Telomeres and Cancer

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Telomeres cap the ends of eukaryotic chromosomes and are indispensable chromatin structures for genome protection and replication. Telomere length maintenance has been attributed to several functional modulators, including telomerase, the shelterin complex, and the CST complex, synergizing with DNA replication, repair, and the RNA metabolism pathway components. As dysfunctional telomere maintenance and telomerase activation are associated with several human diseases, including cancer, the molecular mechanisms behind telomere length regulation and protection need particular emphasis. Cancer cells exhibit telomerase activation, enabling replicative immortality. Telomerase reverse transcriptase (TERT) activation is involved in cancer development through diverse activities other than mediating telomere elongation.

telomerase | telomerase reverse transcriptase | shelterin | CST | promoter mutations

1. Introduction

Cancer is notorious as it can attack any part of the body, rapidly grow beyond its usual boundaries, invade adjoining tissues, and spread to other organs, resulting in uncontrolled proliferation and eventually death. Nearly 10 million deaths were reported in 2020 from cancer, and the risk of getting cancer in a lifetime (before the age of 75 years) is 20% [1]. The most common newly-diagnosed cancers reported worldwide include breast (2.26 million), lung (2.21 million), and colorectal (1.98 million) cancers [1]. Approximately half of the newly diagnosed lung (57%) and pancreatic (52%) cancer cases in the United States are at an advanced or metastatic stage, and the majority of these patients with an early diagnosis of the disease eventually develop tumor progression [2]. The 5-year relative survival rates for advanced-stage cancers, such as lung, colorectal, liver, and pancreatic, remain low, ranging from 3% to 14% even after maximal surgical excision, radiation, chemotherapy, and hormone, immune, and targeted therapies [2]. Thus, none of the standard cancer treatments can completely cure patients at an advanced stage of the disease. Knowledge of the molecular mechanisms influencing tumor growth and invasiveness may lead to novel and effective therapies for the poor prognosis of late-stage cancers.

Cancer formation and progression is a genetic phenomenon with normal cells accruing genomic instability and thereby acquiring the ability to replicate indefinitely, which is the phenotype of immortality [3]. Telomerase, the immortality enzyme, is ubiquitous in all mammalian embryonic tissue and remains active in germs cells but is down-regulated in most somatic tissues [4]. As telomerase activity determines cellular proliferation, it must be tightly regulated to prevent the induction of carcinogenesis [5]. Telomerase reverse transcriptase (TERT), the catalytic subunit responsible for enzyme activity in telomerase, is the rate-limiting factor of human telomerase enzyme

activity [5]. Two of the critical telomere-specific proteins involved in the regulation and maintenance of the telomere length are the shelterin and CST complexes [6].

2. Telomeres, a Genetic Time Bomb or a Biological Clock

Human telomeres comprise a hexameric nucleotide repeat sequence (TTAGGG) that is initially double-stranded DNA (dsDNA) but ends with a single-stranded DNA (ssDNA) overhang (G'-overhang). The extended 5′ to 3′ strand contains the G-rich telomeric repeats and is referred to as the G-strand, while the 3′ to 5′ strand is defined as the C-strand [7][8]. During the cell division cycle, the eukaryotic DNA polymerase is unable to completely replicate the sequences at the chromosomal ends. This is because RNA primers attach at the lagging strand during the synthesis of Okazaki fragments, and the resulting shedding RNA leads to telomere shortening [9]. The so-called "end replication problem" results in eventual apoptosis, cellular senescence, and cell cycle arrest [10][11]. Additionally, chromosomes lacking the "capping structure" tend to get truncated and fused with other chromosomes [12]. As such, telomeres are also considered a genetic time bomb or a biological clock for cellular aging [13].

As approximately 50–200 bases are lost from the terminal sequence of chromosomes each time a cell divides [14], and more than a couple of trillion telomere sequences are in the human genome, the spatiotemporal expression of telomerase must be tightly regulated in humans. Apart from the shedding RNA and the generation of the 3′ overhang by the sequence-specific exonuclease activity to resect back the 5′end of telomeres [15], telomere shortenings can occur, irrespective of cell replication, due to accumulative oxidative stress [16], host age [17], gender [18], sex hormones [19], and lifestyle factors, such as the lack or presence of exercise [20], obesity and weight loss [21], smoking [22], and unhealthy diets [23]. However, short telomeres not only result in genomic loss [24][25], shorter lifespan [26][27] and contribute to diseases such as coronary heart disease [28], heart failure [29], osteoporosis [30], diabetes [31], but can also result in genomic instability and elevated telomerase activity, leading to a potential cancer predisposition factor [32]. Hence, proper telomere maintenance is critical for human life.

