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Pharmacogenomic Approach of Telomerase in Cancer: Importance of End Zone Variability

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1. Introduction

Sixty-five years ago, the term *telomere* was introduced by Muller to describe the terminal elements of linear chromosomes (McClintock 1941). In 1985, Greider and Blackburn discovered the telomere synthesizing enzyme telomerase in Tetrahymena (Greider and Blackburn 1985). Three years after, the human telomeric DNA repeat sequence, TTAGGG, was identified (Moyzis, Buckingham et al. 1988). Since then, it has become apparent that several aspects of telomere biology are relevant to human cancer, offering potential opportunities for clinical intervention. In the work by Blackburn, Greider and Szostak, that was awarded the 2009 Nobel Prize in Physiology and Medicine (Zakian 2009), the structure of the chromosome ends was discovered to be specialized nucleoprotein structures that comprise the ''end zone" of chromosomes, called telomeres.

Several recent studies have highlighted the remarkable importance of telomeres in clinical medicine. These advances provide an opportunity to revisit some of the concepts and data that provide a link between telomeres and the diverse pathology that is now linked to telomere dysfunction. Here, we review the telomere and telomerase biology and the pharmacogenomic implications in human cancer, considering the importance of telomerase in tumor development.

2. Telomeres and telomerase biology

Telomeres are terminal protein-DNA complexes forming capping structures that function to stabilize chromosomal ends and prevent them from being recognized by the cell as DNA double strand breaks. Functional telomeres require sufficient numbers of telomeric DNA repeats, as well as the correct assembly of telomere associated protein complexes (Blasco and Hahn 2003).

Telomeres consist of tandem repeats of a DNA sequence (TTAGGG in all vertebrates) bound by a six-protein complex, known as shelterin. Shelterin encompasses the Pot1-TPP1 heterodimer, the telomere-binding proteins TRF1 and TRF2, and the interacting factors Rap1 and Tin2 (de Lange 2005). Telomeric chromatin is also enriched in epigenetic marks that are characteristic of constitutive heterochromatin, such as histone tri-methylation and DNA

hypermethylation, which act as negative regulators of telomere length and telomere recombination (Blasco 2007).

Telomere shortening below a certain threshold length or alterations in the functionality of the telomere-binding proteins can result in loss of telomeric protection, leading to end-to-end chromosome fusions, cell cycle arrest and apoptosis. Telomeres also perform other functions, which include the transcriptional silencing of genes located close to the telomeres (subtelomeric silencing), as well as ensuring correct chromosome segregation during mitosis. Shortening of telomeres is associated with each round of cell division because of the inability of conventional DNA polymerases to replicate the ends of linear chromosomes, the so-called 'end replication problem'. Telomerase is a cellular enzyme capable of compensating this progressive telomere attrition through *de novo* addition of TTAGGG repeats to the chromosome ends (Greider and Blackburn 1985). Telomerase encompasses a catalytic subunit with reverse transcriptase activity (Tert) and an RNA component (Terc) that acts as a template for DNA synthesis (Blasco 2007).

Telomeres also solve the end protection problem. The DNA regions of the end zone are not blunt ended. Instead, they terminate in a long single-stranded run of the G-rich sequence on the 3' strand, called the 3' G-rich overhang. This long single-stranded tail of DNA is postulated to fold back onto itself to invade the duplex portion of telomeres, forming the t-loop. The t-loop effectively sequesters the free ends of chromosomes, protecting them from being sensed as double strand breaks (Griffith, Comeau et al. 1999).

High telomerase expression is a feature of pluripotent stem cells and early stages of embryonic development, although telomerase activity is also present in adult stem cell compartments (Blasco 2005). Telomerase activity in adult tissues, however, is not sufficient to prevent telomere shortening associated with ageing.

The specificity of shelterin for telomeric DNA is through direct recognition of the TTAGGG sequence by three of its components. In particular, TRF1 and TRF2 bind to the double stranded region of telomeric DNA, while Pot1 binds to the TTAGGG repeats of the G-overhang. TRF1 and TRF2 recruit the other four components of shelterin: Tin2 (a TRF1 and TRF2 interacting factor), Rap1, TPP1 and Pot1. These last two proteins form a heterodimer. Shelterin can form a stable complex in the absence of telomeric DNA (de Lange 2005). Mutations in the different components of telomerase (Tert, Terc and Dkc1), as well as in some shelterins (Tin2), have been linked to rare human genetic diseases, such as dyskeratosis congenita, aplastic anaemia and idiopathic pulmonary fibrosis. These diseases are associated with the presence of short/dysfunctional telomeres and they all exhibit a characteristic failure in the regenerative capacity of tissues and severe skin hyperpigmentation (Mitchell, Wood et al. 1999; Armanios, Chen et al. 2007).

Adult stem cells reside at specific compartments within tissues, which are enriched in cells with the longest telomeres. In young or adult organisms with sufficient telomere reserve, adult stem cells efficiently repopulate tissues and repair lesions as needed. In old organisms, however, stem cell telomeres may be too short, and this could impair the mobilization of stem cells and the ability to repair tissues efficiently (Flores, Canela et al. 2008).

