The Role of Telomeres and Telomerase Complex in Haematological Neoplasia: The Length of Telomeres as a Marker of Carcinogenesis and Prognosis of Disease

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Abstract: Human telomeres (discovery of telomere structure and function has been recently awarded The Nobel Prize) consist of approximately 5–12 kb of tandem repeated sequences $(TTAGGG)_n$ and associated proteins capping chromosome ends which prevent degradation, loss of genetic information, end-to-end fusion, senescence and apoptosis. Due to the end-replication problem, telomere repeats are lost with each cell division, eventually leading to genetic instability and cellular senescence when telomeres become critically short. Stabilization of the telomeric DNA through telomerase activation, unique reverse transcriptase, or activation of the alternative mechanism of telomere maintenance is essential if the cells are to survive and proliferate indefinitely. Telomerase is

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expressed during early development and remains fully active in specific germline cells, but is undetectable in most normal somatic cells. High level of telomerase activity is detected in almost 90% of human tumours and immortalized cell lines. The hematopoietic compartment may develop genetic instability as a consequence of telomere erosion, resulting in aplastic anaemia (AA) and increased risk of myelodysplastic syndrome (MDS) and acute myeloid leukaemia (AML). Genetic instability associated with telomere dysfunction (i.e. short telomeres) is an early event in carcinogenesis. The molecular cytogenetic method telomere/centromere fluorescence *in situ* hybridization (T/C-FISH) can be used to characterize the telomere length of hematopoietic cells. This review describes recent advances in the molecular characterization of telomere system, the regulation of telomerase activity in cancer pathogenesis and shows that the telomeric length could be a potential clinical marker of hematologic neoplasia and prognosis of disease.

Introduction

As early as the 1930s, H. J. Muller (Nobel Prize 1946) (Muller, 1938) and B. McClintock (Nobel Prize 1983) (McClintock, 1941) had observed that the structure at the chromosome ends, the so-called telomeres, seemed to prevent the chromosomes from attaching to each other. They suspected that the telomeres could have a protective role, but how they operate remained an enigma. A major problem in biology – how the chromosomes can be copied in a complete way during cell divisions and how they are protected against degradation, was solved by E. H. Blackburn, C. W. Greider and J. W. Szostak and subsequently, they have won The Nobel Prize in Physiology or Medicine 2009. This fact emphasizes the importance of telomere structure and maintenance discovery.

Telomeres are highly specialized nucleoprotein structures at the ends of chromosomes and they are essential for the preservation of genome integrity (de Lange et al., 1990). The length of telomeres reflects the history of cellular proliferation and concomitantly limits the mean life span of normal somatic cells (Harley et al., 1990). The gradual shortening of telomeres as a result of the cell division of hematopoietic stem cells (HSCs) and aging can be one cause of immune insufficiency, exhausted haematopoiesis and the increasing probability of occurrence of malignant transformations.

Telomerase is a unique reverse transcriptase, which is inactivated in most somatic cells and reversely reactivated in tumour cells (Ly et al., 2003). Increased expression of telomerase is detectable in almost 90% of all malignant tumours (Belair et al., 1997). Major telomerase activity is generally variable in relation to the cellular fraction and grade of proliferation. Marked contraction of telomeres together with increased telomerase activity has been described in solid tumours as well as in hematologic malignancies (Swiggers et al., 2006).

Immature population of CD34+CD38-HSCs can be identified *in vitro* based on their kinetic growth potential and length of the telomeres (Brümmendorf and

Balabanov, 2006). The molecular cytogenetic method T/C-FISH can be used to characterize the dynamics of telomere length as a marker of HSCs turnover *in vivo*. Disease with primary malignant transformation of HSCs (such as chronic myeloid leukaemia – CML) can be recognized in the early stage of disease according to increasing HSC turnover prior to subsequent exhaustion of the compartment of normal hematopoietic cells. Telomere erosion probably correlates with the severity, duration and prognosis of the disease as well as with the response to treatment.

This review deals with the biological, diagnostic and prognostic value of the telomere system and its significance in normal and malignant hematopoietic cells.

Structure and function of telomeres

It was in the 1930s that telomeres (from the Greek *telos* = end and *meros* = part) were first recognized as essential structures at the ends of the chromosomes and were shown to be important for chromosomal stability. The first telomeric sequence was identified in 1978 in the protozoa *Tetrahymena* – a single cell organism that has at a certain stage of development many identical minichromosomes with twice as many telomeres (Blackburn and Gall, 1978).

