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Quantitative telomerase activity in circulating human leukocytes: utility of real-time telomeric repeats amplification protocol (RQ-TRAP) in a clinical/epidemiological setting

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Abstract

Background: There is accumulating evidence from the epidemiological field of telomere biology that telomere length plays an important role in the pathophysiology of cardiovascular disease. The RNA-dependent DNA polymerase, telomerase, is essential in regulating telomere length by acting as a reverse transcriptase. However, the relationship between telomerase activity and telomere length in cardiovascular disease is unclear. This is due, in part, to the paucity of information on the utility of a quantitative and routine assay for the determination of telomerase activity in circulating blood leukocytes.
Methods: We used a validated, high-sensitive real-time quantitative telomeric repeat amplification protocol (RQ-TRAP) to determine telomerase activity in circulating blood leukocytes.
Results: The present investigation demonstrated direct and reliable detection of telomerase activity of circulating blood leukocytes.
Conclusion: The present investigation suggests the feasibility of using RQ-TRAP assay in routine screening of telomerase activity in blood specimens typically collected in a clinical/epidemiological setting.
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Introduction

The telomere-telomerase complex plays an important role in human disorders including cardiovascular disease (1) and carcino/tumorigenesis (2, 3). Critical shortening of the telomere is prevented by telomerase activity that consists of de novo synthesis of telomeric DNA. Population-based studies of telomere length can help obtain additional information on factors directly influencing telomere length, such as telomerase activity. Various methods are used for the determination of telomerase activity in association with germline, immortal, and activated (including tumor) cells. However, at present, routine detection of telomerase activity in mammalian/somatic cells (including circulating human leukocytes) in a population-based setting is rare, owing to its low basal activity.

Very low levels of telomerase activity have been observed in circulating mononuclear blood cells from otherwise healthy subjects using qualitative and semi-quantitative assays (4–7). The telomeric repeat amplification protocol (TRAP), using polyacrylamide gel and radioactivity to detect telomerase activity in leukocytes, does not have the sensitivity to detect basal levels (4, 5). Also, it is not adequate for processing large number of samples as required for epidemiological studies. The semi-quantitative assay, TRAP-ELISA, has also been used in protein extracts from circulating mononuclear blood cells from patients with hepatocellular carcinoma and healthy subjects, showing an association of telomerase activity with hepatocellular cancer (6, 7).

Recently, a real-time quantitative SYBR Green high-sensitivity telomeric repeat amplification protocol (RQ-TRAP) for rapid quantitation of telomerase activity has been developed (8, 9). This procedure may serve as an alternative for the study of telomerase regulation and its relevance to somatic cell physiology, particularly in population studies. Furthermore, the sensitivity of the RQ-TRAP assay has been demonstrated to be greater than the TRAP-ELISA assay (9). We evaluated the applicability/utility of RQ-TRAP to determine telomerase activity in circulating human leukocytes in blood samples collected in a manner that mimics routine blood collection/storage typically performed for clinical/epidemiological investigations.

Table 1 Cycle thresholds (Ct) for telomerase activity of the cell extracts.

Samples	Ct (mean ± SE)			
	Day 0	Day 1	Day 3	Day +10 (frozen)*
Participant 1	27.62 ± 0.12	29.19 ± 0.07	28.31 ± 0.14	28.66 ± 0.02
Participant 1 HI	39.54 ± 0.11	34.29 ± 0.53	34.36 ± 0.79	34.22 ± 0.89
Participant 2	27.36 ± 0.10	29.62 ± 0.53	28.91 ± 0.09	27.18 ± 0.40
Participant 2 HI	37.81 ± 1.08	36.54 ± 2.37	38.14 ± 1.13	36.86 ± 2.03
Participant 3	28.80 ± 0.24	27.49 ± 0.20	28.35 ± 0.23	28.99 ± 0.14
Participant 3 HI	35.25 ± 0.86	36.87 ± 1.25	36.11 ± 1.52	35.36 ± 1.00

Whole blood was kept at 4°C at all times and prepared for quantitative telomerase detection (QTD) at day 0, day 1, and day 3. The Ct was set at 0.05. Each sample was evaluated in duplicate and the heat inactivated (HI) results are presented. Lower Ct indicates higher telomerase activity. *The buffy coat was frozen at day 0.

