Telomeres and telomerase in oncogenesis (Review)

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Abstract. Telomeres are located at the ends of chromosomes and protect them from degradation. Suppressing the activity of telomerase, a telomere-synthesizing enzyme, and maintaining short telomeres is a protective mechanism against cancer in humans. In most human somatic cells, the expression of telomerase reverse transcriptase (TERT) is repressed and telomerase activity is inhibited. This leads to the progressive shortening of telomeres and inhibition of cell growth in a process called replicative senescence. Most types of primary cancer exhibit telomerase activation, which allows uncontrolled cell proliferation. Previous research indicates that TERT activation also affects cancer development through activities other than the canonical function of mediating telomere elongation. Recent studies have improved the understanding of the structure and function of telomeres and telomerase as well as key mechanisms underlying the activation of TERT and its role in oncogenesis. These advances led to a search for drugs that inhibit telomerase as a target for cancer therapy. The present review article summarizes the organization and function of telomeres, their role in carcinogenesis, and advances in telomerase-targeted therapy.

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1. Introduction

Cancer, the second leading cause of death globally, was responsible for an estimated 9.6 million deaths in 2018 (1). An increasing life expectancy extends the period over which oncogenes act on cells and increases the risk of cancer development. The formation and development of cancer is caused by the accumulation of genetic mutations in cells. Cancer is a genetic event in which normal cells accumulate genomic instability and acquire the ability to replicate indefinitely, which is the phenotype of immortality. Telomerase repression and/or short telomeres in human cells are suggested to be a natural evolutionary strategy in the fight against cancer; it functions as a strong barrier to tumor transformation and prevents uncontrolled cell proliferation (2). The basis of oncogenesis is the infinite proliferation of malignant cells, which in most cases is achieved by the activation of telomerase (3).

Telomeres are repeatable (TTAGGG) DNA-protein complexes that protect the ends of chromosomes. They are reduced during cell division in somatic cells. Dysfunctional telomeres may arise as a result of their critical shortening that induce a DNA damage responses (DDR) and cause cellular senescence. If the cells inherit or acquire damage to detect short telomeres they will continue to divide, and the telomeres will continue to shorten and the cells will reach the next phase called the crisis. This leads to the joining of the ends of various chromosomes, pathological mitoses, genomic instability and apoptosis. Some of the cells avoid crisis and activate the telomerase gene, *telomerase reverse transcriptase* (*TERT*), which codes for telomerase, the enzyme responsible for the synthesis of telomere. Telomerase activity allows the cancer cell to have unlimited replication. Although TERT is usually silenced in almost all somatic cells, it is significantly expressed in 85-95% of human cancers (3,4). TERT expression is up-regulated in tumors via multiple genetic and epigenetic mechanisms including: TERT promoter mutations (mainly C228T or C250T), alterations in alternative splicing of TERT pre-mRNA, TERT amplification, epigenetic modifications

through TERT promoter methylation, and/or disruption of telomere position effect (TPE) machinery (1). Rarely, another DNA recombination mechanism called alternative lengthening of telomeres (ALT) is used in ~5-15% of tumors mostly arising from mesenchymal or epithelial tissues (including bone, soft tissues, neuroendocrine systems, and nervous system) (5). The mechanisms that regulate TERT expression and telomerase activity are extensively studied. In addition, telomerase inhibition strategies are used to progressively shorten telomeres and ultimately kill cancer cells. Currently, many drugs that inhibit telomerase in various mechanisms are being evaluated in cancer clinical trials. We present achievements in the field of telomeres and telomerase biology, mechanisms underlying cancer and the development of cancer therapies (6).

2. History

In 1965, L. Hayflick showed that a human diploid cell can divide only a limited number of times (Hayflick limit-about 60 divisions) and there is a gradual inhibition of mitotic activity, called replicative sencence (7). Ołownikow associated the problem of chromosome shortening with sencence (8). Then Szostak and Blackburn (1982), Blackburn (1991) and Greider 1991) described that telomeres shortened 50-200 bp in each division until reaching the critical limit (9-11). The sequential structure of telomeres formed by 5'-TTAGGG-3' repeats and its genomic DNA protection function has been proposed by Moyzis *et al* (12), Makarov *et al* (13), Wellinger and Sen (14). In 2001, Blackburn introduced a telomeric function to maintain chromosome integrity and genome stability (15). In addition, Greider and Blackburn (16), Lendvay *et al* (17), Lingner and Cech (18) presented two telomerase subunits: The telomerase reverse transcriptase catalytic subunit (TERT) and the RNA template (TERC). In 2004, Liu *et al* (19) described that in somatic cells, telomerase remains inactive, but its activity can be found in germ cells and stem cells. In addition, reactivation of telomerase in somatic cells is one way to acquire uninhibited proliferation in cancer. Telomerase activity was detected gradually in approximately 85% of malignant tumors (20,21). In 2013, the presence of C228T and C250T in the TERT promoter mutation in melanoma was reported (22,23). Further studies have shown the presence of these mutations in other cancers, mainly in the central nervous system, bladder, liver, thyroid, and others, described in the following sections of the article. Recent reports explaining the reactivation of telomerase and attempts to inhibit it in malignant cells give hope for its potential use in cancer treatment.

3. Telomere structure and function

Telomeres are nucleoprotein structures located at the ends of chromosomes in eukaryotic cells. Each chromosome has two telomeres and there are 92 telomeres in a diploid human cell. Human telomeric DNA is composed of tandem repeats [10-15 kilobases (kb) at birth] of double-stranded DNA nucleotide sequence 5'-TTAGGG-3', and the final 3' G-rich single-stranded overhang (150-200 nucleotide long), linked by telomere-binding proteins (TBPs) (3).

The telomere spatial structure is created from the 3'G-rich overhang, which invades the homologous double-stranded TTAGGG region and forms a smaller D-loop. Then the larger T-loop is built using protein protective complex called shelterin.

