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Special Review Article

The telomere length dynamic and methods of its assessment

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Abstract

Human telomeres are composed of long repeating sequences of TTAGGG, associated with a variety of telomere-binding proteins. Its function as an end-protector of chromosomes prevents the chromosome from end-to-end fusion, recombination and degradation. Telomerase acts as reverse transcriptase in the elongation of telomeres, which prevent the loss of telomeres due to the end replication problems. However, telomerase activity is detected at low level in somatic cells and high level in embryonic stem cells and tumor cells. It confers immortality to embryonic stem cells and tumor cells. In most tumor cells, telomeres are extremely short and stable. Telomere length is an important indicator of the telomerase activity in tumor cells and it may be used in the prognosis of malignancy. Thus, the assessment of telomeres length is of great experimental and clinical significance. This review describes the role of telomere and telomerase in cancer pathogenesis and the dynamics of the telomeres length in different cell types. The various methods of measurement of telomeres length, *i.e.* southern blot, hybridization protection assay, fluorescence *in situ* hybridization, primed *in situ*, quantitative PCR and single telomere length analysis are discussed. The principle and comparative evaluation of these methods are reviewed. The detection of G-strand overhang by telomeric-oligonucleotide ligation assay, primer extension/nick translation assay and electron microscopy are briefly discussed.

Keywords: telomere - telomerase - measurement - method - cancer - aging

Telomere, telomerase and cancer pathogenesis

Human telomeres are a long tandem array of TTAGGG bases, which are found at the end of each chromosome and which are associated with a variety of telomere-binding proteins. Since 1930s, when Hermann Muller and Barbara McClintock first described the telomere, its significances in the aging process and tumorigenesis have been studied. Normal somatic cells undergo a finite number of cell divisions which culminate in what is described as "replicative

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senescence". The maximum number of divisions is known as 'Hayflick limit' [1]. According to the "Theory of Marginotomy", described by Olonikov in 1970s, telomere shortening acts as an intrinsic clock mechanism counting the lifespan of the cells [2]. This is due to the inability of the conventional DNA polymerase to replicate the 5' end of the lagging strand after degradation of the primer. The actions of putative 5'–3' exonuclease cause further shortening of the 5'end [3–5]. The telomere shortening in each successive division may lead to the formation of 3' end G-strand overhang of the telomere [3–5].

The current model of telomere is a large duplex structure consisting of a telomere (T)-loop and a displacement (D)-loop, associated with telomere-binding proteins. This structure functions as a protective cap, which prevents the chromosome from being recognized as DNA breakage by DNA repair complexes. Subsequently end-to-end fusion, recombination, and degradation occurred, which lead to chromosome instability and cell death [6, 7]. The two major telomere-binding proteins are telomeric repeat binding factor 1 (TRF1) and telomeric repeat binding factor 2 (TRF2). Both may function individually or by interacting with other binding proteins, such as tankyrase, TIN2, hRap1, Mre11/Rad50/Nbs1 DNA repair complex, Ku86 and so on. These binding proteins play an important role in the regulation of the telomere length. TRF1 is a negative regulator of telomere length and the length regulation is dependent on a feedback mechanism involving telomerase. TRF2 is found in the double-stranded T-loop and in the loop-tail junction which probably act as the stabilization of the G-strand overhang at D-loop [8–12].

Telomerase is a ribonucleoprotein consisting of 2 main components, telomere RNA component (TERC; sometimes also referred to as TR or TER) and telomere reverse transcriptase (TERT). The telomerase functions as a reverse transcriptase in the elongation of telomere length, in which the TERC act as a template for telomere synthesis, and TERT catalyze the elongation [13, 14]. The telomerase activity is detected at low levels in normal somatic cells [15]. However, in skin, lymphocytic tissues, endometrial tissue, hair follicles and intestinal crypts active mitotic cells and stem cells express a low level of telomerase activity and is growth regulated [16, 17]. This telomerase activity is regulated at different molecular levels, including transcription, mRNA splicing, and maturation and modification of TERT and TERC. Thus, telomerase is a potential target in the development of anti-cancer therapy [18].

The high level of telomerase activity is detected in about 85% of human tumors and immortalized cell lines [17, 19]. Besides the activation of telomerase, a number of complex events are needed for malignant transformation, including the activation of oncogenes, the inactivation of tumor suppressor genes and uncontrolled mitogenic stimulation [20, 21]. Telomerase activity maintains the length of telomere and conferring tumor cells immortality and high proliferation capacity. Human cells must overcome two barriers to proliferate and achieve immortalization. The first is cellular senescence or mortality stage 1 (M1), resulting in telomere shortening and cell proliferation arrest, but continued cells metabolism. When cells bypass the M1, the telomeres continue to shorten and eventually reach the second proliferation barrier, or 'crisis', or mortality stage 2 (M2). Crisis cells with extremely short telomeres then undergo apoptosis. Rarely, cells escape the crisis and telomerase is activated causing immortalization to occur [22–24]. The current accepted model suggests that telomere length and telomerase activity play a dual role in tumorigenesis. In the early stage of malignant transformation, telomere loss limits cell proliferation and telomerase activation protecting the ends of the chromosome and suppressing tumorigenesis. In the late stage of tumorigenesis telomere loss induces genomic instability and telomerase activation promotes immortalization [25]. Some tumor cells do not express telomerase activity; the stabilization of telomeres is maintained by telomerase-independent mechanism, an 'alternative lengthening of telomeres' (ALT), when the G-strand overhang invade the loop structure and act as the template for its elongation [26, 27].

Dynamics of telomere length

The assessment of telomere length is important in the understanding of biological and clinical significance of the telomere. The telomere length serves as an useful indicator in the study of the chromosomal stability, telomerase activity, proliferative capacity and aging process of the cells. The clinical value of telomeres can be demonstrated in its importance in cancer, premature aging syndrome or segmental progeria, genetic anomalies and age-

related diseases [16, 25, 28]. The dynamics of telomere length have distinct patterns of expression in specific disease progressions. Therefore, it has a great value in the prognosis of the diseases.

Telomere lengths within the same cell are heterogeneous [29]. Studies showed that the length of telomere in specific chromosome arms is very similar in different tissues of same individual, but vary significantly between different individual. Interestingly, certain chromosomes, for example 17p are consistently short in all individuals studied [30]. In germ cells, the telomeres range from 10 to 14 kilobases (kb) in length, while in somatic cells they are several kb shorter [31]. The average telomere length of CD34+ human hemopoietic progenitor cells from fetal liver, cord blood, peripheral blood and bone marrow are 11 kb, 10.4 kb, 7.4 kb and 7.6 kb, respectively [32]. In the absence of telomerase activity, the cells lose 30 to 150 bases (b) of telomeres in each successive cell division, and reach 5 to 10 kb in old or presenescent cells [33, 34]. When the cells reach the senescent phase or M1, the telomere is 5 to 8 kb in length. The short telomeres determine the replicative senescence of cells, in which a specific group of chromosomes with the shortest telomeres is responsible for induction of replicative senescence [35]. In successive telomere shortenings, the cells enter the crisis phase or M2, the telomere length is shorter than in M1, range from 1.5 to 2 kb. However, there is a contradictory report that shows that the telomere in crisis is about 15 kb in length [22].