Telomeres are highly specialized structures on the chromosomes' terminals and they ensure chromosome integrity together with associated proteins as well as protecting them from enzymatic degradation, loss of genetic information, chromosomal fusion, senescence and apoptosis (McGrath et al., 2007; Cheung and Deng, 2008). Telomeres also influence the localization of chromosomes in the nucleus, the pairing of homologous chromosomes in the early phase of cell division and their movement during division (Sen and Gilbert, 1988; Wellinger and Sen, 1997).

In vertebrates, telomeres consist of a hexamer tandem repetition of nucleotides 5'-$(TTAGGG)_n$-3' and of associated proteins. The actual number of repetition copies is species-related and fluctuates according to the life span of a cell. Human telomeres have approximately 500 to 2,000 copies, i.e. 3,000 to 12,000 pairs of bases (de Lange et al., 1990). The terminal regions almost invariably consist of short, direct repeats. These repeats usually contain cluster of 2–4 G residues and the strand that contains these clusters (the G strand) always forms the extreme 3'-end of the chromosome (Wellinger and Sen, 1997). Telomere DNA, together with associated proteins form cap structures which consist of a T-(telomere) and D-(displacement) loop which protects the ends of the telomeres. The T-loop is finished as a single-stranded 3'-overhanging segment which inter-calculates into double-stranded DNA and forms a D-loop (Griffith et al., 1999) (Figure 1). The function of this structure is probably related to the correct segregation of sister chromatids, regulation and expression of other genes (Lippman et al., 2004).

Telomere associated proteins (TRF1, TRF2, POT1, hRAP1, Ku, MRE11, RAD50, ERCC1, XPF, HUS, tankyrase) are important primarily for the stability and regulation of telomere length, whereas some participate even in the reparation of

damaged DNA. Multiple additional proteins bind to TRF1 and TRF2 and thus form complexes that regulate the homeostasis of telomeres (Collins and Mitchell, 2002). Shelterin is a significant protein complex that consists of six subunits (TRF1, TRF2, POT1, hRAP1, TIN2 and TPP1) and participates in forming a cap structure at the end of chromosomes (de Lange, 2005). A higher-order telomere nucleoprotein complex facilitates dynamic change between the cap and loose structure. It presents two states that must be well regulated. The tightly packed loop structure is the limiting factor for the elongation of telomeres as it prevents the access of telomerase and other proteins participating in the elongation of telomeres (Figure 1).

Hayflick and Moorhead (1961) were the first who observed the phenomenon of limiting cell division. A mechanism of progressive shortening of telomeres was first theoretically proposed by Olovnikov (1973). Afterward Harley et al. (1990) experimentally proved this phenomenon as an end-replication problem during which the telomeres of somatic cells shorten as a result of incomplete replication of the linear DNA. Chromosomes of normal cells lose approximately 50–100 bp from their 5'end during each cell division (Harley et al., 1990; Ram et al., 2005).

Telomerase complex

Telomerase is a unique ribonucleoprotein enzymatic complex of which the catalytic site is formed by an RNA template component (*hTR* or *hTERC*) and reverse transcriptase (*hTERT*), and associated proteins (dyskerin, tankyrase, Hsp90, L22, p23, hStau) are also present (Aisner et al., 2002; Collins and Mitchell, 2002). The actual telomerase reaction mechanism can be described simply as a three-step action. The short telomere sequence at the 3'matrix end of the chromosome binds to the RNA domain in the first step. Elongation, which is a template-directed addition of nucleotides, occurs in the second step; translocation, which enables repeated use of the same binding site, occurs in the last step. The RNA template has 445 nucleotides with a repeated sequence consisting of 11 nucleotides 5'-(CUAACCCUAAC)-3', which is complementary to the sequence of the telomere (TTAGGG) (Feng et al., 1995). The actual binding domain consists of 5 nucleotides. The 3' terminal DNA telomere has the conformation of a T-loop during different cell phases. This structure loosens when a cell enters the S-phase thus enabling the replication of the 3' terminal (Ray et al., 2002). The plasticity of telomeres together with the activity of associated proteins helps correct the formation and telomerase function.

Studies on murine and human cells have proved that the removal or reduced regulation of the RNA (*hTR*) subunit leads to a loss of telomerase activity, erosion of telomeres and inhibition of cellular growth (Autexier et al., 1996; Niida et al., 1998). Mutation of the *hTR* gene has been described in distinct autosomal dominant disorder dyskeratosis congenita (Vulliamy et al., 2001; Mason, 2003) but also in AA (Vulliamy et al., 2002).

