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Telomere and Telomerase in Cancer: Recent Progress

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1. Introduction

Telomeres are specialized structures at the ends of all eukaryotic chromosomes and have special biological functions. They protect chromosomes from nucleolytic degradation, end-to-end fusions, and to be recognized as damaged DNA. Telomeres also contribute to the functional organizations of the chromosomes within the nucleus, participate in the regulation of gene expression, and most importantly, they serve as a molecular clock that controls the replicative capacity of the cells (Allsopp et al., 1992; Bailey et al., 2006; Chan & Blackburn, 2004; Hahn, 2005; O'sullivan & Karlseder, 2010).

In the last decade substantial progress has been made in human telomere biology. Humans, like other vertebrates, have telomeres composed of repetitive 5'-TTAGGG-3' sequences (Meyne et al., 1989; Moyzis et al., 1988). Human telomeres end in a 3' overhang composed of TTAGGG track which is 50-500 nucleotides long (Huffman et al., 2000; Makarov et al., 1997; Wright et al., 1997). Electron microscopy studies of the psoralen cross-linked human telomeres have revealed that telomeres terminate in a lariat-like structure termed a t-loop. Binding of TRF1 and single stranded binding protein suggested that this structure is formed by invasion of the 3' telomeric overhang into the duplex telomeric repeat array (Griffith et al., 1999) (Figure 1A and 1B). The fundamental aspect of the t-loop is thought to be the sequestration of the chromosome ends thereby providing a solution for the telomere end protection problem (de Lange, 2006; Griffith et al., 1999). Indeed, disruption of t-loop can result in telomere dysfunction and induce DNA damage response (d'Adda di Fagagna et al., 2003). Human telomeric DNA is found as associated with nucleosomes, six-subunit protein complex called shelterin, and a number of other chromosomal factors that are not part of the shelterin (de Lange, 2005, 2006; Diotti & Loayza, 2011).

1.1 Shelterin

Shelterin complex represses DNA damage signaling responses at the chromosome ends. It also participates in the formation of t-loops and regulation of telomere length (de Lange, 2005, 2006; Palm & de Lange, 2008) (Figure 1A and 1B). This complex consists of six subunits including TRF1, TRF2, TIN2, Rap1, POT1, and TPP1. The expression of shelterin is ubiquitous and it is present at telomeres throughout cell cycle. It is estimated that hundreds of copies of shelterin are found throughout the double stranded telomeric DNA and unlike

other telomere associated non-shelterin proteins; its function is limited only to telomeres (de Lange, 2005, 2006; Takai et al., 2010).

Shelterin binds to double stranded telomeric DNA via its DNA binding proteins TRF1 (TTAGGG-repeat-binding factor 1) and TRF2 (TTAGGG-repeat-binding factor 2) (Broccoli et al., 1997; Chong et al., 1995). TRF1 is the first identified subunit of shelterin complex that particularly binds to the double stranded telomeric DNA mainly as a dimer form that is mediated by its TRF homology (TRFH) domain (Bianchi et al., 1997; Zhong et al., 1992). The TRFH domain also participates in the recruitment of other proteins to the telomere (Y. Chen et al., 2008). In the shelterin complex TRF1 interacts only with TIN2 (S.H. Kim et al., 1999). In telomerase-positive mammalian cells, TRF1 works as a negative regulator of telomere length (Iwano et al., 2004; Okamoto et al., 2008) and supporting of this, the absence of TRF1 from telomeres results in telomere elongation (Iwano et al., 2004; Smith & de Lange, 2000; Smogorzewska et al., 2000; van Steensel & de Lange, 1997).

TRF2, which is a 500 amino acid protein, shows sequence similarity to TRF1. Like TRF1, TRF2 also has a TRFH domain allowing its homodimerization (Broccoli et al., 1997; Fairall et al., 2001). It has been demonstrated that the TRF2 facilitates the t-loop formation *in vitro* (Stansel et al., 2001). TRF2 interacts with other three members of the shelterin complex, TIN2, Rap1, and POT1, and behaves as a negative regulator of telomere length (Smogorzewska et al., 2000; Smogorzewska & de Lange, 2004; Palm & De Lange, 2008). Both TRF1 and TRF2 are subject to post-translational modifications including phosphorylation, SUMOylation, and PARsylation; however, the functional consequences of these modifications have yet to be clarified (Palm & de Lange, 2008).

TIN2 (TRF1-interacting nuclear protein), which is a relatively small protein molecule, associates with TRF1 via its innermost region and at the same time it interacts with TRF2 and TPP1 through its amino terminal domain (S.H. Kim et al., 1999; Palm & de Lange, 2008). Since TIN2 is located in the middle part of the shelterin complex, it can function as a bridge that connects the double stranded telomeric DNA binding proteins to those bind to the single stranded telomeric DNA (S.H. Kim et al., 1999, 2004; Ye et al., 2004a). Rap1 (repressor / activator protein 1) is incorporated to the shelterin complex through binding to the TRF2 in approximately 1:1 ratio, and therefore, its presence on telomeres is TRF2 dependent (B. Li et al., 2000; Takai et al., 2010). Rap 1 plays an important role in telomere length regulation and it is thought that the Rap1 may be important for protective function of shelterin (de Lange, 2005, 2006).

TPP1 (POT1-TIN2 organizing protein, PTP, PIP1, TINT1), both increases DNA binding activity of POT1 and it also connects POT1 to TIN2 by means of both its POT1 interaction and TIN2 interaction domains (Hockemeyer et al., 2007; D. Liu et al., 2004; Loayza et al., 2004; Ye et al., 2004a; Ye et al., 2004b). The last member of the shelterin complex called POT1 (protection of telomeres 1) was identified in the database through its homology with the DNA binding domain of TEBP α . Similar to TEBP α , POT1 binds to single stranded telomeric sequences by its two OB fold domains (Baumann & Cech, 2001). It has been shown that the POT1 binds to single stranded TTAGGG, and it has been predicted that it can bind telomeric sequences both at the 3' single stranded end and the displaced TTAGGG repeats at the base of the t-loop, called D-loop (Loayza et al., 2004). POT1 has a fundamental role in telomere

length regulation, since it functions as a terminal transducer of telomere length control (Loayza & de Lange et al, 2003).

