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Abstract

The key step of carcinogenesis is the malignant transformation which is fundamentally a telomere biology dysfunction permitting cells to bypass the Hayflick limit and to divide indefinitely and uncontrollably. Thus all partners and structures involved in normal and abnormal telomere maintenance, protection and lengthening can be considered as potential anti-cancer therapeutic targets. In this Point of View, we discuss, highlight and provide new perspectives from the current knowledge and understanding to position the different aspects of telomere biology and dysfunction as diagnostic, preventive and curative tools in the field of cancer.

Telomere function: Protecting chromosomes ends and controlling cell proliferation

Large genomes are difficult to regulate when circular. Evolutionarily, linear chromosomes may have arisen to more easily manage large genomes [1] . Two challenges arise with the linearization of chromosomes compared to circular ones: chromosomes ends resemble double-strand DNA breaks and the asymmetric characteristic of DNA replication of the lagging and leading strands induces a shortening of chromosome ends (telomeres) at each DNA replication. The amount of DNA lost corresponds to the length of the RNA substrate used to prime synthesis of the Okazaki fragments plus the distance from the position of RNA primer alignment to the end of the telomere [2-4].

To overcome the first challenge, prokaryotes with linear genomes such as Streptomyces possess palindromic hairpin structures and specific terminal proteins, while mammalian cells developed a highly conserved nucleoprotein complex that protects chromosome ends against the DNA Damage Response (DDR) machinery, thereby preventing undesired DNA repair and chromosome end fusions [5]. The protective nucleoprotein complex arises through protein-telomere and protein-protein interactions of the shelterin proteins (TRF1 and TRF2 (telomeric repeat-binding factor 1 and 2, bind double-stranded DNA), POT1 (protection of telomeres 1, binds single-stranded DNA), Rap1 (repressor/activator protein 1, binds TRF2), TIN2 (TRF1-interacting protein 2), and TPP1 (formerly known as **T**INT1[6], **P**TOP[7] and **PIP1**[8]) [9]. This results in the adoption of a specific telomeric (T) loop structure [10] which represses six pathways: ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) signaling, classical non-homologous end-joining (NHEJ), alternative-NHEJ, homologous recombination, and resection [11]. Additional mechanisms contributing to end protection include ironically, DNA

damage and repair proteins involved in the post-replicative processing and reestablishment of end protection. The CST complex (CTC1/STN1/TEN1) which is implicated in C-strand synthesis and the maintenance of a proper telomeric heterochromatic state, in part via telomere-associated telomeric repeat-containing RNA (TERRA) also regulates chromosome end protection [12]. The contribution of specific G-quadruplex structures that may form at G-rich telomeres in regulating telomere protection is not well characterized [13].

Regarding the second challenge, not much is known about the mechanism in prokaryotes, but highly dividing eukaryotic cells compensate the loss of telomeric DNA typically by reverse transcription of an inherently associated RNA sequence by the reverse transcriptase, telomerase, a ribonucleoprotein possibly acquired from viral reverse transcriptases [14]. Thus the action of telomerase could be viewed as a "rescue" response to the problem of telomeric DNA loss, rather than a mechanism which prevents telomere erosion. Maintaining a mechanism that allows telomere loss and gain provides a system to regulate cell growth in multicellular organisms i.e. a way to suppress cancer and to impart a limit to proliferation that is characterized by dysfunctional organs or cells upon lifelong exposure to stresses and mutagenesis. Mechanistically, when the telomere length reaches a short size limit, cells enter into senescence in a p53-dependent fashion [15] previously defined as the Hayflick Limit (Figure 1. A.) [16]. Otherwise, in the absence of p53, cell division is maintained with dramatic consequences on chromosome stability that can lead to cell death but may also result in cancer.

Two ways to maintain telomeres length / Two possible ways to fight cancer

Approximately 85 to 90% of cancer cells express telomerase to bypass the Hayflick limit [17]. Telomerase expression is tightly regulated, principally at the transcriptional level of the core protein subunit TERT while the RNA template is rarely limiting. The absence of CAAT and TATA elements in the TERT gene promoter prevents constitutive activation of its transcription. However, analysis of the full promoter shows the presence of several modules ensuring its tissue-specific expression, such as hormone receptor binding sites and stress responsive elements [18], and the epigenetic profile shows no specific marks [19]. Interestingly, several elements of the TERT gene promoter are targets of factors that are deregulated in cancer, such as c-myc [20]. It has to be noted that only a few TERT molecules are necessary to ensure telomere maintenance (estimate a few hundred ribonucleoproteins per cell) [21, 22]. Alternative splicing of the TERT transcript adds another level of control on telomerase [23], showing again that a very low amount of TERT transcript is sufficient for function. This suggests that a full up-regulation leading to a high copy number of hTERT transcripts by all mandatory transcription factors may not be required, and that aberrant and/or promoter mutation driven and/or unusual epigenetic changes (commonly observed in cancers) causing transcription "leakage" may be adequate for telomerase function [24].