The catalytic subunit of telomerase *hTERT* is a polypeptide consisting of 1,132 amino acids (Nakamura et al., 1997). The ectopic expression of *hTERT* increases the replication life span of human fibroblasts, corneal epithelial cells and endothelial cells (Bodnar et al., 1998; Yang et al., 1999) during unchanged differentiation without alternating their karyotypes or activating known oncogenes (Jiang et al., 1999; MacKenzie et al., 2000). While *hTR* is expressed in relatively identical amount in embryonic and somatic tissues, the expression of *hTERT* is precisely regulated and undetectable in many somatic cells (Kolquist et al., 1998). This means that the expression of *hTERT* is the limiting step in telomerase activation.

The regulation of telomerase is multifactorial. The *hTERT* promoter differs from other gene promoters as it lacks a TATA or CAAT box and consists of a domain rich in GC instead, which represents a binding site for the *SP1* transcription factor. The *hTERT* promoter even provides other binding sites for various transcription factors and hormone responsible elements (Ram et al., 2005). Gene *hTERT* even participates in the regulation of several oncogene expression and the expression of tumour suppressor genes such as *c-MYC* (Wang et al., 1998), *WT1* (Lee and Haber, 2001) and *MZF-2* (Fujimoto et al., 2000).

Some studies demonstrated a key role of the *hTERT* component in the preservation of telomeres and cell proliferation (Harley and Kim, 1996; Hahn and Meyerson, 2001). Cell lines of immortal tumour cells express a high level of telomerase activity, whereas significant minority does not express *hTERT* or telomerase at all (Bryan et al., 1995). In those cells, the secondary mechanism known as the alternative lengthening of telomeres (ALT) is assumed to preserve the length of telomeres (Londono-Vallejo et al., 2004).

ALT mechanism

The ALT mechanism has been described in tumour cells, which present insufficient telomerase activity. It is found in approximately 10% of malignancies, which is much less frequent than the path of telomerase activation. There were even cases described, where the ALT mechanism is represented at a higher rate, for example 25.9% in liposarcomas (Costa et al., 2006).

However, very little is known about the mechanisms underlying ALT in mammalian cells.

Several types of ALT mechanism models were suggested such as the process of homologous recombination, i.e. telomere DNA of homologous chromosomes is used as template for the elongation of telomeres (Dunham et al., 2000). Consequently, a single stranded 3' overhanging terminal intercalates in another telomere region and forms a structure resembling a replication fork. Elongation of the 3'end then occurs as common DNA replication (Henson et al., 2002). It has been suggested that T-loop structure may prime the telomere elongation. In addition, Muntoni at al. (2009) have demonstrated that intra-telomeric duplication of DNA sequences occurs in ALT-positive cells. ALT cell lines and tumours uniquely

contain nuclear structures termed ALT-associated promyelocytic leukaemia bodies (APBs). In addition to telomeric DNA and telomere-specific proteins, APBs contain the recombination proteins RAD51p and RAD52p supporting the notion of recombination as mechanism for telomere maintenance in these cells (Yeager et al., 1999).

The occurrence of the ALT mechanism depends on the type of neoplasia. It should be noted that tumour cells using both mechanisms of telomere elongation have been described as well as cells which do not use any of known or yet unknown mechanism of elongation (Johnson et al., 2005; Costa et al., 2006).

Telomeres and telomerase complex of normal HSCs

The limited mean life span of mature blood cells requires their continuous production, which exceeds 10^{12} cells per day in adult individuals. The replication capacity of early foetal cells is expected to be of the order of 50–200 doublings. Even 50 doublings represents a tremendous proliferative potential, which can, in theory, yield up to 10^{15} cells (Lansdorp, 1995).

Most HSCs and progenitor cells are in a resting phase (G_0). The length of the telomeres in HSCs gradually shortens as a result of proliferation, differentiation and cell aging. It has been proved that stem cells which were obtained by the separation from bone marrow aspirate had shorter telomeres in comparison with cells from foetal tissues or possibly, blood cells from the umbilical vein (Lansdorp et al., 1994).