In cancer cells, the telomere length has a wide range of variability, and its equilibrium depend on the balance between the telomere shortening from cell division and telomere elongation result from telomerase activity [36]. The telomere length is also affected by the presence of associated protein, replication history and age of the patients as well [36, 37]. Many experiments show that tumor cells have extremely short and stable telomere, and its stability is achieved by the activation of telomerase [19, 38]. However, recent data suggests that the tumor-derived and immortalized cell lines show their clonal heterogeneity in telomerase activity and telomere length. The level of telomerase component and telomerase activity is not predictive of telomere length [39]. Clinical evidences demonstrate the clonal heterogeneity of telomere and telomerase activity in tumor cells. Kim et al. showed that the telomere length in colorectal carcinoma (7.12 kb) is shorter than normal controls (9.25 kb) and colonic polyps (9.41 kb). The study showed no significant correlation between the telomere length and telomerase activity [40]. Counter *et al.* showed that the late stage of chronic myeloid leukemia (4.4 kb) is shorter than its early stage (7.9 kb), and the telomerase is activated late in the progression of the disease [41]. In contrast, some tumor types have longer telomere than normal, for example, intracranial tumors, basal cell carcinomas of the skin, and renal cell carcinoma [42]. This suggests that the cells have not yet undergone enough divisions to induce significant telomere shortening, and the further elongation is caused by telomerase activation [42]. Maruyama *et al.* showed that the telomere length of intestinal metaplasia (5.89 kb) and gastric adenoma (4.62 kb) is statistically shorter than normal control (6.23 kb), but the gastric carcinoma (6.21 kb) has no significant difference compared to normal. This study showed that the telomerase activity is expressed only in the early phase of gastric carcinogenesis, from intestinal metaplasia through adenoma [43].

The evaluation of telomere length is important in clinical diagnosis and prognosis. Although many studies had been done, the exact role of telomere shortening and the length variability in different cells types are still elusive and further studies are needed.

Methods of telomere length measurement

There are plenty of methods to measure telomere length, including southern blot, hybridization protection assay, fluorescence *in situ* hybridization, flow cytometry, primed *in situ*, quantitative-polymerase chain reaction and single telomere length analysis. These methods are described in detail below. There are three methods described for the measurement of telomeric 3'-overhang, telomeric-oligonucleotide ligation assay, primer extension/nick translation assay and electron microscopy. These methods are briefly reviewed (Table 1).

Southern blot (SB)

Southern blot (SB) was described by Southern in 1975, and it is now widely used for analyzing the structure of DNA and telomere [44]. The DNA is

Table 1 Methods of telomere measurement

Methods	Modifications	References
Southern blot		[44, 45]
	Modified SB	[46]
	Slot blot	[47]
Hybridization protection assay		[48]
Fluorescence *in situ* hybridization		[50]
	PNA-FISH	[29, 49, 51, 52]
	FISH with modified probes	[53]
	TELI-FISH	[54]
	T/C-FISH	[55]
	Fiber-FISH	[56, 57]
Flow cytometry-FISH		[58–64]
	Modified flow-FISH	[62, 65]
	Multicolor flow-FISH	[66]
Primed *in situ*		[67–70]
	Dideoxy-PRINS	[71, 72]
	Double strand-PRINS	[73]
Quantitative-polymerase chain reaction		[74, 75]
Single telomere length analysis		[76–78]
Primer extension/nick translation		[3, 4]
Electron microscopy		[79]
Telomeric-oligonucleotide ligation assay		[80]

cut into fragments by restriction enzyme, *Hinf*1 or *Rsa*1. Then, it is separated by electrophoresis in an agarose gel and transferred onto a nitrocellulose or nylon gel. The fragmented DNA is hybridized with labeled probes specific for telomere sequences, $(CCCATT)_n$, either isotopic (^{32}P-labeled) or non-isotopic, chemiluminescence (digoxienin-, biotinylated-, or alkaline phosphatase-labeled). The size and abundance of the telomere / terminal restriction fragment (TRF) is measured quantitatively by densitometer [37, 44]. The *Hinf*1 or *Rsa*1 is used because both restriction enzymes are frequent cutters and therefore can minimize the size of the subtelomeric region.

The TRF analysis by SB has several disadvantages. The extracted DNA for SB analysis should be unfragmented and pure. This is technically difficult and the unfragmented, high molecular weight of DNA is difficult to handle due to it high viscosity. The TRF represents the mean length of telomere of all chromosomes. Besides, the TRF includes the unknown length of the subtelomeric region, in which the TRF value is dependent on the restriction site of the subtelomeric region by the restriction enzyme. Thus, TRF does not provide the information on the actual length of telomere [37]. However, when several frequent cutter enzymes are used together, the subtelomeric region might be reduced to a minimal size and the accuracy might be increased.

Improvements and combinations with other methods have been described to compensate for the shortcoming of the SB [45–47]. The improved SB in telomere measurements is based on two-dimensional calibration of DNA sizes, pulsed-field electrophoresis, high-resolution images, and mathematical calculations [46]. The accuracy of this improved SB is up to ± 0.3 kb [47]. The SB requires at least 1 µg of DNA to measure DNA length, which can be difficult to achieve if the number of cells is small. A variation of the SB method - slot blots, has been described, in which

the measurement of telomere was based on the ratio of telomere to centromere content (TC ratio). This method can measure the minimum of 800 whole cells, or 9 ng of purified DNA, and thus increased the assay sensitivity and DNA isolation is not needed when the cell numbers are too small. However, this method has large variations up to ± 1 kb, and thus it is not suitable for monitoring small changes in telomere length [47].

Although TRF analysis by SB has plenty of disadvantages, it is still the most widely used method in the study of telomere length. It serves as a standard of control and comparison to most of the newly developed methods, as described later.

Hybridization protection assay (HPA)

The detection of telomere repeats by hybridization protection assay (HPA) was developed by Nakamura *et al.* [48]. The authors conducted a comparative study of telomere repeats measurement by TRF analysis and HPA. The results showed several advantages of HPA over TRF analysis. The HPA procedures included the preparation of genomic DNA or cell or tissue lysate and hybridization with acridinium ester (AE)-labeled probe for telomere and *Alu* sequence, measurement of the chemiluminescence by a luminometer, and determination of the ratio of telomere to *Alu* sequences. Their study showed a telomere to *Alu* sequences ratio of 0.01 which corresponds to approximately 2 kb of mean TRF.