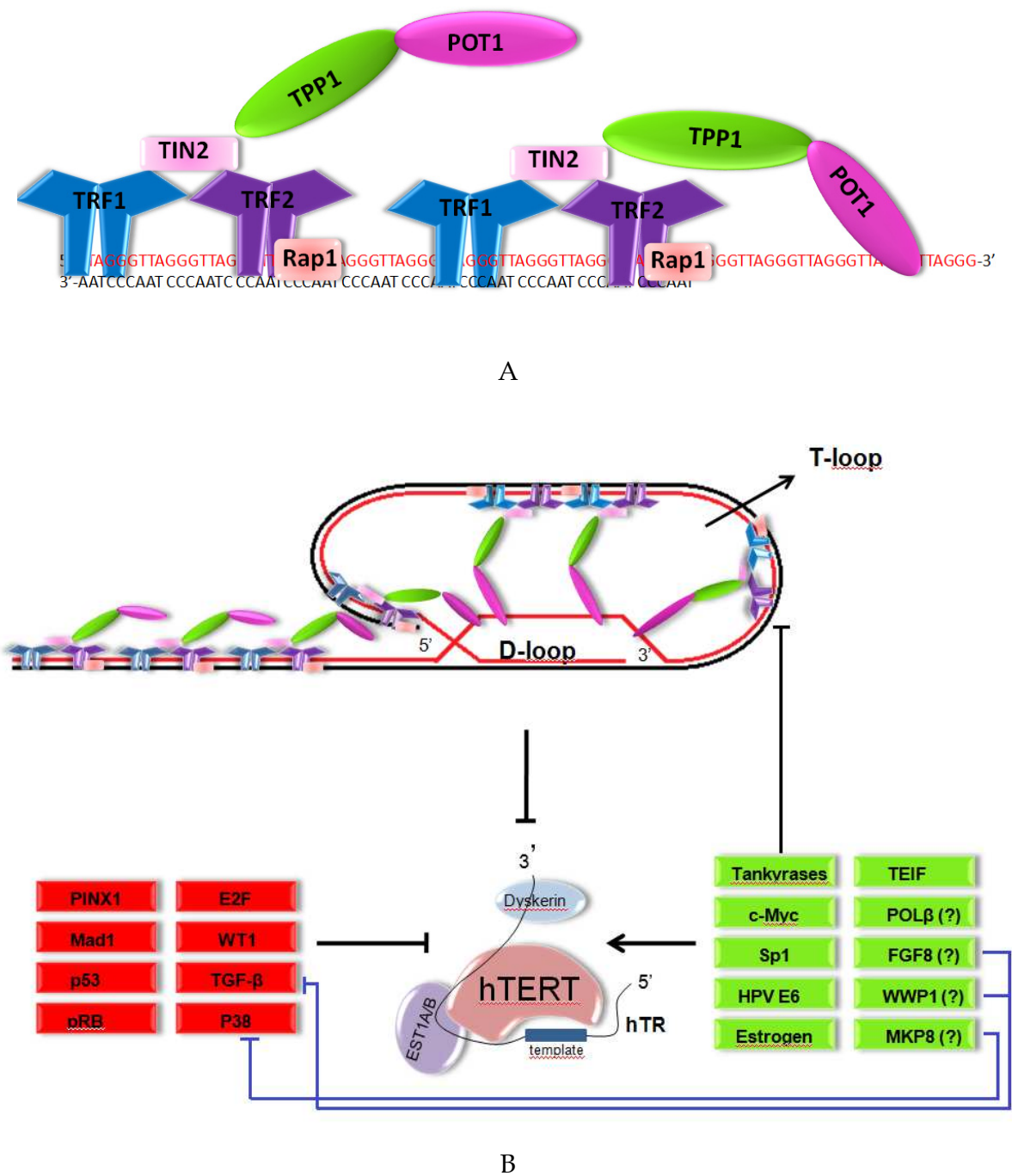


Fig. 1. A. Schematic representation of human shelterin complex composed of six protein subunits: TRF1, TRF2, Rap1, TIN2, TPP1 and POT1. B. Illustration of t-loop structure of human telomeres accomplished by shelterin complex. Known/candidate negative and positive regulators of the human telomerase enzyme are shown as red and green rectangulars, respectively. Blue lines show the possible effects of candidate proteins determined in our study (Gümüř-Akay et al., 2009).

1.2 Non-shelterin telomeric proteins

In addition to the shelterin complex, human telomeres consists of a large number of other proteins including Ku70/80, XPF/ERCC1, Apollo, the MRN complex, RecQ helicases, Tankyrase, and PINX1. Unlike shelterin complex that is located at telomeres throughout the cell cycle, these non-shelterin proteins only transiently associate with chromosome ends (Palm & de Lange, 2008). These proteins interact with the shelterin complex mainly by associating with TRF1 and TRF2. All of the non-shelterin telomere associated factors mediate telomere function in chromosome stability and contribute to telomere length regulation (de Lange, 2006; Palm & de Lange, 2008).

2. Structure of human telomerase enzyme

In human somatic cells, telomeres are approximately 10 kb long (Allshire et al., 1989; de Lange et al., 1990; Harley et al., 1990; Moyzis et al., 1988) and gradually shorten ~50-300 bp per cell division because of the end replication problem and post replicational processing by exonuclease that degrades telomere strand at the 5'-end (Huffman et al., 2000; Makarov et al., 1997; Olovnikov, 1973). If this telomere erosion is not balanced by elongation, telomeres will progressively shorten; eventually lead to genomic instability and cell death. Hence, the long-term proliferation of human cells requires mechanisms to counteract telomere attrition that occurs in each cell division. The most widely used way for telomere maintenance is based on the enzyme called telomerase which was discovered by Carol Greider and Elizabeth Blackburn in 1984 (Greider & Blackburn, 1985). Nobel Prize in Physiology or Medicine 2009 was awarded to Elizabeth Blackburn, Carol Greider and Jack Szostak for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase (http://nobelprize.org/nobel_prizes/medicine/laureates/2009/).