A non-negligible proportion of cancers, 5-10%, do not express telomerase but maintain their telomere length via inter-chromosomal recombination: a process called Alternative Lengthening of Telomere (ALT) in which sister chromatids exchange their telomeres in a non-reciprocal way [25]. The process of ALT including the mechanisms and factors that regulate ALT is not as well defined as the process and regulators of telomerase-mediated telomere maintenance. ALT-based telomeric recombination generates an abundance of

extrachromosomal telomeric DNA of both linear and circular forms [26]. Various features characteristic of ALT cells have been defined, providing evidence for the ALT pathway, but very few factors that regulate the ALT pathway and that can potentially be considered as therapeutic targets have been identified. In mice, ALT-positive tumors can be induced by genetic deletion of telomerase, and these ALT tumors are more sensitive to the inhibition of PGC1□□a master regulator of mitochondrial biogenesis and function, or SOD2, a target of PGC1□ than telomerase-positive tumors[27]. The importance of characterizing the ALT phenotype of cancer is also highlighted by Flynn and colleagues [28] who have recently shown that ALT cancer cell lines are selectively sensitive to inhibition of a DDR pathway component, ATR, compared to telomerase-positive cancer cell lines, providing the first tool to treat ALT cancers (Figure 1. B.).

Telomerase : the unpredictable target

Telomere maintenance is essential for replicative immortality characteristic of cancer cells. The discovery that the majority of cancers maintain their telomeres via telomerase led researchers to investigate telomerase as a potential cancer-specific therapeutic target. Telomerase inhibition leads to anti-proliferative effects on cells [29, 30], but unfortunately, these effects are elicited only when telomeres reach a critically short length. Thus they are observed in a cell-division and time-dependent manner incompatible with cancer treatments. Another consideration of telomerase as a therapeutic target is the crucial role of the enzyme in telomere maintenance in specific normal cells such as gametes, hematopoietic stem cells and other highly proliferative cells [31]. Nevertheless, as telomerase-positive cancer cells typically have shorter telomeres than normal cells [32], preventing telomere maintenance/elongation by inhibiting

telomerase could be considered as mid/long term treatment in combination with conventional chemotherapeutic therapies to prevent relapse and ensure remission (see Figure 1. C.). The association of TERT with the RNA template is required for telomere maintenance in the nucleus and constitutes an interesting therapeutic target. Reports showing that oxidative stress induces an active trafficking of telomerase from nuclei to mitochondria [33] is suggestive of a telomere-independent function of telomerase in the mitochondria. The potential effects of targeting telomerase on alternate telomerase functions are not well understood, moreover, it is not clear if the RNA template is required and/or is the same for these functions [12]. Long term inhibition of telomerase and the functional specificity potentially provided by the RNA template should be studied to ensure that targeting of possibly crucial extra-nuclear functions of the enzyme has no harmful side-effects.

Lastly, a few anti-telomerase drugs remain in clinical trials, and include Imetelstat, an inhibitor of telomerase, and GV1001, a telomerase peptide vaccine. Although they have a biological activity as assessed in phase I clinical trials, to date their efficacy in patients has not been achieved[34, 35]. Trials to determine if the efficacy of these anti-telomerase drugs can be improved in combination, by optimizing delivery, or for certain types of tumors, are still ongoing[30].

The shelterin complex and TERRA as therapeutic targets

The limitations and potential challenges of targeting telomerase, and the fact that telomerase inhibition would not target the one sixth of cancers that use the ALT pathway, oriented researchers to investigate telomeres as a therapeutic target. Telomeres are

nucleoprotein structures composed of a telomeric single-stranded overhang that invades the double-stranded telomeric sequences, both coated by specific proteins that regulate this T-loop structure and its function. The T-loop protects telomere extremities against exonucleases and the number of bound TPP1/POT1 complexes is dictated by the lengths of the double-stranded and single-stranded regions of the telomere, thus serving as a measure of telomere length [36]. A recent study has also shown an active regulation of the overhang of the leading strand during the replication of the telomeric region by a controlled resection of the matrix strand [37]. Telomere length is also indirectly correlated with, and monitored by levels of TERRA, the telomeric repeat-containing RNA that is transcribed from the subtelomeric region toward telomeres. First, TERRA can inhibit telomerase activity to promote telomere shortening. Secondly, TERRA can promote Exo1-dependent resection at chromosome ends to initiate telomere shortening. TERRA also increases euchromatin formation or decreases heterochromatin formation to regulate telomere length [38]. More recently, mounting evidence confirms that TERRA is generally upregulated in ALT cells[39]. Thus TERRA could serve as a marker for screening ALT cancers, and possibly also as a target for ALT cancers[40]. Regulation and monitoring of telomere length by various mechanisms independently of telomerase, including via TERRA expression levels, highlights additional anti-cancer therapeutic opportunities (Figure 1. D.). Moreover, experimentally inducing telomere deprotection (Figure 1. E.) by altering shelterin components [41], manipulating telomere sequences [42, 43] or using G-quadruplex stabilizing ligands [13, 44] elicits the activation of the DDR machinery to provoke end-to-end fusions, leading to cell death (Figure 1. F.). Tumor specificity of such an approach may be enhanced by the high division rate of cancer cells in which the dynamics of deprotection/re-