Materials and methods

Blood sample collection

Peripheral blood was collected from three unrelated, healthy volunteers at the Children's Hospital Boston, MA, USA. All volunteers were male, aged between 32 and 44 years and gave informed consent. The procedures were approved by the Committee for the Protection of Human Subjects at Children's Hospital Boston. Four heparin tubes, each containing 2 mL of blood, were drawn from each participant. To mimic delivery of blood specimens obtained for population-based studies, buffy coats were processed as follows: the buffy coat of the first blood tube was isolated and processed immediately, the buffy coat of the second blood tube was isolated after overnight storage at 4°C, the buffy coat of the third tube was isolated after weekend (3-day) storage at 4°C, and the buffy coat of the fourth tube was isolated immediately after blood drawn and then stored at –80°C for at least 10 days before processing. To further evaluate the effect of long-term storage on telomerase activity, a frozen buffy coat was stored at –80°C for over 1 year (1-year BC). Leukocytes were counted using the Advia 120™ Hematology Analyzer (Bayer Diagnostic Division, Tarrytown, NY, USA).

RQ-TRAP assay

Cell extracts for telomerase activity assays were prepared following manufacturer's instructions (Allied Biotech, Inc., Ijamsville, MD, USA). In brief, cells were lysed in 1X lysis buffer and incubated at 4°C for 30 min. The lysate was then centrifuged at 12,000 × *g* for 20 min at 4°C, and the supernatant collected. The protein concentration of the cell lysate was determined using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc., Rockford, NY, USA) according to the manufacturer's instructions. Telomerase activity was determined using the quantitative telomerase detection (QTD) kit (Allied Biotech, Inc., Ijamsville, MD, USA), an optimized and previously validated SYBR Green real-time PCR method, according to the manufacturer's protocol. Standards, inactivated samples and no-template-reactions were also assayed on every plate for quality control purposes, and to minimize inter-plate variability. A serial dilution-calibration curve was also performed on each plate to further minimize inter-plate variability, as recommended by the manufacturer. Each sample was analyzed in duplicate as described previously (9). Melting curve analysis was performed on each run to verify specificity and identity of the PCR products. All real-time amplifications were performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Telomerase activity was expressed relative to telomere short repeat (TSR) control template, and interpreted relative to 293T cells (a transformed human renal epithelial cell line) as per the manufacturer's specifications.

Results

The average total-cell count (mean ± SE, n = 12) for all participants was 1236 ± 64, and the average cell distributions (% ± SE, n = 12) for neutrophils, eosinophils, basophils, lymphocytes, and monocytes were 52.6 ± 2.0, 6.4 ± 0.5, 0.9 ± 0.2, 24.4 ± 1.5, and 8.5 ± 0.5, respectively. The mean protein concentration (mean ± SE, n = 12) of the cell lysate was 1.073 ± 0.030 g/L.

Table 1 shows the cycle thresholds (Ct) for leukocyte extracts processed at day 0, 1, 3, and +10 time-points. In addition, the Ct for the 1-year BC lysate was (mean ± SE) 27.4 ± 0.5. Of note, these Ct values were comparable across the various time-points that they were analyzed. The heat-inactivated (HI) leukocyte extracts and no-template-control reactions consistently exhibited a Ct of 35 cycles or higher, or null-amplification (at least > 5.6 cycles as compared to the respective active lysate) (Table 1). Our observed control-Cts were within the range specified by the manufacturer of 33 cycles for the blank assay and virtually identical to those reported previously (9). The correlation coefficient (R^2) for the TSR control calibration curve with serial dilution was 0.997 (Figure 1). The inter-assay variability was < 1%. Furthermore, the linearity of the RQ-TRAP assay with respect to the non-HI cell lysate of a sample was verified by per-

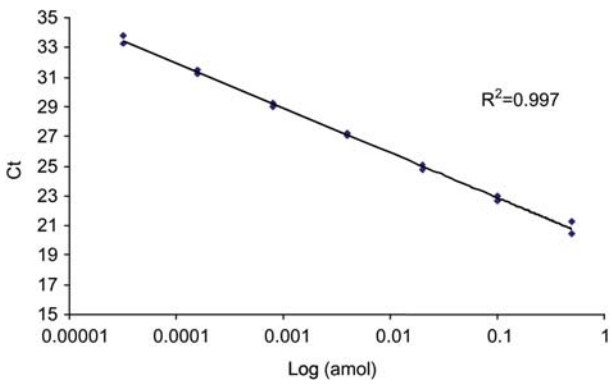

Figure 1 Standard curve of TSR control template with serial dilution.
Each concentration of the control template was analyzed in duplicate.