Telomerase is a specialized reverse transcriptase that uses its RNA template to elongate the telomeres by addition of 5'-TTAGGG-3' repeats to the terminal 3' overhang (Greider & Blackburn, 1985). Human telomerase enzyme is composed of two core subunits involving RNA subunit (hTR) and catalytic telomerase reverse transcriptase (hTERT). In addition to these main components, studies have identified several telomerase-associated proteins required for proper functioning *in vivo*; such as dyskerin, hNop10, hGar1, that are potential components of the holoenzyme complex (Cristofari & Lingner, 2006; Shay et al., 2001). Telomerase components show significant size and sequence diversity among different species especially in the RNA moiety. However, certain parts of both subunits seem to be functionally conserved during evolution.

2.1 hTR

Human telomerase uses single stranded RNA as a template for *de novo* telomeric repeat synthesis. hTR is encoded by the *hTERC* gene located on chromosomal region 3q26 (Feng et al., 1995; Soder et al., 1997). This gene is transcribed by RNA pol II. hTR is 451 nucleotide long RNA and shows unique secondary structure with four special structural and functional domains, namely the pseudoknot domain, the CR4/CR5 domain, the H/ACA scaRNA domain, and CR7 domain. These domains have discrete functions such as RNA binding, dimerization, and recruitment of telomerase to the telomeres. Moreover, they mediate interactions between the hTR and catalytic subunit, hTERT, and other

telomerase-associated proteins. Among functional domains only the core domain and CR4/CR5 domains are required for *in vitro* reconstitution of catalytically active telomerase with hTERT. The template region of hTR is about 1.5-2 times the TTAGGG repeat length allowing both its annealing of the 3' overhang and addition of telomeric repeats at the ends of chromosomes. Boundary element prevents reverse transcription beyond the 5' boundary of the template (J.L. Chen et al., 2000; J.L.Chen & Greider, 2006; Cristofari & Lingner, 2006; Zhang et al., 2011).

2.2 hTERT

hTERT is encoded by *hTERT* gene located at 5p15.33 (Bryce et al., 2000). This gene spans approximately 40 kb of the genome and contains 16 exons (Cong et al., 1999). *hTERT* gene produces alternative transcript variants with unknown biological functions (Kilian et al., 1997). Unlike the hTR, sequence of the catalytic subunit of the human telomerase enzyme is conserved across species. hTERT has functional regions that can be grouped into four distinct domains: TERT N-terminal domain (TEN), TERT RNA binding domain (TRBD), the reverse transcriptase domain (RT), and the C-terminal extension (Mason et al., 2011). The TEN domain, also known as the anchor domain of telomerase, potentially facilitates processivity of the enzyme by preventing enzyme-DNA dissociation during translocation and to promote realignment events that accompany each round of telomere synthesis (Wyatt et al., 2007). TRBD domain, located between TEN and RT domains, is implicated in telomerase ribonucleoprotein assembly and RNA binding. This domain contains evolutionarily conserved CP, QFP, T, and VSR motifs required for CR4/5 binding in vertebrates (Bley et al., 2011). The central RT domain is the catalytic part of the enzyme and has seven evolutionarily-conserved RT motifs including 1, 2, A, B', C, D, E motifs. This domain is organized as two subdomains namely fingers and palm. The TERT active site contains three conserved aspartic acids, one of which resides in motif A and the other two within motif C. These residues form a catalytic triad contributing directly in nucleotide addition (Lingner et al., 1997; Nakamura et al., 1997). The motifs located at the C-terminal domain are not well conserved and telomerase specific (Autexier & Lue, 2006; J.L. Chen and Greider, 2006; Cristofari & Lingner, 2006; Mason et al., 2011; Wyatt et al., 2010). This domain is essential for human telomerase function *in vivo*. It has been shown that the C-terminal domain plays an important role in the catalytic activity and processivity of the telomerase (Huard et al., 2003).

3. Regulation of human telomerase activity

During normal human development, telomerase activity should be strictly regulated to meet proliferative needs of specific cellular functions such as tissue repair, while at the same time preserving proliferative barriers against tumorigenesis. Significant research effort in the last two decades has provided important data for our understanding of the complex processes of the telomere length homeostasis and telomerase regulation. Telomerase activity is regulated at many levels including transcription, mRNA splicing, maturation, processing and nuclear localization of both hTR and hTERT subunits, post-translational modifications and correct folding of hTERT, assembly of ribonucleoprotein complex, and accessibility of the telomeres to the holoenzyme (Cong et al., 2002; Ćukušić et al., 2008).

However, regulation at the *hTERT* level, particularly at the transcription level, seems to be the primary and rate limiting step in the telomerase activity control (L. Liu et al. 2004; Shay et al., 2001)

Several positive and negative regulators of the *hTERT* gene have been identified (Figure 1B). Some of the positive regulators of the *hTERT* promoter are oncogene *c-myc*, the transcription factor Sp1, the human papillomavirus 16 (HPV 16) protein E6, and steroid hormones (e.g. estrogen) (Cong et al., 2002; Ćukušić et al., 2008; L. Liu et al., 2004). By means of microcell-mediated chromosome transfer studies, it has been shown that the normal somatic cells have transcriptional repressors that inhibits *hTERT* transcription. The chromosomal regions that contain genes encoding these repressors have been determined as 3p21.3 in renal cancer cell line RCC23, 3p12-21.1 in breast cancer cell line 21NT (Oshimura & Barrett, 1997), 10p15.1 in hepatocellular carcinoma cell line (Nishimoto et al., 2001), and 6p in human papilloma virus immortalized keratinocyte cell line and cervical cancer cell line (Steenbergen et al., 2001; L. Liu et al., 2004). In addition to these chromosomal regions, certain factors have been demonstrated to repress telomerase activity including antiproliferation factors such as transcription factor Mad1, the tumor suppressor proteins p53, pRb, E2F, the Wilms' tumor 1, the myeloid cell-specific zinc finger protein 2, and differentiation factors, such as interferon-gamma and transforming growth factor β (Cong et al., 2002; Ćukušić et al., 2008; L. Liu et al., 2004). In addition to the transcription factors, *hTERT* expression is also regulated epigenetically (Cong et al., 2002; L. Liu et al., 2004).