Shelterin has three core subunits: Telomere repeat factor (TRF)-1, TRF2, which recognize and bind duplex TTAGGG repeats, and human protection of telomeres 1 (POT1) which is responsible for recognizing single-stranded TTAGGG overhangs. These three proteins are additionally connected by: TRF1-interacting protein 2 (TIN2), TINT1/PTOP/PIP1 protein (TPP1), and repressor-activator protein 1 (Rap1) (Fig. 1).

TRF1 controls the replication of telomeric DNA, TRF2 participates in the formation of T-loops, prevents the activation of DDR pathways and non-homologous end joining (NHEJ) of telomere (24,25). POT1 (in association with TPP1) combines with 3' single-stranded overhang and inhibits ATR-mediated DDR by preventing the recruitment of replication protein A (26). RAP1 affects the selective binding of TRF2 to telomeric DNA (27). TIN2 combines TRF1 and TRF2 with the TPP1/POT1 heterodimer and with telomeric DNA, improves complex stabilization (28).

The shelterin protein complex plays a fundamental role in homeostasis and telomere end stabilization, and protects chromosome ends from inappropriate DNA repair by preventing the activation of DDR pathways and non-homologous end joining (NHEJ) (29).

The basic function of telomeres is to protect the ends of chromosomes against degradation and loss of genetic information. During cell division, telomeres are shortened and do not bear genetic information essential for the cell. The process is the result of combining the phenomenon of end replication with DNA processing at the ends of chromosomes. During semiconservative replication, the delayed strand (resulting from the combination of Okazaki fragments) after the removal of the RNA primer has an incomplete 5' end. The resulting gap cannot be filled because the DNA polymerases responsible for the replication process synthesize the polynucleotide chain only in the 5' to 3' direction.

Telomeres also protect chromosomes against abnormal recombination, chromosome fusion (prevents chromosomal aberrations, including translocations, duplications, and deletions), or their degradation as a result of an exonuclease attack (30-32). They are a molecular clock that, after exceeding the limit of divisions, directs the cell to the path of replicative senescence or apoptosis. With each division, the telomeric sequence is shortened by approximately 50-150 bp in human somatic cells in cell culture. The time at which the telomere will be critically shortened may vary. It is a consequence of the heterogeneous distribution of telomeres in different chromosome arms and the specific rate of telomere shortening in individual cell lines (33). Research results indicate that the shortest telomere can be a factor stopping further cell division. Telomere shortening causes telomere conformational changes and loss of T-loop formation, promotes genomic instability and, in combination with other oncogenic changes, can potentially stimulate cancer initiation (34).

Telomeres are also involved in functions such as regulation of gene expression through transcriptional silencing of genes located close to the telomeres, which is called telomere position effect (TPE), or those located at long distances from telomeres, termed TPE over long distances (TPE-OLD) (35,36).

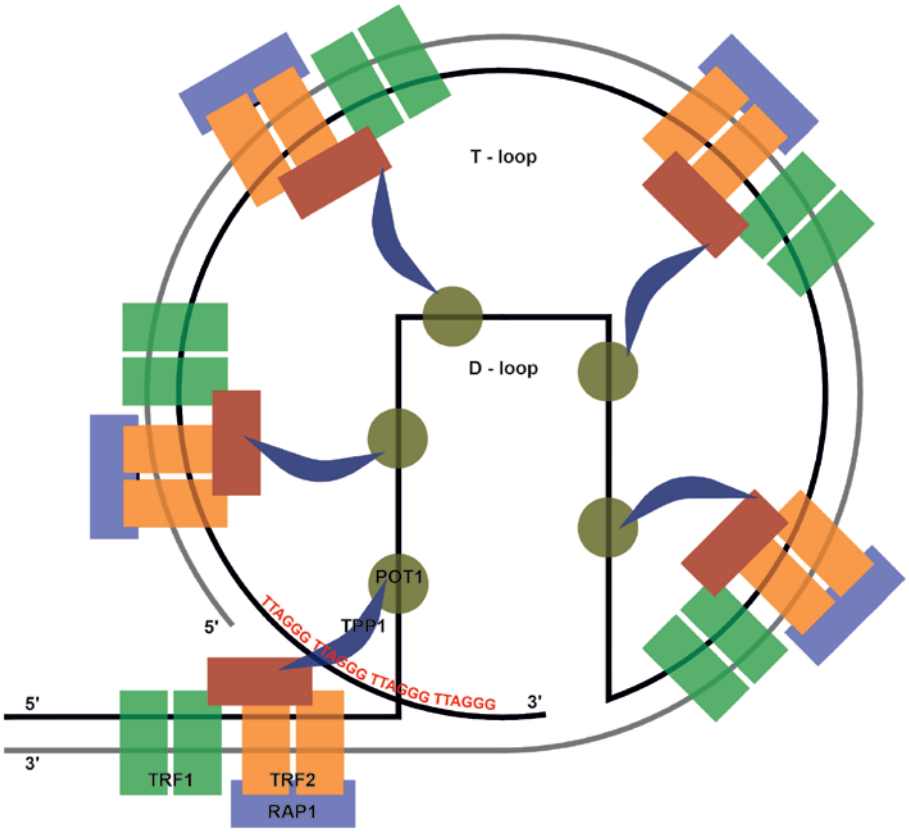

Figure 1. Telomere structure. Telomeric DNA contains tandem repeats of DNA sequence 5'-TTAGGG-3', terminal 3' G-rich overhang and shelterin complex of six subunits: TRF1, TRF2 and POT1 (proteins responsible for recognition of TTAGGG telomeric repeats), and TIN2, TPP1 and RAP1 (complex stabilizing proteins). The telomere structure forms two loops, the T-loop and the D-loop. TRF1, telomere repeat factor-1; TRF2, telomere repeat factor-2; POT1, protection of telomeres-1; TIN2, TRF1 interacting protein-2; RAP1, repressor/activator protein 1; TPP1, TINT1/PTOP/PIP1 protein (POT1-TIN2 organizing protein).

4. Telomerase

Most of the telomeric DNA is copied in the replication process. However, telomere deficiency is supplemented by telomerase-catalyzed elongation.

