Review Article

Telomere length as a potential biomarker of coronary artery disease

Joyeeta Bhattacharyya[1], Keichiro Mihara[2], Deborshi Bhattacharjee[1] & Manjarí Mukherjee[1]

[1]*Department of Cardiac Research, Cumballa Hill Hospital & Heart Institute, Mumbai, India &* [2]*Department of Hematology & Oncology, Research Institute for Radiation Biology & Medicine, Hiroshima University, Hiroshima, Japan*

Coronary artery disease (CAD) is a multifactorial disease whose prevalence remains unabated especially in developing countries. Both lifestyle factors and genetic predisposition contribute to this disorder. Though notable achievements have been made in the medical, interventional and surgical management of CAD, the need for its prevention is more important. Among other modalities, this calls for defining evidence-based new biomarkers, which on their own or in combination with other known biomarkers may predict the risk of CAD to enable institution of appropriate preventive strategies. In the present communication, we have discussed the usefulness of shortening of telomeres as a potential biomarker of CAD. Clinical research evidence in favour of telomere shortening in CAD is well documented in different ethnic populations of the world. Establishing a well-standardized and accurate method of evaluating telomere length is essential before its routine use in preventive cardiology.

Key words Ageing - Biomarker - coronary artery disease - shortening - telomerase - telomere

Introduction

Cardiovascular disease (CVD) in general and coronary artery disease (CAD) in particular is a leading cause of death and disability[1,2]. According to the statistics released by the World Health Organization in early 2015, an estimated 17.5 million people died from CVD in 2012, representing 31 per cent of all global deaths[2]. Of these deaths, an estimated 7.4 million were due to coronary heart disease and 6.7 million were due to stroke. Over three-quarters of CVD deaths take place in low- and middle-income countries[2]. The largest increase in the number of deaths due to CAD and stroke is expected to occur in the south-East Asia region by 2030[1]. Physical inactivity, unhealthy diet, tobacco use and lifestyle disorders such as type 2 diabetes mellitus (T2DM) and hypertension are known risk factors, raised levels of serum low-density lipoprotein (LDL) cholesterol, triglyceride (TG), lipoprotein(a), high sensitivity C-reactive protein (hs-CRP) and homocysteine are some of the recognized biomarkers of CAD[3,4]. However, none of these explain the total burden of CAD in a population. Therefore, search for potential biomarkers for early diagnosis and treatment of the disorder either independently or in combination with other markers continues to be an important goal of researches in preventive cardiology. With reference to a spate of research on telomere length (TL) in various disorders, recent population studies

reported shortening of telomeres in patients affected with CAD[5-9]. This interesting finding prompted us to present the case of 'telomere shortening' as a potential biomarker of CAD in the present communication.

Telomere biology

Telomeres are terminal ends of linear chromosomes, which in all vertebrates consist of tandem repeats of 'TTAGGG' sequences and associated proteins. These form a cap and thereby protect chromosomes from double strand breaks, fusion, recombination and degradation[5]. Telomeres also play a role in the organization of the cellular nucleus. Their structure allows the end of linear DNA to be replicated completely. The length of telomeres varies among different species. Human telomeres are 8-14 kbp long. The telomeric DNA consists of noncoding tandemly repetitive sequences, with the exact replicate sequence varying from one species to the other. DNA polymerases replicate only in a 5' 3' direction by extending existing polynucleotide chain. The leading and the lagging DNA strands follow a different mechanism of DNA replication. DNA fragments, termed Okazaki fragments are formed to replicate the lagging strand by means of DNA polymerase elongating several RNA primers in succession. DNA sequences finally replace the RNA primers. The gap formed due to the removal of the terminal RNA primer on the lagging strand is filled in by extension of the next Okazaki fragment. Because there is no template for the 'last' Okazaki fragment beyond the 5' end of the chromosome, one strand cannot be synthesized till the extreme end. This end replication problem causes a progressive decrease of chromosomal DNA at the 3' ends as cell cycle progresses[5]. Although many questions still need answers, chromosomes must be processed and refolded into a stable structure before cells can proceed to the next step in the cell cycle. Free chromosome ends such as single-strand ends or blunt ends trigger a DNA damage response resolved by recruitment of DNA repair proteins. The need to remodel free ends into functional telomeres following replication requires efficient DNA damage responses and DNA repair reactions. Depending on the magnitude and the efficiency of DNA damage responses in a particular cell, these chromosome ends are at risk for other chromosomal abnormalities. Eukaryotic cells have a limited potential for growth in primary cultures and undergo senescence after a defined number of cell divisions which forms the basis for cellular ageing[6,7]. Tumour cells, however, exhibit unlimited growth and proliferation capability leading to cellular immortalization. There appears to be a relationship between telomere maintenance,