When telomeres have shortened down to a critical length, they are recognized as DNA damage, activating a p53-mediated DNA damage signaling response that prevents the mobilization of the stem cells out of their niches. Decreased stem cell mobilization reduces the probability of accumulating abnormal cells in tissues, thus providing a mechanism for cancer protection. However, the ultimate consequence of impaired mobilization of the stem cells will be organ failure owing to tissue degeneration. By using mouse models over-

expressing telomerase, some investigators showed that elevated TERT expression increases stem cell mobilization. Under these conditions of higher mobilization, the fitness of the tissues would be maintained for longer times, therefore increasing the lifespan. The probabilities of initiating a tumor, however, are also higher, especially if telomerase reactivation occurs in a context of mutations in tumor suppressor genes (Serrano and Blasco 2007).

3. Telomere shortening: Consequences

Normally, cells respond to shortened or uncapped telomeres either by entering an irreversible cell cycle arrest, termed replicative senescence, or by undergoing apoptosis. Such responses involve both the Rb and p53 tumor suppressor pathways and likely evolved to prevent replication of mutation-prone cells harboring unstable chromosomes. If these checkpoints fail, chromosomal instability may occur and with it the potential for developing oncogenic mutations. If left unchecked, however, such instability will likely become lethal to the cell, thereby presenting an additional barrier to cell growth. Although some level of genetic instability is thought to be required for tumor initiation and progression, the vast majority of human cancers stabilizes their telomeres either by activating the enzyme telomerase or, in a minority of cases, by an alternative pathway, termed alternative lengthening of telomeres, or ALT, that seems to involve recombination (Reddel 2003).
It would be advantageous in long-lived organisms for cells to be limited in the maximal number of cell divisions permitted, in order to ensure appropriate growth and DNA repair early in life, but not so many divisions that could lead to the early onset of diseases, such as cancer (Wright and Shay 2002). Therefore, selection for enhanced DNA repair and maintenance would be highly desirable during reproductive years, but this could be lost in later life (Kirkwood and Austad 2000). In our modern 'protected environment', humans are living on average twice as long and are dying of heart disease, cancer and neurodegenerative disorders. Thus, normal cells in the context of genotoxic injuries have innate and probably highly conserved defense mechanisms that initiate programs leading to growth arrest or apoptosis, perhaps in part as a mechanism to prevent cancer.

3.1 Short telomeres activate DNA damage signals leading to cell cycle arrest in normal human cells

Normal human somatic cells have a limited lifespan *in vitro*. This was first demonstrated in human fibroblasts by Hayflick and Moorhead in 1961 (Hayflick and Moorhead 1961). Since then, it has been demonstrated that cultured normal human fibroblasts go through finite numbers of population doublings. Toward the end of a cell's lifespan, cell proliferation slows down and finally stops and the cell enters a state of irreversible growth arrest. The timing of growth arrest is determined by the number of population doublings the cells have undergone, not by the calendar time they have stayed in culture. In addition to fibroblasts, other somatic cells show replicative senescence, including epithelial cells, endothelial cells, lymphocytes, smooth muscle cells, and astrocytes, which serves as an intrinsic mechanism to prevent normal somatic cells from replicating indefinitely (Bierman 1978; Evans, Wyllie et al. 2003). Senescent human cells have increased p53 activity with the involvement of several DNA double strand break repair and checkpoint factors, such as ATM (Atadja, Wong et al. 1995). These multiple DNA damage response factors are assembled at the short telomeres in senescent cells, indicating that dysfunctional short telomeres trigger the response (d'Adda di

Fagagna, Reaper et al. 2003; Zou, Sfeir et al. 2004). The sustained DNA damage response, signaling through p53, can induce both G1 and G2 phase arrest. Numerous studies have also demonstrated that human fibroblasts or epithelial cells undergoing natural replicative senescence have elevated protein levels of hypophosphorylated Rb, p16INK4a, as well as p21CIP1, or decreased hyper-phosphorylated Rb, compared with early and proliferating cells (Atadja, Wong et al. 1995; Beausejour, Krtolica et al. 2003). Telomere-mediated senescence is therefore induced by activation of the multiple DNA damage responses, which then leads to cell cycle arrest in normal human cells.

Several lines of evidence have shown that replicative senescence also occurs *in vivo* and is thought to be associated with cellular aging. An inverse correlation between donor age and the number of population doublings at which human cells senesce has been demonstrated (Martin, Sprague et al. 1970). Moreover, comparisons between different species showed that cells from organisms with longer lifespan *in vivo* achieve more population doublings *in vitro (Rohme 1981)*. Furthermore, cells from humans with premature aging syndromes have shorter lifespan in culture than those from age-matched controls (Smith and Pereira-Smith 1996). These data suggest that there is a genetic basis that controls the cells' replicative lifespan. However, although telomere-associated senescence is apparently an intrinsic barrier to cellular immortalization, which represents an early first step in the multi-stage process of cancer development, accumulation of senescent cells during aging *in vivo* seems to create a microenvironment that predisposes to cancer (Campisi 2005). Telomere shortening or dysfunction, therefore, presents a close association with cancer.