3. The Shelterin Complex

Telomeres are protected by a highly conserved mammalian nucleoprotein complex called shelterin (telosome). This nucleoprotein complex can minimize telomere fragility by enabling DNA replication at the telomeric repeats [33][34]. The shelterin complex can allow DNA to form a lasso-like structure with a telomeric loop (T-loop) and a displacement loop (D-loop) that then shields the 3′-end from DNA damage and blocks the activation of the DNA repair mechanisms, such as ataxia-telangiectasia Rad3-related (ATR)-mediated DNA damage kinase signaling and ataxia-telangiectasia mutation (ATM) kinase cascades, as well as unwanted repair reactions [35]. The shelterin complex anchors to both ssDNA and dsDNA [33][36][37][38]. The shelterin complex and other protein complexes specific to the telomere can detect and react to changes in telomere length in order to maintain the proper length of telomeres [39][40] (**Figure 1**). However, in cancers, mutations in the shelterin complex that cause telomere dysfunction and dysregulation are very common [41].

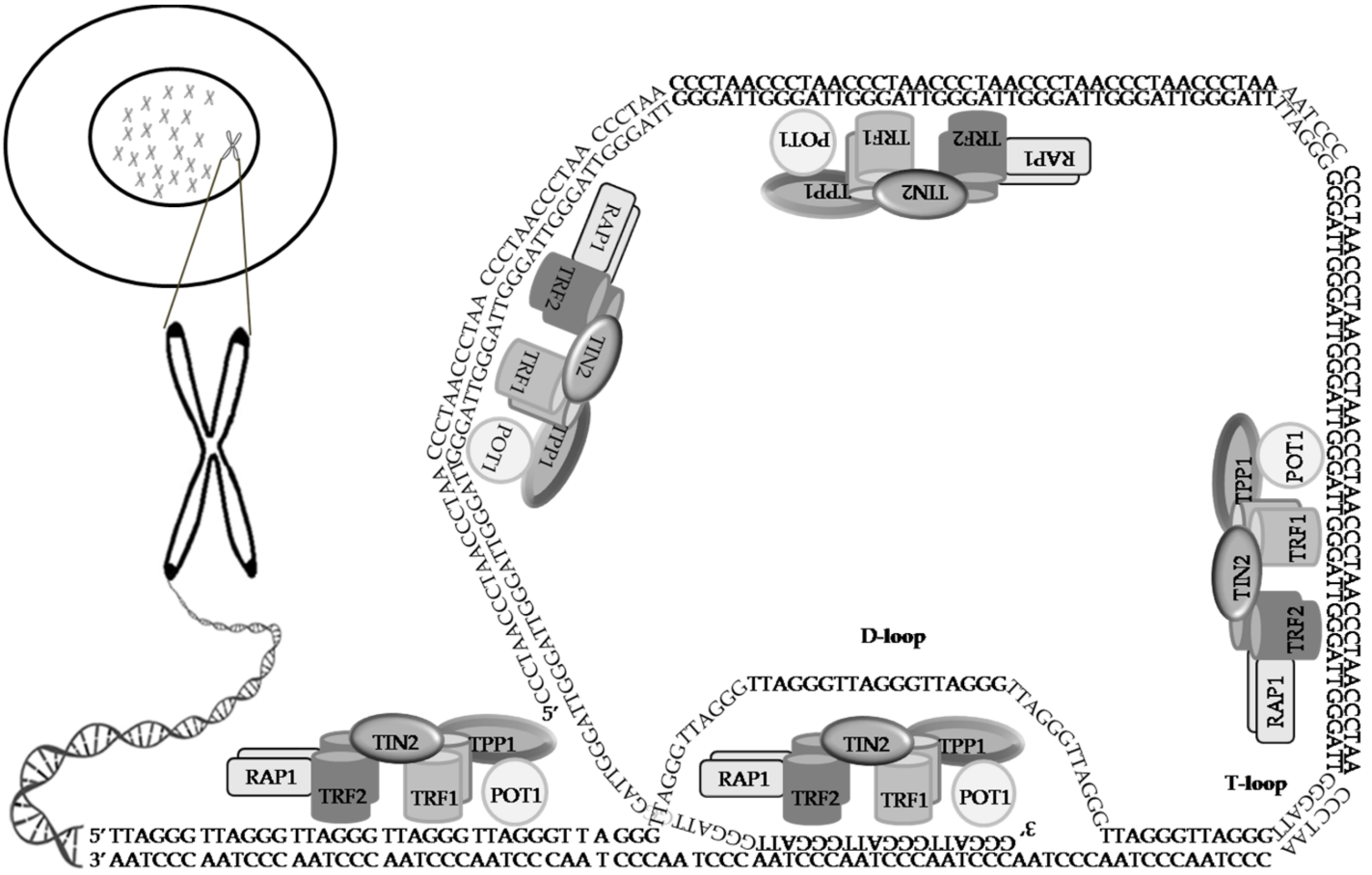

Figure 1. A graphic presentation of telomeric DNA and the proteins that form the shelterin complex. Telomeres are capping structures and are situated at the ends of linear chromosomes. Telomeric DNA, TTAGGG at the chromosome ends, and the complementary DNA strand sequence AATCCC form an extended region of dsDNA ending with a ssDNA G-rich overhang. The 3′ G-rich overhang enables telomeric DNA to form a secondary structure in which the 3′ single-stranded overhang folds back and displaces a strand in the homologous dsDNA TTAGGG region, to create a D-loop that protects the 3′-end from being identified as damaged DNA, thereby preventing the activation of the ataxia-telangiectasia mutation and Rad3-related (ATM/ATR) damage response pathways. The shelterin complex comprises six telomeric proteins: TRF1, TRF2, RAP1, TIN2, POT1, and TPP1. The complex enables the telomeric 3′-overhang/G-tail to fold into a lasso-like structure with a telomeric loop (T-loop) that protects the 3′-end from being recognized for DNA damage and blocks the DNA damage response.

