REVIEW ARTICLE

Measurement of Leukocyte Telomere Length with Various Modifications to Cawthon's Method: A Review

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ABSTRACT

Telomeres are non-coding folded-loop DNA structures that are capped the chromosomes' ends in order to protect them against any damage. Leukocyte telomere length (LTL) shortening represents a biological ageing marker that is linked to the morbidity as well mortality. Cawthon's method of measuring LTL using quantitative real-time PCR has emerged and become phenomenal due to its important features such as affordability, rapidity, and convenience. However, various modifications were introduced to Cawthon's method, such as the number of replicates, PCR profile, concentration of primers, and quality control steps. This review addresses all these modifications and their effects on the measurement of LTL and provides an overview for the researchers who want to assess the LTL in their studies. In addition, some effects of LTL on human health issues are elaborated in this review.

Keywords: Leukocyte telomere length (LTL), Peripheral blood, Quantitative real-time PCR, Cawthon

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INTRODUCTION

The deoxyribonucleic acid (DNA) is encoding the genetic information of eukaryotes and is present in all chromosomes. During mitotic cells division, many identical, non-coding, repetitive specialized DNA structures represent natural ends of whole chromosomes and protecting coding genetic information from any loss. These specialized DNA structures are called telomeres (T) (1). In humans, telomeres have the shelterin complex, which is a protein complex primarily incorporate six main proteins (2). This shelterin complex is crucial into protecting telomere length (TL) via the engagement of TL maintenance mechanism and thereby preventing various DNA damage responses (3).

Moreover, the TL of peripheral blood leucocytes (LTL) obtains significant importance as a prospective biological ageing marker (2). At birth, LTL varies among individuals and reaches up to 15 kilo-base pairs (kb). However, the adult LTL is about 11 kb on average, and this number decreases gradually with a rate of 30-35 base-pair (bp) annually (4). This shortening of LTL is clearly evident throughout infancy due to the accelerated somatic growth (5, 6). In addition, various mechanisms are capable of affecting LTL, such as oxidative stress and geno-toxicity. In particular, oxidative stress produces reactive oxygen species (ROS) and the latter can impact LTL by inducing gene mutation influencing telomere structure and function. Other elements e.g. cigarette smoking, physical inactivity, obesity, unhealthy diet as well as psychological stress can also affect TL (7). Despite this, the reduction in LTL cannot be a straightforward one-direction mechanism. This is due to the presence of reverse transcriptase telomerase that adds new nucleotides to telomeres' ends (2).

Human individuals are commonly at risk of a higher mortality rate when their TLs are short, as the latter is significantly associated with cardiac and infectious diseases (8). Furthermore, accelerated shortening of TL can cause senescence, apoptosis, and alteration of somatic cells in different body tissues. Therefore, TL measurement is crucial to understand telomere biology and its serious effects on the ageing process and pathophysiology of diseases at the molecular levels (9).

Although the mean terminal restriction fragment (TRF) length method is the traditional method of assessing TL in the human DNA specimens (10), this method needs a massive quantity of DNA and takes many days to get results in addition to variation in TL by about 5% according to the specific restriction enzymes used. Thus, Cawthon et al. (9) has introduced a new method for measuring TL, which is quite uncomplicated and responsive to quick large throughput processing of many specimens. Briefly, Cawthon's strategy was to measure the difference between ratio of the copy number of telomere repeat (T) and the copy number of single copy gene (S) of the sample DNA and compare it to the (T/S) ratio of reference DNA (11). Cawthon has selected the 36B4 gene, which is found on chromosome 12 and responsible for acidic ribosomal phosphoprotein, due to its validation in gene-dosage researches (12). The mean TL is proportional to this T/S ratio (13). Since 2002, various modifications have been introduced into Cawthon's method, such as the selected single copy gene (SCG), amount of DNA in each reaction, running the reactions on the same or different plate, the number of replicates, and the PCR profile (9, 14-23).

This review is mainly focused on assessing these modifications and their effects on the outcome of the experiments. Also, this article assesses the quality control steps in these studies and compares the measured LTL among different populations.