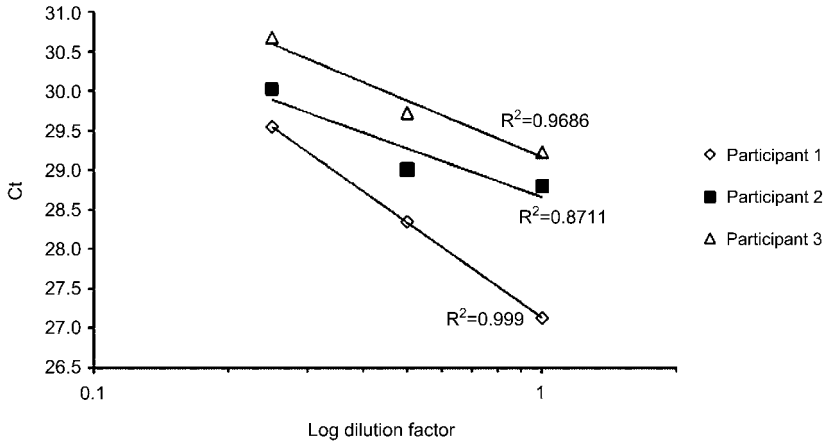

Figure 2 Cycle thresholds (Ct) for telomerase activity following serial dilution of the cell extracts.

forming a standard curve using serial dilutions (1, 0.5, and 0.25 μL template volume). R^2 between 0.871 and 0.999 were obtained (Figure 2). Most importantly, comparable levels of telomerase activity were detected in frozen cell extracts (+10 days, and 1-year BC) as compared to those stored at 4°C, thus demonstrating the robustness and utility of the RQ-TRAP protocol used for the detection of telomerase activity in circulating leukocytes. This shows the feasibility of this technique in population (epidemiological) based settings for disease association/prediction.

Discussion

The majority of published studies on the determination of telomerase activity emphasize immediate preparation of samples. Often times, immediate preparation of cell extracts is not feasible in blood specimens routinely collected in clinical/epidemiological settings due to logistic/delivery considerations. Hence, the present observations provide evidence that there is no observable reduction in telomerase activity following long-term storage at –80°C. In fact, it is generally believed that telomerase activity is particularly labile due to degradation of the RNA component (10–12). Our data show that if leukocytes/buffy coats are isolated, stored and processed as described, telomerase activity is not significantly affected. Therefore, this assay provides the opportunity to evaluate archived frozen samples, as well as prospectively collected samples.

The TRAP-ELISA technique allowed for an increase in the sensitivity of telomerase activity measurements, enabling the detection of low levels in unstimulated somatic cells such as leukocytes (4–7). However, the RQ-TRAP assay is quantitative, correlates with TRAP-ELISA (9) and lends itself to high-throughput screening and analysis. There is no consensus on the units that best represent telomerase activity. Units such as ''arbitrary units'' (13), ''percentages'' (9, 14), ''absorbance ratios'' (15), ''relative activity'' (16), and undefined ''units'' or ''activity'' (17, 18) have been used by various authors to describe telomerase activity. Our results are presented as Ct to allow for comparability with the original study reported by Wege et al. (9). Using this format, an increase in Ct levels represents a decrease in telomerase activity.

In conclusion, the present study demonstrates the utility of the SYBR Green high-sensitivity RQ-TRAP assay for the detection and quantification of very low basal levels of telomerase activity in circulating human leukocytes. It also suggests the feasibility of this method for screening blood samples routinely collected in clinical/epidemiological studies for telomerase activity.

Conflict of interest disclosures

None declared.

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