In HPA, intact or unsheared DNA is not required, but sheared DNA with lower limit of 10 ng is recommended, and unpurified DNA in cell or tissue lysates with lower limit of 1000 cells can be measured; in comparison, the TRF analysis required purified DNA as intact as possible. The TRF analysis is time-consuming, while the HPA is rapid and simple, because the procedure requires only approximately 45 min. Furthermore, the TRF may include unknown lengths of subtelomeric sequences, and the real telomere length is unknown; while the telomere measurement by HPA may not included subtelomeric sequences. The TRF analysis is inaccurate when the smear pattern of TRF autoradiogram is quantified by densitometry and HPA avoid this. In comparison to TRF analysis, the HPA is safer to use because no radioactive probes are used. However, the HPA does not give detailed information about telomeres at the cell level or chromosome level, and the size of telomeres can't be measured directly. HPA is convenient for clinical examination of telomeres in small numbers of cells in washings or body fluids or tissues obtained by endoscopy or needle biopsy [48]. However, modification of HPA is needed, particularly in the context of statistical analysis and technical handling, in order to increase its accuracy and improve it disadvantages, and thus make it more applicable in experimental and clinical approaches.

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization (FISH) allows the direct labeling of oligonucleotide probe to the telomere sequences at individual cell level [49]. The standard procedures of FISH involved the metaphase preparation of chromosome and DNA denaturation, the hybridization with synthetic telomeric-specific fluorescein isothiocyante (FITC)-conjugated or Cy3-labeled oligonucleotide probes, counterstaining with 4', 6'-diamidino-2-phenylindole (DAPI) or propidium iodine (PI) and finally visualization by fluorescence microscopy or with a digital imaging system, such as a CCD camera, which capture the image and quantify the fluorescent signals [49, 50]. The FISH methods are extensively employed in cytogenetics studies of specific genes. There are several reasons that make it suitable in telomere measurements, for instance, the small size of telomere-specific probes increase their penetration into the cells and the renaturation is not needed because the probes used are single stranded make it more applicable for analyzing of telomeres [49]. The quantifying of telomere length by FISH method is called quantitative-FISH (Q-FISH).

Lansdorp *et al.* developed a quantitative-FISH by using peptide nucleic acid (PNA) oligonucleotide probes (PNA-FISH) instead of oligonucleotide probe [49]. The PNA probe resulting duplexes are more stable than the DNA/DNA or DNA/RNA duplexes formed by the conventional oligonucleotide probes, because the charged phosphate-deoxyribose backbone is replaced by uncharged repeating N-(2-amino ethyl)-glycine backbone linked by peptide bonds [51]. The digital images were recorded with a CCD camera on a fluorescence microscope, and analyzed quantitatively

[29, 49]. In comparison to conventional oligonecleotide probes, although they produce higher background fluorescence, the PNA probes exhibit more intense staining of most telomeres, and thus improve it sensitivity. When fetal livers, bone marrows and chronic myeloid leukemia cells were tested, the result showed that the mean telomere fluorescence intensity (TFI) is well correlated with the expected mean TRF [29, 49]. Poon *et al.* used a digital image microscopy system for measurement of the TFI in metaphase cells following PNA-FISH [52]. The images of Cy3 and DAPI fluorescence are separated by the computer program TEL-TELO. The TFI is proportional to the number of hybridized probes and showed a good correlation with mean TRF. Poon *et al.* proposed several improvements including an improved microscope focusing process and segmentation of telomeres in interphase nuclei without chromosome segmentation can increase the accuracy and efficacy of analysis [52]. Although the prerequisite of thousands of live cells are needed in cell culture for both Q-FISH and TRF analysis, one of the advantages of Q-FISH is that the cell harvesting can be done easily because very few metaphase cells (< 30 cells) are needed for Q-FISH analysis. In comparison, the large amount of cells (> 100.000 cells) and a serial of complex molecular biological techniques are needed for TRF analysis [52].

Hacia *et al.* designed the modified version of oligonucleotide probes for the FISH assay in detection of telomere sequences [53]. The modified oligonucleotides are composed of 18 nucleotides in length and are complementary either to C-rich or G-rich telomere sequences. The modified oligonucleotides were made on a PE Biosystems 391 DNA synthesizer. The modified probes are designed either to increase its target affinity by enhancing duplex stability (2'-OMe ribose sugars and 5-(1-propynyl) pyrimidine residues) or inhibit the formation of inter- or intramolecular structures which may interfere with target binding (7-deazaguanosine and 6-thioguanosine residues). The result showed that the modified probes increased affinity for target binding in comparison to conventional probes, and thus enhanced the telomere staining potential. The modified oligonucleotide probes gave comparable result with PNA. The modified probes can easily be synthesized and are more cost-effective in contrast to PNA. The authors proposed that such probes could be used as an alternative method to PNA in the assessment of telomere length [53].

Meeker *et al.* described the measurement of telomere length by combined FISH and immunostaining (TELI-FISH) [54]. This allows the measurement of telomere length in standard formalin-fixed, paraffin-embedded human tissue specimens and simultaneous identification of cell species. Very few cells (~10-15 cells) are required in TELI-FISH. In their study, human cancer cell lines with known telomere lengths are studied and it is applied to human prostate tissue microarray with matched normal/tumors pairs. This enabled the direct comparison of telomere length of normal and malignant cells, in which the undesired cells types can be ignored. The result showed a good correlation with the mean TRF [54].

Perner *et al.* developed the telomere/centromere-FISH (T/C-FISH) to measure the telomere length of every single chromosome arm [55]. The centromere of chromosome 2 serves as an internal reference, the metaphase images are captured and karyotyped by ISIS software. The ratio of fluorescence intensity of absolute telomere to reference centromere are calculated and correlated with mean TRF, and thus telomere length is quantified. This method can be applied only to proliferating cells, but not in resting cells because metaphase imaged can be captured and karyotyped for future analysis [55].

Recently, Yan *et al.* developed fiber-FISH method in telomere measurement on chromatin/DNA fiber preparations [56]. The telomere-specific PNA green probe and 1q subtelomere-specific red probe co-hybridized on the chromatin fiber. The size of the 1q subtelomere probe is known to be 100 kb. When it hybridized to the fiber, its visible length becomes variable because of the nature of the variant stretching degree of the released chromatin fiber. However, the target sequence that is very close to the subtelomere probe supposes to have similar stretching degree. Thus the known size subtelomere probe as internal control plays important role to judge the degree of release of the DNA fibers and calibrate the target sequence in practice [57]. In other words, the variation of the signal length on the fiber is not a matter for the measurement of telomere length as long as a good reference probe with known size is used. In the examination of

