Original Article

Pre-Analytical Storage Temperature Affects Telomere Length and mRNA Expression of *hTERT*

Mohit Mehndiratta[1], Almeida Edelbert Anthonio[2], Ravoori Saaideswar Rao[3], Rajarshi Kar[4], Seema Garg[5], Bindiya Gupta[6], Amita Suneja[7], Dinesh Puri[8]

[1]Associate Professor, [2]Post-graduate Student, [4]Assistant Professor, [8]Director-Professor and Head, Department of Biochemistry, [6]Associate Professor, [7]Director-Professor and Head, Department of Obstetrics and Gynaecology, UCMS and GTBH, New Delhi; [3]Tutor, Department of Biochemistry, Government Institute of Medical Sciences, Greater Noida, Uttar Pradesh, India; [5]Professor and Head, Department of Biochemistry, AIIMS, Nagpur, Maharashtra, India

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ABSTRACT

Introduction: Telomere length (TL) is a biomarker of cellular ageing and an important marker of stress in many diseases. Physical conditions like collection method, storage of samples, and technique of isolation could affect TL and affect the final results of a study. The present study aimed to study the effect of pre-analytical storage temperature on telomere length (TL) and mRNA expression of *hTERT* in blood.

Methodology: As a part of the standardization procedure for estimation of TL and mRNA expression of *hTERT,* 20 whole blood samples were analysed. Each sample was divided into three aliquots of 200 µl each within two hours of sample collection. They were stored at room temperature (25°C), 4°C, and -20°C, respectively. DNA was extracted after a period of 24 hours from storage time point using column-based kit. Telomere length was measured by using Multiplex Real Time Polymerase Chain Reaction. RNA was extracted using RNA extraction reagent. cDNA was synthesized and mRNA expression was determined using delta-delta Ct method.

Results: The T/S ratio (TL) of the samples stored at 25°C and 4°C were comparable (50.35±25.87 and 53.02 ± 53.37) while it was higher at -20°C (155.14±90.80). mRNA expression of *hTERT* was reduced in samples stored at -20°C (1.38 fold) compared to 25°C, while it was increased 1.47-fold at 4°C when compared with 25°C.

Conclusion: Temperature is an important pre-analytical variable that affects TL and the mRNA expression of *hTERT*.

Keywords: *hTERT*, Pre-analytical variables, Telomere length, Temperature

INTRODUCTION

Telomeres are the guanine rich non coding repetitive segments (TTAGGG) of DNA present at the ends of eukaryotic chromosomes[1] varying in length from 500 bp to 20 kbp.[2] They play an important role in cell division and maintenance of chromosomal stability.[3] Telomere length (TL) shortens with every cell cycle and once it reaches a critical value (Hayflick limit) the cell ceases to divide and undergoes apoptosis.[4] This could give rise to problems, especially in embryonic cells, but this is not so, mainly because of the presence of a telomere length maintaining enzyme telomerase. Telomerase consists of a reverse transcriptase/TERT which adds TTAGGG sequences at the chromosome ends using RNA template (TERC).[5] Homologous recombination and soft copy switching can also address this issue.[6] Over expression of telomerase can increase the life span of a cell by 20 cycles[7] while its inhibition in neoplastic cells can lead to cell death. [8]

Telomerase is highly active in embryonic cells and cancer cells while its expression is low in normal somatic cells, which leads to their ultimate senescence and death. Telomerase repression in somatic cells is thought to be an evolutionary response to prevent carcinogenesis due to the high basal metabolic rate and replication rate in warm blooded animals.[9] Therefore, telomeres can be regarded as biologic clocks and serve as markers of cellular ageing.

Many factors are known to affect telomere length.

Oestrogen is known to prevent telomere attrition[10] while variations are seen in pathological stress,[11] chronic inflammatory conditions, mental stress [12] etc, on the other hand increased telomerase activity is seen in cancer cells. A study in mice demonstrated that telomerase reactivation reversed tissue degeneration. [13]

Whole blood DNA is frequently used for TL measurements.[14] Initially telomere restriction fragment (TRF) analysis was the most widely used method, which is being replaced by PCR based assays[15,16] which are more rapid, scalable, and cost effective with the only drawback being that standardization is difficult and results from different centres may not be directly compared. Furthermore, there is evidence that qPCR-based TL quantification may also be affected by various pre-analytical variables like sample storage temperature, period of storage, DNA isolation method,[17] and freeze-thaw cycle of samples.[14]

Under ideal conditions samples once collected should be immediately placed into appropriate storage but majority of the times this does not happen. This delay factor between sample collection and processing could impact the results.

It is important to determine whether procedure of sample storage introduces any change in the parameters to be observed. The main aim was to study the effect of pre-analytical storage temperature on telomere length and mRNA expression of *hTERT* in peripheral blood.