The shelterin complex is composed of six subunits, namely the telomere repeat-binding factor 1(TRF1), telomere repeat-binding factor 2 (TRF2), repressor activator protein 1(RAP1), TRF1-interacting nuclear factor 2(TIN2), TINT1/PTOP/PIP1(TPP1), and the protection of telomeres-1(POT1) [42][43].

4. The CST Complex

The CST complex comprises telomere-specific proteins that regulate telomere length replication and maintenance. The CST complex was initially identified in Saccharomyces cerevisiae and later in vertebrates [44][45][46].

4.1. Yeast CST Complex

Saccharomyces CST complexisa trimeric nucleoprotein complex composed of cell division control protein 13(CDC13), suppressor of CDC thirteen 1 (STN1), and telomeric pathway with STN1 (TEN1) [47]. During cell budding, the CST complex is known as the CDC13-STN1-TEN1 complex; however, fission yeast only contains STN1 and TEN1 [48]. Deletions affecting CDC13, STN1,or TEN1 make budding yeast cells unviable [47]. Therefore, the yeast CST complex is crucially important and may possess evolutionarily conserved functions in DNA replication [49]. Yeast CST complex is structurally related to the heterotrimeric replication protein A (RPA)-complex [45], which is a heterotrimeric ssDNA-binding protein complex composed of replication factor A1 (Rfa1), Rfa2, and Rfa3 (**Figure 2**A) [50].

4.2. Human CST Complex

As with yeast, the human CST complex comprises the conserved telomere maintenance component 1 (CTC1), STN1, and TEN1 (**Figure 2**B), and each subunit is present in the stoichiometric ratio of 1:1:1 [51][52][53]. It localizes at the chromosomal ends, preferentially to G-rich and repetitive elements [54], and can maintain telomere length [46][55]. Human CST is an RPA-like ssDNA-binding protein that has primarily been characterized as a telomere replication factor [56]. RPA is crucial for replication, repair, and recombination and is involved in multiple protein–protein interactions [57], telomere metabolism [45], and chromosome maintenance [58]. The human RPA complex comprises RPA70, RPA32, and RPA14 [50][59]. (**Table 1**).