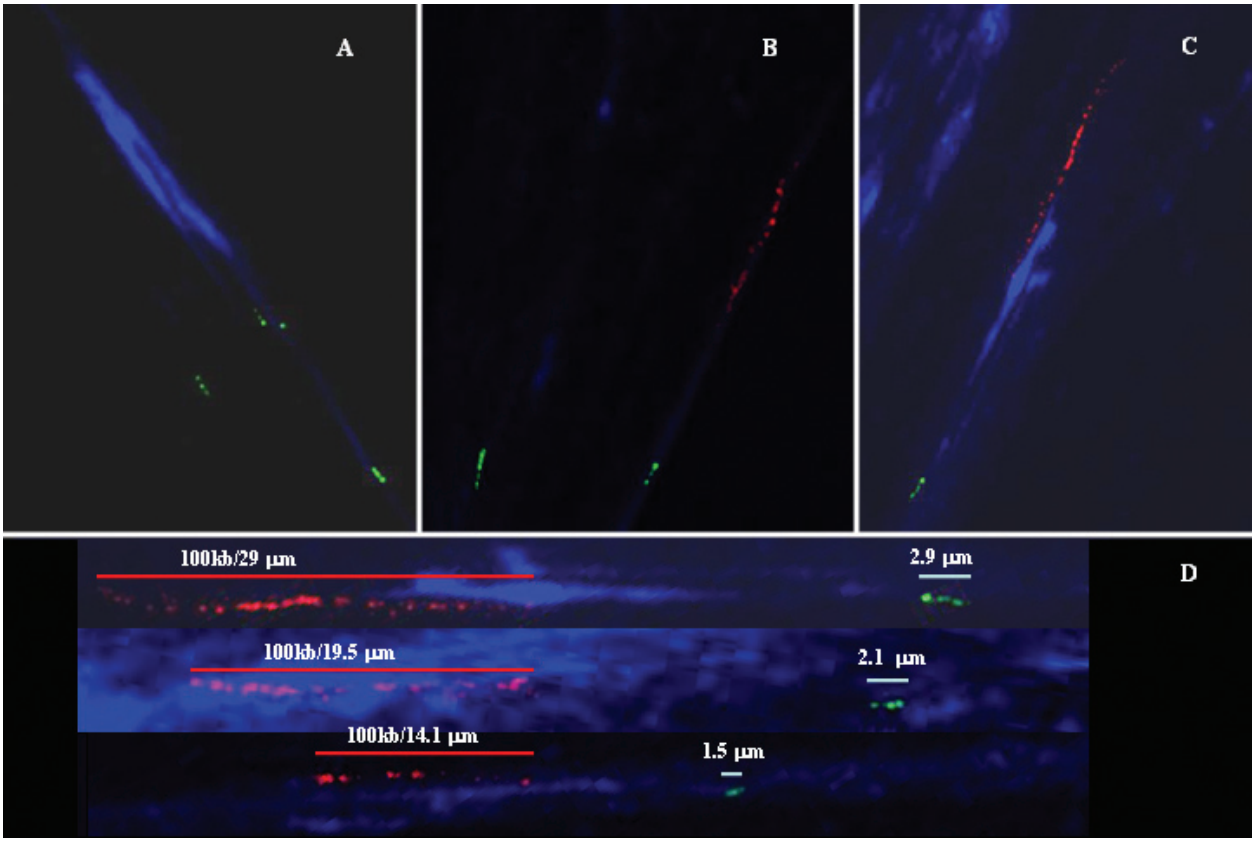

Fig. 1 Measurement of telomere length using fiber-FISH. (A): Telomere signals detected by PNA probe on chromatin fiber preparations. (B) and (C): Co-hybridization of telomere probe (green) and 1q subtelomere probe (red) on the fibers. (D): Measurement of signal length on the fibers with different stretching level. From presentation of Yan J. *et al.* in The American Society of Human Genetics 54th Annual Meeting, Toronto, Canada, October 26-30, 2004 [56].

1q in a lymphoblast cell line, the length of measurable telomere and subtelomere signals aligned on the fibers ranged from 0.9–2.9 μm and 13.8–29 μm, respectively. The average telomere length in the 1q from the lymphoblast cell line is 8.4 kb with SD 1.9 kb (Fig. 1). It implies 3.4–7.2 kb/μm (average 5.5 kb/μm) of the visible length of the probes hybridized on such fibers according to the various stretching degree of the fibers. The fiber-FISH are able to measure the length of particular telomere, and thus useful as a reference to the telomere length measured by Q-FISH [56].

Flow cytometry - fluorescence *in situ* hybridization (flow-FISH)

The Lansdorp's team combined FISH with flow cytometry (flow-FISH) in the measurement of telomere lengths within the immunologically characterized cell populations [58, 59]. The six basic steps of flow-FISH protocol are summarized: cell separation and preparation, DNA denaturation, hybridization with PNA probes, washes to remove excess probes, DNA counterstaining, and finally acquisition and analysis by flow cytometry [60]. Each of these steps is important for accuracy and reproducibility and the recommended protocol was described by Baerlocher *et al.* [60]. In the study by Hultdin *et al.*, the accuracy is increased by using an internal cell line control, which serves as a standard for telomere length to normalize the relative telomere length to the DNA index of G_0/G_1 phase cells for compensating the differences in hybridization steps [61]. Various cell lines and clinical samples from bone marrow, blood, lymph nodes and tonsils were tested and their values of telomere length were well correlated with mean TRF [61]. In contrast to Q-FISH, the flow-FISH allows the analysis of

telomeres in cycling and non-cycling cells instead of metaspread preparation and the entire procedure can be achieved in a single day [58, 60]. Furthermore, the different subpopulations of cells can be processed at the same time this is useful when certain cell populations present in low frequencies are difficult to purify [62]. The telomere length measurement by flow-FISH in the studies of nucleated blood cells of normal individual or patients with hematological disorders showed its usefulness [60, 63, 64]. However, flow-FISH is feasible only when large number of cells are available.

The simultaneous analysis of telomere length and cell phenotype is technically difficult because only few fluorochromes with suitable emission spectra tolerate the conditions when DNA denaturation and PNA hybridization take place [61]. Schmid *et al.* overcame it by measuring the telomere length in cell subsets characterized by expression of two surface antigens [62, 65]. In their presented protocol, the differential expression of two surface antigens, telomere length and DNA content are measured simultaneously [62]. The results are well correlated with mean TRF [60]. Baerlocher *et al.* used the automated multicolor flow-FISH in the measurement of telomere length by a Hydra 96-well microdispenser device [66]. This allowed the quantification of samples when the differences in telomere length are very small (0.5 kb) and when only few cells (1000 cells) are available. The automation is useful in large scale analysis, when multiple samples are measured simultaneously [66].