telomerase expression and extension of cellular lifespan in mammalian cells[8,9]. Telomere shortening occurs with repeated cell divisions and, when the length is reduced to a critical point, the resulting genomic instability leads to further genetic abnormality that in most of the cells promotes cellular death or apoptosis, a hallmark of cellular ageing[10]. Telomere shortening is a mechanism that prevents replication error that would cause mutations in DNA. Once the telomeres are shortened, due to the multiple cell divisions, it will no longer divide. This replicative senescence is also termed as Hayflick limit or Mortality Stage I[10] . Telomerase, the TL modulator ribonucleoprotein, is ordinarily inactive in differentiated somatic cells, but its activity can be detected in tumour cells. The telomerase activation in malignant cancers is an imperative step in tumorigenesis. The cells gain the capability of indefinite propagation to become immortal[8].

The primary role of telomerase seems to be telomere lengthening. Alteration in telomerase expression is associated with many degenerative diseases, ageing and cancer-related functions. Other than telomere maintenance in the nucleus, several studies have shown that telomerase may also have extracurricular activities[11,12], including sensitizing cells to oxidative damage leading to apoptosis in mitochondria, DNA repair, regulation of gene expression, chromatin organization and cell growth. It also has a role in stem cell maintenance and modulation of Wnt signaling[11,12]. These activities appear to require TERT (telomerase reverse transcriptase) instead of TERC (telomerase RNA component) and are thus free of typical telomerase activity. In the mitochondria, telomerase shows RNA-dependent DNA polymerase activity that is independently of TERC and uses tRNA as a template. Telomerase plays a role in the regulation of apoptosis[12]. It regulates gene expression by small interfering RNA (siRNA). Telomerase sensitizes the DNA of mitochondria to hydrogen peroxide (H_2O_2), which causes oxidative damage to mitochondrial (mt)-DNA[12]. Increased levels of oxidatively modified proteins and lipoproteins, *e.g.* oxidized LDL and lipid peroxidation products, have been seen in people with CAD[13]. Association of shorter telomeres with CVD could be a result of such an extracurricular activity of telomerase.

In addition to telomerase, a large number of factors help maintain telomere integrity. Shelterin proteins recruit a host of other factors to the telomere including PinX1. Overexpression of PinX1 results in decreased

telomerase activity, telomere shortening and induction of crisis[11]. A strong association was found between PinX1 and coronary intima media thickness in a large genome wide study[14].

Factors affecting telomere length in coronary artery disease

Telomeres shortening can often be caused by adopting an unhealthy lifestyle. Ageing, degenerative disease and stress are potential regulators of TL. As with the ageing process, cardiovascular disorders, most notably atherosclerosis, diabetes as well as insulin resistance, are found to be closely associated with telomere shortening. TL is also influenced by psychological stress (Table I). Patients with short telomeres show the lowest telomerase activity and equally high oxidative stress. Hypothalamus, pituitary, adrenal activation due to stress, elevates glucocorticoid production, which increases reactive oxygen species (ROS) level, resulting in shortened lifespan and increased risk for age-related disease, including CVD[15,18]. Animal models have shown that shortened telomeres with reduced telomerase activity are closely associated with an increase in inflammatory mediators, greater susceptibility to oxidative damage,

stroke, blood-brain barrier permeability, as well as tight junction disturbances[38,39]. Cellular stress- or age-related disorder is linked to telomere shortening[40]. Mitochondrial dysfunction results in (ROS) production which initiates genomic instability and telomere shortening[41,42]. The persistent activation of p53 induces further mitochondrial damage, and a vicious cycle continues. Improving mitochondrial function may assist in healthy ageing[41,42].