Apart from the mechanisms summarized above, telomerase is also regulated by the shelterin and non-shelterin proteins localized at the telomeres. This type of regulation based on the accessibility of telomere substrates to the enzyme, telomerase. Several studies have shown that shelterin may affect the telomerase action, particularly, as a negative regulator (Smogorzewska & de Lange, 2004; de Lange, 2005, 2006; Palm & de Lange, 2008) (Figure 1B). Shelterin recognizes telomere length via its ability to accumulate all along the double stranded telomeric DNA. The longer the telomeric DNA, the more shelterin complex associated with it. This results in a t-loop formation and decreases the chance of the telomerase to reach the telomeric end (de Lange, 2006; Smogorzewska & de Lange, 2004). All of the shelterin subunits behave as a telomere length regulator and their amount at telomeric DNA affects elongation of telomeres. For example, the absence of TRF1 from telomeres results in telomere elongation (Iwano et al., 2004; van Steensel & deLange, 1997), whereas increased amount of TRF1 on telomeres leads them to become shorter (van Steensel & deLange, 1997). Similarly, as telomeres get longer, the number of TRF2 on telomeres increases. It has been shown that the overexpression of TRF2 results in telomere shortening, implying TRF2 also can modulate telomere length (Smogorzewska et al., 2000).

Since telomerase ribonucleoprotein functions on the single stranded 3' overhang, shelterin complex should perform its negative regulatory functions at this place (de Lange, 2005, 2006). POT1, which is the only member binding to the single stranded telomeric sequences plays an important role in the effect of shelterin on telomerase (de Lange, 2005, 2006). According to the counting model for telomere length regulation, POT1 is the key player that functions as a terminal transducer of telomere length control (Loayza & de Lange, 2003). When telomeres become elongated, they are occupied with more shelterin, thereby the

chance of POT1 to associate with single stranded 3' overhang also increases, which in turn leads to blockage of telomerase (Figure 1B).

While shelterin complex seems to be responsible for negative telomerase regulation (Smogorzewska & de Lange, 2004; de Lange, 2005; Palm & de Lange, 2008), unexpected results showed that the POT1-TPP1 complex may activate telomerase processivity (Tejera et al., 2010; Wang et al., 2007). In order to explain the two opposite functions of the TPP1-POT1 complex, three-state model of telomere length regulation has been proposed by Wang et al. (2007). When POT1-TPP1 coat the 3' end of the G-overhang, it makes the telomeres inaccessible to the telomerase. On the other hand, it has been suggested that the TPP1-POT1 complex probably function together to positively recruit telomerase to telomeric ssDNA through the TPP1 OB fold (Xin et al., 2007). At second state, TPP1-POT1 are removed from their binding sites by an as yet unidentified mechanism such as post-translational modification and disruption of the shelterin complex. The POT1-TPP1 complex then act as a telomerase processivity factor during telomere elongation. As the telomere length reaches a certain threshold, the newly synthesized repeats bind shelterin complexes, and the 3' end of the G-overhang is again bound by POT1-TPP1, thereby preventing telomerase action (Bianchi & Shore, 2008; Wang et al., 2007).

As mentioned before, non-shelterin telomere associated factors mediate telomere function in chromosome stability and contributes to telomere length regulation. For example, poly-ADP-ribose polymerase called tankyrase binds to TRF1 and adds ADP-ribose chain to the Glu residues located at the N-terminal domain of the protein. This post-translational modification leads to separation of TRF1 from telomeres. Therefore, tankyrase acts as a positive regulator of telomere length (Hsiao & Smith, 2008; Smith & de Lange, 2000). Another shelterin accessory factor PINX1, for instance, functions as a telomerase inhibitor and participates in TRF-1 mediated telomere length control (Soohoo et al., 2010; Zhou et al., 2001).

Unfortunately, the mechanisms involved in control of telomerase activity seem to be a big puzzle. It is clear that the understanding the mechanisms involved in telomere length and telomerase regulation, and identification of novel candidate molecules would certainly have implications for understanding the molecular basis and management of human cancers. We have determined the chromosomal localizations of putative unidentified telomerase activator(s) and/or repressor(s) by high resolution comparative genomic hybridization (HR-CGH) in highly telomerase expressing gastric tumor samples. We have found that genomic imbalances including 1q+, 8p+, 8q+, 10q+, 17p-, and 20p+ are associated with the higher telomerase activity. Our results suggest that 1q24, 8p21-p11.2, 8q21.1-q23, 10q21-qter and 20pter-p11.2 may contain putative telomerase activator(s), whereas the 17p12 region may harbor candidate telomerase suppressor(s) (Gümüş-Akay et al., 2009).

WWP1 (WW domain containing E3 ubiquitin protein ligase 1) gene located at 8q21 seems to be a candidate telomerase regulator. It is known that the TGF- β (beta), inhibits positive regulatory effect of c-Myc on *hTERT* promoter by increasing Smad3 phosphorylation (H. Li et al., 2006a, 2006b). Komuro et al. (2004) have shown that WWP1 protein could block the transcriptional activities of TGF- β by inducing nuclear export of Smad7 and eventually enhancing degradation of TGF- β type I receptor through ubiquitination. Our results have suggested that increase in WWP1 gene copy number could have caused blockage of TGF- β -

mediated hTERT repression. We have suggested that the *POLB* and *MKP8* genes located at 8p11.2 and 8p12, respectively, might be putative telomerase activators (Gümüř-Akay et al., 2009). *POLB* encodes a DNA repair enzyme called β -pol which is an error-prone polymerase (Bergoglio et al., 2001; Canitrot et al., 2000). TEIF (Telomerase Transcriptional Element Interacting Factor) is one of the newly identified transcriptional activators of *POLB* gene. This transcription factor can also bind to the promoter region of *hTERT* and activates its expression. Transcriptional regulation of both *POLB* and *hTERT* by the same transcription factor and increased activity of these two enzymes in cancers suggest a functional link between β -pol and telomerase (Tang et al., 2004; Zhao et al., 2005). The *MKP8* gene at 8p12 encodes a mitogen-activated protein kinase phosphatase-8 that inhibits P38 by dephosphorylation (Vasudevan et al., 2005). It has been shown that the P38 bind to the *hTERT* promoter and repress its transcription (Chang et al., 2005). Therefore, MKP8 might be another putative telomerase activator. *FGF8* encoding Fibroblast Growth Factor 8 is located at 10q24. Repression of FGF8 protein has been reported to be necessary for TGF- β mediated transcriptional suppression (Takayashiki et al., 2005). Our results have insinuated that the FGF8 might regulate telomerase activity via TGF- β pathway.