3.2 Telomere dysfunction leads to genomic instability in checkpoint defective cells

Broken chromosome ends tend to fuse with their sister chromatids or other broken chromosomes, leading to formation of anaphase bridges, cycles of chromosome breaks and further fusions during subsequent cell divisions (McClintock 1941). Telomere dysfunction initiates chromosomal instability through such breakage-fusion bridge (BFB) cycles. The p53 and Rb/p16INK4a pathways are crucial for DNA damage responses, cell cycle regulation, as well as apoptosis, which are indispensable for maintaining genomic stability. Most cancers have alterations in either or both of these pathways. Inactivation of the p53 and p16INK4a/Rb pathways can be accomplished *in vitro* by expression of viral oncogenes, such as HPV (human papillomavirus) E6 and E7 or adenovirus E1B and E1A (Moran 1993; Moran 1993) to stimulate the sustained inactivation of p53 and Rb genes, observed in cancers. Such alterations extend the lifespan and allow the cells to override cell cycle checkpoints and continue to proliferate. However, as telomeres continue to shorten with further cell divisions, the chromosome ends are no longer protected and become unstable. At this stage, the cells enter a mortality barrier, termed crisis (Wright and Shay 1992), which is characterized by wide-spread cell death and extensive chromosomal instability (Ducray, Pommier et al. 1999). However, a low percentage of cells may survive the crisis period by a mutation or an epigenetic event that activates a telomere maintenance mechanism. This may involve activation of telomerase, which elongates telomeres by synthesizing telomeric DNA and maintaining pre-existing telomeres, or an alternative telomere lengthening (ALT) mechanism, which involves copying of DNA sequences from telomere to telomere by means of homologous recombination. The resulting telomere preservation allows the cells to continue their long-term proliferation to become immortalized (Figure 1) (Dunham, Neumann et al. 2000).

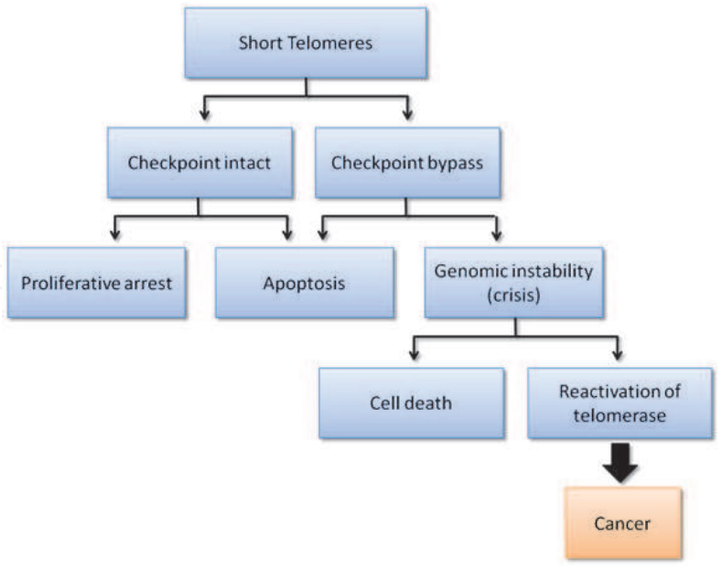

Fig. 1. Telomeres shorten with cell proliferation when not balanced by telomere synthesis. In healthy somatic cells, critically short telomeres activate a checkpoint that induces either apoptosis or the proliferative arrest of replicative senescence. In the absence of checkpoint function, telomeres may become substrates for aberrant DNA repair. Occasionally, spontaneous activation of telomerase during the crisis phase of genomic instability stabilizes and allows maintenance of the rearranged genome, conferring indefinite renewal capacity.

Critically short telomeres are hotspots for erroneous recombination. Whereas very few chromosome end-to-end fusions are observed in senescent normal human cells (Benn 1976; Zou, Sfeir et al. 2004), the frequencies of chromosome end-to-end fusions, reach a peak during crisis and decrease after this period due to telomere preservation (Ducray, Pommier et al. 1999). This is because the two centromeres are pulled in opposite directions during mitotic anaphase, forming a bridge between the daughter cells. The bridge can cause cell death independently of p53 and Rb genes, partly because the bridge compromises the integrity of cellular and nuclear membranes. The broken ends trigger DNA recombination by rejoining to other broken or unprotected chromosome ends. This can produce additional aberrations, such as structurally stable translocations and unstable dicentrics, which can undergo another round of BFB. Repeated cycles of sister-chromatid fusion-bridge breakage may, therefore, generate multiple copies of the same gene on the same chromosome in the same cell, while causing the loss of the genes in its sister cells after several rounds of cell divisions (Gisselsson, Jonson et al. 2001). In summary, regardless of how telomeres become disrupted, when telomeres become dysfunctional and can no longer protect the chromosome ends, they elicit a DNA damage response to activate the p53 and p16INK4a/Rb pathways, which prompt the cell to undergo irreversible cell cycle arrest (if both p53 and pRb checkpoints are intact) or p53-mediated apoptosis (if only the p53 checkpoint is intact) (d'Adda di Fagagna, Reaper et al. 2003). In cells defective in both p53 and p16INK4a/Rb pathways, excessive shortening of telomeres, coupled with the freedom to continue proliferation, promote aberrant fusions of unprotected chromosome ends and trigger massive chromosomal instability, increasing the risk of malignant transformation (Feldser, Hackett et al. 2003). Thus, telomere shortening can be both a barrier and a facilitator for cancer development, depending on the integrity of checkpoint response.

4. Role of telomere-mediated genomic instability in carcinogenesis

Carcinogenesis is a multistep process, characterized by a stepwise accumulation of genetic and molecular abnormalities. These events generally follow exposure to carcinogens and result in the selection of clonal cells with uncontrolled growth capacities (Hanahan D 2000). Thus, cancer develops through a series of stepwise events, from preinvasive histological changes to invasive disease. The earliest events are mutations, deletions, or polysomy at the genomic level, but these do not systematically lead to changes in cell morphology or tissue structure. Current knowledge indicates that cancer progression from premalignancy to malignancy is slow and multiple mutations are required to reach a full metastatic potential (Schedin and Elias 2004).