Shorter leucocyte telomeres in people prone to coronary heart disease could indicate the cumulative effect of other cardiovascular risk factors on TL. The association of shorter telomeres with an increased risk of coronary heart disease has a genetic basis that exacerbates or retards the TL[28,29]. In the genetic disorder dyskeratosis congenita, telomere shortening is accelerated[30], and patients have premature onset of many age-related diseases and early death. It is also longer in African Americans than in whites of European descent. Although at the start of life, no sex difference in the length of telomeres is seen[43], it is longer in women than in men. It is seen that men have shorter lifespan and greater telomere shortening[44]. This has led to speculation that sex-specific telomere shortening is one cause of sex-specific mortality. Survival advantage could be the result of variation in telomere maintenance alleles on the X-chromosome[45].

It was found in the elderly twins that the twin with shorter TL was more likely to die before the twin with longer TL[46]. TL is heritable and modified by paternal age at the time of conception[31]. Obesity, lack of exercise, smoking and psychological stress are some of the major factors that intensify the process (Table I).

Individual differences in biological ageing, as shown by shortening of TL, could affect susceptibility to coronary heart disease and might serve as a predictor of the disease[6,7]. Shorter TL may serve as a potential marker for the presence of atherothrombotic and haemorrhagic stroke and for the risk of post-stroke death[47].

Oxidative stress could also be a possible contributor for the shorter TL in high-risk individuals for CAD. One of the many benefits of statins in CAD is that it may retard the shortening of telomeres. Short-telomere individuals might benefit most from statin treatment. The anti-ageing effects of statins are shown to be linked to their ability to inhibit telomere shortening by reducing either directly and indirectly oxidative telomeric DNA damage, as well as by a mechanism involving telomere capping proteins[37].

Table I. Factors affecting telomere length	
Factors	References
Shortened because of	
Ageing	6,7
Stress	15
Smoking	16
Decreased physical activity	17
Low telomerase activity	6,7
Oxidative stress	18
Obesity	19
Inflammation and degenerative diseases	9
Cardiovascular disorders	20-27
Genetic	28-31
Intact due to	
Healthy diet and lifestyle	32
Increased physical activity	17
Decrease in calorie intake	32
Vitamins and antioxidant	18,33-35
Reduced smoking	16
Intake of omega 3 fatty acid	36
Statins intake	21,37

Table II. Studies showing telomere length is shorter in patients with coronary artery disease

Disease	Sample size	Country	Telomere length	Outcome	References
MI	Nested case-control study, 14,916 participants	USA	Shortened	Increased risk of incident in MI	20
CHD	Nested case-control study, 484 participants	West of Scotland (WOSCOPS)	Shortened	Predictor of future coronary events	21
CAD	Prospective cohort study, 608 participants	USA	Shortened Same length Lengthened	45 per cent patients 32 per cent patients 23 per cent patients	22
CVD and obesity	989 participants	Northern Sweden	Shortened	Associated with obesity (only in women)	19
Ageing and CVD	Cardiovascular health study, 419 participants	USA	Shortened	Diseases of ageing and progression to CVD	23
CAD	Prospective cohort study, 608 participants	USA	Shortened	Lowest omega 3 fatty acid showed fastest telomere shortening	36
Chronic heart failure	Case-control study 803 participants	USA	Shortened	Signify severity of disease and atherosclerotic disease manifestation	25
CAD	Case-control 476 participants	India	Shortened	Associated with CAD. However not related with number of coronary artery stenosis	55
CAD	Cross-sectional study of 944 outpatients with stable CHD	USA	Shortened	Physical inactivity leads to shorter telomere length	17

CAD, coronary artery disease; CVD, cardiovascular disease; CHD, coronary heart disease; MI, myocardial infarction

