Special Article “Green Banana”*

The Epidemiology of Human Telomeres: Faults and Promises

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There are neglected but growing problems in the epidemiological field of telomere biology. The focus of the field has been on leukocyte telomere dynamics, which ostensibly register the accruing burden of oxidative stress and inflammation. Important as they are, studies that have examined associations between leukocyte telomere length and indices of aging and diseases of aging also include many that are compromised by poor epidemiological and laboratory methodology. The shortcomings of these studies muddle findings, undermine conclusions, and compromise the ability of the field to attain its goals, which include a better understanding of human aging. Specific steps are delineated to resolve these problems. They include a call for an impartial evaluation of the two major methods (Southern blots and quantitative polymerase chain reaction) currently in use to measure telomere parameters and a proposal for a working model to test the potential connections of leukocyte telomere dynamics with human aging and longevity.

Key Words: Aging—Skeletal muscle—Telomere—Life span—Human—Longevity.

EPIDEMIOLOGY is an exploration of associations that relate to the human condition. A frequent criticism that is leveled at findings linking telomere parameters to environmental factors and clinical manifestations of aging-related disorders in humans is that these associations do not show evidence of causality. Although of some merit, this criticism does not invalidate findings and their relevance for a better understanding of human aging, as well as potential clinical applications.

The identification of major mutations in telomerase, which may cause diseases such as dyskeratosis congenita (1) and pulmonary fibrosis (2), has helped to explain the phenotypic manifestations of these monogenetic diseases in humans. However, such diseases hardly explain telomere biology in the general population. For obvious reasons, human experimentations that demonstrate causality would be ethically untenable. Therefore, associative data are essential for hypothesis testing in the general population. Without this type of research, scientific and medical communities are left with extrapolations from model organisms to humans, which should not be accepted at face value (3).

Although the fundamentals of biology apply to all life forms, the more distant a model organism is from the human species, the less likely it mirrors the human condition. This has been demonstrated, for instance, in the divergence of transcriptional regulation of homologous genes between mice and humans (4). More specific to human aging is the paradoxical link between insulin-like growth factor 1 (IGF-1) and longevity, which serves as a cautionary tale of how extrapolations from model organisms to humans may actually cause confusion. Mutations that affect the insulin/IGF-1 pathway, the IGF-1 receptor (IGF-1R) in particular, have been shown to increase longevity in nematodes and flies (5). In contrast, studies of the IGF-1 system in humans depict an entirely different picture. Based on findings derived from model organisms, one would anticipate that lower IGF-1 levels would engender better health, ultimately expressed in increased longevity. This is not the case; a lower IGF-1 level is a risk factor for cardiovascular disease and is associated with frailty in the elderly population (6–8).

What is needed in the field of aging, telomere studies included, is the recognition that basic and clinical research efforts are mutually complementary—part and parcel of the same overall venture.

LEUKOCYTE TELOMERE DYNAMICS (TELOMERE LENGTH AT BIRTH AND ITS ATTENTION RATE AFTERWARD)

Given that most epidemiological investigations have used leukocytes (or lymphocytes), the focus here is on studies that have used these readily available cells to explore associations and test hypotheses. From the epidemiological perspective, the most striking feature of telomere length is that it is highly variable among individuals, even after adjustment for age. Three potential causes of this variability are: (i) high inter-individual variations in telomere length at birth (9–12); (ii) high inter-individual variations in telomere attrition after birth (9,13); and (iii) variations that are attributed to the techniques used to measure telomere length. At birth, inter-individual variations in leukocyte telomere length amount to as much as 4 kb (11,12); during adulthood the variations in length might be as wide as or wider than those at birth (13–20). Based on cross-sectional and longitudinal analyses, during infancy and early...
childhood, leukocyte telomeres are shortened at a fast pace (>0.2 kb/year) (9,10,18), whereas during adulthood the average rate is roughly 0.03 kb/year, but with a wide range (13–17).

Although telomere length and attrition rate might vary among subsets of circulating leukocytes, in the final analysis, at any time during the individual’s life span these parameters reflect birth telomere length and replicative history of hematopoietic stem cells (HSCs) and progenitor cells (PCs). Telomere attrition in HSCs arises from asymmetric proliferative activity (a stem cell dividing into one daughter stem cell and one committed PC, which ultimately gives rise to peripheral leukocytes) and symmetric proliferative activity (a stem cell dividing into two daughter stem cells to maintain the HSC pool) (21,22). Inflammation would increase the demand on PCs and ultimately HSCs to divide. The inflammatory response may primarily involve subsets of leukocytes. However, at any given time and for whatever duration, the demand by a leukocyte subset on PCs to increase the number of their divisions would ultimately impact telomere dynamics in HSCs and therefore telomere length in all leukocytes.