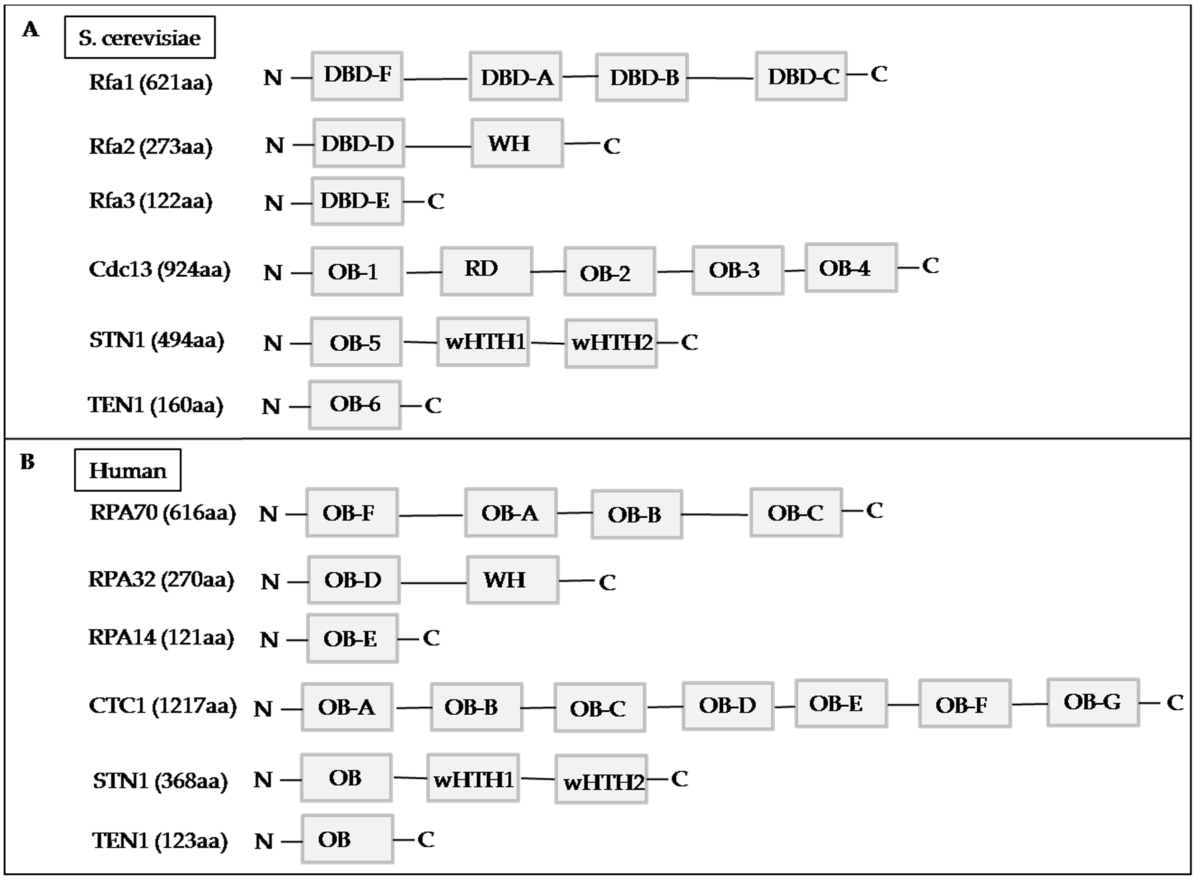

Figure 2. Comparison of (**A**) *S. cerevisiae* Rfa and CST with (**B**) human RPA and CST. Domain structures of Rfa and CST. DBD: DNA-binding domain;OB: OB-fold domain; RD: recruitment domain; TR2: the single RAD51-binding domain; WH: winged helix domain; wHTH: winged helix-turn-helix domain.

Table 1. Comparison of CST and RPA.

CST.	Component	aa	OB	wH	wHTH	Functions	References
						1. Binding to ssDNA.	[52][60][61][62][63][64]
	CTC1	1217	7	0	0	2. Binding to ssDNA-dsDNA junctions.	[65]
	STN1	368	1	0	2	3. Recognize different specialized DNA structures at DNA replication and breakage sites.	[66]
	TEN1	123	1	0	0	4. Acting synergistically with ATR to maintain telomere length and genome stability.	[67]
						5. Stimulating Polα.	[68][69][70]
						6. Helping in C-strand fill-in.	[63][71]
						7. Preventing the accumulation of G4.	[66]
						8. Preventing telomeric DNA damage.	[58][61]
						9. Interacting with the MCM and disrupting binding of CDT1 to MCM, leading to decreased origin licensing.	[72]
						10. Interacting with AND-1.	[73]

CST.	Component	aa	OB	wH	wHTH1	Functions	References
						11. Inhibiting telomerase.	[65][70][71][74][75]
RPA						1. Binding ssDNA.	[76]
	RPA70	616	4	0	0	2. Activating the ATR signaling.	[77]
	RPA32	270	1	1	0	3. Activating the helicase.	[78]
						4. Unwinding G4.	[79]
						5. Involved in DNA replication, recombination, and repair.	[80]
	RPA14	121	1	0	0	6. Activating BLM's bidirectional DNA unwinding.	[81]
						7. Modulating the fork remodeling enzyme activity. [76]	[82]
		[77]				8. Enhancing primase. [78]	[83]

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Checkpoint kinase 1(CHK1), which induces cell cycle arrest to allow DNA repair, fork stabilization, or replication start [84]. 3: Activating the helicase: RPA binding stimulates the accumulation of the human DNA helicase B on chromatin in replication stress [85].4. Unwinding G4: RPA binding promotes WRN activity and multiple RPA binding makes WRN a super-helicase on G4 unwinding [79]. 5. Involvement in DNA replication, recombination, and repair: BLM forms a complex with topoisomerase IIIα, RPA, and several factors involved in functions related to DNA replication, recombination, and repair [80]. 6. Activating BLM's bidirectional DNA unwinding [81]. 7. Modulating the fork remodeling enzyme activity: SMARCAL-1(SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1) is a fork-remodeling enzyme. RPA binds to ssDNA at the fork junction, creating an optimal DNA-protein substrate for SMARCAL-1. However, when the RPA binding to the ssDNA formed at the leading strand stimulates the SMARCAL1-mediated fork remodeling activity, the RPA binding at the lagging strand inhibits the SMARCAL1 activity [82]. 8. Enhancing primase: RPA enhances primase activity at forks [83] (**Table 1**).