Tumor cells acquire the so-called hallmarks of cancer during this carcinogenic selection process (Hanahan D 2000). Cell immortality is one of the most prevalent of these major acquired features. Immortality involves the stabilization of telomere lengths, which is achieved by telomerase activation in about 80% of cases of human tumors. However, the specific changes in telomere length, telomeric proteins and telomerase expression that occur during the multistep carcinogenic process remain undetermined and thus represent a field of active research.

One of the core concepts in cancer research is that genomic instability helps drive development of human cancer (Maser and DePinho 2002). Rapid evolution of genomic alterations in genetically unstable cells makes them advantageous in natural selection by acquiring new features. Of the two categories of genomic instability, microsatellite instability exists only in a small subset of solid tumors, whereas chromosomal instability is present in most cancers. Chromosomal instability includes numerical instability reflected by alterations in chromosome numbers, and structural instability, which is characterized by continuous generation of new structural chromosome aberrations. Regarding the role of chromosome aberrations in cancer development, it is well recognized that chromosome aberrations have oncogenic potential. First, chromosome translocations can result in the formation of fusion genes or deregulation of gene transcription at or near the translocation points, as demonstrated in most leukemia and many soft-tissue tumors. Second, gains or losses of chromosome elements or whole chromosomes can lead to large scale genomic imbalances or alterations in gene dosage in human cancers (Albertson, Collins et al. 2003). These abnormalities persist along with continued acquisition of additional abnormalities with tumor progression towards late stage malignancies. It is known that chromosomal instability is an important mechanism leading to genomic rearrangement and imbalances that provide a platform for continuous selection of aberrant cells for cellular immortalization and cancer development. The mouse model with the depletion of telomerase RNA component (mTerc) shows increased incidence of spontaneous malignancies in late generation animals (Rudolph, Chang et al. 1999; Maser and DePinho 2002).

Extensive studies have shown that telomeres in normal human somatic cells shorten with cell divisions *in vitro* as well as *in vivo*. In cultured human fibroblasts, this progresses at a rate of 50-200 bp per population doubling (Harley, Futcher et al. 1990; Allsopp, Vaziri et al. 1992). *In vivo* studies have also shown that the average telomere lengths in normal somatic cells shorten at an estimated rate of 15-40 bp per year and that telomere erosion declines with age (Hastie, Dempster et al. 1990). One major mechanism leading to telomere shortening is associated with the end-replication problem intrinsic to linear chromosomes.

To date, numerous experiments have demonstrated that telomeric DNA is indeed progressively lost with cell divisions in most human somatic cells that lack telomerase

activity. In addition, some studies also suggest that oxidative stress contributes to telomere shortening. Under conditions of additional oxidative stress induced by hydrogen peroxide treatment, human cells show preferential accumulation of single-strand breaks within the telomeres. The effects of oxidative stress on telomere erosion has important significance in the study of telomere dynamics in aging and cancer, since numerous oxidants are produced *in vivo* due to normal metabolism and extracellular stresses and oxidative stress increases with the aging process *in vivo* (Saretzki and Von Zglinicki 2002; von Zglinicki 2002).

Proof that telomeres shortening and cellular aging are causally related was demonstrated (Bodnar, Ouellette et al. 1998). The introduction of telomerase into normal telomerase silent cells was sufficient to bypass senescence, activate telomerase activity and lead to cell immortalization. It was further shown that ectopic expression of telomerase (TERT) in pre-senescent cells or in cells between senescence and crisis could be immortalized with ectopic introduction of TERT, demonstrating that telomeres are mechanistically important in both senescence and crisis. In the absence of intact critical checkpoint pathways, genomic instability occurs when telomeres are short, leading to end-to-end fusions, anaphase bridges, the development of aneuploidy and eventually to telomerase reactivation. One possibility is that the re-expression or upregulation of telomerase in cancer reduces the ongoing chromosomal instability that occurs in cells in crisis to a level compatible with both viability and sufficient instability to generate mutational evolution of the malignancy. In summary, telomere shortening may be a common underlying cause of chromosomal rearrangements in cancer (Bodnar, Ouellette et al. 1998). In a recent study, DePinho and colleagues determined whether entrenched multi-system degeneration in adult mice with severe telomere dysfunction can be halted or possibly reversed by reactivation of endogenous telomerase activity. These authors engineered a knock-in allele encoding a 4-hydroxytamoxifen (4-OHT)-inducible telomerase reverse transcriptase-oestrogen receptor (TERT-ER) under transcriptional control of the endogenous TERT promoter. Homozygous TERT-ER mice presented short dysfunctional telomeres and sustained increased DNA damage signalling and classical degenerative phenotypes upon successive generational matings and advancing age. Telomerase reactivation in such late generation TERT-ER mice extended telomeres, reduced DNA damage signalling and associated cellular checkpoint responses, allowing resumption of proliferation in quiescent cultures and eliminating degenerative phenotypes across multiple organs including testes, spleens and intestines. The authors demonstrated that telomere damage acts as a driver of age-associated organ decline and disease risk and that the marked reversal of systemic degenerative phenotypes in adult mice supports the development of regenerative strategies designed to restore telomere integrity (Jaskelioff, Muller et al. 2011).