Oxidative stress may influence telomere attrition in HSCs and PCs in several ways. Like other somatic cells, HSCs are sensitive to oxidative stress (23); damage to these cells would entail more symmetric proliferative activity to replenish the HSC pool, resulting in enhanced telomere attrition. In addition, oxidative stress also heightens the loss of telomere repeats per replication (24), which would further augment the overall loss of telomere attrition in HSCs. Increased oxidative stress may also shorten the biological life of peripheral leukocytes, increasing demand on PCs and HSCs to replicate.

Thus, the cumulative burden of inflammation and oxidative stress over the individual’s life span is registered in telomere dynamics of HSCs and their proxies, peripheral leukocytes. This model is at the core of many epidemiological studies that have measured leukocyte telomere length (10,39–41), which are capable of concurrently measuring telomere signals from cell subsets, and the second relates to the ability of current methodology to detect small changes in telomere lengths over time. Very few repositories have sequential samples of leukocyte DNA (blood or buffy coats) that have been collected longitudinally from the same individuals. This lack of sequential samples might cause most researchers to resort to prospective studies. What then is the optimal follow-up time period to allow the detection of meaningful impact of genetic and environmental factors, and perhaps therapeutic interventions, on leukocyte telomere attrition? To answer this question, let’s consider two individuals, A and B.

A’s and B’s leukocyte telomere lengths are 7 kb and 8 kb, respectively. Suppose that the coefficient of variation (CV) for a given method that measures telomere length is 1%. For A’s and B’s telomeres, this amounts to the respective values of 0.07 kb and 0.08 kb. Suppose that A’s telomere attrition rate is 0.02 kb/year and that of B is 0.04 kb/year. In terms of the follow-up time, in telomere year equivalence (the unit length of telomere lost in the course of 1 year), a 1% deviation from the real value of telomere length amounts to an overall error of 3.5 years for A and 2.0 years for B. In reality, the CV of methods that measure telomere length may considerably exceed the 1% value.

It follows that prolonged follow-up periods and large cohorts will be required to attain credible inter-individual variations in telomere attrition rates. What is more, the methods used in epidemiological studies to measure telomere length may not be sufficiently reliable, not only in precision but also in other respects to accomplish their ultimate goal. That goal, of course, is to decipher the links between leukocyte telomere dynamics and aging, diseases of aging, and longevity in humans.

The two major methods currently in use to measure telomere length in epidemiological/clinical research are Southern blot analysis (37) and real-time quantitative (Q)-PCR (38). Other less widely used methods are quantitative fluorescence in situ hybridization (Q-FISH) and flow-FISH analyses of telomere length (10,39–41), which are capable of concurrently measuring telomere signals from cell subsets and from different chromosomes. These methods require intact cells (nuclei), as well as metaphase cells (in the case of chromosomal analysis), which might limit their applicability to epidemiological investigations and longitudinal evaluations. Moreover, their CVs are apparently >5%.
The Southern blot analysis requires a considerable quantity of DNA (~2–3 μg per analysis) and is cumbersome, labor intensive, and costly. Each DNA sample must be tested for integrity, because the method measures the lengths of the TRFs, which might be compromised in DNA samples that are substantially degraded. In addition, the restriction enzymes generate TRFs that consist of an admixture of canonical (strictly TTAGGG) repeats of telomeres and some noncanonical sequences at the proximal telomeric region. Both theoretical considerations and some published data point to restriction site and length polymorphisms in the proximal telomeric regions, although the extent of this problem is unknown for the general population. In addition, data are often reported as the mean of the TRFs, yet the TRF distribution might not always be Gaussian.

In contrast, the Q-PCR method to measure telomere content is rapid, relatively simple, and with high throughput. Moreover, it requires small amounts of DNA (<100 ng) per sample, and because it measures telomere repeat content rather than telomere length, it can be used to analyze degraded DNA samples. Therefore, the Q-PCR seems more advantageous than the Southern blot analysis for epidemiological/clinical settings, but what appears advantageous in theory may not hold in practice. By its very nature, Q-PCR can easily generate artefactual findings. Minute changes in the efficiency of the PCR of the T and the S might be amplified into substantial variations in the accumulating products, with considerable impact on the T/S ratio. The original communication describing the Q-PCR method indicated that the CV (presumably assaying the same samples measured on different plates) of the T/S was 5.8% (38). Of a special concern is the practice of reporting T/S ratios in kilobases by using a conversion factor. This factor had been derived from the slope of a regression line describing the relationship between the T/S ratios and the mean TRFs in ‘reference’ samples. No evidence has been provided that the conversion factor is constant. In principle, because of a host of variables that are intrinsic to the PCR and the DNA samples, the conversion factor might differ in DNA samples derived from different groups, e.g., old versus young people.

Much has been made of the low cost and convenience of the Q-PCR method, but a 5.8% CV calls into question its adequacy for most epidemiological studies exploring the links between leukocyte telomere length and human aging. Although the reported CV for the Southern blot analysis has been as high as 12% (43), with sufficient experience, it can be reduced to <2% (19). In addition, the ability to profile the distribution of telomeres of various lengths sets the Southern blot apart from the PCR method.

