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REVIEW

Beginning at the ends: telomere and telomere-based cancer therapeutics

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Abstract

Telomeres, which are situated at the terminal ends of chromosomes, undergo a reduction in length with each cellular division, ultimately reaching a critical threshold that triggers cellular senescence. Cancer cells circumvent this senescence by utilizing telomere maintenance mechanisms (TMMs) that grant them a form of immortality. These mechanisms can be categorized into two primary processes: the reactivation of telomerase reverse transcriptase and the alternative lengthening of telomeres (ALT) pathway, which is dependent on homologous recombination (HR). Various strategies have been developed to inhibit telomerase activation in 85–95% of cancers, including the use of antisense oligonucleotides such as small interfering RNAs and endogenous microRNAs, agents that simulate telomere uncapping, expression modulators, immunotherapeutic vaccines targeting telomerase, reverse transcriptase inhibitors, stabilization of G-quadruplex structures, and gene therapy approaches. Conversely, in the remaining 5–15% of human cancers that rely on ALT, mechanisms involve modifications in the chromatin environment surrounding telomeres, upregulation of TERRA long non-coding RNA, enhanced activation of the ataxia telangiectasia and Rad-3-related protein kinase signaling pathway, increased interactions with nuclear receptors, telomere repositioning driven by HR, and recombination events between non-sister chromatids, all of which present potential targets for therapeutic intervention. Additionally, combinatorial therapy has emerged as a strategy that employs selective agents to simultaneously target both telomerase and ALT, aiming for optimal clinical outcomes. Given the critical role of anti-TMM strategies in cancer treatment, this review provides an overview of the latest insights into the structure and function of telomeres, their involvement in tumorigenesis, and the advancements in TMM-based cancer therapies.

Keywords Telomere · Telomerase · Cancer · Telomere maintenance mechanisms · TERT · ALT · Anti-cancer therapy

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Introduction

Telomeres are nucleoprotein complexes located at the terminal sections of chromosomal arms. They primarily safeguard the terminal regions of telomeric DNA against the cellular DNA repair mechanisms (Shay and Wright 2019; Cohen and Bryan 2022). In each cell cycle of somatic cells, the DNA sequences of chromosomal termini are attrited. After a limited number of divisions, this phenomenon causes the cells to enter the replicative senescence and finally die (Victorelli and Passos 2017). In all proliferating normal cells, telomere length is gradually diminished, attributed to factors such as oxidative stress, exonucleolytic trimming, and other cellular processes, alongside the inherent insufficiency of lagging strand DNA replication. Cancer cells have adopted mechanisms for preventing telomere shortening. Hence, therapeutic strategies have been developed against telomere maintenance of malignancies (Xu and Goldkorn 2016).

Mammalian telomeres are characterized by a conserved hexameric tandem repeat sequence denoted as $(TTAGGG)_n$. This sequence is organized into a configuration that resembles a lariat, referred to as a T-loop and is associated with a complex of six different proteins (Phan 2010). As shown in Fig. 1, the looped configuration begins with nucleolytic processes at the ends of telomeric DNA. The process culminates in forming an elongated single-stranded overhang characterized by a high concentration of guanine bases. This overhang then undergoes a folding process, resulting in the establishment of a T-loop, which subsequently invades the double-helical configuration of the telomeric DNA, thereby facilitating the creation of a displacement loop, commonly referred to as a D-loop (Webb, Wu et al. 2013; Tomáška, Cesare et al. 2020). The Shelterin complex, comprising six distinct proteins, is closely associated with telomeric DNA. This assembly is crucial for properly functioning and maintaining chromosome ends in mammalian cells, playing a vital role in safeguarding the integrity of telomeric regions (Zinder, Olinares et al. 2022; Hu, Yan et al. 2024). The single-stranded TTAGGG sequence-binding protein, known as protection of telomeres 1 (POT1), functions in conjunction with the double-stranded TTAGGG sequence-binding telomeric repeat-binding factors 1 and 2 (TRF1/2 or TERF1/2) (Glousker, Briod et al. 2020; Kallingal, Krzemieniecki et al. 2024). This collaboration is further enhanced by the involvement of three additional proteins. These include a subunit of the shelterin complex that also acts as a telomerase recruitment factor, known as the adrenocortical dysplasia homolog protein (ACD, which is also referred to as TPP1, PIP1, and PTOP), the TERF1 interacting nuclear factor 2 (TINF2, also known as TIN2), and the TERF2 interacting protein (TERF2IP, also called RAP1). Together, these proteins are assembled at the telomere through their interactions with TRF1 and TRF2, thereby playing a crucial role in the maintenance and protection of telomeres (Nandakumar and Cech 2013; Smith, Pendlebury et al. 2020; Lim and Cech 2021; Zhang,