The loss of repetitive telomere sequences in normal hematopoietic cells is evident during the first year of life (stem cell performs 15–30 cell divisions). The erosion of telomeres is slower until 50 to 60 years of age and then they speed up again after this period (Verfaillie et al., 2002). Although the telomere length is determined by age and is heterogeneous in humans, it seems that it is primarily genetically determined (Slagboom et al., 1994). Very similar lengths of telomeres were detected in granulocyte and lymphocyte cells of monozygotic twins, whereas there were obvious differences in the length of telomeres in dizygotic twins. Different lengths of telomeres were most evident in individuals who were not related (Rufer et al., 1999), and have been proved to correlate with the cell age even in different cells of the same individual, e.g. telomere shortening is much faster in lymphocytes than in granulocytes (Akbar et al., 2004). Edelstein-Keshet et al. (2001) and Shepherd et al. (2004) used the telomere length in granulocytes as a marker of telomere length in HSCs for calculation of their previous replications.

Most circulating HSCs exhibit low telomerase activity. Conversely, lymphocytes in clonal expansion possess substantial telomerase activity (Allsopp et al., 2002). Thymic T-cells exhibit a much higher telomerase level in comparison with the same cells in lymph-nodes or in resting phase of the peripheral lymphocytes (Weng et al., 1996). Whereas telomere loss with ageing corresponded to 33 bp per year in T-cells, telomere shortening was slower in B cells, corresponding to 15 bp/year.

Separation of adult B-lymphocyte subpopulations based on CD27 expression revealed that telomere length was almost 2 kb longer in CD19+CD27+ (memory) compared with CD19+CD27- (native) cells (Martens et al., 2002). The elongation process is probably important for preservation of the long term B-cell immune response. The reduction of telomere length *in vivo* in peripheral lymphocytes is specific for B- and T-cells (Son et al., 2000).

Telomeres and telomerase complex in haematological malignancies

Regulation of the cell cycle and the actual process of apoptosis through the *TP53* gene and pRb/p16^{INK4a} pathways are essential for the preservation of genome stability. Most cancers exhibit a disorder of one or possibly both signal pathways (Sherr, 1996; Sherr and McCormick, 2002). If telomeres are dysfunctional or lose the protective structure of the caps and thus cannot protectively elongate the chromosomal ends, they cause a response to DNA damage via the p53 and pRb/p16^{INK4a} pathways. Those pathways induce either cell cycle arrest or apoptosis. If both control mechanisms of DNA damage are impaired (cells bypass barrier of mortality – M1 senescence), extensive shortening of telomeres with the continuing proliferation results in aberrant fusion of the unprotected chromosomal ends. This stage is identified as the secondary barrier of mortality (M2) or crisis (Wright and Shay, 1992) (Figure 2). This period is characterized by extensive cellular death and chromosomal instability (Ducray et al., 1999). A very low percentage of cells (10^{-9}–10^{-5}) can overcome the critical period in which the mutation and epigenetic changes activate telomere supportive mechanisms (telomerase activation and the ALT mechanism). The result of telomere protection is elongation of the proliferation period up to immortality of the cell.

Investigations dealing with telomere length describe erosion of the telomere region, which correlates with the occurrence and severity of some hematopoietic diseases such as AA (Ball et al., 1998; Brümmendorf et al., 2001), MDS (Terasaki et al., 2002; Sieglová et al., 2004), CML (Brümmendorf et al., 2000) and CLL (chronic lymphatic leukemia) (Březinová et al., 2010).

The hematopoietic cells of patients with AA exhibit shorter telomeres in comparison with age-matched control cells and also show correlation between the loss of telomeres and duration of the disease. Telomere erosion probably provides information on the disease stage. The telomere length in leukocytes in patients with AA, who have been treated by immunosuppressive therapy, does not differ from control leukocytes whereas significantly shortened telomeres have been detected in untreated patients as well as in patients without any response to therapy. In this disease, especially in the chronic phase, increased telomerase activity has also been detected in the population of bone marrow mononuclear cells (Polychronopoulou and Koutroumba, 2004).

In patients with MDS, only slightly increased telomerase activity is observed in bone marrow cells, in contrast to very high level of telomerase activity in primary

acute leukaemia (Ohyashiki et al., 1999). Significantly eroded telomeres have been detected in patients with MDS with an abnormal karyotype. Shortened telomeres have been associated with the disease progression and were correlated with specific chromosomal aberrations (deletion 5q, monosomy 7 and trisomy 8) (Sieglová et al., 2004). The low expression of *hTERT* and increased level of hnRNPB1 (nucleoprotein binding telomeric cDNA) are associated with a poor prognosis and transformation into acute leukaemia.