Telomerase, an enzyme made of protein subunits and RNA, comprises a catalytic subunit with reverse transcriptase activity (TERT-telomerase reverse transcriptase), an RNA template (TERC-telomerase RNA component) with a sequence complementary to the sequence of the telomere, and accessory proteins such as discerin, NHP2 ribonucleoprotein, NOP10 ribonucleoprotein, and GAR1 (localization factor). The TERT subunit binds to TERC and the protein complex. Telomerase is recruited to single stranded telomeric DNA through interaction with the telomere-localizing protein TPP1. Additional factors such as the chaperones HSP90 and p23, a WD-repeat-containing protein 79 called TCAB1, as well as the ATPases pontin and reptin are also involved in this process. SRSF11 (TERC-blinding protein) stabilizes the telomerase-telomere complex (37) (Fig. 2).

TERT synthesizes telomeric sequences using TERC as a template. The expression or activity of TERT is regulated at many stages by various factors. Induction of TERT mRNA expression requires binding of the transcription factors c-MYC and SP1 to the E-box (5'-CACGTG-3') and five GC boxes (5'-GGGCGG-3') (38). Other transcription factors such as E2F, AP-1, estrogen response element (ERE) for estrogen receptor α binding, and CCCTC binding factor are involved in the activation of TERT transcription (38). The phosphatidylinositol-3 kinase (PI3K)/AKT pathway enhances TERT activity at the post-translational level via TERT phosphorylation by AKT (39).

Telomerase is activated in germline, hematopoietic, stem and mitotically active, and rapidly regenerating cells. In contrast, telomerase activity is very low or absent in somatic cells, although telomerase activity has been found in normal human blood cells, proliferative basal skin layer, endometrial tissue, intestinal crypt proliferative zone, and hair follicles (40-44).

Telomerase repression and/or shorter telomeres in human cells prevent uncontrolled cell proliferation (2). Lack of telomerase leads to a progressive shortening of telomeres during division because of the nature of DNA polymerase. When the telomere length reaches a critical size, DNA damage occurs and the stage of growth arrest called replicative senescence is activated.

By contrast, cells with strong proliferative potential are characterized by high telomerase activity. These cells include stem cells and embryonic and progenitor cells of the hematopoietic system, skin, and intestinal crypts. Telomerase activity is closely related to the life stages of the body. The enzyme is active during embryonic development.

Cancer cells are characterized by high telomerase activity, which enables cells to divide indefinitely. Telomerase is active in

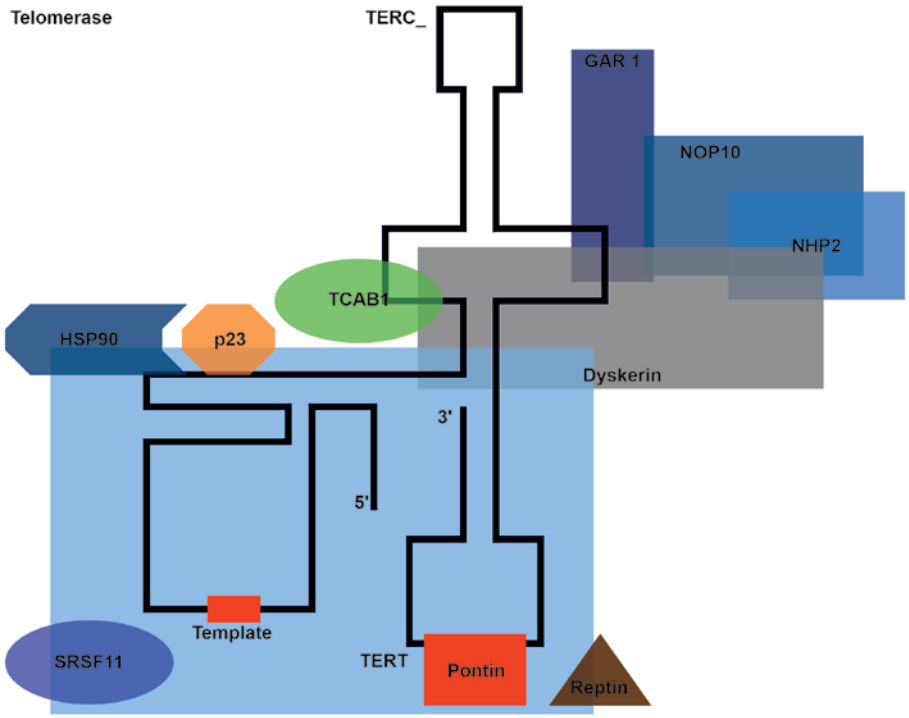

Figure 2. Telomerase structure. Human telomerase is composed of TERC-telomerase RNA component (RNA template), TERT-telomerase reverse transcriptase (catalytic subunit with reverse transcriptase activity) and the accessory proteins. NHP2, non-histone protein 2; NOP10, nucleolar protein 10; GAR1, glycine arginine rich 1; TCAB1, telomerase Cajal body protein 1; HSP90, heat shock protein 90; SRSF11, serine and arginine rich splicing factor 11; *TERC*, telomerase RNA component; *TERT*, telomerase reverse transcriptase.

85-95% of cancers (3,4). The exception is cancer cells possessing an active Alternative Lengthening of Telomeres (ALT) pathway. ALT, which is the ability of cancer cells to extend telomeres in the absence of telomerase, is based on homologous recombination using telomeric DNA as a matrix (45). ALT activation correlates with the presence of mutations in the genes encoding α-thalassemia/mental retardation X-linked chromatin remodeler and death domain associated protein in both tumors and cell lines (46). This process is observed in aggressive, difficult to treat tumors of mesenchymal origin, which account for approximately 5-15% of all cancers (47).

TERT induction and telomerase activation not only create unlimited cancer cell proliferation potential by stabilizing telomere length (telomere lengthening-dependent), but also cause oncogenic effects independently of the telomere lengthening function. The telomere lengthening-independent functions of TERT, which significantly contribute to cancer initiation or progression, include its effects on mitochondrial and ubiquitin-proteasomal function, DNA damage repair, gene transcription, microRNA (miRNA) expression, RNA-dependent RNA polymerase activity, and epithelial-mesenchymal transition (48-56). These TERT activities physiologically affect the processes that ultimately lead to cell aging; however, they also drive cancer development by conferring survival, proliferation, motherhood, and invasive phenotypes.

