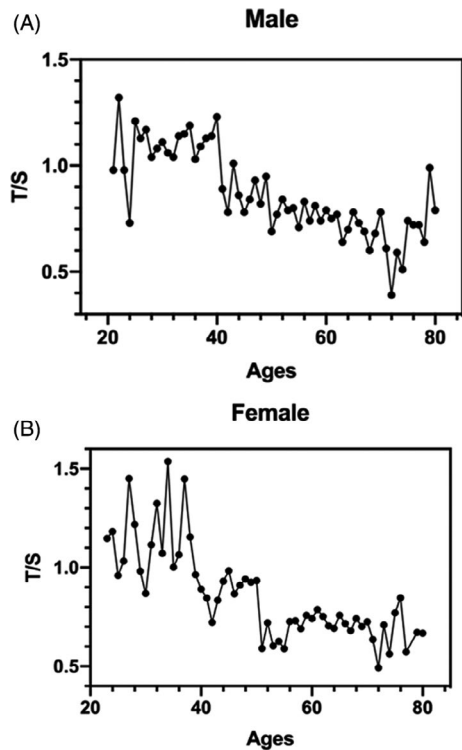


FIGURE 6 A, Comparative telomere length decreases with increasing age in the male population. Figure 6A shows the variation of mean telomere length in male samples of different age-groups. Average telomere length decreases gradually. Male reach their lowest average telomere length at 72 years of age. B, Comparative telomere length decreases with increasing age in the female population. Figure 6B shows the variation of mean telomere length in female samples of different age-groups. Average telomere length decreases gradually

3.2 | Copy number of the internal gene was stable in the population

The high-copy internal gene screened from the genome database had no copy number variation across the population and no non-specific amplification on the sex chromosome (X chromosome) indicating that it could be used as a stable internal control gene.

No variation in the copy number of YH-1 was detected in the population. We screened the genome database for a stable high-copy gene (300 copies) called YH-1 was then used as the internal reference gene. The results of the blast search showed that YH-1 has no copy number variation in the population. Also, Figure 2A showed the common stable keep-housing gene 36B4 was used as a control to test the stability of YH-1 in one hundred blood samples. Results showed that the average CT of 36B4 was approximately 29.22 ± 1.17 and the average CT of YH-1 was approximately 20.86 ± 1.17 . The change in YH-1 was consistent with that of 36B4.

To test the reliability of our system, we examined copy number differences in YH-1 across thirty males and female blood samples. Figure 2B showed that the average ΔCT of YH-1 in females was approximately -8.25 ± 0.25 and the average ΔCT of YH-1 in males

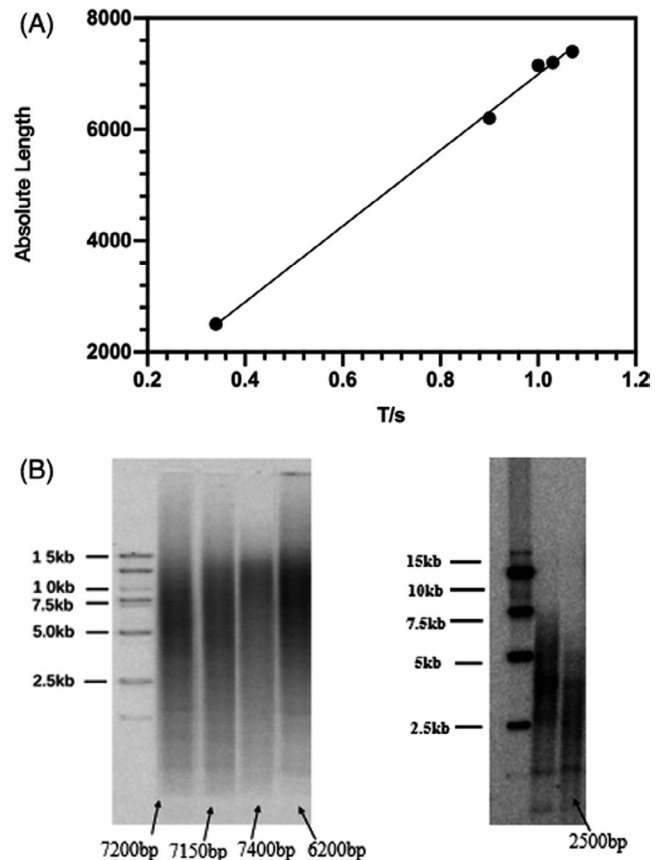


FIGURE 7 A, TRF method was used to verify the accuracy of the detective result. Figure 7A shows the telomere length detected by TRF method and qPCR method. In the figure, the abscissa represents the qPCR test results, and the ordinate represents the TRF test results. Two different methods have similar results for the same sample. B, Detecting telomeres absolute length using TRF method. Figure 7B shows the use of TRF method to detect the telomere length of the sample. The leftmost lane in the figure is a marker strip. According to the density of different lanes in the electrophoresis, the telomere lengths of different samples can be obtained

was approximately -8.38 ± 0.23 . These data indicated that the copy number of YH-1 was stable across the sexes.