Primed *in situ* (PRINS)

Primed *in situ* (PRINS) is an effective method for detecting long repeat sequences of DNA, such as α-satellite in centromere and mouse telomere (> 100 kb). The synthetic oligonucleotide primers, $(CCCTAA)_7$ are annealed or hybridized to the metaphase or interphase spread chromosomes. When the thermostable DNA polymerase and fluorescence labeled nucleotides are added, elongation of the primers occurs. Finally, the fluorescence signals are detected and analyzed by fluorescence microscope with computer software [67, 68]. The simultaneous extension of the primers at many sites will produce sufficient fluorescence intensities to be detected. However, the human telomere is short and the uneven primer annealing may lead to insufficient telomere detection [68]. Several modifications were introduced to increase labeling and annealing efficacy. Multiple cycles of amplification were used to increase the hybridization and elongation of primers, and thus enhance the signal of telomere detection [69]. Next, multicolor labeling for different chromosomes and enzymatic detection instead of fluorochromes were introduced [70]. Later, the dideoxy-PRINS were designed when one or more dideoxynucleotides are added, for example, ddGTP are used in C-rich telomeric strand. This result in more specific labeling of telomere sequences by reduces non-relevant priming from random break in chromosomal DNA [71, 72].

In recent years, Yan *et al.* developed the double-strand PRINS method [73]. Two primers, $(TTAGGG)_7$ and $(CCCTAA)_7$, were used to label both forward and reverse telomeric DNA strands. The two directional labeling resulted in an intensified signals production. In comparison to the classic single-strand labeling, the labeling efficacy increased from 78% to 95%; when the chromosome arms were evaluated, the labeling efficacy was increased from 88% to 99% [73]. Moreover, this study had shown that the digoxigenin-labeling yielded a higher detection power in comparison to the biotin-labeling. The withdrawal of dGTP from G-rich primed reaction or dCTP from C-rich primed reaction avoid the self-annealing between the two complementary primers [73]. The comparative studies suggested that the PRINS is more cost effective and simpler than FISH in the detection of telomere [68, 73].

The modified oligonucleotide probes in the FISH detection of telomere which previously described are suggested to be applied to PRINS [51]. The high target affinity of 2'-OMe-ribose- and 5-(1-propynyl) pyrimidine-modified probes could be suit for PRINS in the detection of telomere [51]. However, the experimental studies are needed to prove it usefulness in telomere measurement.

Quantitative polymerase chain reaction (Q-PCR)

Cawthon described the measurement of telomere by quantitative polymerase chain reaction (Q-

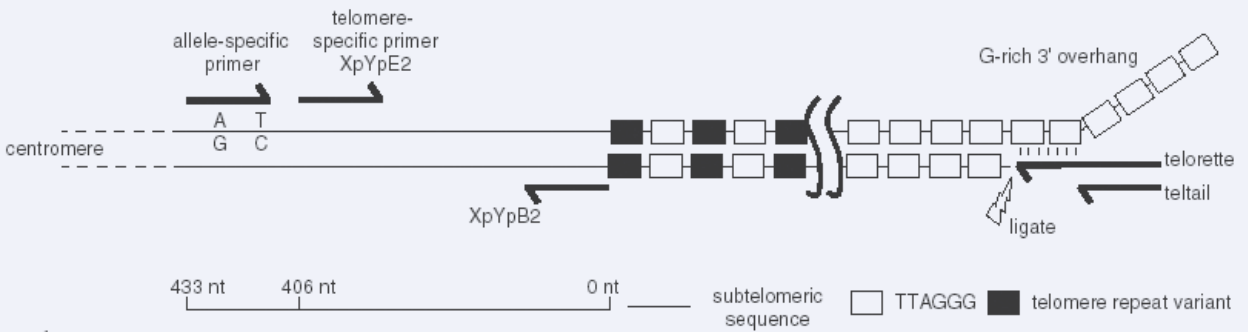

Fig. 2 The STELA involve the annealing and ligating of 'telorette' to telomeric G-rich strand 3'-overhang. The PCR amplification is performed using primer 'teltail' together with a telomere- or allelic-specific upstream primer in the subtelomeric region. Reproduced with permission from Baird D.M. [76].

PCR) [74]. Previously, it was thought that the measurement of telomere by PCR amplification with oligonucleotide primers, TTAGGG and CCCTAA is impossible, because the hybridization may occur between these primers and only primer dimer-derived products are generated [74, 75]. In their presented study, the primers are modified and are composed of repeated pattern of six bases containing four consecutive paired bases followed by two mismatched bases. In this way, the two primers will not be hybridizing with each other and the PCR-amplified telomere products are free of primer-derived products.

In Q-PCR, the ratio of telomere repeat copy number (T) to single copy gene copy number (S) is determined. This ratio (T/S ratio) is proportional to the average telomere length, and the relative telomere length is measured quantitatively [74, 75]. Cawthon carried out the experiments by using AmpliTaq Gold DNA polymerase on an Applied Biosystem Prism 7770 Sequence Detection System [74]. Gil *et al.* modified the conditions and reagents of this protocol when they used FastStart Taq polymerase on a Roche LightCycler [75].

In comparison to TRF analysis and Q-FISH methods, the measurement of telomere by Q-PCR does not include the subtelomeric region which is highly variable between individuals, from 2.5 to 6 kb. In the HPA assay, the ratio of telomere to *Alu* sequences is determined, and the relative length of telomere is subsequently quantified; however, the level of inter-individual variation in the copy number of *Alu* sequence and centromeric sequence is unknown, thus the results may be inaccurate. The use of single copy gene avoids this problem. The Q-PCR is suitable for genetic and epidemiological studies, since the T/S ratio vary approximately 2.5 fold among age- and sex-matched individuals [74].

Single telomere length analysis (STELA)

Baird *et al.* developed single telomere length analysis (STELA), a PCR-based method that measures the telomere lengths at the individual chromosome level [76]. The first step involved the annealing of 'telorette', a linker which compose 7 repeats of TTAGGG followed by 20 nucleotides non-complementary tail to telomeric G-rich strand 3'-overhang. Secondly, the telorette is ligated to the 5' end of C-rich strand of telomere (Fig. 2). Thereafter the PCR amplification is performed using 'teltail', a primer which is identical to the non-complimentary telorette tail, together with a chromosome-specific upstream primer in the subtelomeric region [76, 77]. The upstream primer can be allele-specific when the polymorphisms in this region are studied, for example, the AT (adenine, thymine)- and GC (guanine, cytosine)- allele telomere loss rates are examined in Werner syndrome fibroblast cells [75, 77]. The STELA was first developed for analysis of short arms of the human sex chromosome, the XpYp telomere and suggested that this method can be applied to telomeres in human and other species [76]. A recent study on strain-specific telomere length of *Caenorhabditis elegans* and telomere erosion rates in Werner syndrome fibroblast cells showed its validity in telomere analysis at the single chromosome level [78]. The result showed good correlation with mean TRF (Fig. 3). The STELA