Approximately 80% of patients in the chronic phase of CML exhibit a reduced telomere length and slightly increased telomerase activity (Bakalova et al., 2004). During CML progression into the acute and blastic phase, increased genome instability occurs in the vast majority of patients resulting in an increase of cytogenetic changes and thus increased telomerase activity and telomere shortening. The correlation between telomere length in the diagnostic period and shortened telomere length in the period of accelerated phase was described (Ohyashiki et al., 1997; Boultwood et al., 2000).

The above mentioned facts suggest that telomere length can be regarded as a prognostic factor in patients with haematological malignancies.

Telomere/telomerase in transplant biology and telomere/anti-telomerase therapy

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment option for a variety of malignant and non-malignant disorders (Copelan, 2006). Hematopoietic reconstitution after allogeneic marrow transplantation relies on a relatively small number of HSCs compared with the estimated stem cell pool in the donor (Nash et al., 1988). It is reasonable to speculate that extreme proliferative demand on limited number of stem cells would result in significant telomere shortening. Furthermore, short telomeres might limit the cells' remaining replicative capacity. This may be of special significance after HSCT. To our knowledge, Notaro et al. (1997) first demonstrated significant telomere shortening in human peripheral blood granulocytes of HSCT recipients as compared with their donors. Shortened telomere lengths have been found in various hematopoietic cell subsets after allogeneic HSCT (Akiyama et al., 2000; Mathioudakis et al., 2000). Telomere shortening in long-term survivors may be determined by the initial telomere loss during the repopulation period as well as by host-related factors such as chronic graft-versus-host disease (GVHD) (Baerlocher et al., 2009). Nevertheless, other studies on telomere loss after HSCT are desirable.

Recently, new discoveries in telomere structure and the functions of telomere associated proteins have led to an increasing interest in targeting telomeres instead of telomerase in anti-cancer therapy. One strategy is to target telomere associated proteins that regulate telomere function, e.g. triggering telomere loss and inducing apoptosis or senescence through inhibition of the telomeric DNA-binding protein

TRF2 (Biroccio et al., 2006). The 3' G-rich overhang can also fold into a 4-stranded DNA structure, termed G-quadruplex, so as to render telomeres inaccessible to telomerase action. Another strategy is to stabilize this G-quadruplex structure (Salvati et al., 2007). These and other approaches for targeting either telomeres or telomerase might also shed some light on their effects on anti-telomerase therapy for hematologic neoplasia.

Possibilities of telomere length determination and telomerase complex detection

The molecular cytogenetic method T/C-FISH (a variation of quantitative FISH) can be used to characterize the dynamics of telomere length in the dividing bone marrow cells of patients with leukaemia and it quantifies telomere length using Peptide Nucleic Acid (PNA) probes for the telomeric and centromeric sequence. Principle of T/C-FISH is measurement of the absolute intensity of fluorescent signals on the p- and q-telomeres of each individual chromosome and of the reference centromeric signal on chromosome 2 (Perner et al., 2003) (Figure 3). The basis for telomere length analysis is the measurement of fluorescence hybridization signal intensities (Cy3-labeled PNA) in 4',6-diamidino-2-phenylindole (DAPI) stained chromosomes. Digital images (twenty metaphases from each individual) are recorded with a CCD camera attached to a fluorescence microscope, and analyzed quantitatively by appropriate software.

Studies evaluating this method of measuring telomere length have been presented recently (Mayer et al., 2006; Göhring et al., 2008). Mayer et al. (2006) measured the length of the telomeres in dividing cells of peripheral blood in 205 blood donors of different ages and sexes. They proved a significant loss of telomeres in relation to age. The median telomere length was shorter in male population as compared to the female population. Further, they noticed the variability of telomere lengths in cells from various donors but also in cells from the same donor.

Göhring et al. (2008) described the mean telomere length and average value of each chromosome arm in 18 patients with secondary AML with a complex aberrant karyotype and they found significantly shorter telomeres than those of the healthy controls (mean length in patient was 7.5 kb, controls were 9.6 kb, $p<0.005$).