METHODS

We would like to state that for all work involving DNA and RNA handling, fresh DNAase/RNAase free micro-centrifuge tubes and pipettes tips were used. All necessary standard precautions were taken to avoid contamination and maintain sample integrity.

DNA isolation and TL assay: DNA was extracted using commercially available column-based kit (QIAamp DNA Blood Mini Kit, Qiagen). Telomere length was measured as per the method described by Cawthon[16] with minor modifications by using Multiplex Real Time Polymerase Chain Reaction (CFX Connect™ Real Time System, Biorad). It was quantified using a spectrophotometer (NanoDrop 2000c). 100 ng of DNA was used per reaction. Relative telomere length was determined by quantitative multiplex real time PCR. Telomere (T) PCR and a single copy gene (S) i.e. β-globin gene PCR was performed as a multiplex PCR setup using dye-based chemistry (Dy NAmo ColorFlash SYBR Green qPCR kit, Thermo Scientific). The Ct values thus obtained were used to determine the T/S ratio which is a measure of relative telomere length. Sequence of primers (Sigma) used is given in table 1.

***hTERT* expression:** RNA was extracted using RNA extraction reagent (RiBoZoL, Amresco). The extracted RNA was stored in 70% ethanol solution at -20°C. This was followed by air drying of the pellet and re-dissolving it in nucleus free water according to the manufacturer's protocol. cDNA was synthesized from the extracted RNA using commercially available cDNA Synthesis Kit (Verso cDNA synthesis kit, Thermo Scientific). mRNA expression of *hTERT* was determined by using delta-delta Ct method (Biorad CFX Connect™ Real Time System). 18s was used as a control. Sequence of primers (Sigma) used is given in table 1.

Study design: This observational study was approved by the institutional ethics committee on human research. As a part of the standardization procedure for estimation of TL and mRNA expression of *hTERT*, 20 samples were analysed. Each sample was divided into three aliquots of 200 µL each within two hours of sample collection. They were stored at room temperature (25°C), 4°C, and -20°C, respectively. Repeated freeze thaw cycles were avoided. Room temperature was measured with a digital thermometer and was maintained between 25-26°C. Deep

Table 1: Sequence of primers used in PCR for estimation of telomere length and *hTERT* expression

Telg (Telomere F)	ACACTAAggTTTgggTTTgggTTTgggTTTgggTTAgTgT
Telc (telomere R)	TgTTAggTATCCCTATCCCTATCCCTATCCCTATCCCTAACA
Hbgu (β-globin F)	CggCggCgggCggCgCgggCTgggCggCTTCATCCACgTTCACCTTg
Hbgd (β-globin R)	gCCCggCCCgCCgCgCCCgTCCCgCCggAggAgAAgTCTgCCgTT
hTERT F	GCAAGTTGCAAAGCATTGGA
hTERT R	ACCTCTGCTTCCGACAGCTC

freezer temperature was monitored with preinstalled thermocouples and was in the range of -19°C to - 21°C. DNA and RNA were extracted after a period of 24 hours from storage time.

Statistical analysis: Given the small sample size, statistical tests of significance to compare the groups were not used and only descriptive results are given. TL is expressed as a ratio (T/S) as described by Cawthon[16] and values expressed as mean of the 20 samples per group that were analysed. Relative mRNA expression of *hTERT* was calculated by ddCt method and expressed as fold change in expression.

RESULTS

A total of 20 samples were analysed for TL (as T/S) and mRNA expression of *hTERT*. The T/S ratio of the samples stored at 25°C and 4°C were comparable (50.35 ± 25.87 and 53.02 ± 53.37) while it was higher at -20°C (155.14 ± 90.8) (Table 2). mRNA expression of *hTERT* was reduced in samples stored at -20°C (-1.38) compared to 25°C, while an increase of 1.47 times were obtained in samples stored at 4°C compared to 25°C as shown in figure.

Table 2: Effect of temperature on telomere length

Temperature	TL (as TS)
25°C /RT	50.35 ±25.87
4°C	53.02 ± 53.37
–20°C	155.13 ± 90.8

DISCUSSION

In our knowledge, this study is the first description of the effect of temperature on telomere length and h*TERT* expression from peripheral blood cells on stored blood samples until it is finally processed (pre-analytical phase). Most of the studies[7,8] which have been done mainly focussed on the quality and quantity of the DNA extracted using different commercially available kits and studied the effect of repeated freeze-thaw cycles and prolonged storage of blood at low temperatures. In this study, the same blood samples were exposed to three different temperatures (25°C, 4°C, and –20°C) for the same amount of time and avoiding repeated freeze-thaw cycles.