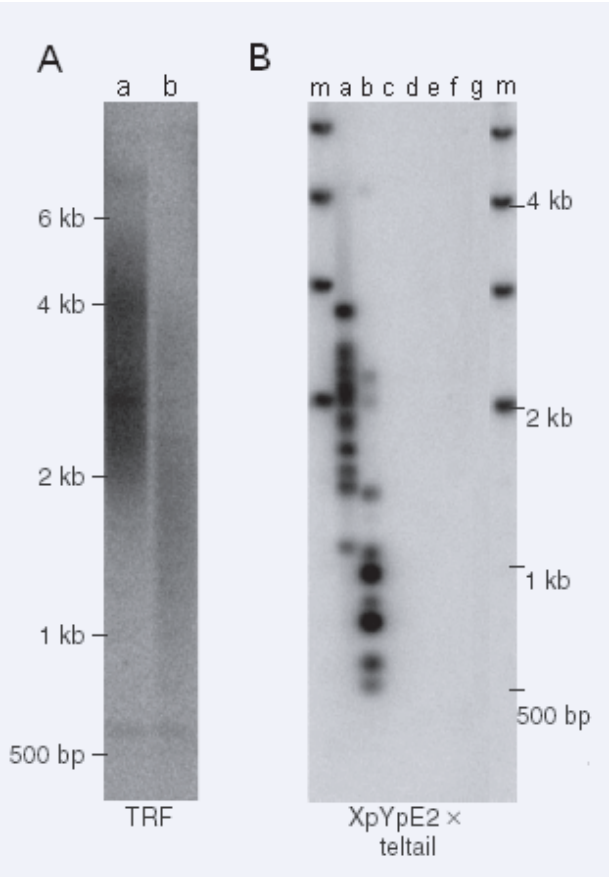

Fig. 3 (A) TRF analysis of 2 subclones of K1 human thyroid cancer cell lines, with mean TRF of 2.9 kb and 1.5 kb. (B) STELA of same subclones of K1 human thyroid cancer cell lines with a) and b) telorette ligated; c) and d) irrelevant telorette ligated; e) no telorette ligated; f) and g) 3'-overhang digested by nuclease and telorette ligated. TRF is a continued DNA smear, while STELA contains many DNA bands reflecting the higher accuracy. Reproduced and modified with permission from Baird D.M. [76].

can readily measure the telomere lengths ranging from 406 b to 20 kb. When the exact position of each telomere molecule is inferred from telomere-variant repeat mapping and together with control experiments of similarity in intensity of each band, it is proposed that the PCR amplification of STELA is highly telomere-specific. The STELA measurement of XpYp telomere of human fibroblast cells are consistently lesser than mean TRF and the relationship between these two are linear, suggested that the unknown length of subtelomeric sequences are not included in STELA [76].

Methods of G-rich strand 3'-overhang measurement

A long telomeric G-rich strand 3'-overhang are found in the human chromosome end. Electron microscopy reveals that about 42% of the telomere ends form a large duplex loop structure, which depends on the presence of 3'-overhang and telomeric repeat factor 2 protein [6, 7]. Besides, the rate of telomere shortening during cell division is proportional to the size of the telomeric 3'-overhang [79]. The study of the 3'-overhang is needed in understanding how the telomeres are being protected by hiding in the loop structure, and how the telomerase accesses the hidden telomeres, and is therefore useful in exploitation of target for anticancer therapy.

Several methods are described for the quantitative measurement of the telomeric 3'-overhang, namely primer extension/nick translation (PENT), telomeric-oligonucleotide ligation assay (T-OLA) and electron microscopy (EM). By using PENT, 85% of the human umbilical vein endothelial cells (HUVEC) are detected to have 130–210 nucleotides of 3'-overhang [3, 4]. In the study by EM, the BJ fibroblasts, mammary epithelial cells, IMR 90 lung fibroblasts, and HUVEC have 156±7, 263±7, 187±4, and 322±14 nucleotides of 3'-overhang respectively [79]. In contrast, the T-OLA enables to detect 3'-overhangs with as few as 24 nucleotides to 360 nucleotides in various tested cell lines, for instance HUVEC, human fibroblasts, peripheral blood lymphocytes, HeLa and U937 cells. However, this study showed that cells with < 90 nucleotides are predominant within the broad spectrum of length distribution [80]. These studies showed that the T-OLA is able to detected shorter telomeres in comparison to other methods, which is necessary for studying the 3'-overhang of senescent cells, tumor cells and certain cell lines, in which short telomeres can expected [37]. The details of these methods are not discussed here.

Conclusion

The assessments of telomere length is the main approach in understanding the biology of telomeres and their role in physiological aging and var-

ious pathological processes, especially malignant transformation and progeria. The telomerase activity, replicative history and genetic stability of the cells are reflected in the dynamic of telomeres length. Although there are plenty of methods for the evaluation of telomere length, as described above, each has its specific features and can applied to different approaches. However, further modifications and improvements of these methods are necessary to compensate their shortcoming and make them more cost-effective, simple, sensitive, suitable for larger output and thus more applicable not only to experimental research but also to clinical diagnosis and prognosis.

There are a variety of questions that remain to be solved, for example, the heterogeneity of the of telomere length in various cell types, the dynamics of telomere length and the various factors which contribute to its changes, the structure and functions of G-strand overhangs and so on. Further investigations are needed, which will lead to the improvement of our current telomere model. This is a prerequisite step for researchers to exploit a target-specific anti-tumor therapy.