Swiggers et al. (2006) used quantitative FISH (Q-FISH) in examining 13 patients with previously untreated AML. The median patient age was 59 (32–75 years) and this group were characterized by having three or more gains and/or losses of parts of chromosomes, terminal deletions, or nonreciprocal translocations. Patients with AML exhibited obvious losses of telomeric DNA or a reduced fluorescent signal in comparison with the control group (without alteration of the karyotype). The median length of telomeres was 16 a.f.u. (arbitrary fluorescence units), which corresponds to a size of 4 kb. Critical shortening of telomeric DNA and thus telomere dysfunction plays an important role in the process of chromosomal instability in patients with haematological malignancies.

The determination of telomerase activity is most often performed using a polymerase chain reaction (PCR) based assay designated for Telomeric Repeat Amplification Protocol (TRAP) method (Kim et al., 1994; Březinová et al., 2010). The sensitivity of this method is high – activity can be assessed in material containing 100–500 ng of total protein. As presented by Kim et al. (1994), an extract quantity representing 10^3 cells gave a reproducibly positive signal in the TRAP assay with a current lower limit of 10^2 cells for detection of telomerase activity at 27 PCR cycles.

Methods of telomere length measuring (such as southern blot, hybridization protection assay, flow cytometry, primed *in situ*, quantitative-PCR and single telomere length analysis) are reviewed in Lin and Yan (2005).

Conclusion

The telomere and telomerase system is critical for the biology of normal HSCs. Natural telomere losses and the alteration of telomerase activity participate in many processes including differentiation of progenitor cells, immune senescence and senescence, ineffective haematopoiesis and malignant transformation.

It is highly probable that the viability of cells and chromosomal stability are affected by critically shortened telomeres rather than by the mean length of all telomeres (Hemann et al., 2001). According to the "telomere checkpoint model", the presence of a minimal number of telomeric repetitions on each chromosome is required. Telomeres play a crucial role in signalling cell cycle progression and subsequently, cell division.

The length of telomeres measured by T/C-FISH method can serve as a genetic damage marker and could be a potential clinical marker of neoplasia progression.

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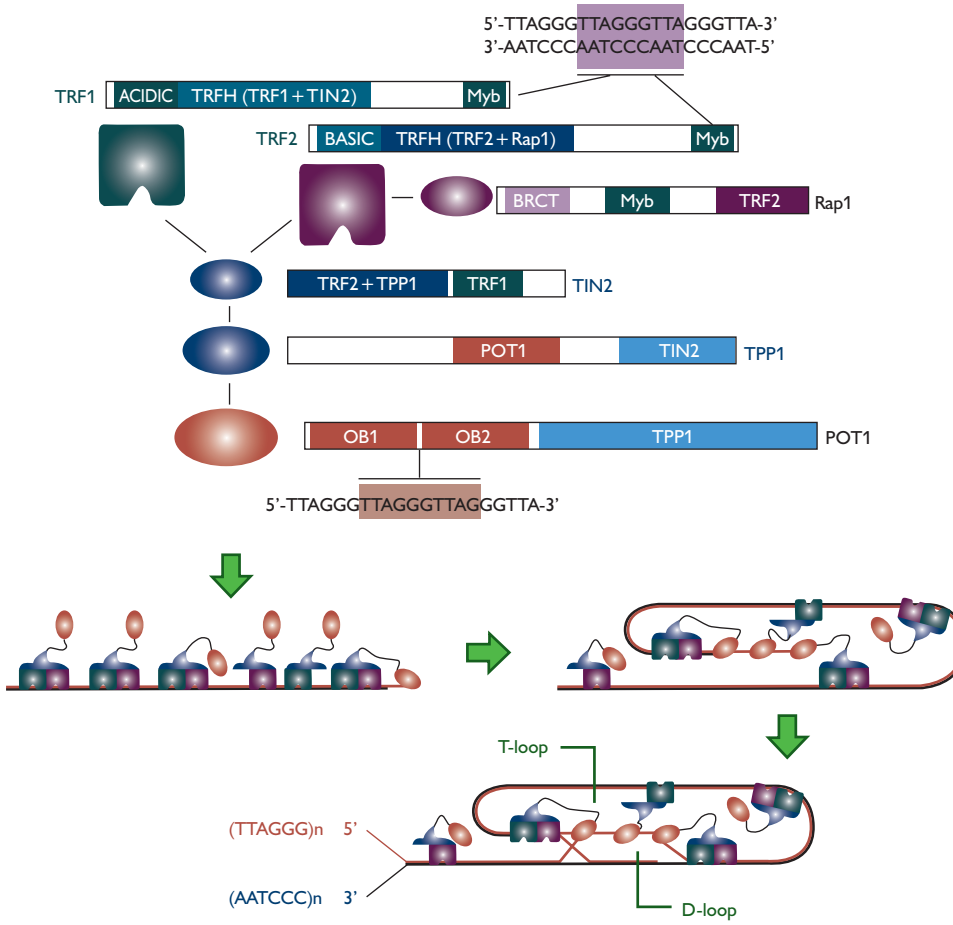

Figure 1 – New view of telomere structure. Duplex structure of telomeres consists of a T-loop and D-loop, associated with telomere-binding proteins. Reproduced and modified from de Lange (2005).