4. Telomeres and telomerase in cancer

Telomerase activity is undetectable in most normal human somatic cells. However, it is expressed during early development and remains fully active in specific germ-line/embryonic stem cells and cancer cells (Hahn et al., 2005; N.W. Kim et al., 1994). In addition, modest levels of telomerase activity are determined in proliferative tissues with high renewal potential such as the bone marrow, skin, gastrointestinal tract and testis as well as in activated lymphocytes (Yui et al., 1998; K. Liu et al., 1999). Normal human somatic cells have a limited replicative potential and after 50-70 doublings they become senescent. This is known as the Hayflick limit (Hayflick & Moorhead, 1961). This raises the question of what is the mechanism accounting for the finite division capacity of normal somatic cells? Although the whole mechanism has yet to be understood, one of the main determinants of cellular replicative capacity is the progressive loss of telomeric repeats occurs in each cell division (Allsopp et al., 1992; Harley et al., 1990).

At the Hayflick limit one or a few critically shorten telomeres, which lose their capping function and activate DNA damage checkpoints, trigger an irreversible growth arrest known as a Mortality stage 1 (M1). Senescence involves p53 and pRb pathways and leads to the termination of cell proliferation (Wright et al., 1989; Shay et al., 1991). Senescence, therefore, can be accepted as a tumor suppressor mechanism (Campisi, 2005). Cells that can bypass this replicative senescence by inactivation of important cell cycle checkpoint genes (e.g. p53) continue to divide and lose their telomeres further until reaching the crisis stage or Mortality stage 2 (M2) (Wright et al., 1989; Shay et al., 1991; Zou et al., 2004). Cells that are able to reactivate/up-regulate their telomerase enzyme can escape from crisis and become immortalized, which is generally believed to be a critical step in cancer progression (Counter et al., 1992; N.W. Kim et al., 1994; Shay & Bacetti, 1997; Shay & Wright, 2010) (Figure 2). Although most cancer cells (80-90%) express a high level of telomerase activity, the remaining uses alternative lengthening of telomeres (ALT) by recombination to maintain their immortality (Bryan & Reddel, 1997).

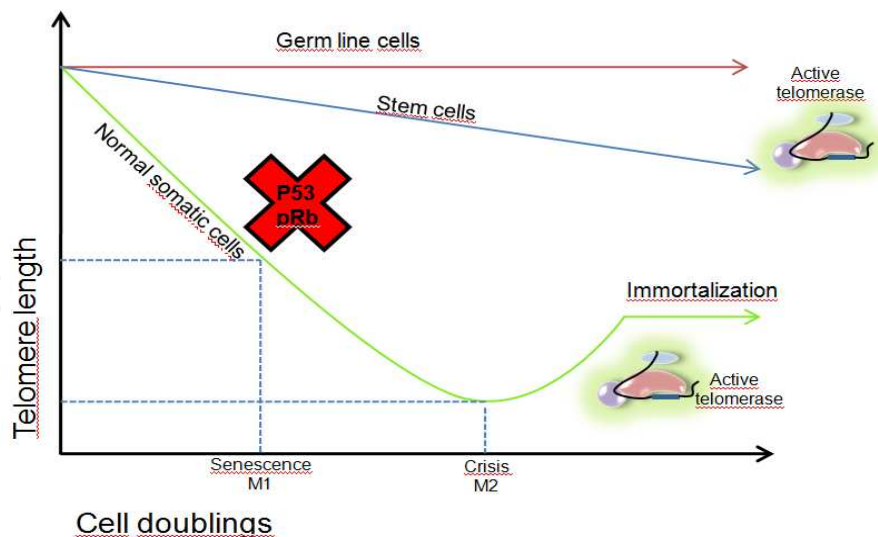


Fig. 2. The telomere hypothesis of replicative senescence and cancer.

Telomere length is maintained in germ cells and stem cells by active telomerase. In the absence of active telomerase enzyme one or a few critically shortened telomeres trigger an irreversible growth arrest known as a Mortality stage 1 (M1). Cells that can bypass this replicative senescence by inactivation of important cell cycle checkpoint genes (e.g. p53) continue to divide and lose their telomeres further until reaching the crisis stage or Mortality stage 2 (M2). Cells those are able to reactivate/upregulate their telomerase enzyme can escape from crisis and become immortalized.

The pivotal role of telomere and telomerase biology in cancer has been well documented by means of cellular and animal models (Artandi & Depinho, 2000). In 1994, N.W. Kim et al. showed that telomerase could be detected in about 90% of all human tumors examined, and a couple of years later other groups have demonstrated that the *hTERT* transfected cells never show a growth arrest (Bodnar et al., 1998; Counter et al., 1998). After that time, the number of articles on telomeres, telomerase and cancer has increased tremendously. All of the studies indicate that telomerase activity is almost universally an essential requirement for cellular immortalization and unlimited proliferative characteristic of cancer cells. In many human primary cancers increased or up-regulated telomerase activity have been reported, and increased telomerase activity has also been demonstrated to be related to poor prognosis. Thus telomerase activity emerges as an attractive target for both cancer diagnosis and treatment (Artandi & Depinho, 2010; Donate & Blasco, 2011; Granger et al., 2002).