protection of telomeres during their replication provides a therapeutic window. Also, anti-proliferative effects of telomere uncapping strategies appear to be restricted to cancer cells, suggesting that the telomere cap may differ in cancer cells versus normal cells[45-48] . Deregulated expression of shelterin proteins has been reported in some human tumors, and Pot1 was found to be mutated in chronic lymphocytic leukemia (CLL) [49-51], suggesting that the telomere cap in cancer cells may be different than in normal cells and that it may be possible to specifically target telomere function in cancer cells. Pot1-mutated CLL cells harbor telomeric abnormalities consistent with telomere uncapping[51]. There is also an uncharacterized mechanism of short telomere perception/detection during the cell cycle, i.e by one or more checkpoints triggered by the presence of at least five uncapped or dysfunctional telomeres, preventing the cell to re-enter in the cell cycle and provoking p53-mediated senescence [15]. Thus, affecting a cell' perception of its telomere length by manipulating the checkpoint signaling could induce senescence (Figure 1. G.). Interfering with factors that regulate and/or monitor telomere length would finally constitute a means to restore the Hayflick limit in cancer cells.

Telomere lengthening as an indicator of transformation: a diagnostic tool

Telomere lengthening as a consequence of telomerase or ALT activation could be considered as a potential indicator of a cellular transformation event (Figure 1. H.). Indeed, with the increased sensitivity of experimental assays over the last 30 years, it is now feasible to detect very low activity levels of any kind of enzyme. For example, the PCR-based assay for telomerase could be adapted and optimized to detect any abnormal level of telomerase activity or transcription that would reveal a transformation in progress with a direct correlation

between sensitivity of the test and precocity of the diagnosis (Figure 1. I.). To detect a potential induction of the ALT pathway as an indicator of transformation, extrachromosomal telomeric c-circle DNA could be measured. C-circles are composed of a full-length telomeric C-rich strand hybridized to a partial G-rich strand and are the most specific and quantifiable marker of ALT reported to date, being 750-fold more abundant in ALT-positive compared to ALT-negative cells (i.e. telomerase-positive cancer cells and telomerase-negative normal cells) and can be measured using blood (and tissue) from ALT-positive cancer patients (Figure 1. J.) [26]. C-circle intermediates of an ALT-based mechanism are similar, in principle, to cancer cell-derived DNA □a□i□□e□ec□e□in pla□ma an□w□ic□i□un□er in□en□e inve□iga□ion a□a 'liqui□ biop□' for cancer [52].

Additional methods to detect increased telomerase activity or telomere lengthening that could reveal a transformation event in progress could be implemented using a routine blood test. But, as telomerase expression or activity already exists in healthy humans in lymphoid and hematopoietic nests, complementing such an assay with standard biological and biochemical blood measurements to rule out increases in telomerase caused by increased number of immune cells due to an infection for example, should be adopted. For lymphomas and leukemias that are considered as "liquid tumors", measuring telomerase transcripts by qRT-PCR would be feasible, but for solid tumors, the isolation of Circulating Tumor Cells (CTC) from the blood would be required. However, the presence of CTCs is variable and is also associated with metastasis. Thus the detection of telomerase activity or transcripts from cells isolated from the blood would reveal rather a progression from primary to metastatic cancer. Maintaining this monitoring during the remission phase would also be a powerful tool to allow early detection of

any relapse of the cancer likely resulting from the development of therapeutic resistance, notably by switching from telomerase-mediated to ALT-mediated telomere lengthening. Such monitoring could permit adjustment in treatment. Lastly, mutations and loss of the ATRX chromatin remodeling complex is associated with ALT cancers[53-55] consistent with ATRX being a repressor of telomere homologous recombination in ALT cells[56]. These results highlight how genetic screening may be of clinical use in ALT cancer diagnosis, monitoring and treatment.