4.3. CTC1

CTC1 and CDC13 are the largest subunits of the human and budding yeast CST complexes, respectively. As the CDC13 null strain cannot be generated in yeast cells [86], it is believed that CDC13 is essential for cell viability. Although STN1 and TEN1 are highly conserved [87], the genomic sequences and functions of CDC13 in yeast and that of CTC1 in humans are different [53]. Structurally, CDC13 consists of OB1, OB2, OB3, and OB4 (**Figure 2**A). The roles of these OBs include ssDNA binding, protein–protein interactions, DNA polymerase α-primase binding, and CDC13 homo-dimerization [88][89][90]. However, a recent study analyzing the crystal structures of the CST complex of *Kluyveromyces lactis* suggests that OB2 and OB4 are required for the CDC13–STN1 interaction that assembles CST in a 2:2:2, instead of1:1:1, stoichiometry [91].

Human CTC1 has OB-A, OB-B, OB-C, OB-D, OB-E, OB-F, and OB-G (**Figure 2**B). The C terminus of CTC1 (OB-D through OB-G) acts as a platform to assemble STN1 and TEN1 [51]. STN1 (STN1 OB and the first winged helix-turn-helix [wHTH1] domain of STN1) interacts with CTC1 at two interaction sites, CTC1 OB-G and CTC1 OB-E, respectively (**Figure 2**B) [51]. Structural analyses have shown that CTC1 OB-G is similar to the OB-C of RPA70 and not CDC13 [92]. The CDC13 recruitment domain (RD) contains numerous phosphorylation sites [93][94][95][96][97]. Phosphorylated CDC13 RD enhances the ever shorter telomere 1 (Est1), a component of the yeast telomerase holoenzyme binding and telomerase recruitment to telomeres [93][94]. Est1 is present in humans and a report shows that the expression of Est1 is significantly reduced in B-chronic lymphocytic leukemia [98]. Dephosphorylated CDC13 RD promotes CST complex assembly to bind and cap the ends of chromosomes [94][95][96][99]. Human CTC1 represses the elongation of telomerase by binding to telomerase-extended telomeres thus preventing telomerase activity [71]. CDC13–Est1 and POT–TPP1 are essential in directing telomerase to the chromosomal ends [100][101]. CTC1 interacts with TPP1 to compete with TPP1–POT1 for binding at the telomeric 3′ tail and sequestrate the single-stranded telomeric overhang to inhibit the telomerase extension reaction [53][71]. In humans and yeast, the CST complex prevents 3′ overhangs via boosting the fill-in synthesis [75][102]. Yeast CDC13 deficiency causes genome stability and unstable chromosomes [103]. Dyskeratosis congenita (DC) and Coats plus syndrome (CPS) are two uncommon diseases associated with mutations that affect the CST complex. CPS is an autosomal recessive, systemic disorder characterized by intrauterine growth retardation, bilateral exudative retinal telangiectasias, intracranial calcifications, intracerebral cysts, extra-neurological features, including osteopenia with a tendency of fractures and gastrointestinal bleeding, and portal hypertension [104]. Symptoms of DC include increased cancer incidence, bone marrow failure, lacy reticular pigmentation of the upper chest and/or neck and oral leukoplakia [105]. Changes that occur as a result of CTC1 and STN1 mutations include telomere DNA replication defects, genome instability, defects in interactions with Polα, chromosome breakage, and an accumulation of the ssDNA gaps of telomeric DNA [47][106][107].

4.4. STN1

Human STN1 was initially named as Polα accessory factor44 as STN1 has been shown to enhance primase and up-regulate the recruitment of Polα for lagging strand DNA replication [68][108]. Structurally, the yeast STN1 consists of an OB-5 domain and two wHTH motifs, wHTH1 and wHTH2, which may involve Polα and CDC13 binding [51]. The N-terminus of STN1 binds to TEN1, while the C-terminus associates with both CDC13 and Pol12 (the B subunit of Polα) [109][110].The STN1 and TEN1 are enlisted to telomere ends via direct association with CDC13.

Both STN1 and TEN1 display relatively poor telomeric DNA-binding affinities [111]. In humans, STN1 functions as an adapter between TEN1 and CTC1 [55], and the STN1 N-terminal interacts with CTC1 OB-G and the C-terminal with CTC1 OB-E [51]. Fluorescence investigation has demonstrated that the STN1-binding sites are prone to DNA breakage in STN1 deficient cells under replication stress, leading to chromosome fragmentation [54].

4.5. TEN1

Of the CST components, TEN1is the smallest with a single OB fold [51]. Yeast TEN1 may promote the activity of CDC13 and bind to telomeric ssDNA to enhance the DNA-binding activity of CDC13 [112]. TEN1 in humans is to stabilize the binding of CTC1-STN1to ssDNA and to support C-strand fill-in after G-strand extension by telomerase [65]. Human TEN1 attachment to CTC1 OB-G is facilitated by the OB of STN1 [51]. Human TEN1mutant strain proteins are unable promote the binding of CDC13 to telomeres in vitro, indicating that TEN1 improves the telomeric DNA-binding activity of CDC13 that then negatively affects the telomere length [65]. Knockout TEN1 cells show gradual telomere shortening comparable to that resulting from telomerase deficiency [65], indicating that TEN1 is crucial for the maintenance of telomere length. In addition to ensuring telomere stability [52], TEN1 and STN1 can rescue replication fork stalling during replication stress [55][58][113].