METHODS

This review included studies that followed Cawthon's method (9) to measure LTL from peripheral blood using qPCR between 01/01/2006 and 30/06/2021. Leukocyte telomere length, peripheral blood, quantitative real-time PCR, and Cawthon were the keywords in the search process. Original articles, which presented initial modification or a substantial refinement of the existing Cawthon's method and were published in peer-reviewed journals, included in this review. Besides that, studies using the monochrome multiplex qPCR method produced by Cawthon in 2009 and other methods of measuring LTL were excluded. The data were compiled through literature databases such as Google Scholar and PubMed. Out of 31 articles, ten articles corresponding to the inclusion and exclusion criteria with at least one of these modifications were included in this review. Different modifications were assessed, such as the chosen SCG along with TL, the quantity of DNA and total volume of every reaction, concentrations of primers, serial dilution of DNA, same plate for TL and SCG or different plate, the number of replicates, and the PCR profile. In tandem with that, this review focused on quality control steps and compared the measured LTL in these selected studies.

RESULT AND DISCUSSION

Ten studies were included in this review that chose the 36B4 gene, as the SCG along with telomere. The pair primers of T were T1 (5′-GGTTTTTGA GGGTGAGGGTGA GGGTGAGGGTGAGGGT-3′) and T2 (5′-TCCCGACTATCCCTA TCCCTATCCCTA TCCCTATCCCTA- 3′), while the pair primers of 36B4 gene were 36B4d (5′-CCCAT TCTATCATCAA CGGGTACAA- 3′) and 36B4u (5′-CAGC AAGTGGGAA GGTGTAATCC-3′).

Quantity of DNA, Concentrations of Primers, and Total Volume of Reaction

While Cawthon was using 35 nanograms (ng) of DNA in every PCR reaction, these studies showed that the highest amount of DNA was 25 ng (14, 21), and the lowest amount of DNA was 1.6 ng (16). In addition, four studies were using 5 ng (17, 19, 20, 22), and the rest were using 10 ng (18), 15 ng (23), and 20 ng (15), respectively. However, most of these studies added similar concentrations of T and S primers to that of Cawthon's study (T1=270nM, T2=900nM, 36b4u=300nM, 36b4d=500nM) (9). In particular, the concentrations of T primers were commonly 270 nM and 900 nM for T1 and T2 primers, respectively. Also, the 36B4u primer was mainly reported in the concentration of 300 nM, while the 36B4d primer was frequently used with 500 nM as shown in (Table I). According to Cawthon's method (9), the total volume of each reaction (T or S) was 30 microliter (uL). In contrast, neither of these selected studies were using that volume, and instead, they were using the reaction mixture volume of 10 uL (16, 17, 19, 20, 22), 20 uL (14, 15, 23), and 25 uL (18, 21), respectively. In summary, these variations in the amount of DNA and total volume of reaction mixture lacked any effect on the values of T/S ratio in these studies.

PCR Profile

In Cawthon's experiment, all PCR reactions were carried out on thermal cycler manufactured by Applied Biosytems (9, 18). The PCR profile for both T and S reactions composed of initiation at 95°C for 10 min, 18 cycles of 95°C for 15 s, 54°C for 2 min for T reactions, and 30 cycles of 95°C for 15 s, 58°C for 1 min for S reactions. All of the selected studies were using thermal cyclers from Applied Biosytems with PCR plates (16, 17, 19-23) except one study that was using Corbett Research Rotor-Gene 3000 that holds standard PCR tubes (15). However, one study did not report the name of the thermal cycler (14). Moreover, all of these studies applied the exact temperature of Cawthon's initiation step, as shown in (Table I). Although more than half of these studies selected Cawthon's initiation duration (14-16, 18, 20, 21), the rest used various durations ranging from 5 min (19, 22) to 5 hours (17). For T and S reactions, seven studies (14, 16, 18, 19, 21-23) and five studies (14-16, 19, 22) were followed the same Cawthon's T and S reactions, respectively. Only two studies reported different parameters of T and S reactions (17, 20). In tandem with that, neither of these selected studies

Table-I: Primers and their concentrations, amount of DNA, total reaction volume, PCR profile, Status and No. of replicate