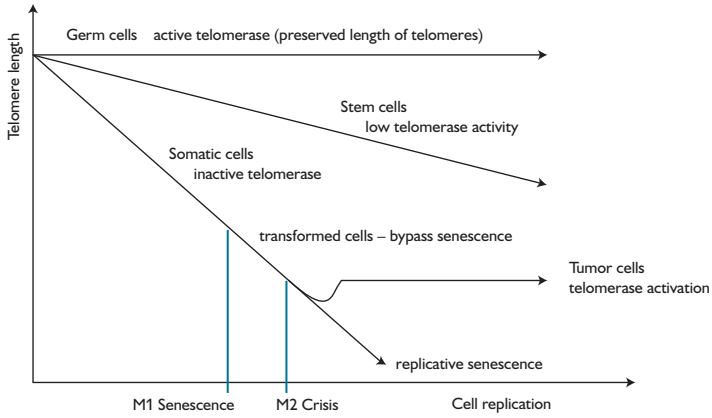

Figure 2 – The telomere-telomerase hypothesis. Normal cells erode their telomeres gradually, until they reach the minimal Hayflick limit (~3 kb), a stage termed barrier of mortality – M1 senescence. An extremely short telomere will be recognized as a double-stranded break with activation of DNA-damage response proteins. In the absence of cell-cycle checkpoint pathways (e.g. p53 and or p16/Rb), cells bypass M1 senescence and telomeres continue to shorten eventually resulting in crisis, also called the barrier of mortality – M2 crisis. This stadium is characterized by many uncapped chromosome ends, end-to-end fusion, mitotic catastrophe and high fraction of apoptotic cells. The telomerase can be reactivated or up-regulated, resulting in indefinite cell proliferation. This immortalization is a potentially rate-limiting step in carcinogenesis.

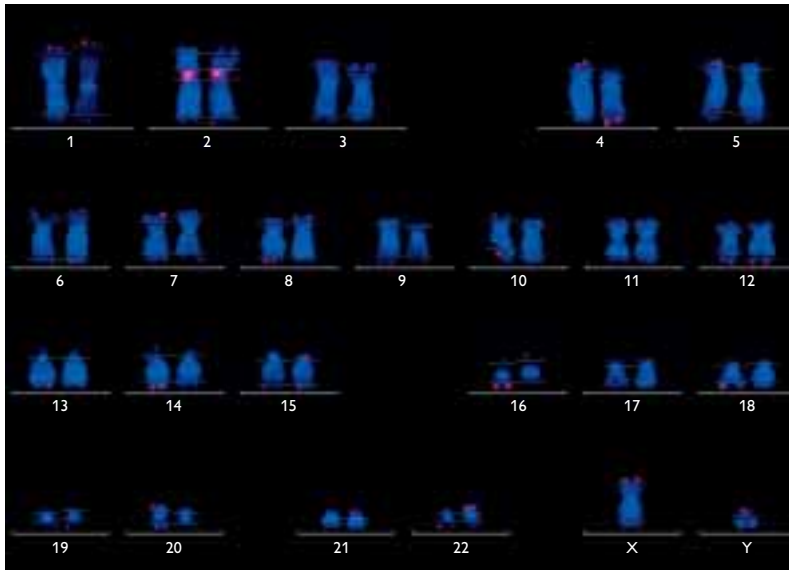

Figure 3 – T/C-FISH image of telomeres – peripheral karyotype of patient (65-year old men) with chronic lymphocytic leukaemia (CLL). Chromosomes in metaphase spreads were hybridized to Cy3-labeled PNA probes for telomeric sequences and the chromosome 2 centromere (serving as internal reference) and stained with 4',6-diamino-2-phenylindole (DAPI). Center of Oncocytogenetics, Institute of Clinical Biochemistry and Laboratory Diagnostics, Prague