Several signalling pathways (mainly c-MYC, NF-κB, B-Catenin) are involved in the transcriptional reactivation of TERT in cancer cells. Additionally PI3K/AKT kinase pathway enhances TERT activity at the posttranslational level via phosphorylation. TERT activation occurs as a result of c-MYC binding to the E-box (5'-CACGTG-3') in the TERT promoter region. There is an increase in vascular cell viability and stimulation of c-MYC-dependent oncogenesis potential as a result of induction of transcriptional activity of TERT, stabilization of c-MYC levels on chromatin and c-MYC ubiquitination and proteosomal degradation (57). NF-κB controls the transcription of TERT via NF-κB binding sites in the TERT promoter specific for p50 and p65.

This pathway induces TERT transcription activities and additionally leads to repression of ROS-dependent activation, modulation of TERT nuclear translocation, recruitment of IL6 and TNF alpha, and upregulation of MMP. Repression of ROS-dependent activation, inflammation and cancer progression occurs as a result of these activities (54,58,59). Wnt/B-Catenin is another pathway involved in the regulation of TERT. Activation of Wnt-depend reporters results in stem cell pluripotency, cell proliferation, and cancer progression (60).

5. Telomeres as tumor suppressors

Divisions of human cells that lack telomerase activity lead to shortening of telomeres and disruption of their spatial structure (they lose the ability to form T loops). Telomere shortening is a natural consequence of cell division due to the 'end replication problem' and leads to critically shortened telomeres that trigger DDR. The DNA damage detection pathways by chromosomal instability and p53 or p16-RB pathway activation are induced, and the cell enters a stage named replicative senescence. This is thought to be an effective barrier against cancer by blocking proliferation and genetic mutations resulting from DNA replication. Telomeres act as tumor suppressors. If the cells acquire a mutation in the gene encoding p53, which is

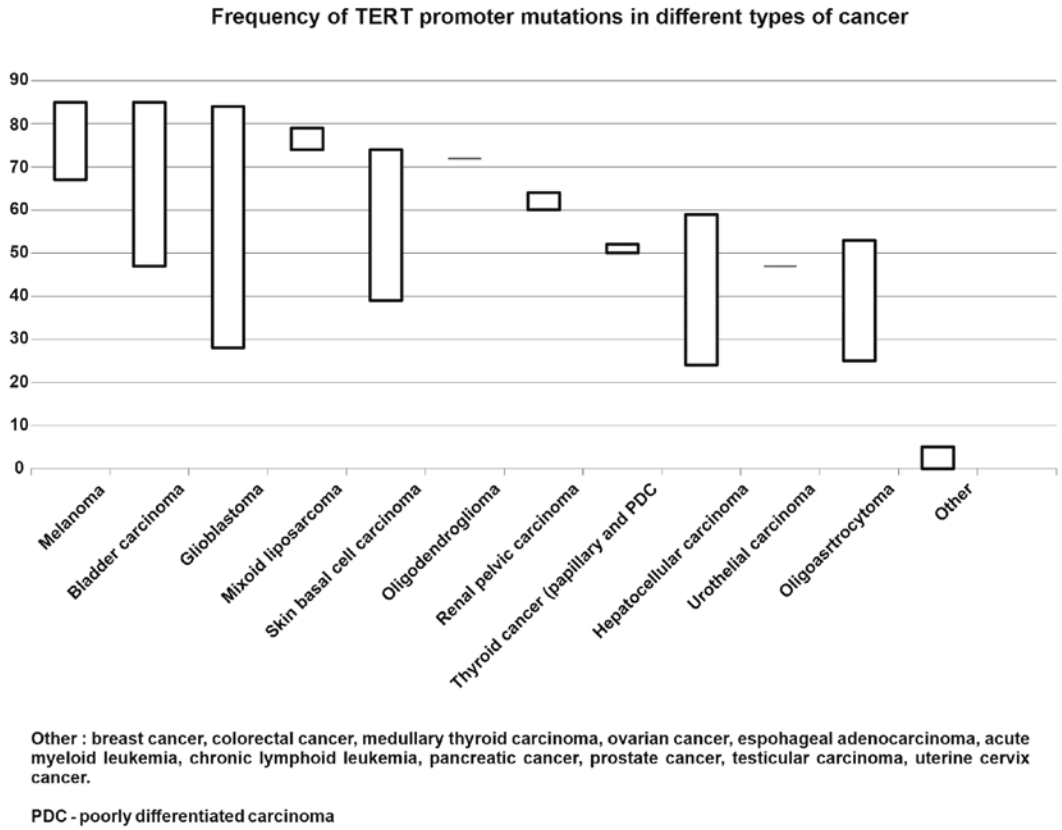

Figure 3. Frequency of TERT promoter mutations in different types of cancer. Data were obtained from a previous study (37). TERT, telomerase reverse transcriptase; PDC, poorly differentiated carcinoma.

responsible for detecting short telomeres or other checkpoint proteins, they can overcome senescence. The cells will continue to proliferate until telomeres become critically short and then will be directed to apoptosis. As a result of various disorders, some cells (~$1/10^7$) activate telomerase or ALT and acquire the potential for endless proliferation, which makes them immortal (31). Most often this condition is achieved by upregulation or reactivation of telomerase. The rare telomerase negative immortalization pathway termed ALT involves DNA recombination to maintain telomeres.

6. Mechanisms activating TERT transcription and telomerase in human cancer

The *TERT* gene is located on the short arm of chromosome 5. The 433-bp genomic region encompassing 52 CpG sites located immediately upstream of the *TERT* core promoter region may bind to transcription factors or repressors (61). This region upstream of the *TERT* promoter is unmethylated in normal human cells, whereas it is methylated in malignant cells. There is also evidence that the unmethylated region upstream of the promoter core sequence is responsible for binding to the repressor (62). Transcriptional regulation of the *TERT* gene occurs at many levels and is mediated by various positive and negative factors or signaling pathways (4). These factors control the *TERT* gene and ensure inhibition of TERT activity in most normal cells, as well as its expression at the right time and place in a small number of cell types such as activated lymphocytes or stem cells. This balance may be disturbed in malignant cells. A typical example is the Myc/Max/Mad1 protein. Endogenous expression of the cellular c-MYC oncogene may result in dissociation of the Mad1/Max repressor from the E-box complex, leading to de-repression of the *TERT* gene and telomerase activation (63).