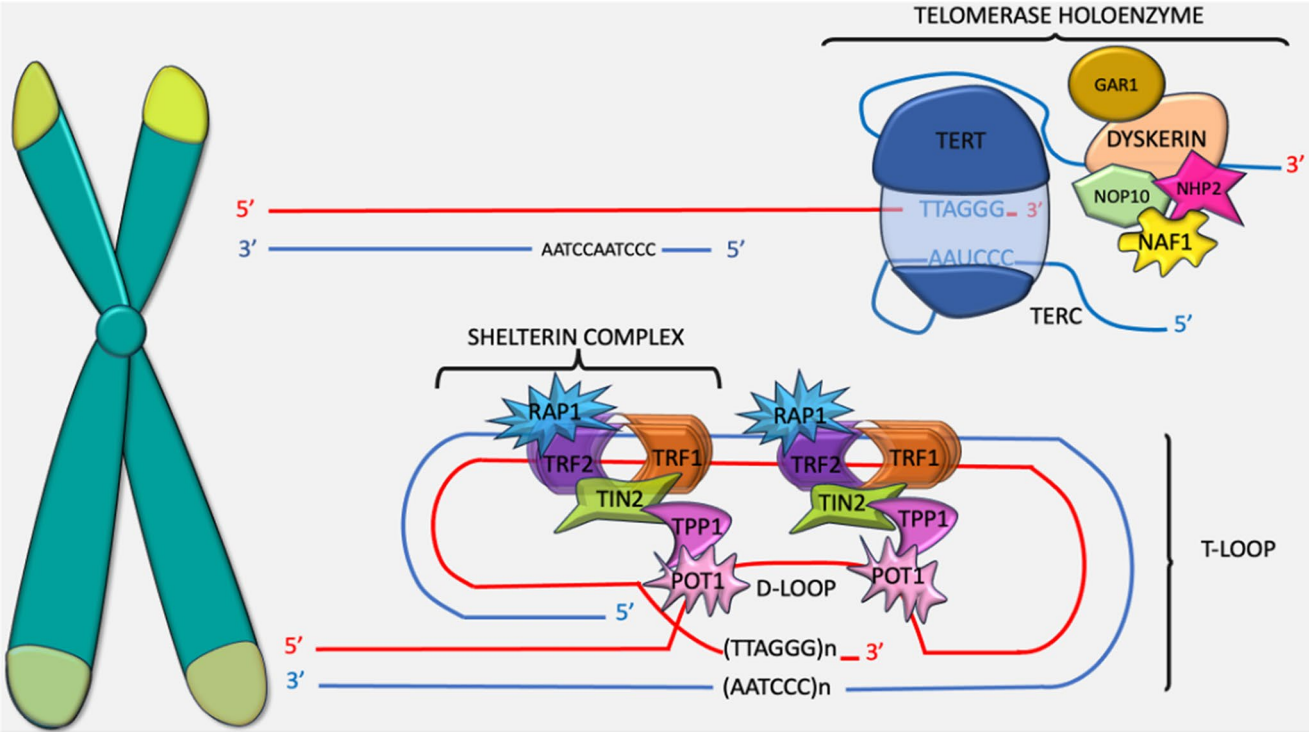

Fig. 1 Schematic presentation of telomeres and the main components of telomerase

Hou et al. 2023). A comprehensive overview of these six members of the shelterin complex and their characteristics is provided in Table 1.