A study was done in human umbilical venous endothelial cell culture where the cells were cultured at temperatures of 37 °C, 39 °C, and 42°C for a period of 24 hours. Analysis showed that the mean TL did not change, whereas TL distribution altered at 42°C, long telomeres decreased and mid-sized telomeres increased in TL distribution at 42°C. Telomerase activity did not show any heat associated alterations, *TERT* was up regulated alongwith temperature elevation (39°C and 42°C).[5]

In another multicentric study, it was noted that freezing and thawing of blood and storage at 4°C for up to 4 days did not affect TL values, while the T/S ratio of samples stored at either 4°C or room temperature differed after 24 hours with greatest change in T/S ratio observed at room temperature. At 4°C, day 4 T/S ratio was increased relative to day 1. For samples stored at RT, a similar but smaller increase was observed on day 4. In contrast, storage for 8 days or more resulted in noticeably high T/S ratio at room temperature.[18] In another study using pooled left-over samples, it was demonstrated that freezing had minor effect on TL quantification except for DNA extracted from one kit which showed a 27% higher TL abundance from frozen sample.[14]

Based on these studies and the present study, it can be safely said that pre analytical variables do play an important role in the quality/quantity of DNA extracted which will influence the various parameters that will be studied from that DNA and therefore, standard protocols must be in place so that variables can be accounted for and various laboratory findings be correlated with each other.

CONCLUSION

Temperature is an important pre-analytical variable that affects TL and the mRNA expression of *hTERT*. We would like to suggest that more detailed studies with preferably larger sample sizes be undertaken however, based on the present study we recommend the following:

- Uniform sampling procedure to be followed all throughout the study.
- Storage conditions of all samples should be similar.
- Avoid repeated freezing-thawing of samples.

Implementation of standard sample collection and processing criteria will lead to more comparability of results done in different parts of the world and avoid any erroneous interpretation.

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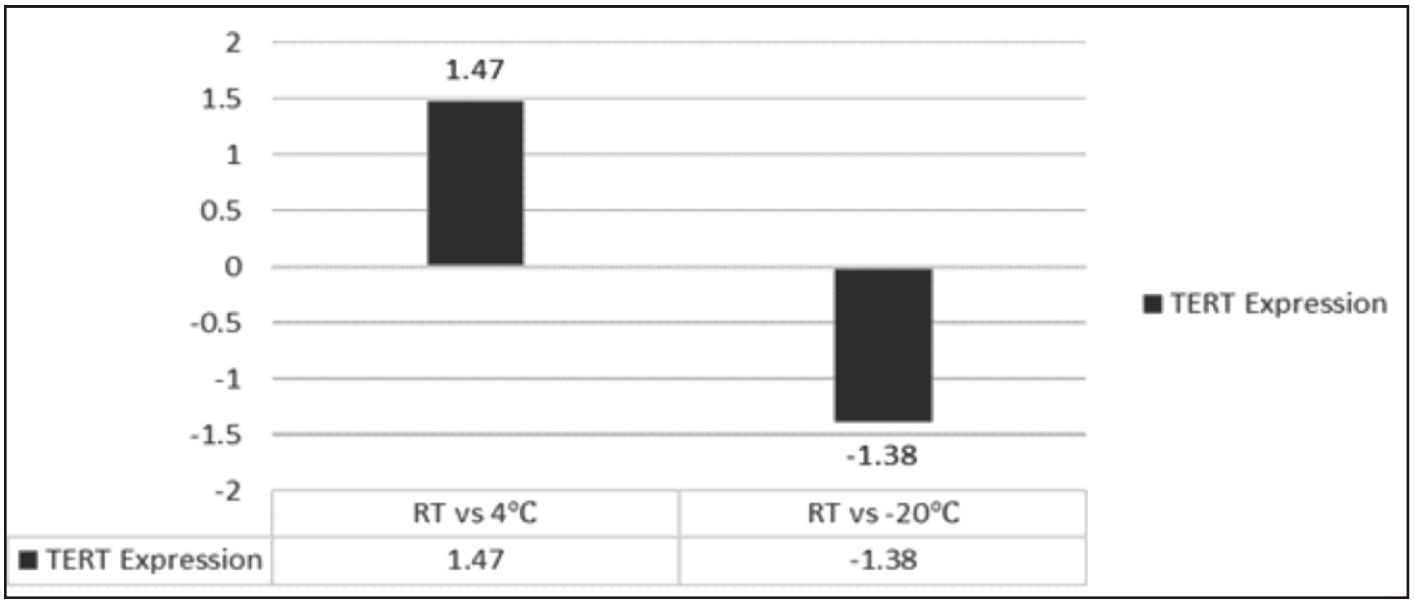

Figure: Graph showing the difference in *hTERT* expression.

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Corresponding Author

Dr Rajarshi Kar, Department of Biochemistry, 2nd Floor, University College of Medical Sciences and GTB Hospital, Dilshad Garden, Delhi-110095.

email: rajarshi.kar@gmail.com