Epigenetic factors responsible for DNA methylation, histone acetylation, methylation, and phosphorylation are another group of factors modulating TERT transcription. As mentioned, *TERT* promoter methylation is required for the expression of TERT and activation of telomerase in cancer cells (62).

Some viruses may code for proteins that act as cofactors to stimulate *TERT* transcription as well. These include Epstein-Barr virus, cytomegalovirus, Kaposi sarcoma-associated herpesvirus, human papillomavirus, hepatitis B virus, hepatitis C virus, and human T-cell leukemia virus-1 (64).

TERT expression and telomerase activity in tumors are also affected by TERT promoter mutations, which occur mainly at two active points of chromosome 5, C228T and C250T. The incidence of TERT promoter mutations varies from undetectable to over 90% in various human malignancies. The highest TERT promoter mutation rates (up to 80-90%) occur in glioblastoma, melanoma, bladder urothelial carcinoma, and brain lower-grade glioma. An intermediate mutation frequency range is observed in liver hepatocellular carcinoma and thyroid carcinoma. The lowest level of mutation frequency (<10%) is detected in kidney, lung, prostate, and gastrointestinal cancers and in leukemia (65). The mutation frequency of the TERT promoter varies depending on the type of cancer (Fig. 3) (22,37,66-72). The C228T mutation is more common than the C250T mutation. These mutations form

the binding site for E-twenty six (ETS) transcription factors. In addition, GABPA and GABPB1 (ETS family members) binding proteins form heterotetramers that bind to the de novo ETS site and activate *TERT* transcription (73).

The presence of the TERT promoter mutation is negatively correlated with telomere length and is associated with older patients (4). Telomere damage causing their dysfunction and genomic instability, which are often observed in old age, may be due to short telomeres (3).

Some viruses may code for proteins that act as cofactors to stimulate TERT transcription as well. These include Epstein-Barr virus, cytomegalovirus, Kaposi sarcoma-associated herpesvirus, human papillomavirus, hepatitis B virus, hepatitis C virus, and human T-cell leukemia virus-1 (64). Targeted activation is one of the key mechanisms of carcinogenesis through a virus. For example, HPV E6 is a viral oncoprotein that forms a complex with E6AP and c-Myc. This complex binds to the E-box in the TERT core promoter and induces TERT activation. Another well-studied cofactor is the CMV early protein 72, which by interacting with Sp1 activates TERT transcription (64). Recent studies indicate that rearrangements and insertions of the oncogenic viral genome at the TERT locus are new mechanisms underlying increased expression of TERT by hijacking enhancers (74). Recent evidence suggests that viral DNA integration at the TERT locus may trigger an additional TERT regulatory mechanism; an exogenous viral enhancer was found to drive endogenous TERT transcription (74). Enhancers regulate gene transcription by interacting with gene promoters, regardless of their position relative to the place of transcription initiation. These factors make DNA more accessible to the transcription machine. Functionally, rearrangements and onco-viral DNA cause active enhancers to affect the TERT gene and increase TERT expression (75).

High-throughput new generation sequencing of human cancers has also provided genomic information on *TERT* amplification and the important role of this genomic aberration in telomerase activation during cancer development. Barthel *et al* (65) analyzed more than 6,000 cancer patients and found that 4% of the studied tumors show *TERT* gene amplification with high frequency in ovarian cancer, adrenal cortical cancer, esophageal cancer, and lung cancer. In addition, the highest telomerase activity was found in tumors with *TERT* amplification.

7. TERT promoter mutations-cancer-specific biomarkers in diagnostics/screening

Telomerase repression in combination with shorter telomeres are protective mechanisms against cancer. Human somatic cells achieve malignant transformation through TERT gene de-repression/telomerase reactivation in most cases. The assessment of cancer-specific expression of TERT or telomerase activation is the experimental field for the potential clinical application of cancer tests (3).

However, there have been difficulties in using reliable tests to evaluate TERT or telomerase activity for diagnostic or screening purposes.

On the one hand, false positive results of TERT expression found in lymphocytes associated with inflammation or infiltrating tumors. On the other hand, telomerase and mRNA TERT are temperature sensitive, so managing them is difficult and at the same time requires high quality tissue samples. An additional problem is the specificity of the available TERT antibodies for immune-histochemical staining or immunoblotting (4).

Researchers have mainly focused on assessing TERT promoter mutations in various cancers. The presence of the TERT promoter mutation in human malignancies and its absence from normal cells creates new cancer-specific markers. DNA stability enables the routine use of mutation analysis. Detection of the mutant TERT promoter in plasma, urine, and cerebral spinal fluid (CSF) serves as a useful biomarker in hepatocellular carcinoma, bladder cancer, and glioma, respectively (76-78). In addition, the detection of methylated CpG in the TERT promoter region in the feces may be useful for the diagnosis of gastrointestinal cancer (79). Evaluation of the methylated TERT promoter in CSF may be a useful non-invasive diagnostics tool for predicting metastases to the meninges (80). In addition, analysis of circulating oncogenic miRNAs targeting TERT expression can be a useful diagnostic tool. miRNA assessment in blood can be a valuable biomarker in cancer (81).

8. TERT promoter mutations-prognostic factors in cancer/sign of aggressiveness

Abnormal expression of TERT and hypermethylation of the TERT promoter serve as prognostic factors in many types of human cancer. The presence of the TERT promoter mutation is an unfavorable prognostic factor (metastasis/survival) in melanoma and glioma (82,83). In thyroid cancers, mutation of the TERT promoter is associated with poor biological characteristics and a low rate of survival, and includes differentiated thyroid cancers with aggressive clinical behavior, poorly differentiated thyroid cancers (PDTC), and anaplastic thyroid cancers (ATC) (84,85). The association of TERT promoter hypermethylation with poor results and cancer prognosis in brain tumors and adrenal cortical cancer has also been reported (86,87).