CDC13, STN1, and TEN1 are essential for cell viability and regulating telomere length. Subunit mutations resulting in loss-of-function can cause an accumulation of telomeric ssDNA and result in abnormal elongation of the telomeres, indicating that these three subunits are critical to the health of organisms with the CST complex [6][51][60][74]. The interactions between POT1-TPP1 and CST can significantly affect the telomere length and may result in telomere length dysregulation and cancer development, such as familial glioma [114], melanoma [115], chronic lymphocytic leukemia [116] and breast cancers [117][118], stomach cancers [117], and parathyroid cancers [119].

5. Telomerase: Breaking through the Limitation of Replication

Telomerase, the enzyme responsible for lengthening the telomeres, can extend the cellular lifespan or induce immortalization [1]. Typically, in healthy adult somatic cells, telomerase is inactive to avoid uncontrolled cellular proliferation [2], whereas in approximately 90% of human tumors, telomerase is up-regulated or reactivated to help tumor cells survive and multiply [120]. However, developing embryos, reproductive cells, activated immune cells, bone marrow, and adult stem cells show high telomerase activity [18].

6. Telomerase-Based Anti-Cancer Strategy

The fundamental concept of cancer immunotherapy is based on manipulating the host immune system to attack the cancer cells. Although there are several novel cancer immunotherapy strategies, vaccine-based strategies are the most attractive and promising ones. However, it is very difficult to target tumor-associated antigens on the surface of tumor cells but not on that of the normal cells because of the heterogeneity and overlapping expression of these antigens in both cancers and healthy tissues [121]. As cancer cells lacking telomerase can undergo

spontaneous remission, telomerase inhibition in most cancers may shed light on a potentially successful therapeutic strategy [122]. As telomerase is an HLA class-I antigen and can stimulate a cell-mediated immune response by inducing cytotoxic T-cells, numerous novel approaches have recently been developed to attenuate/inhibit the functions of the telomerase that impact cancer. Vaccination against telomerase is tolerable and safe and has been shown to induce excellent immunological responses associated with increased survival in several cancer types.

6.1. GV1001

The GV1001, an HLA class II-restricted peptide vaccine, is composed of 16 amino acids ($TERT_{611–626}$:EARPALLTSRLRFIPK) derived from the hTERT active site [32][123]. GV1001 was the first TERT peptide vaccine to be evaluated for treating advanced pancreatic cancer, lung carcinoma, melanoma, and liver carcinoma in clinical trials [123][124][125][126][127][128][129][130]. GM-CSF can enhance immunological response through the recruitment and maturation of dendritic cells and the activation of macrophages, neutrophils, and NK cells [131]; therefore, GV1001in combination with GM-CSF can result in a high frequency of immune responder s [32][124]. GV1001 can induce an efficient hTERT-specific T-cell activation and penetrate within tumor cells through the cell membrane [132]. Therefore, it can recognize the antigen-presenting cells that are internalized in the tumor and lymph nodes [124]. GV1001 can induce cancer cell apoptosis [133][134][135] and down-regulate heat shock proteins, hypoxia-inducible factor-1, and vascular endothelial growth factor to enhance its anti-tumor effect [132][134][136]. Although GV1001 is theoretically suitable for most cancers, a report suggests that the GV1001 vaccination is not effective in cutaneous T-cell lymphoma [137], and another report indicates that GV1001 cannot induce any specific immune responses in patients with advanced HCC [125], and the addition of GV1001 to chemotherapy (gemcitabine and capecitabine) did not show any significant clinical benefits [128]. Patients with tuberculosis or receiving tuberculin may not be suitable for GV1001 vaccination because the evoked immune response against mycobacterial peptides may be so dominant as to suppress the immune response against the hTERT peptide [126].

6.2. GX301

The GX301 vaccine contains four immunogenic peptides ($hTERT_{540–548}$: ILAKFLHWL; $hTERT_{611–626}$: EARPALLTSRLRFIPK; $hTERT_{672–686}$: RPGLLGASVLGLDDI, and $hTERT_{766–780}$: LTDLQPYMRQFVAHL) that can bind both HLA class I and II; GX301 also contains two complementary adjuvants, Montanide ISA-51 and Imiquimod. Each GX301 administration consists of four intradermal injections (a fixed hTERT peptide dose, 500 µg)—one injection for each hTERT peptide—given at the same time and followed by topical application of imiquimod [138]. Montanide can protect the degradation of the peptides by tissue proteases, enhance peptide uptake by intradermal dendritic cells, induce interferon- γ release by innate immunity cells, and increase the expression of major histocompatibility complex (MHC) by tumor cells [139]. Imiquimod can activate the Toll-like receptor-7 and receptor-8 and induce the activation and maturation of dendritic cells [140]. The immunogenicity of GX301 was demonstrated in an ex vivo study in which circulating T-cell responses to its hTERT peptides were detected in all subjects [138]. A phase I trial of GX301 has provided evidence of vaccine-specific immune response in patients with stage IV prostate and kidney cancer, and prolonged progression-free survival and overall survival