3.3 | Establishment of a multiplex PCR detection system

A multiplex PCR system was constructed to detect the copy number of telomeres and the internal reference gene. This system was found to reduce the error from different tubes and improving the accuracy of the detection results.

Figure 3 showed that irrespective of whether the internal and telomeres gene were amplified simultaneously, the internal gene did not significantly interfere with the amplification of the telomere gene. Figure 4A and B showed the standard curves of the telomere and internal reference genes were detected as normal. Analysis of

the standard curves from the multiplex fluorescence PCR system showed good correlation.

3.4 | 293T cells were selected as the standard

A previous report showed that in immortalized cell lines such as 293T and HeLa, telomere enzyme activity is higher than normal differentiated cell lines resulting in the ability to maintain constant telomere lengths in different cell generations^{13,14}. Therefore, both 293T and HeLa cells were used as standards.

By testing the telomere lengths in different generations of 293T cells and HeLa cells, Figure 5 showed that telomere length did not change with the number of generations compared to normal differentiated HUVEC cells.

3.5 | Testing of 1500 samples

Using the multiplex qPCR system detection system, we detected the telomere lengths of 1500 random volunteers at different age stages and established a small age database of the characteristics of the Chinese population.

Results suggested that telomere length was negatively correlated with age. Figure 6A and B showed comparative telomere length dropped significantly after the age of 40 in both men and women while Figure 6A showed telomere lengths began to rise after 72 in males.

3.6 | The TRF method verified the accuracy of the detection results

To test the accuracy of the detection results of the multiplex PCR system, a TRF method was used to detect the same sample as the qPCR.

Figure 7A and B showed the comparative length of telomeres from the two different detection methods was highly consistent, demonstrating that the telomere data detected by a qPCR method were reliable.

4 | DISCUSSION

Telomere length is a promising biomarker for age-associated diseases and many cancer¹⁵. Several techniques including TRF, analysis by Southern blotting, qPCR, quantitative telomere/centromere fluorescence *in situ* hybridization (T/C-FISH), fluorescence *in situ* hybridization combined with flow cytometry (Flow FISH), single telomere length analysis (STELA), and whole-genome sequencing (WGS)¹⁶ have been used to detect the lengths of telomeres. In the normal population, the dynamic variation of telomere length was not significant¹⁷ and so the accurate detection of telomere length is particularly important. Average telomere length is negatively correlated with age, and the difference in the population is

about 2–3 times. However, due to the limitation of qPCR accuracy and the possible inter-hole error, there are still many challenges in the individual detection. Therefore, by selecting high-copy internal reference genes and optimizing the detection system, the internal reference genes and telomere were placed in the same tube for detection, which corrected the possible problems such as inter-pore errors and unstable detection results in the past. In the test samples, the copy number of telomeres and the internal gene differed by several dozen (the copy number of telomeres are in the tens of thousands, while the copy number of internal genes are in the hundreds). The SYBR green signal consists of the telomere and internal gene product signals. However, the proportion of the total signal due to the internal reference gene is relatively low and so can be ignored. A one-tube detection method for telomeres and the use of internal reference genes could effectively reduce errors and improve the accuracy of the detection results.

Through the above methods, the newly developed qPCR technology in this study has two advantages: Firstly, it can accurately detect the annual variation of individual telomere length and, secondly, it can accurately grasp the variation trend of telomere length of different age-groups.

A more accurate telomere length detection method could be used to uncover correlations between disease, aging, and changes in telomere length^{18–20}. Providing researchers a more reliable and convenient platform to perform qPCR for the accurate detection of telomere length.

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CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

AUTHOR CONTRIBUTIONS

J.H.X., G.Z.S., Y.X.Z., and K.L. conceived ideas and designed experiments; G.Z.S. and H.C. lead experiments (with help from B.Y., Y.X.Z., H.C., and J.H.X.); G.Z.S., J.H.W., and K.L. conducted data analysis and interpretation; J.H.X., J.H.W., and G.Z.S. wrote the paper and provided feedback to all authors. G.Z.S. and H.C. are the first co-authors of this article.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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