The relationship between the coexistence of TERT promoter and *BRAF V600E* mutations and a poor disease course has been reported in thyroid cancer and melanoma (88,89). The synergy in the coexistence of TERT promoter and *BRAF V600E* mutations is most likely caused by the activation of the mitogen-activated protein kinase (MAPK) and/or PI3-Akt pathways, which upregulate ETS transcription factors and trigger the expression of TERT. Recently, Liu *et al* (90) demonstrated that *BRAF V600E* enhances the activation of the MAPK pathway, leading to the FOS-mediated expression of GABPB, which binds to TERT mutated promoters and induces TERT expression. In addition, a significant relationship was observed between TERT promoter mutations and RAS mutations that often occur in PDTC and ATC (85).

Liu *et al* (91) reported that KRAS mutations increase TERT mRNA expression by activating the RAS/MEK pathway, which contributes to the aggressive phenotype of non-small cell lung cancer (NSCLC).

9. Telomere shortening in cancer and its potential advantage

Although tumors with telomerase activation acquire the ability to extend the telomere, telomere length is shorter in prostate

Table I. Main research areas using telomerase as a therapeutic target.

Author, year	Strategy	Factor		Target	Mechanism	Effect	(Refs.)
Frink *et al*, 2016; Burchett *et al*, 2014; Shammas *et al*, 2008; Dikmen *et al*, 2005; Tokcaer-Keskin *et al*, 2010; Burchett *et al*, 2017; Koziel *et al*, 2015; Wu *et al*, 2017	Antisense oligonucleotides (ASO)	GRN163L (Imetelstat)		RNA template of telomerase component (TERC)	TERC competitive antagonist. Blocking recruitment to telomeric DNA. Expression inhibition.	Telomerase inhibition	(94,96-102)
Chhabra *et al*, 2018; Schrank *et al*, 2018 Pitman *et al*, 2013; Puri *et al*, 2014; Wojdyla *et al*, 2014; Weng *et al*, 2010	Uncapping mimicking	T-oligos		Telomere 3' overhang region.	Shelterin complex proteins dissociation. Ataxia telangiectasia mutated (ATM) pathway activation. Cytotoxic effects-inducing DNA damage responses (DDR).	Cycle arrest, apoptosis	(103-108)
Guzman *et al*, 2018; Zhou *et al*, 2016; Mitomo *et al*, 2008; Zhang *et al*, 2015; Melnik *et al*, 2015; Yang *et al*, 2015; Nguyen *et al*, 2017;	Expression modulators	TERT/TERC-miRNA TERT/TERC anti-miRNA		Suppressor miRNAs Oncogenic miRNAs	TERT/TERC-targeted suppressor miRNAs. TERT/TERC targeted oncogenic anti-miRNAs	Post-transcriptional gene silencing. Expression repression	(109-115)
Mizukoshi *et al*, 2019; Staff *et al*, 2014; Fenoglio *et al*, 2013; Fenoglio *et al*, 2015; Lilleby *et al*, 2017; Kotsakis *et al*, 2014	Anti-telomerase immunotherapy	Peptide Vaccines	GV1001 GX301 UV1 Vx001	HLA, HLA-A	CD4+/CD8+ T cells stimulation	[116-121] [116,126,127]	(116-121)
Mizukoshi *et al*, 2019; Su *et al*, 2005; Khoury *et al*, 2017; Galati *et al*, 2018; Salazar-Onfray *et al*, 2013		GRNVAC1 TAPCells vaccine		TERT-Targeting Dendritic cells	Antigen presentation by dendritic cells transfected with TERT-mRNA		(116,122-125)
Mizukoshi *et al*, 2019; Thalmensi *et al*, 2016; Yan *et al*, 2013		DNA Vaccines	phTERT INVAC-1	HLA	CD4+/CD8+ T cells stimulation		(116,126,127)
Jordheim *et al*, 2013	Reverse transcriptase inhibitors	AZT azidothymidine		DNA elongation	Replication termination	Induction of telomere shortening/Inhibition of tumor cell proliferation	(129)
Pascolo *et al*, 2002		BIBR 1532		Reverse transcriptase	Non-competitive inhibition		(130)
Kim *et al*, 2002; Gomez *et al*, 2016	G-quadruplex stabilization	Telomestatin BRACO-1910 RHPS4		Telomeric DNA	Telomerase to telomere endings access blocking	Telomeres erosion induction/Cell cycle inhibition	(131,132)
Nemunaitis *et al*, 2010; Schepelmann *et al*, 2007	Gene therapy	Telomelysin		TERT positive cells	Chimeric gene introduction	Cancer cell lysis	(133,134)

cancer than in normal tissues (92). Recent genome-wide analyses have shown that 70% of the cohorts have shorter telomeres than normal samples (65). Cancer cells with short telomeres show upregulation of interferon-stimulated genes, which is likely to contribute to the malignancy of the tumor. In addition, truncated telomeres facilitate cancer evolution, resulting in moderate chromosome instability (93). Theoretically, genetic or pharmacological inhibition of telomerase activity in TERT-positive cancer cells should have an antitumor effect. In addition, we would expect the anticancer effect of telomerase inhibition to appear earlier in cancer cells with shorter telomeres. In fact, a short telomeric length may be a predictive biomarker of the efficacy of telomerase inhibitors (94). The Imetelstat telomerase inhibitor increases the median progression-free survival and overall survival of NSCLC patients with short telomeres (95).

10. Telomeres as a possible therapeutic target

Telomerase is expressed in most types of cancer and in cancer stem cells and is the focus of cancer treatments. Normal human cells have lower telomerase activity and usually have longer telomeres than cancer cells. The main point of anti-telomerase therapy is the selective destruction of cancer cells while minimizing the effect on normal cells (due to the presence of longer telomeres compared to cancer cells). Many therapeutic approaches have been adopted to achieve this goal (Table I).