Authors/ Year	Primers (nM)		DNA (ng)	Total Reaction Volume (uL)	Initial Denature			Annealing		Cycles No.	Thermal Cycler	Replicate Status and No.
						Temp (°C)	Time	Temp (°C)	Time			
Cawthon et al. (12)	T1	270	35	30	T	95	10 min	95	15 s	18	Prism 7700 Sequence Detection System (Applied Biosytems)	Separated
	T2	900						54	2 min			Triplicate
	36B4u				S	95	10 min	95	15 s	30		
	36B4d	300						58	1 min			
		500										
Brouilette et al. (22)	T1	300	20	20	T	95	10 min	95	15 s	25	Corbett Research Rotor-Gene 3000	Separated
	T2	300						58	1 min			Duplicate
	36B4u	300			S	95	10 min	95	15 s	35		
	36B4d	500						58	1 min			
Shen et al. (14)	T1	2.4	25	25	T	95	10 min	95	15 s	30	ABI 7500 (Applied Biosystems)	Separated
	T2	2.4						54	2 min			Duplicate
	36B4u	2.4			S	95	10 min	95	15 s	40		
	36B4d	2.4						60	1 min			
Wang et al. (18)	T1	270	5	10	T	95	5 min	95	15 s	40	Applied Biosystems 7900HT	Separated
	T2	900						54	2 min			Duplicate
	36B4u	300			S	95	5 min	95	15 s	40		
	36B4d	500						58	70 s			
Han et al. (17)	T1	270	5	10	T	95	5 h	95	15 min	40	Applied Biosystems 7900HT	Separated
	T2	900						54	2h			Triplicate
	36B4u	300			S	95	5 h	95	15 min	40		
	36B4d	500						58	70 min			
								72				
Liu et al. (20)	T1	300	10	25	T	95	10 min	95	15 s	35	Applied Biosystems 7500	Separated
	T2	800						54	2 min			Duplicate
	36B4u	300			S	95	10 min	95	15 s	35		
	36B4d	300						60	1 min			
Zee et al. (21)	T1	100	15	20	T	95	15 min	95	15 s	40	Applied Biosystems 7900HT	Together Duplicate
	T2	900			&			54	2 min			
	36B4u	300			S							
	36B4d	700										

CONTINUE

Table-I Cont.: Primers and their concentrations, amount of DNA, total reaction volume, PCR profile, Status and No. of replicate

Authors/ Year	Primers (nM)		DNA (ng)	Total Reaction Volume (uL)	Initial Denature			Annealing		Cycles No.	Thermal Cycler	Replicate Status & No.
						Temp (°C)	Time	Temp (°C)	Time			
Gramatges et al. (15)	T1	270	1.6	10	T	95	10 min	95	15 s	30	Applied Biosystems 7900HT	Separated
	T2	900						54	2 min			Triplicate
	36B4u	500			S	95	10 min	95	15 s	40		
	36B4d	500						58	1 min			
Nan et al. (16)	T1	270	5	10	T	95	5 min	95	15 s	30	Applied Biosystems 7900HT	Separated
	T2	900						54	2 min			Triplicate
	36B4u	300			S	95	5 min	95	15 s	40		
	36B4d	500						58	70 s			
Anic et al. (13)	T1	2.4	25	20	T	95	10 min	95	15 s	30	N.A.	Separated
	T2	2.4						54	2 min			Triplicate
	36B4u	2.4			S	95	10 min	95	15 s	40		
	36B4d	2.4						60	1 min			
					S	95	10 min	95	15 s	35		
								58	1 min			
Qin et al. (19)	T1	300	5	10	T	95	10 min	95	15 s	40	Applied Biosystems 7900HT	Together
	T2	300			&			60	60 s			Duplicate
	36B4u	300			S							
	36B4d	300										

Note: N.A: data not available from the study; nM: nano-molar; L: liter; uL: micro-liter; ng: nano-gram; T1: Telomere Forward; T2: Telomere Reverse; T: Telomere; S: 36B4

used Cawthon's number of cycles. Remarkably, 25-40 cycles were the range for the T reactions, with 30 cycles represented the primary choice. For the S reactions, 35-40 cycles were the range in these studies, and 40 cycles were marked as the best choice. The reason behind the difference in the number of cycles for T and S reactions (30 vs 40 cycles) is that the PCR of S requires approximately nine cycles greater than the PCR of T to produce the same fluorescent signal of the T PCR (9).