Estimation of telomere length

TL measurement techniques used in epidemiological/clinical research include Southern blot analysis of the terminal restriction fragments (TRFs) length[48]; quantitative polymerase chain reaction (qPCR)[49,50] and fluorescence *in situ* hybridization (FISH)[51,52]. Southern blot analysis to estimate mean TRF length generally involves digestion of genomic DNA with restriction enzymes. Determination of average TL by qPCR involves the comparison of copy number of telomere repeats with that of a single-copy gene (36b4) in a given sample (T/S ratio). The T/S ratio is proportional to the average TL[49,50]. The measurement of telomeres in vertebrate DNA by PCR amplification with oligonucleotide primers intended to hybridize to the TTAGGG and CCCTAA repeats was considered impossible until recently because only primer dimer-derived products were expected. However, a primer pair that removes this problem was found that allows easy measurement of telomeres in a closed tube, fluorescence-based assay[49].

The FISH technique for determination of telomere repeats includes quantitative FISH (QFISH) using digital fluorescence microscopy or flow cytometry (flow-FISH). Flow-FISH is less time consuming than QFISH where TL measurements are performed on individual metaphase chromosomes. The flow-FISH technique can measure average TL in each cell and TL can be determined in specific cell populations. Southern blot and qPCR methods are more extensively used to measure leucocyte TL (LTL) than flow-FISH. The Southern blot method requires more DNA (μg quantities), is costly and more laborious. The qPCR method, on the other hand, requires less DNA. However, lack of good reference standards may make absolute TL measurement difficult. In the flow-FISH technique, a high variability in the length of alphoid centromere among individuals makes this method unreliable. Telomerase activity can, however, be tested by Telomeric Repeat Amplification Protocol (TRAP) assay[53]. The telomerase activity can be detected in extracts of cellular protein by TRAP assay. Thus, there remains the need for an easy, inexpensive and high throughput method to measure TL. Besides CAD and other clinical disorders, LTL is considered to be a biomarker of human ageing[54]. Short LTL was found to be associated with women[19]. Furthermore, investigations in twins suggested that short LTL was associated with their decreased survival[46,55,56].

Shortening of telomeres in coronary artery disease

Research shows that telomere shortens in length over an individual's lifetime that may contribute to ageing and development of several diseases including CVD (Table II), diabetes, depression, pulmonary fibrosis, dementia and osteoarthritis.

Heart tissue is also affected by age. CAD is an age-related disorder[57] where cellular senescence is a major feature for the development of atherosclerotic plaques[58-60]. *In vitro* studies have shown that induction of senescence causes expression of certain molecules which are entailed in atherogenesis[60]. As shown by previous studies, in many cell types, telomere shortening is the confounding factor for cellular ageing[15]. Almost all patients with CAD have shortening of telomeres. Endothelial injury occurring as a result of liberation of free radical produced by hypertension, diabetes mellitus, cigarette smoking, as well as increased serum levels of low-density lipoproteins may contribute to telomere shortening. Studies have also shown that suppression of the oxidative stress slows down this shortening process[33,34]. Telomere shortening was highly accelerated in the regions that were easily prone to atherosclerosis[58-60]. Increased cellular turnover may result in cellular senescence associated with telomere shortening and may participate in coronary atherogenesis.

A large body of evidence in ethnic populations across the world[20-26,36,54,61-66] including Caucasian whites, Japanese and Asian Indians[61] has demonstrated the association of shortened telomeres with CAD. One study showed LTL to be a valuable prognostic marker for carotid atherosclerosis[54]. This study provided preliminary evidence for a prospective association of LTL with risk assessment of carotid atherosclerosis. As atherosclerosis is a systemic disease, the prevalence of carotid atherosclerosis increases the prevalence of coronary atherosclerosis. Events originating from one artery have a strong prediction on the occurrence of events in the other artery[65]. Variation in the *TERT* gene also acts as a predictor of CAD in certain racial groups[66]. Middle-aged men with shortened telomeres appeared more prone to future coronary events[64] including myocardial infarction (MI)[20]. Besides CAD and other clinical disorders, LTL is considered to be a biomarker of human ageing[54].