Since cancer cells have to bypass senescence and crisis, it is consistent to assume that the cancer cell that first became immortal by upregulating telomerase would have had short telomeres (Figure 2). For example, in most cases of preneoplasia, it has been shown that cells have very short telomeres. In prostate cancer for example, telomere shortening is detected in low grade prostatic intraepithelial neoplasia (PIN) lesions (Koeneman, Pan et al. 1998; Meeker, Hicks et al. 2002). This indicates that the tumor initiating cells are likely to originate from a subset of transient amplifying cells which may have critically shortened telomeres.

Telomere length abnormalities are nearly universal in preinvasive stages of human epithelial carcinogenesis. Indeed, telomere shortening occurs in most cases of early stage bladder, cervix, colon, oesophagal and oral cavity cancer. Similar results were found for prostate cancer; the majority of high-grade PIN lesions (Prostatic intraepithelial neoplasia)

examined had much shorter telomeres than adjacent, apparently normal epithelial cells. This was also observed in fully invasive prostate carcinomas (Meeker, Hicks et al. 2002; Meeker, Hicks et al. 2004). Markedly or moderately shortened telomeres have also been found in lesions of ductal carcinoma *in situ* (Meeker and Argani 2004). Unlike normal breast epithelium, a moderate telomere shortening was observed in benign secretory cells in about half of histologically normal terminal duct lobular units. These findings clearly support a role for telomere shortening in pre-invasive stages, as well as in invasive cancer. Overall, these observations suggest that telomere length abnormality is one of the earliest and most frequently acquired genetic alterations involved in the multistep process of malignant transformation.

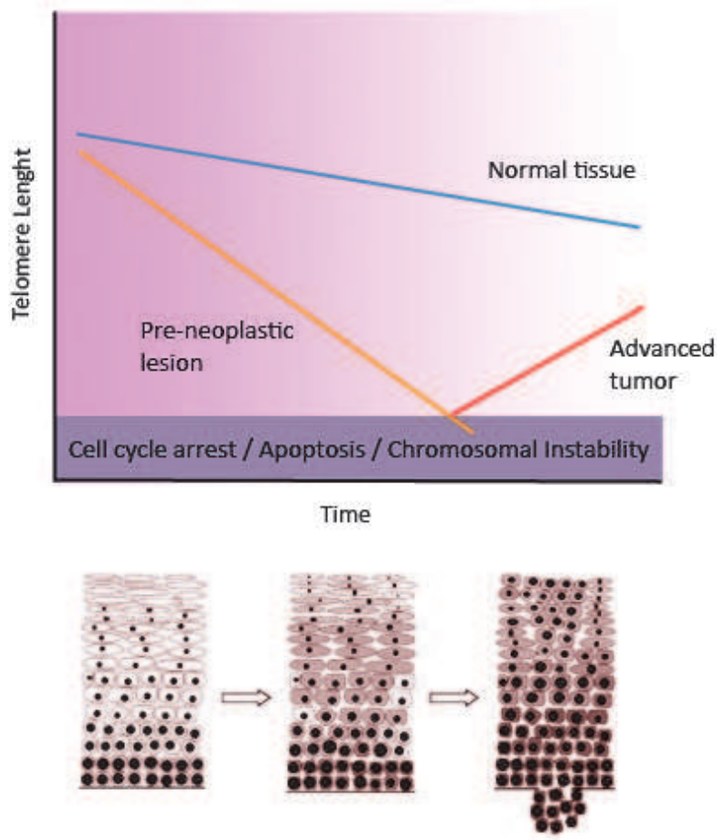

Fig. 2. Schematic representation of telomere length associated with multistep carcinogenesis.

As mentioned above, telomere shortening appears to be a signal for replication arrest in many cells. However, in some cells, rather than inducing cell cycle arrest, the substantial shortening of telomeres results in cycles of end-to-end fusions and BFB (Chin, de Solorzano et al. 2004; Lantuejoul, Soria et al. 2005). In most cells, damage-surveillance mechanisms detect genomic instability and induce cell death. Occasionally, however, one or a few cells escape this protective mechanism, leading to accumulation of multiple genomic and epigenomic aberrations and thus acquisition of additional cancer hallmark features (Chin, de Solorzano et al. 2004).

5. Heterogenity in telomere lengths - impact on cancer development and prognosis

It is known that telomere length varies considerably among individuals. To date, the major conclusions on human telomere lengths include that there are large differences in telomere lengths between the arms of the same chromosomes, as well as between the same arms of homologous chromosomes in the cells of the same individual; different individuals have different profiles of telomere length heterogeneity, which seem to be largely inherited; and different tissues of the same individual have similar telomere length heterogeneity (Lansdorp, Verwoerd et al. 1996; Graakjaer, Bischoff et al. 2003; Nordfjall, Larefalk et al. 2005).

There is growing evidence that TL (Telomere Length) carries information that may be of clinical importance for cancer patients. It is well established that TL is important for senescence in normal cells. Malignant cells in general have shorter telomeres than their normal counterparts and there seems to be a connection between telomere shortening, genetic aberrations and risk of transformation (Hackett and Greider 2002). The interest of investigating TL as a potential clinical biomarker in cancer has grown considerably in recent years (Ohyashiki, Sashida et al. 2002; Bisoffi, Heaphy et al. 2006).