In conclusion, and summarized in Figure 1, as telomeres and telomerase play a pivotal role in the transformation process of malignant cells, they have the potential to be central in cancer prevention, diagnosis and treatment. Pathways regulating telomere protection and telomere synthesis should be considered separately. While high efficiency and rapid treatment should target telomere monitoring and protection pathways, targeting telomere lengthening regulated by telomerase or ALT should be envisioned as long term therapies, particularly to prevent relapse. Lastly, monitoring telomere lengthening in blood cells (by measuring telomerase expression/activity or ALT markers) could be valuable as a diagnostic tool.

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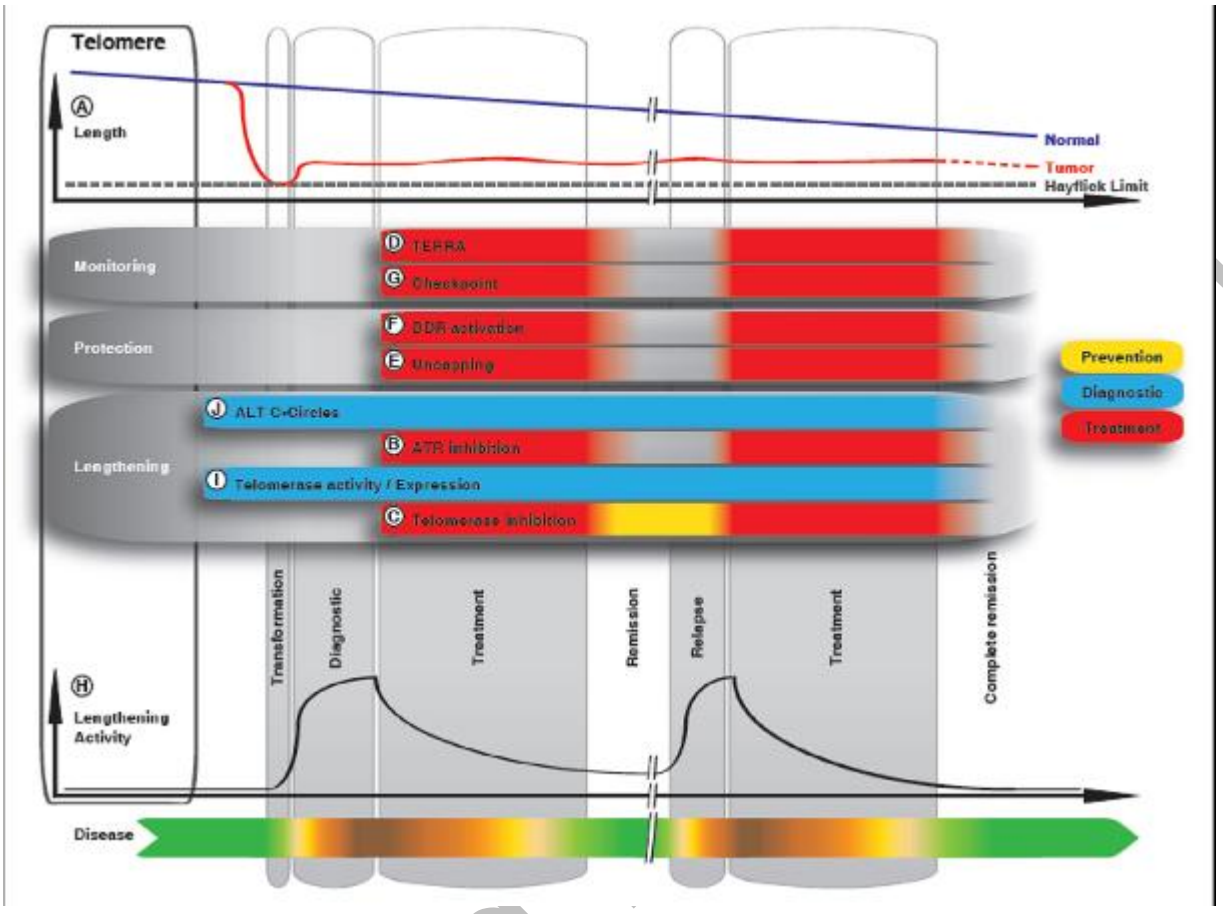

Figure 1: (A) Telomere length as a function of cell division in normal and tumor cells. When the telomere length reaches a critically short length cells enter into senescence (Hayflick Limit). Cancer cells acquire alterations allowing telomere maintenance and indefinite cell proliferation. Telomere biology hallmarks (C-G, I-J) that could be monitored, that are implicated in telomere protection, or in telomere lengthening (highlighted in grey) and that could be used as diagnostic tools (blue boxes), as targets for treatment (red boxes) and the prevention of relapse (yellow box) are indicated. (H) Schematic representation of the evolution of the disease over time with an arbitrary color code reflecting acuteness of the disease (bottom) and the increase in lengthening activity that permits diagnosis and detection of relapse.