were observed in patients showing a full pattern of vaccine-specific immunologic responses [138]. A phase II, randomized, parallel-group, open-label, multicenter trial (EudraCT: 2014-000095-26 and ClinicalTrials.gov Identifier: NCT02293707) has demonstrated that all the patients showed good immune responses to at least one of the peptides. The overall response was more for the multi-peptide vaccines than the single-peptide vaccines [141], suggesting that the four GX301 peptides endow a cumulative epitope pattern wide enough for inducing telomerase-specific peripheral T-cell reactivity in most individuals. A phase II, multicenter, randomized, parallel-group, open-label trial (EudraCT:2014-000095-26 and ClinicalTrials.gov Identifier:NCT02293707) was designed to comparatively analyze the safety and immunological response to GX301 regimens in castration-resistant prostate cancer patients with response/disease stability after docetaxel chemotherapy. Although the results indicate that the GX301 cancer vaccine is safe and 95% of the patients showed at least one vaccine-specific immune response, the overall survival did not differ between immunological responders and non-responders [142].

6.3. UV1

UV1 is a second-generation, multi-peptide vaccine constituted by three hTERT-derived peptides ($hTERT_{652–665}$: AERLTSRVKALFSVL; $hTERT_{660–689}$: ALFSVLNYERARRPGLLGASVLGLDDIHRA and $hTERT_{691–705}$: RTFVLRVRAQDPPPE) [124]. In phase I and IIa trials, UV1 was administered along with GM-CSF for six months in patients with metastatic prostate cancer in combination with radiotherapy and androgen deprivation treatment (ADT). A total of 85.7% of patients showed an immune activation and 64% showed reduced levels of the prostate-specific antigen (PSA). In addition, 45% of the patients showed no evidence of the disease at the end of the trial [143]. Several checkpoint inhibitors, including Ipilimumab (anti-CTLA-4) or pembrolizumab (anti-PD-1) in melanoma patients (NCT02275416 and NCT03538314, respectively) and ipilimumab in association with nivolumab (anti-PD-L1) in patients affected by mesothelioma (NIPU trial, NCT04300244) have been singly or multiply used in combination with UV1 in clinical trials. The results showed that the treatment of UV1 together with these checkpoint inhibitors were safe and well-tolerated, and no severe allergic reactions were observed [144][145][146]. The NIPU trial is still ongoing and the primary end-point is expected to be analyzed in 2022.

6.4. Vx-001

Vx-001 is a peptide-based cancer vaccine consisting of two peptides: hTERT-derived low-affinity cryptic hTERT peptide: TERT 572 (RLFFYRKSV; ARG-Vx001) and its optimized mutant hTERT peptide: TERT 572Y (YLFFYRKSV; TYR-Vx001), which has an enhanced affinity to MHC class I molecules as the first amino acid was replaced with a tyrosine residue [147]. The antitumor efficacy and safety of Vx-001 has also been investigated in phase I/II clinical trials for different cancers, such as melanoma, bile duct cancer, breast cancer, and lung cancer. Results of these trials show that Vx-001may elicit a specific and possibly optimal cytotoxic T cell response against hTERT-expressing tumor cells and has improved clinical outcomes in clinical trials without any relevant toxicity [148][149][150][151].

Collectively, the hTERT-vaccine clinical trials indicate that these immunotherapies may represent a promising approach in cancer treatment. Apart from the TERT peptide vaccines, several novel immunotherapies, including the dendritic cell-based tumor vaccine, such as GRNVAC1 [151] and GRNVAC2 [152][153]; Tumor Antigen Presenting

Cells (TAPCells) vaccines [154]; DNA vaccines such as phTERT [155], INVAC-1 [156]; adenovirus type 6 of an anticancer vaccine expressing hTERT, such astheV934/V935 vaccine [157]; gene-modified T-cell therapy, such as the use of tumor antigen-specific T-cell receptors originating from tumor-specific T cells or their clones [158][159]; the use of a chimeric antigen receptor (CAR) [160][161]; the molecules inhibiting Ras farnesylation [162], and hTERT-expressing human umbilical endothelial cells (HUVEC-TERTs) [163], may be effective without prominent toxicity.