Serial Dilution of DNA and Standard Curve

Based on Cawthon's method (9), a reference DNA sample might serially be diluted in the PCR plate in order to get a standard curve that could measure the relative T/S ratio and inter-plate variability (9, 22). A DNA specimen's cycle threshold (Ct) represents how many PCR cycles are needed for a fluorescent signal to cross the threshold. Besides that, any pooled DNA specimen is able to initiate a standard curve if only the cycle threshold values of measured specimens adhere to the cycle threshold values range of that pooled DNA. Thus, a reference DNA specimen was serially diluted by approximately 1.68-fold to form five points with a range of (100-12.64 ng) DNA, with the middle point nearly matched the DNA quantity of the measured samples. The standard curve correlation coefficient (R_2) of Telomere was 0.98, and 36B4 gene was 0.99 in Cawthon's protocol (9). The R_2 in seven out of these ten studies were ≥0.98 for both Telomere and 36B4 gene standard curves (14, 17, 18, 20-23), as shown in (Table II). One of these studies showed an R_2 value of 0.95 (15), while the lowest R_2 value was reported in another study with 0.83 and 0.79 for Telomere and 36B4 gene standard curves, respectively (19). However, only one study did not mention the R_2 value of the standard curves (14). Overall, these studies have shown that proper serial dilution of DNA can produce an excellent standard curve with a highly accurate correlation coefficient.

Quality Control

According to Brouilette et al. (15), calibrator sample and non-template control sample were included in every run for both the T and S assays done in duplicate and separated plates to compare results across all runs (15). Additionally, whole analyses were carried out by personnel blinded to case-control status (15). Similarly,

Table-II: Characteristics of Standard Curve

Authors/ Year	Amount of DNA (ng)	Dilution Factor	No. of Points	Telomere R^2	36B4 R^2
Cawthon et al. (12)	5 – 0.63	1.68	5	0.98	0.99
Brouilette et al. (22)	100 – 1.56	2	7	0·95	0.95
Shen et al. (14)	2 – 0.4	1.3	5	>0.98	>0.98
Wang et al. (18)	50 – 1.25	1.7	8	0.98	0.99
Han et al. (17)	30 – 1.25	1.9	6	0.98	0.99
Liu et al. (20)	40 – 0.625	4	5	≥0.98	≥0.98
Zee et al. (21)	80 – 2.5	2	6	-0.99	-1.00
Gramatges et al. (15)	N.A.	N.A.	N.A.	≥0.98	≥0.98
Nan et al. (16)	20 – 1.25	1.75	6	0.83	0.79
Anic et al. (13)	2 – 0.4	1.3	5	N.A.	N.A.
Qin et al. (19)	20 – 0.625	2	6	≥0.98	≥0.98

Note: N.A: data not available from the study; R^2: correlation coefficient of standard curve.

Shen et al. (21) used these quality control steps and got the intra-plate and inter-plate variability about 19% and 28%, respectively (21). However, the intra-plate variability in triplicates of Cawthon' methods was only 9.4%. Furthermore, Wang et al. (22) showed that the coefficient of variation (CV) was 0.9% within duplicates of the T assays and 2.4% in S assays (22). Interestingly, Han et al. (17) revealed these variations between plates further extensible (17). In particular, the inter-plate CV of the T and S triplicates were 4.62 and 3.21%, respectively (17). Also, the intra-plate CV of T and S reactions were 3.02 and 2.07%, respectively (17). Two studies were only reported the intra-plate CV of 5% for triplicates of both assays (16) and 0.83 of T assay as well as 0.79 for the S assay (19).

In contrast to Cawthon's protocol and the previously mentioned studies, Zee et al. (23) was the first person to run duplicates of both T and S assays on each DNA specimen alongside the same PCR plate, and the assays were done under similar PCR conditions (23, 24). This modification was carryout out to further eliminate the inter-plate variation besides the other steps in Cawthon's method (23). In addition, this modification could help to minimize the variation in the threshold and Ct values, thereby reducing the T/S ratio variability (25). Interestingly, the CV of T, S, and T/S ratio were <4%, <2%, and <5%, respectively. Additionally, R_2 for the standard curve of T was -0.99 and R_2 for the standard curve of S was -1.00 (23). These outcomes demonstrated the robustness of this modified protocol (23). Similar to the previous study, Qin et al. (20) did whole specimens for the T and S reactions in duplicate side by side on the PCR plate and showed that the inter-plate and intra-plate CVs were 8.9% and 4.6%, respectively (20).