4.1 Telomere dysfunction and its consequences in carcinogenesis

As mentioned above, telomeres are critical for maintenance, protection and stabilization of linear chromosomes, enabling continuous cell division. In the absence of telomere maintenance mechanisms progressive telomere shortening is thought to be the major cause of replicative senescence. Critically shortened telomeres lose the protection of telomere-binding proteins, leading to telomere uncapping, and this is known as telomere dysfunction. In normal cells, telomere dysfunction activates DNA damage checkpoint pathways, particularly p53 and RB pathways, leading to stable growth arrest or

programmed cell death called apoptosis (d'Adda di Fagagna et al., 2003; d'Adda di Fagagna et al., 2004; Zou et al., 2004).

On the contrary, cells derived from tumors can divide indefinitely in culture by means of their tumor maintenance mechanisms, most prominently by telomerase. It has been shown that the dysfunctional telomeres can lead to chromosome fusions before the reactivation/upregulation of telomerase, which has been proposed as a mechanism of chromosome instability in cancers (Gisselson et al., 2001; Maser & Depinho, 2002; Murnane, 2010). Comparative genomic hybridization techniques have demonstrated extensive focal amplifications and deletions in human cancers which are now known to harbor oncogenes and tumor suppressor genes, respectively. This raises the question whether these chromosomal loci contain genes that induce carcinogenesis or they are the consequences of the genomic instability seen in cancer cells. Copy number alterations comes from aCGH analyses of tumors from telomerase-deficient mice (TERC $-/-$ p53 $+/-$) have suggested that chromosome breakage-fusion-bridge process caused by telomere dysfunction might provide a major mechanism driving amplifications and deletions in human cancer genomes (reviewed in Artandi & Depinho, 2010).

4.2 Telomerase as diagnostic and prognostic marker in cancer

Even though morphological assessment remains the gold standard for cancer diagnosis, there has been continuous seeking for new molecular markers that are less subjected to evaluation bias (E. Hiyama & K. Hiyama, 2003). Given that both of its crucial role in the carcinogenesis process and its uniqueness to all types of cancers, analysis of telomerase activity has been anticipated as a diagnostic and/or prognostic marker for this disease.

After the development of PCR-based telomerase assay called telomeric repeat amplification protocol (TRAP) by N.W. Kim et al. (1994), investigation of telomerase activity in different tumor tissues and their normal counterparts has become easier. TRAP assay can detect as few as 10 telomerase positive cells (Wright et al., 1995) in a variety of biological samples, including solid tissue samples (Gümüř-Akay et al., 2007), blood, urine (Hess & Highsmith, 2002), fine needle aspirates (E. Hiyama & K. Hiyama, 2004), ascites, and peritoneal washing fluids (Hess & Highsmith, 2002; Ozmen et al., 2008). In its original form, TRAP assay was not able to measure the telomerase activity quantitatively, and serial dilutions of the tissue extracts and use of internal control were recommended in each assay. However, modified and more user-friendly versions of this method have been developed to improve its sensitivity and quantitative detection of telomerase activity, including TRAP-ELISA (enzyme-linked immunosorbent assay) (Gonzalez-Quevedo et al., 2000; Wu et al., 2000), TRAP-ELIDA (enzymatic luminometric inorganic pyrophosphate detection assay) (Xu et al., 2001), and real-time TRAP assay (Shim et al., 2005). The main disadvantages of detecting telomerase activity by TRAP assay are the contamination of telomerase expressing normal cells with the tissue of tested and the possibility of presence of PCR inhibitors in some tissue extracts (E. Hiyama & K. Hiyama, 2002, 2003, 2004). To avoid false-positive results due to contamination of cell samples with lymphocytes, K. Hiyama et al. (1995) suggested using of 1000 cell equivalents of cell lysate per assay, since proteins extracted from 1000 adult lymphocytes do not show detectable telomerase activity. Nevertheless, this assay is an indispensable method enabling sensitive and quantitative measurement of the telomerase activity.

In addition to the TRAP assay that directly assesses the enzymatic activity, detection of transcript levels of *hTERT* or *hTR* by RT-PCR and detection of hTERT and hTR by *in situ* hybridization techniques are also used in telomerase investigations (Heaphy & Meeker, 2011; E. Hiyama and K. Hiyama, 2002, 2003, 2004). Expression studies of both *hTERT* and *hTR* via RT-PCR allow quantitative measurement of these subunits, although mRNA levels of *hTERT* may not always correlate with telomerase activity due to alternative splicing variants and post-translational modifications of this subunit and *hTR* is constitutively expressed in most of the cell types (Cong et al., 2002; Heaphy & Meeker, 2011). *In situ* hybridization studies of both hTERT and hTR enable detection and visualization of these subunits at single cell level, however, detection of transcripts does not always correlate with telomerase activity and quantitative analysis is almost impossible (Heaphy & Meeker, 2011; E. Hiyama and K. Hiyama, 2004).

The increase in telomerase in cancer cells generally occurs very early during tumorigenesis; i.e. at the preneoplastic or *in situ* stage, telomerase activity may be useful for early detection of cancer, particularly in cytology samples. In some instances telomerase activity might not be very high, but it could show correlation with the tumor progression. Due to space limitations, here we focus predominantly on two types of malignancies that we had experiences: gastric and ovarian cancer. Readers who are interested in the clinical utility of telomerase in other types of cancer can find well-written articles in the literature.

4.2.1 Telomerase in gastric cancer

The prevalence of telomerase activity in gastric cancer has been reported as 70-98% (C.H. Chen and R.J. Chen, 2011). Telomerase activity in gastric lavage fluid has been suggested as a potential tumor marker that might help for early diagnosis of gastric cancer rate (S.C. Wong et al., 2006). Telomerase activity has also been reported in the peritoneal lavage of patients with gastric cancer and shown to be associated with advanced stages of the disease or peritoneal metastasis (Hu et al., 2009; Mori et al., 2000). Clinicopathological significance of telomerase activity in human gastric cancer is controversial. Some investigators have indicated that the telomerase activity in tumor tissues correlates well with depth of invasion and tumor differentiation (Usselman et al., 2001; Yoo et al., 2003). On the other hand, other groups, similar to us, have shown no relation between clinical or histological factors and telomerase activity (Furugori et al., 2000; Gümüş-Akay et al., 2007; Kameshima et al., 2000).