The vast majority of studies in the field have been performed on tumor samples, but there are also investigations on blood cell TL and its possible relation to cancer risk and prognosis. When tumor DNA is studied, TL reflects the cumulative result of a variety of tumor-associated factors with impact on telomere homeostasis. TL in peripheral blood is often used as a surrogate for TL in other healthy tissues. The fact that TL in peripheral blood may carry clinical information for cancer patients has been indicated by a growing number of studies. One question to be solved, however, is whether altered blood TL can contribute to disease development, or whether the TL alterations arise as a consequence of the disease. The latter case would suggest that an altered blood TL is the result of unknown mechanisms associated with the presence of a malignant tumor in the body. Previous data indicate that blood TL may serve both as a marker for cancer risk and as an independent prognostic marker for survival. Thus, both tumor TL and TL in peripheral blood may carry information with important clinical implications.

In hematological malignancies, there are convincing data suggesting that short telomeres in malignant hematopoietic cell populations indicate progressive disease and poor survival [reviewed in (Ohyashiki, Sashida et al. 2002; Bisoffi, Heaphy et al. 2006)].

A number of studies have reported that TL in solid tumors has potential to be used as a prognostic indicator. Several different tumor types have been investigated regarding tumor TL and clinical outcome. The majority of studies have found associations between altered tumor TL, i.e. attrition or elongation, and a poor outcome. Several studies have reported associations between reduced telomere content and poorer survival in both breast and prostate carcinomas. Similarly, in sarcoma, short TL was linked to genomic instability and poor survival (Bisoffi, Heaphy et al. 2006). A number of studies indicated that telomere alterations are associated with parameters of clinical outcome in patients with lung cancer (Shirotani, Hiyama et al. 1994; Hirashima, Komiya et al. 2000; Frias, Garcia-Aranda et al. 2008). A study (Frias, Garcia-Aranda et al. 2008) indicated a significant poor clinical outcome in lung patients presenting telomere shortening, a finding that emerged as an independent prognostic marker in multivariate analysis. In other tumor types, such as prostate cancer, it has been suggested that reduced telomere content is associated with poor

clinical outcome and markers of disease progression. Thus, reduced telomere content values conferred a relative hazard of 5.02 compared with tumors with greater telomere content (Fordyce, Heaphy et al. 2005). Also, studies in breast cancer have shown that telomere attrition is associated with parameters of increased risk and poor outcome, with low telomere content conferring an adjusted relative hazard of 4.43 (Bisoffi, Heaphy et al. 2006; Fordyce, Heaphy et al. 2006).

In contrast, long telomeres, or a high tumor to non-tumor TL ratio, have been coupled to tumors of more advanced stages and a worse prognosis in colorectal carcinoma, Barrett carcinoma and head and neck tumors (Patel, Parekh et al. 2002; Gertler, Rosenberg et al. 2004; Garcia-Aranda, de Juan et al. 2006; Gertler, Doll et al. 2008). Although not entirely consistent, most studies have indicated that tumor TL alterations are associated with a worse clinical outcome. The type of alteration linked to a poorer survival (short vs. long TL) might depend on the tumor type. At the present time, the underlying mechanisms remain unclear. As for short TL and its association to a poorer outcome, it is logical to consider the relation between short TL and genomic instability.

5.1 Blood telomere length as a potential predictor for cancer risk and prognosis

There is a need for reliable, easily measurable biological markers for risk and prognosis assessment of malignancies. In recent years, a growing number of studies have focused on analyzing telomeres in peripheral blood cells in relation to cancer risk. Since blood is an easily accessible biological sample, blood TL is an interesting candidate as a biological marker. However, previous reports from this research area are not unambiguous. In renal, lung, bladder, head and neck and oesophagus carcinomas, short TL in peripheral blood have been associated with increased cancer risk (Wu, Amos et al. 2003; McGrath, Wong et al. 2007; Risques, Vaughan et al. 2007; Jang, Choi et al. 2008). Similar data have been reported for malignant lymphoma (Widmann, Herrmann et al. 2007). Regarding breast cancer, a study in sister sets found a non-significant association between short TL and increased cancer risk (Shen, Terry et al. 2007). In contrast, in newly diagnosed women with spontaneous breast tumors, cancer risk increased with increasing TL (Svenson, Nordfjall et al. 2008). Hence, the majority of studies have found blood TL alterations in cancer patients when compared to healthy controls. It might be that the type of TL alteration differs depending on the tumor type. Differences in the methodology of measuring TL might also contribute to the discrepancy. In addition, the type of blood cells that are analyzed can vary between different studies.

The biological mechanisms behind the reported association between blood TL alterations and increased cancer risk are not clear. It is not evident whether the TL alterations observed in peripheral blood reflect a secondary event caused by the cancer disease, or if the alterations are in fact part of the cancer etiology, or both. Oxidative stress has been associated with tissue aging, as well as telomere shortening, and it has been proposed that the cumulative burden of oxidative stress through life is registered in the telomeres of leukocytes (von Zglinicki 2002; Balaban, Nemoto et al. 2005). Accordingly, it has been speculated that blood TL may act as a surrogate marker of tissue dysfunctions. In 2003, Wu et al. measured TL in peripheral lymphocytes and found an association between short TL and increased risk of bladder, head and neck, lung and renal cell cancers (Wu, Amos et al. 2003). The authors proposed telomere dysfunction as a potential predisposition factor for cancer development. It cannot be excluded that the disease itself, or responses to the disease, can originate a systemic effect which directly or indirectly affects the telomere length of

peripheral blood cells. The immune response to the tumor might need to be considered. Some inflammatory molecules, such as cytokines and reactive oxygen species, may have an impact on leukocyte TL. A number of cytokines have shown potential to activate telomerase, whereas oxidative stress, as mentioned above, might increase telomere attrition (Akiyama, Hideshima et al. 2002; von Zglinicki 2002). The proliferation rate of immune cells, and hence the telomere attrition rate, may also differ depending of the state of the immune system, which in turn might be altered due to the presence of a tumor.