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References

1. **Hayflick L, Moorhead PS.** The serial cultivation of human diploid cell strains. *Exp Cell Res.* 1961; 25: 585–621.
2. **Olonikov AM.** A theory of marginotomy. *J Theor Biol.* 1973; 41: 181–90.
3. **Makarov VL, Hirose Y, Langmore JP.** Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell* 1997; 88: 657–66.
4. **Wright WE, Tesmer VM, Huffman KE, Levene SD, Shay JW.** Normal human chromosomes have long G-rich telomeric overhangs at one end. *Gen Dev.* 1997; 11: 2801–9.
5. **Hao YH, Tan Z.** The generation of long telomere overhangs in human cells: a model and its implication. *Bioinformatics* 2002; 18: 666–71.
6. **Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T.** Mammalian telomeres end in a large duplex loop. *Cell* 1999; 97: 503–14.
7. **Greider CW.** Telomeres do d-loop-t-loop. *Cell* 1999; 97: 419–22.
8. **Broccoli D, Smogorzewska A, Chong L, de Lange T.** Human telomeres contain two distinct Myb-related protein, TRF1 and TRF2. *Nat Genet.* 1997; 17: 231–5.
9. **Bilaud T, Brun C, Ancelin K, Koering CE, Laroche T, Gilson E.** Telomeric localization of TRF2, a novel human telobox protein. *Nat Genet.* 1997; 17: 236–9.
10. **Enrique S, Fermin AG, Predrag S, Paul PW van Buul, Blasco MA.** Mammalian Ku86 protein prevents telomeric fusions independently of the length of TTAGGG repeats and the G-strand overhang. *EMBO Reports* 2000; 11: 244–52.
11. **Kim SH, Patrick K, Judith C.** TIN2, a new regulator of telomere length in human cells. *Nat Genet.* 1999; 23: 405–12.
12. **Blasco MA.** Mammalian telomeres and telomerase: why they matter for cancer and aging. *Eur J Cell Biol.* 2003; 82: 441–6.
13. **Stewart SA, Hahn WC, O'Connor BF, Banner EN, Lundberg AT, Modha P Mizuno H, Brooks MW, Fleming M, Zimonjic DB, Popescu NC, Weinberg RA.** Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc Natl Acad Sci USA.* 2002; 99: 12606–11.
14. **Shay JW, Zhou Y, Hiyama E, Wright WE.** Telomerase and cancer. *Hum Mol Genet.* 2001; 10: 677–85.
15. **Masutomi K, Yu EY, Khurts S, Ben-Porath I, Currier JL, Metz GB, Brooks MW, Kaneko S, Murakami S, DeCaprio JA, Weinberg RA, Stewart SA, Hahn WC.** Telomerase maintains telomere structure in normal human cells. *Cell* 2003; 114: 241–53.
16. **Judy MYW, Collins K.** Telomere maintenance and disease. *Lancet* 2003; 362: 983–8.
17. **Greider CW.** Telomerase activity, cell proliferation, and cancer. *Proc Natl Acad Sci USA.* 1998; 95: 90–2.
18. **Cong YS, Wright WE, Shay JW.** Human telomerase and its regulation. *Microbiol Mol Biol Rev.* 2002; 66: 407–25.
19. **Shay JW, Bacchetti S.** A survey of telomerase activity in human cancer. *Eur J Cancer* 1997; 33: 787–91.
20. **Hahn WC, Weinberg RA.** Rules for making human tumor cells. *N Engl J Med.* 2002; 347: 1593–603.
21. **Belair CD, Yeager TR, Lopez PM, Reznikoff CA.** Telomerase activity: A biomarker of cell proliferation, not malignant transformation. *Proc Natl Acad Sci USA.* 1997; 94: 13677–82.
22. **Halvorsen TL, Leibowitz G, Levine F.** Telomerase activity is sufficient to allow transformed cells to escape from crisis. *Mol Cell Biol.* 1999; 19: 1864–70.
23. **Lutsig AJ.** Crisis intervention: the role of telomerase. *Proc Natl Acad Sci USA.* 1999; 96: 3339–41.
24. **Reddel RR.** The role of senescence and immortalization in carcinogenesis. *Carcinogenesis* 2000; 21: 477–84.
25. **Hahn WC.** Role of telomeres and telomerase in the pathogenesis of human cancer. *J Clin Oncol.* 2003; 21: 2034–43.
26. **Bryan TM, Englezou A, Dalla PL, Dunham MA, Reddel RR.** Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med.* 1997; 3: 1271–4.

27. **Grogelny JV, McEliece MK, Broccoli D.** Effects of reconstitution of telomerase activity on telomere maintenance by the alternative lengthening of telomeres (ALT) pathway. *Hum Mol Genet.* 2001; 10: 1953–61.
28. **Johnson FB, Sinclair DA, Guarente L.** Molecular biology of aging. *Cell* 1999; 96: 291–302.
29. **Londono-Vallejo JA, DerSarkissian H, Cazes L, Thomas G.** Differences in telomere length between homologous chromosomes in humans. *Nucleic Acids Res.* 2001; 29: 3164–71.
30. **Martens UM, Zijlmans JMJM, Poon SSS, Dragowska W, Yui J, Chavez EA, Ward RK, Lansdorp PM.** Short telomeres on human chromosome 17p. *Nat Genet.* 1998; 18: 76–80.
31. **de Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM, Vermus HE.** Structure and variability of human chromosome ends. *Mol Cell Biol.* 1990; 10: 518–27.
32. **Engelhardt M, Kumar R, Albanell J, Pettengell R, Han W, Moore MAS.** Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. *Blood* 1997; 90: 182–93.
33. **Zhang XL, Mar V, Zhou W, Harrington L, Robinson MO.** Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Gen Dev.* 1999; 13: 2388–99.
34. **Bodnar AG, Quellete M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE.** Extension of life-span by introduction of telomerase into normal human cells. *Science* 1998; 279: 349–52.
35. **Zhou Y, Sfeir A, Gryaznov SM, Shay JW, Wright WE.** Does a sentinel or subset of short telomeres determine replicative senescence? *Mol Biol Cell.* 2004; 15: 3709–18.
36. **Dahse R, Fiedler W, Ernst G.** Telomeres and telomerase: biological and clinical important. *Clin Chem.* 1997; 43: 708–14.
37. **Saldanha SN, Andrews LG, Tollefsbol TO.** Assessment of telomere length and factors that contribute to its stability. *Eur J Biochem.* 2003; 270: 389–403.
38. **Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW.** Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; 266: 2011–5.
39. **Savre-Train I, Gollahon LS, Holt SE.** Clonal heterogeneity in telomerase activity and telomere length in tumor-derived cell lines. *PSEBM.* 2000; 223: 379–88.
40. **Kim HR, Kim YJ, Kim HJ, Kim SK, Lee JH.** Telomere length changes in colorectal cancers and polyps. *J Korean Med Sci.* 2002; 17: 360–5.
41. **Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S.** Telomerase activity in normal leukocytes and in hematological malignancies. *Blood* 1995; 85: 2315–20.
42. **Albanell J, Bosl GJ, Reuter VE, Engelhardt M, Franco S, Moore MAS, Dmitrovsky E.** Telomerase activity in germ cell cancers and mature teratomas. *J Natl Cancer Inst.* 1999; 91: 1321–6.
43. **Maruyama Y, Hanai H, Fujita M, Kaneko E.** Telomere length and telomerase activity in carcinogenesis of the stomach. *Jpn J Clin Oncol.* 1997; 27: 216–20.
44. **Southern EM.** Long range periodicities in mouse satellite DNA. *J Mol Biol.* 1975; 94: 51–69.
45. **Oexle K.** Telomere length distribution and southern blot analysis. *J Theor Biol.* 1998; 190: 369–77.
46. **Slijepcevic P.** Telomere length and telomere-centromere relationship? *Mutat Res.* 1998; 404: 267–78.
47. **Norwood D, Dimitrov DS.** Sensitive method for measuring telomere lengths by quantifying telomeric DNA content of whole cells. *Biotechniques* 1998; 25: 1040–5.
48. **Nakamura Y, Hirose M, Matsuo H, Tsuyama N, Kamisango K, Ide T.** Simple, rapid, quantitative, and sensitive detection of telomere repeats in cell lysate by a hybridization protection assay. *Clin Chem.* 1999; 45: 1718–24.
49. **Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Rapp AK, Tanke HJ.** Heterogeneity in telomere length of human chromosome. *Hum Mol Genet.* 1996; 5: 685–91.
50. **McNeil N, Reid T.** Novel molecular cytogenetic techniques for identifying complex chromosomal rearrangements: technology and applications in molecular medicine. *Exp Rev Mol Med.* 2000, Available at: http://www-ermm.cbcu.cam.ac.uk/00001940h.htm.
51. **Uhlmann V, Prasad M, Silva I, Luettich K, Grande L, Alonso L, Thisted M, Pluzek KJ, Gorst J, Ring M, Sweeney M, Kenny C, Martin C, Russell J, Bermingham N, O'Donovan M, Sheils O, O'Leary JJ.** Improved in *in situ* detection method for telomeric tandem repeats in metaphase spreads and interphase nuclei. *J Clin Pathol Mol Pathol.* 2000; 53: 48–50.
52. **Poon SS, Martens UM, Ward RK, Lansdorp PM.** Telomere length measurements using digital fluorescence microscopy. *Cytometry* 1999; 36: 267–78.
53. **Hacia JG, Movotny EA, Mayer RA, Woski SA, Ashlock MA, Collins FS.** Design of modified oligonucleotide probes to detect telomere repeat sequences in FISH assays. *Nucleic Acids Res.* 1999; 27: 4034–9.
54. **Meeker AK, Gage WR, Hicks JL, Simon I, Coffman JR, Platz EA, March GE, De Marzo AM.** Telomere length assessment in human archival tissues. Combined telomere fluorescence *in situ* hybridization and immunostaining. *Am J Pathol.* 2002; 160: 1259–68.
55. **Perner S, Bruderlein S, Hasel C, Waibel I, Holdenried A, Ciloglu N, Chopurian H, Neilsen KV, Plesch A, Hogel J, Moller P.** Quantifying telomere lengths of human individual chromosome arms by centromere-calibrated fluorescence *in situ* hybridization and digital imaging. *Am J Pathol.* 2003; 163: 1751-6.
56. **Yan J, Bouchard E, Chen BZ, Drouin R.** Measurement of chromosome specific telomere length on chromatin/DNA fibers. The American Society of Human Genetics 54th Annual Meeting, Toronto, Canada, October 26–30, 2004.
57. **Henry HQ, Tsui LC.** High resolution free chromatin/DNA fiber fluorescent *in situ* hybridization. *J Chromatography A* 1998; 806; 219–29.
58. **Rufer N, Dragowska W, Thornbury G, Roosnek E, Lansdorp PM.** Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. *Nat Biotechnol.* 1998; 16: 743–7.