Based on scatter plots of TRF lengths versus T/S ratios, the original communication describing the Q-PCR method to quantify telomere DNA contended that the different values of TRF lengths of individuals with the same T/S ratios reflect inter-individual variation in restriction site polymorphism (38), but the obverse is as plausible, namely, the variations in T/S ratios for individuals with the same TRF length are largely due to artefactual variations in the T/S ratio. Furthermore, although potentially affecting cross-sectional findings, restriction site or length polymorphisms would not confound longitudinal studies exploring telomere dynamics.

**Putting Experience Into Practice**

The following steps might limit the sources of confusion in the field of telomere epidemiology. First, an impartial comparison of the Q-PCR method versus the Southern blot analysis in measuring telomere parameters across a wide age range is long overdue. A large panel of standard blinded sets of high quality DNA could be established in major organizations such as the National Institute on Aging in the United States or similar establishments elsewhere. Duplicates of these samples could be sent to main laboratories and measures of performance evaluated centrally. In this way, claims could be confirmed or refuted once and for all about the ability of one technique versus another to achieve benchmarks of reproducibility and to demonstrate in cross-sectional analyses associations of telomere parameters with aging-related disorders and mortality in elderly persons. Second, in designing longitudinal studies we must recognize that the current techniques are inherently limited in their ability to detect small differences in telomere length. Third, we must confront the central question of whether or not, as expressed in leukocytes, telomere dynamics has anything to do with the human life span. Several studies have explored the association of telomere length with survival in the elderly population (30,35,43–47). Whether such an association was found, findings generated by a subset of these studies are questionable due to less than optimal cohorts, small numbers of participants, and poor precision of telomere measurements.

The tracking of telomere length from birth to death, with a view to understanding the telomere-longevity connection in humans may provide the answer, but it would require as long as a century of follow-up. Therefore, without investigations based on numerous years of follow-up, we will not know for a long time how the independent or joint input of birth telomere length and telomere attrition afterward might explain associations of leukocyte telomere dynamics with aging-related diseases and longevity in humans. The following proposal is a pragmatic approach among a group of
imperfect alternatives to explore telomere dynamics in a quasi-longitudinal design. It is based on the following core concepts: Telomere length is equivalent in tissues, both proliferative and nonproliferative, of the human fetus and newborn (11,48). After birth, telomere length in proliferative cells, including leukocytes, undergoes attrition. In contrast, telomere length in skeletal muscle—a postmitotic tissue—is probably unchanged or changes very little after birth; it hence represents birth telomere length in all tissues, including leukocytes. By measuring telomere length relative to (uninjured) skeletal muscle and in leukocytes at any age, we might be able to more accurately explore connections of birth telomere length and telomere shortening afterward, independently or jointly, with aging-related diseases and longevity in humans. This approach will provide a within-subject baseline from which relative or absolute decrease in telomere length can be more accurately estimated and then compared across individuals. Although muscle biopsies are clearly nonroutine in epidemiological/clinical research, they pose only minimal risk when taken from superficially located and easily accessible sites. In addition, samples of muscle, blood, and other tissues might also be obtained during autopsies, specifically for the measurements of telomere parameters.

As far-fetched as this paradigm sounds, there is evidence to support it, given that telomere length in skeletal muscle is considerably longer than in proliferative tissues of adults (49). If the model is proven valid, it might help to answer whether, for instance, exceptionally old humans are born with long telomeres, their telomeres undergo slow attrition after birth, both of these possibilities, or neither. Similarly, the question—still unanswered—is whether the longer life span of women than men in most advanced societies relates to leukocyte telomere dynamics, given that women have longer leukocyte telomeres than men have (14,19,36). These and many other enigmatic matters would be wide open for detailed inquiry.

CONCLUSION

In taking on the big question of human aging, we must recognize not only the potential of telomere epidemiology but also its limitations. Like aging itself, leukocyte telomere length is fashioned by many genes that interact with the environment. Accordingly, an association between a given variable and telomere length might occasionally be observed in one population and not in another—not because of statistical power and technique issues, but because of differences between populations and their environments. Moreover, although a single association that is replicated in diverse populations does not amount to evidence of causality, a cluster of associations that tests a pivotal hypothesis from different perspectives may provide a proof of concept and generate sufficient confidence about the validity of the hypothesis. In the end, novel paradigms that enable the testing of associations between leukocyte telomere dynamics and indices of human aging can resolve fundamental questions that are at the heart of gerontology, but this will only happen after we confront and resolve current challenges for telomere epidemiology. Without resolution, dogmas and rancor will prevail and the field will continue to be a source of confusion and conflict.

NOTE


Main attributes of a Green Banana are a provocative title and brief abstract (less than 150 words) that presents the idea in essence with crisp journalistic wording. Diagrams, models, and figures are preferred over tables and text to illustrate concepts and convey ideas, with no more than 30 references that support the plausibility of the hypotheses presented. We acknowledge that many Green Bananas may fail to ripen and may quickly rot, but if only one grows sweet, we believe the cost worth the effort.

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