It was previously reported that the inter-individual TL variation was reduced in the healthy aged individuals (≥85 years) (Halaschek-Wiener, Vulto et al. 2008). The authors had hypothesized that healthy old individuals without age-related diseases, such as cancer or cardiovascular disease, would have unusually long telomeres. The study, however, showed that telomere length in the "normal range" may be most protective against tumor development, since long telomeres may favor escape from senescence, whereas short TL may cause genomic instability.

Studies investigating blood telomere length as a prognostic indicator in malignancies are sparse (Svenson, Ljungberg et al. 2009; Svenson and Roos 2009; Willeit, Willeit et al. 2010).

A recent prospective, population-based study estimated the impact of peripheral blood leukocyte telomere length on overall cancer manifestation and mortality (Willeit, Willeit et al. 2010). This study demonstrated significant inverse correlations between baseline leukocyte telomere length and both cancer incidence and mortality, which emerged as independent of standard cancer risk factors. The authors found evidence of heterogeneous effects according to cancer type, whereby tumors with a high fatality rate tended to exhibit more prominent relationships with telomere length and tumors with a more favorable prognosis showed modest or no associations. These results corroborate with previous evaluations demonstrating a link between short telomere length and lung cancer, bladder cancer, renal cell carcinoma, non-Hodgkin lymphoma and head and neck tumors (Wu, Amos et al. 2003; Broberg, Bjork et al. 2005; McGrath, Wong et al. 2007; Shao, Wood et al. 2007; Jang, Choi et al. 2008; Lan, Cawthon et al. 2009), but failed to obtain significant correlations between short telomere length and colorectal and breast cancer (De Vivo, Prescott et al. 2009; Shen, Gammon et al. 2009; Zee, Castonguay et al. 2009). A variety of experimental and genetic studies support the hypothesis that telomere attrition contributes to the manifestation and dissemination of malignancies. While fully functional telomeres confer protection of the genome, shortened telomeres facilitate chromosomal instability (Calado and Young 2009).

In fibroblast cultures, accruing senescent cells were shown to produce and release high amounts of growth factors, causing an overwhelming proliferation of the surrounding tissue and to secrete metalloproteinase, diminishing intercellular adhesions and potentially favoring metastatic spread of tumors (Krtolica, Parrinello et al. 2001; Liu and Hornsby 2007). Moreover, a significantly higher production of vascular endothelial growth factor in senescent cells may stimulate tumor growth and dissemination by promoting neovascularization (Coppe, Kauser et al. 2006). The association between short leukocyte telomere length and cancer formation may be partially mediated by the aging of the immune system itself. Aging of leukocytes reflected by short telomere length may impair immune surveillance and reduce the clearance of tumor cells.

A study demonstrated that patients with multiple myeloma with high telomerase activity and short telomere length presented a poor prognosis (Wu, Orme et al. 2003). Accordingly, another study reports the same results in adult T-cell leukemia, suggesting that telomerase

activity and low telomere content may be novel markers for the prognosis of these diseases (Kubuki, Suzuki et al. 2005).

5.2 Telomerase genetic variants

In recent years, a growing number of studies have focused on genetic polymorphisms influencing cancer risk and prognosis (Araujo, Ribeiro et al. 2007; Nogal, Coelho et al. 2007; Teixeira, Ribeiro et al. 2009; Nogueira, Catarino et al. 2010).

Although telomere shortening is inversely associated with age, telomere length has been found to vary considerably in human peripheral blood lymphocytes from individuals of the same age (Graakjaer, Pascoe et al. 2004; Londono-Vallejo 2004).

Rafnar et al (Rafnar, Sulem et al. 2009) demonstrated that polymorphisms in the gene encoding *TERT*, the catalytic subunit of telomerase, were associated with an increased risk for cancer of the lung, urinary bladder, prostate and cervix.

Matsubara and colleagues (Matsubara, Murata et al. 2006) screened the promoter region of *hTERT* for functional polymorphisms and a frequent T to C transition was found 1327 bp upstream the transcription starting site (-1327T/C). Individuals homozygous for the -1327C/C genotype showed lower telomerase activity and shorter telomere length in their peripheral leukocytes compared to the -1327T/T and -1327T/C genotypes.

Matsubara and co-workers (Matsubara, Murata et al. 2006; Matsubara, Murata et al. 2006) demonstrated that the -1327T/C polymorphism within the *hTERT* promoter region has functional roles: the -1327T sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. The relationship of the -1327T/C polymorphism to telomere shortening, telomere length, and telomerase activity was found in normal peripheral leukocytes. Transcriptional regulation of hTERT has a key role in telomerase activity and telomere shortening. Approximately 25% higher promoter activity in the -1327T-sequence was found compared the -1327C-sequence and the T allele was strongly associated with longer telomere length. Thus, *hTERT* T allele with higher hTERT transcriptional activity is associated with more effective extension of the telomeric end during cell division. Another study found an overrepresentation of the -1327C/C genotype in patients with coronary artery disease compared to controls, presenting shorter telomeres compared to other patients with alternative genotypes, indicating that a subgroup of patients is more prone to telomere shortening (Matsubara, Murata et al. 2006).