It is proposed that telomeric sequences act as a protective buffer, preventing the loss of critical genetic information (Jenner, Peska et al. 2022). The primary role of telomeres is to safeguard the terminal regions of chromosomes. This protective function is achieved through two distinct mechanisms. Firstly, telomeres serve as protectors against inappropriate DNA repair processes, thereby shielding chromosomes from irregular recombination and fusion occurrences. Secondly, they inhibit the degradation of genes located near the chromosome ends, a process that could arise from incomplete DNA replication (Martínez and Blasco 2011; Trybek, Kowalik et al. 2020; Cohen and Bryan 2022). Notwithstanding this protective mechanism, a gradual reduction in telomere length is commonly noted. The critical limit of telomere length is linked to its gradual reduction throughout the process of DNA replication. This reduction hinders the shelterin complex proteins from adequately associating with the telomeric DNA sequences, resulting in an inability to perform their protective function at the termini of chromosomes. In the majority of differentiated somatic cells, there is an intrinsic decrease of roughly 30–150 base pairs in telomere length with each cellular division, along with changes in their spatial structure, ultimately leading to a diminished ability to establish protective T loops (Lulkiewicz, Bajsert et al. 2020). The steady shortening of telomere length eventually attains a threshold that activates multiple signaling pathways, initiating the process of cellular senescence. This process results in the halting of cell division, signifying that the cell is approaching its maximum replicative potential, a concept referred to as the Hayflick limit. This limit is a fundamental protective mechanism against tumorigenesis (Shay 2016; Niveta, Kumar et al. 2022). To bypass this state of replicative senescence and maintain telomere length within viable limits, two main approaches are utilized in cancer cells: the enzymatic function of telomerase and the alternative lengthening of telomeres (ALT), a process that is supported by homologous recombination (HR) (Maciejowski and de Lange 2017). Telomerase activation is detected in 85–95% of human cancers, while ALT recombination mechanisms occur in 5–15% of cases (Claude and Decottignies 2020). Recent studies have suggested that dual telomere maintenance mechanisms (TMMs) can coexist within the same cellular structures. It has been argued, however, that this co-occurrence might be attributed to the experimental methodologies used, rather than being a fundamental trait commonly seen in cellular processes (De Vitis, Berardinelli et al. 2018). In this review, we will focus on the mechanisms underlying telomerase

Table 1 Characteristics and function of six members of shelterin complex

Gene	Located on	Protein	Abbreviation	Number of amino acids	Domain	The function of each domain		Contribution/ interaction
						Binds to	Function	
TERF1	8p23.1	TRF1	Telomeric Repeat Binding Factor 1	439	Myb-type DNA-binding domain	Double-stranded telomeric DNA	Regulates telomere length	Contributes to telomere protection and maintenance
TERF2	16q23.3	TRF2	Telomeric Repeat Binding Factor 2	542	Myb-type DNA-binding domain	Double-stranded telomeric DNA	Promotes t-loop formation	Contributes to telomere protection and regulation of DNA repair processes
POT1	7q31.33	POT1	Protection of Telomeres 1	634	Oligonucleotide/oligosaccharide-binding fold (OB-fold)	Single-stranded telomeric DNA	Protects telomeres from degradation	Interacts with TPP1 to regulate telomere length
TERF1IP	14q12	TIN2	TRF1-Interacting Nuclear Factor 2	451	TRFH domain	Bridges TRF1 and TRF2	Form the core Shelterin complex	Interacts with TRF1, TRF2, and TPP1 to stabilize the Shelterin complex
TERF2IP	22q13.2	RAP1	Repressor/Activator Protein 1	399	C-terminal Myb domain	Double-stranded telomeric DNA	Regulates telomere length	Interacts with TRF2 to promote telomere protection and maintenance
ACD	17q21.31	TPP1	TINT1/PTOP/PIP1	544	OB-fold domain	-	Regulates telomerase recruitment and activity at telomeres	Interacts with POT1 to promote telomere protection and length regulation

activation and ALT, as well as their potential therapeutic targets within tumor biology.