Nevertheless, two studies did not mention the CV of their duplicates (18) and triplicates (14). To sum up, the number of replicates is very important in the procedure of quality control as these replicates are capable of measuring variation in the experiment; thus, statistical tests can be used to evaluate these differences. Besides that, averaging across replicates improves the precision of T/S ratio values and supports the detection of smaller changes. Lastly, comparing replicates helps to identify any outlier result that may result from the sample or the experimental procedure.

Table-III: Participants age, T/S ratio, and country for each study

Authors/ Year	Participants Age Range/Mean (Years)	T/S Ratio	Country
Cawthon et al. (12)	5–94	0.69 – ≥1	USA
Brouilette et al. (22)	49.7 (cases) & 49.6 (controls)	0.909 – 1.37	Scotland
Shen et al. (14)	38–60	0.70 – 0.74	USA
Wang et al. (18)	40–75	0.04 – 1.55	USA
Han et al. (17)	57.4 (cases) & 58.8 (controls)	0.85 – 1.52	USA
Liu et al. (20)	52.7 (cases) & 53.1 (controls)	0.72 – 1.14	China
Zee et al. (21)	40–84	N.A.	USA
Gramatges et al. (15)	30–65 (cases) & 30–60 (controls)	0.6 – 1.4	USA
Nan et al. (16)	57.5 (cases) & 57.8 (controls)	N.A.	USA
Anic et al. (13)	21–80 (cases) & 18–88 (controls)	0.49 – 1.74	USA
Qin et al. (19)	58.8 (cases) & 58.8 (controls)	N.A.	China

Note: N.A: data not available from the study

Telomere Length and Effects

Telomere length is variable from one population to another. However, these selected studies showed a different range of T/S ratio as they were from different countries (seven studies from United States (US), two from China, and one from Scotland) as shown in (Table III). Despite the lacking of T/S ratio in 3 studies, the highest and lowest T/S ratios were reported in the US with 1.74 and 0.04, respectively.

Telomere shortening possesses potential roles in the development of malignancy. Particularly, the recurrent deletion of telomeric repeats that happen in every cell division hinders the ability of cellular division later. Furthermore, if telomeres show a serious short length, DNA destruction stimulus is provoked, resulting in a senescent or apoptotic cell. While the senescent cell enters an irreversible and permanent arrest in the G1 phase, they maintain their metabolic activities (26).

The G1 phase checkpoint inhibits the build-up of oncogenic mutations and later malignant transformation (27). Nevertheless, these cells proceed to proliferation once this checkpoint is bypassed, leading to more telomere erosion (28). Thus, if TL becomes only 77 bp, the ends of the chromosome become liable to fusion, causing chromosomal instability, thereby malignant transformation (29). Many studies paid attention to the influences of TL on various diseases and malignancies that burden human life.

For instance, the selected studies in this review have shown various associations between TL and some of these health issues. The findings of Brouilette et al. (15) revealed that the correlation between TL shortening and coronary heart disease was not an outcome of the latter. Two possibilities could explain this association. Firstly, the fact that senescent cells have an important effect on atherogenesis (30), and observation that any in-vitro interruption to telomere action in coronary endothelial cells triggers the release of some substances involved in the formation of atherosclerotic plaques (31), thereby shorter TL may probably have direct contribution to the process of atherosclerosis. Secondly, there is a possibility that genetic background is the reason behind this association. Some studies indicated an inter-individual variation in average TL due to genetic issues (32, 33). This finding explains why those individuals born with shorter TL have a higher possibility of gaining coronary heart disease.

In tandem with that, Zee et al. (23) had investigated the association of mean TL with the possibility of incident myocardial infarction (MI). The outcomes showed that participants with shorter TL were obviously prone to an elevated risk of incident MI. Also, the outcomes of the present study revealed that the TL could inversely be correlated with age and thereby supported the observation that TL biology could show an important influence on the incidence of coronary heart disease (23).

On another aspect, Wang et al. (22) found that male participants were at lower risk for developing Parkinson's disease (PD) if they had shorter TL. This finding ran unexpectedly counter to what everyone expected, as shorter TL could be a marker of oxidative stress (34), and the latter might have been a significant contributor to the pathogenesis of PD.