Oligonucleotides. Therapies based on telomerase-targeted oligonucleotides include GRN163L (Imetelstat). This 13-mer oligonucleotide sequence is a competitive antagonist that binds to the TERC template region. It blocks its recruitment to telomeric DNA and leads to complete inhibition of telomerase activity. Imetelstat has been studied in glioblastoma, bladder, breast, liver, prostate, and pancreatic cancer and shows promising antitumor activity, although it is too toxic as a stand-alone therapy (94). GRN163L treated pancreatic cancer and myeloma cells showed growth similar to untreated cells for the first 3-8 and 3-5 weeks, respectively, but later began to undergo progressive aging and apoptosis (96,97). Significant reduction of rapid cellular attachment and reduction of metastatic lesions has been demonstrated for lung cancer cells expressing A549-luciferase treated with GRN163L (98). Despite promising anti-cancer effects, the use of GRN163L in clinical settings is limited due to its hematological toxicity. Neutropenia and thrombocytopenia require frequent drug holidays, limiting the effectiveness of GRN163L as a therapeutic agent (95). Another problem associated with GRN163L is its harmful effect on mesenchymal stem cells. A change in mesenchymal stem cell morphology, loss of adhesion, and G1 phase arrest of the cell cycle have been demonstrated (99). Although the use of GRN163L alone is currently ineffective, it has been shown to have promising effects in combination with other molecularly targeted drugs or in the sensitization of cancer cells to radiation therapy (100-102).

T-oligos homologous to the 3'-telomeric overhang are also promising oligonucleotides that have demonstrated anti-cancer activity. They dissociate shelterin complex proteins, activate the ataxia telangiectasia mutated pathway, and exert cytotoxic effects by inducing the DDR (103). However, rapid degradation by nucleases and an incomplete explanation of its mechanism of action remain obstacles to the introduction of T-oligos into clinical trials (104). They dissociate shelterin complex proteins, activate the ataxia telangiectasia mutated pathway (ATM) and its downstream effectors p53, pRb, E2F1, cdk2, and p95/NBS1 and exert cytotoxic effects by inducing the DDR. Two models explain the mechanism of anti-cancer action by T-oligo. The first assumes that T-oligo accumulating in the nucleus are detected by homology to the telomere as damaged DNA and DDR activation occurs. The second model assumes the action of T-oligos by dissociating shelter proteins, thereby exposing the telomere overhang and inducing responses to DNA damage (104). T-oligo anti-tumor activity has been demonstrated in vitro in cancers such as melanoma, lymphoma, lung, breast, prostate, pancreas, colorectal and ovarian cancer (104-106). However, rapid degradation by nucleases and an incomplete explanation of its mechanism of action remain obstacles to the introduction of T-oligos into clinical trials (104). Therefore, the efforts of researchers are directed to the search for methods of inhibition by nucleases. In addition, the use of T-oligo in combined therapies has shown promising results. Additive inhibition has been demonstrated in combination with an EGFR (gefitinib) inhibitor in colorectal cancer (107). T-oligo also increases the sensitivity of breast cancer cells to radiation therapy (108).

miRNAs are an endogenous group of oligonucleotides that regulate gene expression at the post-transcriptional level, effectively silencing genes by interacting with the RNA-Induced Silencing Complex. Carcinogenic miRNAs may exist as oncogenic miRNAs or suppressor miRNAs that promote or inhibit the development of cancer, respectively. Several miRNAs (miR-128, miR-138, miR-1182, miR-342, miR-491, and miR-541) negatively regulate the expression of the *TERT* gene, thereby acting as tumor suppressor miRNAs (109). Overexpression of miR-138 has been shown to inhibit cell proliferation, invasion and induce apoptosis in cervical cancer (110). In contrast, a decrease in the level of miR-138 is observed in anaplastic thyroid cancer (111). Another study found that miR-1182 reduced the proliferation and migration of gastric cancer cells (112). In contrast, miR-128 can act as either oncogenic or suppressor miRNA, depending on interaction with various targets (109). TERC-targeted miRNAs may act as telomerase inhibitors and are being tested in clinical applications. Unfortunately, inhibition of telomerase induces an anti-tumor effect when they lead to a critical shortening of telomere, usually after weeks of treatment. In addition, the effect of miRNA reducing telomerase activity in stem cells requires further study. Oncogenic miRNAs such as miR-21 that cause tumor transformation by regulating TERT expression have been identified. The participation of miR-21 in melanoma, colorectal cancer or glioma has been described (113,114). The use of anti-miRNAs that are antisense to target miRNAs and block their action has been reported. miRNA inhibition in cancer treatment remains in the preclinical stages (115).

Anti-telomerase immunotherapy. Telomerase-based vaccines sensitize immune cells to cancer cells expressing TERT peptides as surface antigens via the human leukocyte antigen (HLA) class I and class II pathways. The expansion of $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTL) specific for

oncogenic telomerase causes T cells to kill telomerase-positive tumor cells (116).

TERT-targeted peptide vaccines. GV1001 is the most advanced of all TERT vaccines. It induces specific T cell responses in pancreatic cancer, NSCLC, and melanoma (117). It is a MHC class II restricted peptide vaccine that elicits strong $CD4^+$ and $CD8^+$ T cell responses and cytotoxic T lymphocytes (CTL) activation. Clinical studies have shown that it induces T cell responses in 50-80% of patients with advanced pancreatic cancer and lung cancer without clinical toxicity. The vaccine did not affect bone marrow cells (116). GX301, a vaccine consisting of four peptides derived from TERT, is more effective than single-peptide vaccines; it has been tested in patients with prostate cancer and kidney cancer (118). The results of these studies indicate that multi-peptide vaccines are more effective because they enhance the immune response in more responders than single-peptide vaccines (119). Immune responses to UV1 or Vx-001 vaccines were demonstrated in prostate cancer and NSCLC, respectively (120,121). The first of these induced an immune response in 85.7% of patients and reduced prostate-specific antigen (PSA) levels in 64% of patients with metastatic prostate cancer (120). Similarly, the second vaccine elicited a strong TERT-specific immune response in NSCLC and had a good tolerance profile (121).