59. **Ishikawa F.** FISH goes with the flow. *Nat Biotechnol.* 1998; 16: 723–4.
60. **Baerlocher GM, Mak J, Tien T, Lansdorp PM.** Telomere length measurement by fluorescence *in situ* hybridization and flow cytometry: tips and pitfalls. *Cytometry* 2002; 47: 88–99.
61. **Hultdin M, Gronlund E, Norrback KF, Eriksson-Lindstrom E, Just T, Roos G.** Telomere analysis by fluorescence *in situ* hybridization and flow cytometry. *Nucleic Acids Res.* 1998; 26: 3651–6.
62. **Schmid I, Dagarang MD, Hausner MA, Matud JL, Just T, Effros RB, Jamieson BD.** Simultaneous flow cytometric analysis of two cell surface markers, telomere length, and DNA content. *Cytometry* 2002; 49: 96–105.
63. **Brummendorf TH, Holyoake TL, Rufer N, Barnett MJ, Schulzer M, Eaves CJ, Eaves AC, Lansdorp PM.** Prognostic implications of differences in telomere length between normal and malignant cells from patients with chronic myeloid leukemia measured by flow cytometry. *Blood* 2000; 95: 1883–90.
64. **Brummendorf TH, Maciejewski JP, Mak J, Yong NS, Lansdorp PM.** Telomere length in leukocyte subpopulations of patients with aplastic anemia. *Blood* 2001; 97: 895–900.
65. **Schmid I, Jamieson BD.** Assessment of telomere length, phenotype, and DNA content. In: **Robinson JP, Darzynkiewicz Z, Hyun W, Orfao A, Robinovitch P**, eds, Current Protocols in Cytometry. vol. 1, John Wiley & Sons, 2004, pp. 7.26.1–13
66. **Baelocher GM, Lansdorp PM.** Telomere length measurements in leukocyte subsets by automated multicolor flow-FISH. *Cytometry* 2003; 55A: 1–6.
67. **Therkelsen AJ, Nielsen A, Koch J, Hindkjaer J, Kolvraa S.** Staining of human telomeres with primed *in situ* labeling (PRINS). *Cytogenet Cell Genet.* 1995; 68: 115–8.
68. **Lavoie J, Bronsard M, Lebel M, Drouin R.** Mouse telomere analysis using an optimized primed *in situ* (PRINS) labeling technique. *Chromosoma* 2003; 111: 438–44.
69. **Musio A, Rainaldi G.** Cycling-PRINS. A method to improved the accuracy of telomeric sequence detection in mammalian chromosomes. *Mutat Res.* 1997; 390: 1–4.
70. **Wilkens L, Tchinda J, Komminoth P, Werner M.** Single- and double-color oligonucleotide primed *in situ* labeling (PRINS): applications in pathology. *Histochem Cell Biol.* 1997; 108; 439–46.
71. **Krejci K, Koch J.** An *in situ* study of variant telomeric repeats in human chromosomes. *Genomics* 1999; 58: 202–6.
72. **Malaska J, Sklenickova M, Krejci K, Fajkusova L, Bajer M, Hrstkova H, Fajkus J.** Telomerase activity and expression and telomere analysis *in situ* in the course of treatment of childhood leukemias. *Blood Cells Mol Dis.* 2000; 26: 534–9.
73. **Yan J, Chen BZ, Bouchard EF, Grouin R.** The labeling efficacy of human telomeres is increased by double-strand PRINS. *Chromosoma* 2004; 113: 204–9.
74. **Cawthon RM.** Telomere measurement by quantitative PCR. *Nucleic Acids Res.* 2002; 30: e47.
75. **Gil ME, Coetzer TL.** Real-time quantitative PCR of telomere length. *Mol Biotechnol.* 2004; 27: 169–72.
76. **Baird DM, Rowson J, Thomas DW, Kipling D.** Extensive allelic variation and ultrashort telomeres in senescent human cells. *Nat Genet.* 2003; 33: 203–7.
77. **Cheung I, Schertzer M, Baross A, Rose AM, Lansdorp PM, Baird DM.** Strain-specific telomere length revealed by single telomere length analysis in *Caenorhabditis elegans*. *Nucleic Acids Res.* 2004; 32: 3383–91.
78. **Baird DM, Davis T, Rowson J, Jones CJ, Kipling D.** Normal telomere erosion rates at the single cell level in Werner syndrome fibroblast cells. *Hum Mol Genet.* 2004; 13: 1515–24.
79. **Huffman KE, Levene SD, Tesmer VM, Shay JW, Wright WE.** Telomere shortening is proportional to the size of the G-rich telomeric 3'-overhang. *J Biol Chem.* 2000; 275: 19719–22.
80. **Cimino-Reale G, Pascale E, Battiloro E, Starace G, Verna R, D'Ambrosio E.** The length of telomeric G-strand 3'-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Res.* 2001; 29: e35.