7. Alternative Lengthening of Telomere (ALT)

7.1. ATRX and DAXX

Even with these new therapies, there have been certain cancers that can evade treatment by using an alternative lengthening of the telomere (ALT) mechanism. ALT is a telomerase-independent mechanism that uses recombination-dependent pathways to increase telomere length [164]. ALT is present in non-neoplastic tissues and in stromal, endothelial, and epithelial cells [165] and in approximately 10-15% of cancers [166], and it is common in sarcoma and glioma [167][168]. In the absence of telomerase, the ALT pathway uses a homologous recombination-based DNA replication mechanism to gain immortality. ALT activation required two chromatin-remodeling factors: the α-thalassemia X-linked intellectual disability (ATRX) and the death domain-associated protein (DAXX) [167][169]. DAXX was initially describe as a Fas death receptor binding protein [170]. ATRX is widely expressed and is a multi-functional factor involved in chromatin organization, DNA methylation, and transcriptional regulation [171]. Mutations in ATRX result in α-thalassemia ATRX syndrome, which is characterized by severe developmental delays, peculiar facial hypotonia and a characteristic mouth, intellectual impairment, genital anomalies ranging from undescended testes to ambiguous genitalia, and anemia secondary to α-thalassemia [172]. Patients with this syndrome may present long telomeres, which may be due to either improper maintenance of telomeric heterochromatin, improper resolution of replication stress at telomeres, or both by the mutation of ATRX [173].

7.2. Correlation between the Loss-of-Function of ATRX/DAXX and ALT in Cancer

The mutated *ATRX* gene is frequently detected in several tumors, including adrenocortical carcinoma, gliomas, GBM, neuroblastoma, and osteosarcoma [169], and pancreatic neuroendocrine tumors(panNETs), which are a group of endocrine tumors arising in the pancreas. PanNETs are among the most common neuroendocrine tumors. Functioning panNETs include insulinoma, gastrinoma, vasoactive intestinal peptide tumors (VIPoma), glucagonoma, and others that produce specific hormonal hypersecretion syndromes. Endocrine testing, imaging, and histological evidence is necessary to accurately diagnose panNETs. PanNETs may or may not cause signs or symptoms; however, as most panNETs may have malignant potential, an aggressive therapeutic approach for panNETs, including surgery, locoregional therapy, systemic therapy, and complication control, is required [174]. A report showed that 43% of panNETs contained the mutated *ATRX* or *DAXX* [175]. A correlation between the loss-of-function of ATRX/DAXX and the ALT phenotype in panNETs was found [176] and ATRX was proposed to serve as the primary suppressor of ALT [177]. Furthermore, when ATRX was reintroduced into ALT-positive ATRX-negative cell lines it was found to eliminate ALT-associated phenotypes [178][179]. Gliomas with wild-typeTERT promoters often present ATRX mutations to activate ALT [180]. A fibrosarcoma cell line (HTC75), which is telomerase-positive,

can be converted to an ALT-mediated telomere elongation mechanism through TERT knockout, and the subsequent changes result in telomeric DNA damage and disruption of the ATRX/DAXX complex, indicating a negative correlation between mutations affecting TERT and ATRX/DAXX [167]. Consequently, telomeric DNA damage can reduce the compaction of telomeric chromatin, resulting in the production of altered telomeric DNA sequences. This in turn activates a telomere-specific DDR pathway [12][179], which can stimulate the homology-directed synthesis of telomeric DNA. However, cancer cells can circumvent cell death caused by an absence of telomerase or dysfunction by switching from telomerase-dependent to ALT-mediated telomere lengthening [181][182].

7.3. Targeting Telomerase Activity and the ATRX/DAXX Complex

Direct and indirect approaches to targeting telomerase activity and the ATRX/DAXX complex could prove effective. Direct approaches include immunotherapy specifically targeting TERT tumor-associated antigens, such as anti-sense oligonucleotides (e.g., Imetelstat/GRN163L) and small-molecule inhibitors (e.g., BIBR1532) and small molecule inhibitors could be used to bind telomerase and inhibit telomere elongation. Indirect techniques, such as G-quadruplex stabilizers (e.g., RHPS4, Telomestatin, TMPyP4, CX-3543/quarfloxacin), which are designed to block telomerase activity, are promising [183]. The G-rich oligos, which homolog to the telomeric overhang that forms the G4 structures, cause telomere dysregulation and a decreased proliferation rate, enhance apoptosis, and reduce expression of the TERT within melanoma cells [184].An alternative approach is based on telomere uncapping, using nucleoside analogs(e.g., 6-thio-dG) that rapidly affect telomere dysfunction, quickly triggering cancer cell death [185]. In addition, other factors, such as transcriptional, posttranscriptional, and epigenetic modifications can affect the activation or silencing of TERT; however, the effects are poorly understood in somatic, cancer, and stem cells. Epigenetic regulators, such as non-coding RNAs, histone modification, and DNA methylation, are now seen as crucial components for the regulation of telomeres and telomerase activity [186] and unlocking the epigenetic mechanisms associated with telomerase regulation could see advances in cancer diagnosis, treatment, and prognosis [187]. Convergently, a multipronged treatment strategy can maximize anti-tumor effects.

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