Immunotherapy using TERT-targeting dendritic cells (DC). DCs are the strongest antigen presenting cells and play an important role in inducing immunity. GRNVAC1 is a DC-based cancer vaccine that elicits a polyclonal immune response. Previous clinical studies have shown that GRNVAC1 is effective, safe and well tolerated in patients with prostate cancer and acute myeloid leukemia (122,123).

Another DC-based approach is to produce therapeutic dendritic-like cells called tumor antigen presenting cells (TAPCells) (124). This vaccine has been evaluated in patients with advanced stage melanoma and castration-resistant prostate cancer. It increased the survival of patients with melanoma and extended the doubling time of PSA (125).

DNA vaccines. The genome encoding the TERT peptide can be created using recombinant DNA technology. Plasmids containing these genomes can be delivered to antigen presenting cells, which improves the efficiency of epitope presentation in T lymphocytes. phTERT is a DNA-based vaccine that codes for TERT, whereas INVAC-1 is a plasmid that codes for the inactive form of TERT (126). The phTERT and INVAC-1 vaccines inhibited tumor proliferation and prolonged survival in the HPV-related and melanoma-related tumor model, respectively (126,127).

Gene-modified T-cell therapy. This is another promising method based on the use of T cells genetically modified to produce the T-cell receptor, which recognizes tumor antigens and their epitopes (128).

Nucleosides. Azidothymidine (AZT) was the first reported telomerase inhibitor. AZT inhibits telomerase irreversibly. AZT has a synergistic effect with paclitaxel and significantly enhances its effect. It is used to treat many human cancers associated with viruses, whereas in non-viral tumors, it causes some degree of regression (129). Acyclic nucleoside analogs such us acyclovir, ganciclovir, and penciclovir have been identified as inhibitors or antagonists of telomerase.

Small molecule inhibitors. The BIBR1532 acid is a non-nucleotide small molecule compound that selectively inhibits telomerase activity by non-competitive binding to the active site of TERT (130). Although preclinical studies on breast and prostate cancer cell lines have shown good results, no further inclusion in clinical trials has been reported.

Stabilization of G quadruplexes. Stabilization of G-quadruplexes prevents TERC from recognizing the unfolded single-stranded telomere overhang, thereby inhibiting telomerase activity. Compounds that stabilize G-quadruplexes include telomestatin, RHPS4, and BRACO19 (131). Other compounds that stabilize G-quadruplexes and inhibit telomerase are being investigated. Daunomycin, distamycin A, ascididemin and meridine, berberine, cryptolepine derivatives, and cationic porphyrins are being studied as possible telomerase inhibitors because of their ability to bind and stabilize G-quadruplexes (132).

Gene therapy. Imetelstat (GRN163L) is one of the best known gene therapy molecules, it was previously described in paragraph 10 (133). Other strategies include the introduction of a chimeric gene, namely, the coding sequence of a proapoptotic protein under the control of the TERT gene promoter. Telomelysine is an attenuated adenovirus-5 vector that induces lysis of cancer cells overexpressing TERT (133). Another approach involves the use of viral vectors that have been genetically modified to encode a cytotoxic prodrug activating enzyme (134).

Targeting telomeres and telomerase-associated proteins. Therapeutic approaches based on targeting telomerase-related proteins have been investigated. One of these strategies is targeting tankyrase (the enzyme responsible for the correct separation of chromosomes) with PARP inhibitors. Also interesting is the inhibition of the chaperone HSP90 (required for maturation and activation of telomerase) by Geldanamycin (135). Molecules against TRF1, TRF2, and TIN2 or POT1 have also been analyzed (136).

Telomerase inhibitors derived from natural products and microbial sources. Natural products capable of telomerase inhibition as potential chemotherapeutic agents for cancer treatment have been identified. Natural products from plants with activity against telomerase include polyphenols (curcumin, quercetin, resveratrol, and tannic acid), alkaloids (boldine and berberine), terpenoids (pristimerin and oleanane), and xanthones (gambogic acid and gambogenic acid) (137). Oleic acid is a fatty acid that occurs in various animal and vegetable oils, is classified as omega-9 monounsaturated fatty acid, and has been identified as a human telomerase inhibitor (138). *Actinomycetes spp*. are microorganisms containing benzofuran and benzodipyrane rings that act as telomerase inhibitors. Rubromycins isolated from *Streptomyces collinus*

that have telomerase inhibitory activity have recently been studied (138).

Telomerase and oxidative stress. Studies have reported telomerase damage induced by reactive oxygen species (ROS). Antioxidant enzymes such as peroxiredoxin 1 (PRDX1) and the nudix phosphohydrolase superfamily enzyme (MTH1) protect telomeres against oxidative stress. Cancer cells are more vulnerable to ROS than noncancer cells. Combination therapy with ROS-inducing chemotherapeutic agents and inhibitors of proteins that protect telomeres from ROS (such as PRDX1 and MTH1) could selectively target telomere maintenance in cancer cells (139).

11. Conclusions

Understanding the structure of telomeres and the mechanism of action of telomerase provides hope for the identification of new forms of cancer treatment. The high specificity of telomerase and the potential for inhibiting its activity at various stages underlie its value as a target for cancer therapy. Many *in vitro* studies of telomerase inhibition have been conducted to date, and clinical trials are in the early stages. The combination of telomerase inhibitory agents with cytostatic drugs or radiation therapy increases the effectiveness of existing therapies. The results so far point to the huge possibilities of using telomerase for the development of new targeted therapies and for improving the efficacy of current anticancer drugs. Further studies are required to develop TERT-based cancer therapeutic interventions.

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Authors' contributions

TT and AlK developed the concept. TT and AlK developed the methodology. TT, ArK, SG and AlK were involved in validation. TT, ArK, SG and AlK prepared the original draft. TT, ArK, SG and AlK reviewed and edited the manuscript. TT, ArK and AlK were responsible for visualization. TT and AlK supervised the study. TT and AlK were involved in project administration. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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