It was demonstrated that the mean TL in leucocytes was shorter in patients with severe triple vessel CAD[63] and patients with a history of premature MI[64] as compared to corresponding age- and sex-matched controls. This was at variance to the finding that although telomere shortening was associated with CAD, it was not related to the number of coronary artery stenosis[61]. Similarly, patients who have suffered a premature MI also show shorter telomere compared with age- and sex-matched subjects without such a history[21,64]. Also future coronary events in middle-aged men can also be anticipated in individuals with shorter telomere[21]. A cohort study involving 603 participants found that lowest omega 3 fatty acid in the diet was associated with fastest telomere shortening[36]. Another study involving 803 participants, demonstrated significant shortening of the telomere in chronic heart failure patients[25]. Hence, all these studies suggest telomere shortening as a predictor of future coronary events. There are however, data disagreeing that there is a fundamental association between short telomeres and CVD[27]. Data suggest that changes in telomeres other than shortening can also trigger cell-ageing[15]. The TL varies throughout the life in an individual, and that is independent of the level of cardiovascular risk. In the only animal study, short telomeres seemed to protect apolipoprotein E deficient mice from diet-induced atherosclerosis[67]. When patients with shorter telomeres are treated with statins; their coronary heart disease risk becomes indistinguishable from that of patients with longer telomeres[21]. Diseases of ageing and progression to CVD[23], and obesity particularly in women[19], increase in coronary intima media thickness[24] are also related to shortened telomere.

Conclusion

Besides lipoprotein and proinflammatory markers, TL appears to be a potential biomarker of CAD. Its evaluation may be introduced as a routine test in preventive cardiology practice, but it is essential to use a standard and an accurate method for the purpose. The TL of CAD patients may be compared with the normal range in samples of age, sex and ethnicity-matched asymptomatic people not affected by CAD, and its predictive power assessed over time.

Increased physical activity[17,32], decrease calorie intake, resveratrol, antioxidant and mitochondrial support, avoidance of toxin exposure, reduced smoking[16] and sufficient antioxidant intake[18,35] are some of the factors which are associated with prevention of telomere shortening and maintaining telomere integrity. Drugs that target tumour cells by

blocking their abnormally high levels of telomerase are already in different phases of clinical trials. It has been suggested that altered expression of miRNAs in the ageing heart contributes to the age-dependent turn down in cardiac function[68]. *In vivo* silencing or genetic deletion of miR-34a reduces age-associated death of cardiomyocytes[68]. The study documented PNUTS (also known as PPP1R10) as a novel direct miR-34a target, which reduces telomere shortening, DNA damage responses and cardiomyocyte apoptosis, and improves functional recovery after acute MI[68].

Telomeres and telomerase are a focus of research aimed at understanding of ageing, stress and chronic diseases. Telomere shortening has been demonstrated in white blood cells from patients with coronary heart disease, premature MI, hypertension and diabetes mellitus[69]. Expression of 95 per cent telomerase activity has been detected in pancreatic adenocarcinoma. Thereby, the detection of telomerase activity has been proposed to be a useful tool in the diagnosis of pancreatic cancer[70]. To include telomere as CAD biomarkers, TL measurement variation needs to be reduced that presently restricts our means to perceive consistent result in TL assessment. Second, as TL in senescent lymphocytes is more informative than in leucocytes[71], hence measuring the TL in specific cell populations may be more informative than measuring in mixed populations such as leucocytes. Merging conventional epidemiology with modern genomics at both bench and bedside will enhance our perceptive of CAD and switch discoveries into clinically useful application that can make a valuable impact in public health[72].

Acknowledgment

***Conflicts of Interest*:** None.

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Reprint requests: Dr Joyeeta Bhattacharyya, Department of Cardiac Research, Cumballa Hill Hospital & Heart Institute,
93-95 August Kranti Marg, Mumbai 400 036, Maharashtra, India
e-mail: bjoyeeta23@gmail.com