As mentioned before, although telomerase is repressed in most somatic cells, it can be detected in highly proliferative tissues. We have shown that in addition to the tumor samples (98%), normal gastric mucosa samples (95%) also show appreciable telomerase activity (Gümüş-Akay et al., 2007). According to our results, we suggest that if someone has to study the telomerase activity in tumors of such proliferative/regenerative tissues, care must be taken in order to make correct evaluations and telomerase activity should be tested in both tumor sample and their corresponding normal tissue sample obtained from the same patient. Moreover, we have experienced that the telomerase activity in tumor/normal tissues show great inter-individual variability. Therefore, it is difficult to set a general cut-off level of telomerase activity for gastric adenocarcinoma.

The expression of catalytic subunit hTERT and the methylation status of its promoter have also been investigated in gastric cancer samples. Although it is highly expressed in tumor

tissues, there is a disagreement between the studies evaluating the usefulness of *hTERT* expression as a prognostic factor (Gigek et al., 2009; W. Li et al., 2008; Suzuki et al., 2000). It could be concluded that there is no a strict association between telomerase activity and staging or grading of disease with regard to all of information obtained by different research groups. This may explain why detection of telomerase activity have not reached the clinic in terms of predicting outcome for patients with gastric cancer.

4.2.2 Telomerase in ovarian cancer

In ovarian tumors, telomerase activity has been widely investigated among malignant, borderline, and benign lesions (Datar et al., 1999; Zheng et al. 1997). Studies have shown that telomerase activity is increased in about 74-100% of ovarian cancer subjects (C.H. Chen and R.J. Chen, 2011). Rate of telomerase activity in benign and borderline ovarian tumors have produced diverse results. It has been reported that the majority of cystadenomas do not show telomerase activity, while all borderline tumors express this enzyme (Wan et al., 1997). In contrast, Yokoyama et al. (1998) have found that 40% of ovarian cystadenomas have telomerase activity. We have investigated the applicability of telomerase activity in detecting early recurrences of epithelial ovarian cancer after first-line chemotherapy (Ozmen et al., 2008). We have found that although mean telomerase activity was statistically higher in epithelial ovarian cancer patients than patients with benign disease, there was no relation between telomerase activity levels at second-look surgery and early recurrences of epithelial ovarian cancer.

Buttitta et al (2003) have reported that an immunohistochemical study on archival tissue sections showing a moderate to strong nuclear hTERT staining in 86% of serous epithelial ovarian cancers. A significant correlation between hTERT expression and response to platinum-based chemotherapy has been documented in advanced epithelial ovarian cancer patients. However, the prognostic significance of hTERT in epithelial ovarian cancer is still uncertain. Similar to gastric cancer studies, some authors have reported that hTERT expression does not reflect the clinical outcome (Wisman et al., 2003 as cited in Gadducci et al., 2009; Widschwendter et al., 2004), whereas others have showed a poor survival for patients whose tumors displayed moderate to strong hTERT immunostaining (Brustmann, 2005). It has been suggested that telomerase activity might only be used as a supplementary diagnostic procedure beside conventional pathological diagnosis.

4.3 Telomerase as a target for cancer therapy

Since most of the cancer cells and putative cancer stem or stem-like cells have appreciable telomerase activity (E. Hiyama & K. Hiyama, 2003; N.W. Kim et al., 1994; Shay & Bacchetti, 1997; Shay & Wright, 2010) to circumvent the telomere-dependent cellular senescence, inhibiting the telomerase activity by means of different approaches has emerged as potential cancer therapeutics. When compared to other cancer therapy strategies, anti-telomerase approaches seem to be more promising because of the following three main reasons. Firstly, unlike other molecular genetic changes, which show great differences in different types of cancers, telomerase can be accepted as a hallmark of all cancer types. Secondly, it is thought that cancer cells are less likely to develop resistance to anti-telomerase approaches than they are to other cancer targets. Finally, telomerase is a relatively specific cancer/cancer stem cell target because normal somatic

cells show slight or diminished telomerase activity and generally have longer telomeres. This reduces the probability of therapy-related toxicity to normal tissues. At present, two main telomerase targeting methodologies are presently in clinical trials: A direct inhibition of telomerase, and a number of active immunotherapy targeting against catalytic protein subunit hTERT. In addition to these strategies, agents blocking telomerase expression and biogenesis, telomere disrupting agents, and suicide gene therapy are also under study (H. Chen et al., 2009; Harley, 2008).

There has been several strategies that seek to inhibit telomerase activity at its expression and biogenesis levels, including, single-stranded antisense oligonucleotides and small-interfering RNAs that target hTERT mRNA and hTR (Folini et al., 2005; Glukhov et al., 1998; Kraemer et al., 2003; You et al., 2006), ribozyme cleavage of hTERT mRNA and hTR (Hao et al., 2003, 2005; Lu et al., 2011), expression of dominant-negative hTERT protein (Hahn et al., 1999), and Hsp90 inhibitors (Lee and Chung, 2010). However, agents belonging to this category may be more leaky, or less efficient than direct inhibitors. In addition, similar to the gene-therapy based methodologies, this group of agents also has delivery and stability problems (Harley, 2008).

Telomerase activity can also be inhibited by stabilizing the G-quadruplex structure of telomeres by G-quadruplex-interacting agents, like telomestatin (M.Y. Kim et al., 2002). This group of agents inhibits telomerase activity by fixing the single-stranded telomere substrate into a quadruplex structure so as to blocking telomere accessibility. Although several compounds in this class have demonstrated relatively rapid activity in human tumor xenograft studies in mice, their safety is under question, because they can also target endogenous telomeric sequences or G-rich sequences, which might be lethal for normal cells (Ogenasian & Bryan, 2007). Another telomerase based approach in cancer treatment is the *hTERT* promoter-driven cancer cell suicide. Because of its higher tumor specificity, stronger activity, and universal presence in cancer cells have made the *hTERT* promoter encouraging over other known promoters (Gu & Fang, 2003). Bax, caspase 8, and cytosine deaminase (CD) are among the suicide genes that have been used in *hTERT* promoter-driven cancer cell suicide (Gu et al., 2000; Komata et al., 2002; Yu et al., 2011). However, this methodology has not yet received clinical development support (Harley, 2008).