Similarly, Nan et al. (19) recognized a significant association between the Caucasian population with shorter TL and lower risk of incident melanoma. This outcome supports the finding that telomere-induced senescence may specifically affect the development of melanoma. Also, this emerging evidence interestingly points to an important link between melanoma and PD. Some studies were shown a reduction in cancer (e.g. melanoma) among patients with PD (35, 36). The explanation for this link could be the identical individual risk factors, e.g. red hair colour as well as a family background of melanoma that are shared by PD and melanoma (37, 38). Another explanation could speculate that melanocytes and dopamine-containing nerve cells might have specific molecular mechanisms against cell damage and stress.

Moreover, Han et al. (17) were the first who observed that shorter TL in women could reduce their incidence of melanoma. In contrast, they found that TL shortening might be associated with an elevated incidence of basal cell carcinoma (BCC). Both BCC and squamous cell carcinoma (SCC) refer to non-melanoma skin cancers. However, TL shortening did not affect the SCC incidence (17). In agreement with the previous two studies, Anic et al. (14) found a remarkable correlation between TL and the risk of all types of skin cancers. The results revealed a link between TL shortening and a reduction in melanoma incidence but rise in BCC incidence. These results were corresponding to the previous study (17). Nevertheless, Anic et al. (14) revealed an association between short TL and an elevated incidence of SCC. More studies are required to examine these associations further and investigate the effect of TL on melanoma development.

In another aspect, Shen et al. (21) linked shorter TL with a non-significant elevated possibility of breast cancer development, especially among pre-menopausal females. In contrast, Gramatges et al. (16) exhibited that there was a gradual increase in risk for breast cancer with every increase in TL. Moreover, women with breast cancer significantly revealed longer TL in comparison to unaffected women. Interestingly, healthy females with a family background of breast malignancy revealed longer TL than healthy females without any family background. The latter finding could be used as a potential marker for risk identification of breast cancer. Again, there is an urgent need to research further the correlation between TL and breast malignancy (39).

One of these selected studies demonstrated that the patients with gastric cancer (GC) showed significantly shorter TL compared to their healthy controls (18). Lastly, Qin et al. (20) examined the relation of short TL and the incidence of the colorectal cancer (CRC) (40). Findings showed that shorter TL might be associated with an elevated incidence of CRC among Chinese people (41). Besides that, this association was clear in non-smoker, non-drinker women younger than sixty years old.

Despite the important findings of this review, some limitations related to the pre-analysis were found in these chosen studies. Several studies revealed that specific pre-analytic factors could have an important effect on the reproducibility of TL. Firstly, all but one study (11) failed to mention the freeze-thaw cycles. It is of note that these freeze-thaw cycles result in progressive degradation of the genomic DNA, and the latter gradually degrades with every freeze-thaw cycle with DNA of large size is

more prone to degradation than small-seized DNA (42). Secondly, data regarding the duration and conditions of DNA storage were reported only in two papers (14, 18). Some studies found huge degradation of DNA samples stored at 4℃ and room temperature for a long time. However, DNA can be stored at -20℃ and -70℃ and show the least degradable ability (43) (44). Moreover, Dagnall et al (43) recommended that DNA samples should be stored at −80 °C. Thirdly, only a few of these selected papers specifically commented on the DNA quality and integrity (16-18). Ostensibly, the yield DNA samples from the extraction must pass the basic quality control specifications, and DNA purity of OD260/OD280 ranged between 1.7 and 2.0 is a priority in DNA quality and integrity (45). In addition, the presence of any residual impurities and/or chemicals, which are missed by spectrometry, may have a potential impact on qPCR assays.

CONCLUSION

Measuring TL by quantitative real-time PCR represent the most cost-effective and efficient high-throughput method, and Cawthon's method provides a biologically meaningful measure of LTL. This method generates a T/S ratio corresponding to the mean LTL and has highly consistent results despite the various modifications introduced over these years. These modifications such as the amount of DNA, total reaction volume, concentrations of primers, and serial dilution of DNA missed any significant effect on the outcome of Cawthon's method. Besides that, the robustness of results was evident despite the modifications in quality control steps. However, new studies are obviously crucial that can extensively compare among these modifications in order to get a universal version of Cawthon's method to minimize the variation in LTL as much as possible. Furthermore, additional studies are urgently needed to investigate the critical roles the LTL can play in different diseases and malignancies.

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