A previous study performed by our group indicates an influence of the telomerase genetic variants in overall survival of NSCLC patients. Multivariate Cox regression analysis indicated an increased overall survival for T carrier patients, when compared with CC genotype, after adjustment for tumor histological type, stage, smoking status, age and gender (hazard ratio, HR=0.52). The median estimated cumulative survival was significantly higher in T carrier patients, of 26.5 months, comparing with CC patients, of 19.3 months (Catarino, Araujo et al. 2010).

Tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells capable of invasion, extravasation and metastasis, i.e., an aggressive tumor phenotype. It has been hypothesized that altered telomere length could predispose cells to gain the necessary properties to metastasize and cause recurrent disease, and thereby be a predictor of clinical outcome (Bisoffi, Heaphy et al. 2006; Willeit, Willeit et al. 2010).

6. Concluding remarks

Cancer remains an important public health problem in developed countries. Therapeutic failure and side effects of anticancer therapy are essential issues requiring future research. One of the main aims of clinical or translational research in cancer is the search for genetic factors that could foresee treatment outcomes, in biologic activity and toxic effects. Therefore, the study of tumor and patient genetic profiles, relative to drug-related genes, may offer new opportunities for tailoring treatments.

The assessment of telomere length and telomerase genetic variants could supplement prognosis of survival in the course of cancer and may be a promising molecular marker of treatment response in cancer patients. Furthermore, a better knowledge of the underlying molecular profile of the host and the tumor could facilitate screening for cancer susceptibility and tailoring of chemotherapy in individual patients, choosing those most likely to respond, adjusting doses more precisely in order to reduce adverse effects, and establishing safety profiles based on individual genetic analyses.

Most cancer cells have strong telomerase activity to maintain telomere lengths for long-term cell proliferation. It has been proposed that cancer cells can be killed by inducing critical shortening of telomeres, and hence senescence or apoptosis, through inhibition of telomerase.

In most cancers (80%) unlimited replicative potential is achieved through telomerase reactivation, allowing telomere lengthening. This major feature of cancer cell biology is observed from the earliest stages in most tissues. Early telomerase activation gives a clear advantage to established tumor cells, but also contributes to cancer development. Moreover, the role of telomeres in carcinogenesis could partly underlie individual, tissue-specific predisposition. Indeed, telomere length varies between individuals in the population; some studies suggest that hereditary factors are involved in determining telomere length. Thus, individual telomere length heterogeneity and telomere-driven instability may be central events in early carcinogenesis.

Overall, these observations highlight the need for improved understanding of telomere-driven senescence and of the mechanisms involved in the failure of this process to protect against cancer progression in the early stages of disease onset. In particular, improved understanding of the role and mechanism of action of telomerase in preneoplasia and established tumors is essential for current and future developments of accurate and individually adapted anti-telomerase anti-cancer therapy. Thus, improvement of the methods used to measure telomerase expression and activity will be required. Finally, a combination of techniques – each one adapted for studying telomerase, telomere associated proteins, DNA damage proteins and telomere length and structure – will be necessary for use in future studies, to fully elucidate the molecular mechanisms underpinning telomere-related biology.

A possible prognostic impact of TL abnormalities seems to be tumor type dependent and more data on well characterized tumor materials are required. It is evident that the appearance of a malignancy affects the whole body, and it is possible that the alterations in blood TL seen at diagnosis reflect responses to the tumor. Of most interest is perhaps the fact that blood TL appears to constitute a significant prognostic indicator, but additional studies on different patient cohorts are needed in order to further substantiate this indication. Large longitudinal studies and improved standardized protocols are needed for the future, to fully be able to evaluate TL as a marker in malignancies.

New discoveries pertaining to telomere structure and telomere homeostasis that shed light on the complex relationship between telomeres, telomerase, chromosomal instability and cancer will undoubtedly have an important impact in cancer therapeutics.

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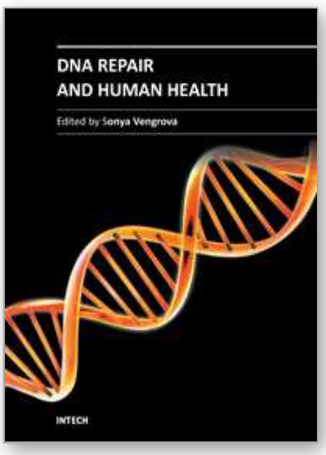

DNA Repair and Human Health

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Over the past decades, great advances have been made in understanding the cellular DNA repair pathways. At the same time, a wealth of descriptive knowledge of human diseases has been accumulated. Now, the basic research of the mechanisms of DNA repair is merging with clinical research, placing the action of the DNA repair pathways in the context of the whole organism. Such integrative approach enables understanding of the disease mechanisms and is invaluable in improving diagnostics and prevention, as well as designing better therapies. This book highlights the central role of DNA repair in human health and well-being. The reviews presented here, contain detailed descriptions of DNA repair pathways, as well as analysis of a large body of evidence addressing links between DNA damage repair and human health. They will be of interest to a broad audience, from molecular biologists working on DNA repair in any model system, to medical researchers.

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