Although gene therapy and antisense strategies have remarkable potential for the treatment of many human diseases, the practicability and clinical utility of these methodologies remain to be confirmed. One of the anti-telomerase approaches that seems to be more practical is the use of small-molecule inhibitors, such as reverse transcriptase inhibitors (Strahl & Blackburn, 1996), BIBR1532 {2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid}, and BIBR1591 {5-morpholin-4-yl-2-[(E)-3-naphthalen-2-yl-but-2-enoylamino]-benzoic acid} to the catalytic component hTERT (Damm et al., 2001). Another group of direct telomerase inhibitors, namely 2'-deoxy and 2'-substituted N-3'→5' NP oligonucleotides (NPS), have been identified, which binds to their complementary sequences on hTR. These oligonucleotides, unlike hTR antisense counterparts, do not activate RNase-H mediated degradation of the hTR. Instead, they inhibit telomerase activity through functioning as a template antagonist (Gryaznov et al., 2001; Shea-Herbert et al., 2002). GRN163 was developed in 2003 as a competitive telomerase inhibitor. This molecule hybridizes to hTR and represses primer binding to telomere. It has been shown that GRN163 suppresses tumor growth without major toxicity (Asai et al., 2003). GRN163 has been further modified by

covalent lipid conjugation in order to improve its pharmacological properties, and named GRN163L. This compound is currently in multiple Phase-I and Phase-I/II clinical trials as potential broad-spectrum anticancer agent (Gryaznov, 2010).

Because of its universal expression and fundamental function in cancer cells, TERT has been regarded as a self antigen useful for developing active telomerase immunotherapy/vaccine strategies. In this approach, TERT immunotherapy products are constructed to stimulate the immune system of the organism to damage and kill TERT expressing tumor cells. In order to achieve this, antigen-presenting cells (APCs) are subjected to peptide fragments or TERT coding gene products *ex vivo* or *in vivo*. Then, APCs cause an activation and propagation of TERT-specific T-cells that kill the cells displaying TERT peptides on their surfaces (Harley, 2008; J.P. Liu et al., 2010). Vaccination with dendritic cells transfected with hTERT mRNA has been shown to induce strong immune responses to multiple hTERT epitopes and is therefore accepted as an attractive approach to more potent immunotherapy (Suso et al., 2011). Clinical trials using telomerase immunotherapy/vaccine approaches involving GRNVAC1, GV1001, Vx001 have been very encouraging, and have involved several types of solid tumors and acute myeloid leukemia (Harley, 2008; Kotsakis et al., 2011; Suso et al., 2011; Vetsika et al., 2011; Vonderheide, 2008).

It is thought that in a well-designed clinical trial, if the p53 statuses of the patients are screened and those patients with an intact p53 are selected, better response rates to the telomerase inhibitors might be expected. On the other hand, tumors deficient for p53 would show high levels of genomic instability so as to gain resistance to other therapeutics in the regimen and could acquire ALT mechanism (K. K. Wong et al., 2006). Although ALT seems to be a potential resistance pathway to telomerase inhibitors, it is unlikely to be a major factor limiting the usefulness of telomerase inhibitors in a clinical setting (Shay & Wright, 2006). The telomerase knockout mouse model has also demonstrated that absence of telomerase and associated telomere dysfunction enhance sensitivity to ionizing radiation and chemotherapeutic agents that generate double strand breaks. Therefore, it is suggested that one particularly attractive clinical trial design would be the potentially synergistic combination of telomerase inhibitors and radiation or break inducing agents (K. K. Wong et al., 2006). In support of this Ward & Autexier (2005) have shown that pharmacological inhibition of telomerase catalytic activity by BIBR1532 can sensitize cells to traditional etoposide, melphalan, and doxorubicin treatments in a telomere length-dependent fashion. Similarly, Incles et al. (2003) demonstrated that a G-quadruplex-interacting agent, BRACO-19, sensitized parental and flavopiridol-resistant human colon carcinoma cell lines.

There are several ongoing phase I and II clinical trials targeting telomerase activity, and phase III clinical trial has been recruited for pancreatic cancer. It is interesting that, none of them telomerase activity has not been used for exclusion or inclusion criteria for patient registration. It also reveals that more accurate, sensitive and robust test for telomerase activity has been requested.

5. Conclusion

Telomeres were first discovered in the 1930s, as the ends of linear chromosomes which have unique molecular structure that prevent end-to-end fusion of different chromosomes. Alexey Olovnikov reported in 1973 that the ends of human chromosomes could not replicate

by previously known replication machineries. This observation implied that cells had an enzyme that could lengthen telomeres. In 1984, Greider and Blackburn discovered that the enzyme telomerase has the ability to lengthen chromosomal ends by its reverse transcriptase activity. In 2009 Blackburn, Greider and Szostak were awarded the Noble Prize in Physiology or Medicine “for the discovery of how chromosomes are protected by telomeres and the enzyme telomerase”.

Thousand studies have been published on telomeres and telomerase, and at the present time it is known that, they are essential for stability of genome as well as regulation of DNA repair pathways. The long and short of it is that they regulate cellular viability and proliferative capacity. Loss of telomerase in differentiated cells limits cell division, while reactivation of telomerase sustains proliferation and transformation. Cancer cells need a mechanism to maintain telomeres if they are going to divide indefinitely, and telomerase solves this problem. This precious function of telomerase causes itself to gain a unique role in cellular immortality and carcinogenesis. In many human primary cancers increased or up-regulated telomerase activity has been reported, and increased telomerase activity has also been demonstrated to be related to prognosis. The discovery of the central role of telomerase in cancer offer entirely new therapy alternatives targeting the telomeres and/or telomerase.

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