Telomerase structure and its activation in in human cancer

The telomerase complex is a ribonucleoprotein holoenzyme that consists of a catalytic component known as telomerase reverse transcriptase (TERT), along with its RNA template, TERC, which is also identified as hTR. This complex is further supported by several associated proteins, including dyskerin (DKC1), NOP10, NHP2, NAF1, and GAR1 (Schmidt and Cech 2015) (Fig. 1). Telomerase exhibits activity during the initial phases of human development; however, its transcriptional expression is notably silenced between the 12th and 18th weeks of gestation (Wojtyla, Gladych et al. 2011). Following the embryonic stage, the majority of human cells demonstrate a transcriptional suppression of TERT. However, germ cells, hematopoietic stem cells, proliferating lymphocytes, epidermal cells, and intestinal epithelial cells possess the ability to express telomerase (Roake and Artandi 2020). TERT induction and telomerase activation contribute to oncogenesis through two primary pathways. The first pathway depends on telomere lengthening, facilitating continuous cancer cell proliferation by preserving telomere length. The second pathway operates independently of telomere lengthening and influences oncogenic processes related to cancer initiation and progression through various physiologically linked mechanisms to cellular aging. These mechanisms encompass mitochondrial functions, the ubiquitin-proteasomal system, DNA damage repair, gene transcription, microRNA (miRNA) expression, RNA-dependent RNA polymerase activity, and the process of epithelial-mesenchymal transition (Low and Tergaonkar 2013; Li and Tergaonkar 2014; Yuan, Larsson et al. 2019; Dratwa, Wysoczańska et al. 2020; Liu, Zhang et al. 2024). Numerous factors influence the activity of TERT across multiple regulatory levels. At the transcriptional level, the expression of TERT is stimulated by a variety of transcription factors that interact with specific sequences located within the TERT promoter region. Notable among these are c-MYC, which binds to the E-box (5'-CACGTG-3'), SP1, which interacts with five GC boxes (5'-GGGCGG-3'), and the estrogen receptor α, which targets the estrogen response element (ERE). Additionally, other transcription factors such as E2F, AP-1, and CCCTC binding factors also play significant roles in modulating TERT expression (Koh, Khattar et al. 2015; Khattar and Tergaonkar 2017; Leão, Apolónio et al. 2018; Dratwa, Wysoczańska et al. 2020). The abnormal increase in TERT gene expression within tumor cells is induced by a range of genetic and epigenetic modifications. These alterations encompass gene amplifications, mutations in the promoter region, variations in the alternative splicing of TERT pre-mRNA, as well as DNA methylation of the TERT promoter. Additionally, modifications such as histone acetylation, methylation, and phosphorylation play a role, alongside the disruption of the telomere position effect (TPE) and the associated TPE-OLD machinery (Wong, Wright et al. 2014; Lewis and Tollefsbol 2016; Kim and Shay 2018; Yuan, Larsson et al. 2019; Yuan and Xu 2019; Dogan and Forsyth 2021).

The amplification of the TERT gene has been found to result in the most significant telomerase activity when compared to other mutations that activate this enzyme (Yuan, Larsson et al. 2019; Dratwa, Wysoczańska et al. 2020). Research conducted by Barthel et al. indicated that TERT gene amplification occurs in approximately 4% of cancer patients, with a notable prevalence in those diagnosed with lung, esophageal, adrenal cortical, and ovarian cancers (Barthel, Wei et al. 2017). The transcriptional activation of the TERT gene, influenced by mutations in its promoter region—particularly the C228T and C250T variants—exhibits varying frequencies that range from nearly 0% to more than 90%, contingent upon the specific type of cancer involved (Heidenreich, Rachakonda et al. 2014; Panebianco, Nikitski et al. 2019; Tornesello, Cerasuolo et al. 2023). Notably, mutations found in promoter regions are more prevalent among older individuals diagnosed with cancer, and these mutations have been linked to shorter telomere length (Liu, Yuan et al. 2016). The TERT promoter exhibits the most elevated mutation rates, reaching as high as 80–90%, particularly in bladder-urothelial carcinoma, glioblastoma, melanoma, and lower-grade glioma of the brain. In contrast, hepatocellular carcinoma and thyroid carcinoma display a moderate frequency of these mutations. Conversely, leukemia, kidney, lung, prostate, and gastrointestinal cancers are characterized by the lowest mutation rates, which are recorded at less than 10% (Barthel, Wei et al. 2017). The C288T mutation has been found to occur more frequently than the C250T mutation, with both mutations aberrantly creating new binding sites for the E-twenty-six (ETS) family of transcription factors (Bell et al. 2015; Panebianco, Nikitski et al. 2019). In terms of prognosis, mutations in the promoter region of the TERT gene, both genetic and epigenetic, have been identified as detrimental factors across a range of malignancies, such as melanoma (Motaparthi et al. 2020; van Ipenburg, Naus et al. 2021; Guo, Chen et al. 2022), glioblastoma (Simon, Hosen et al. 2015; Vuong, Nguyen et al. 2020; Olympios, Gilard et al. 2021), and thyroid cancer (Jin, Xu et al. 2018; Khatami and Tavangar 2018; Huang, Chen et al. 2021). Furthermore, the coexistence of activated mutations in the TERT promoter alongside particular mutations in a few oncogenes and signaling pathways has been linked

to aggressive phenotypes and unfavorable outcomes in certain cancers. Notably, this includes the V600E mutation in BRAF, as well as the aberrant activation of the MAPK, PI3K/AKT, and RAS/MEK signaling pathways (Liu, Qu et al. 2014; Macerola, Loggini et al. 2015; Liu, Yin et al. 2017; Liu, Zhang et al. 2018; Trybek, Walczyk et al. 2019; Kim, Kim et al. 2022a). Moreover, numerous viruses have been identified as stimulators of TERT transcription, including hepatitis B and C viruses (HBV and HCV), human papillomavirus (HPV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human T-cell leukemia virus-1 (HTLV-1), and Kaposi sarcoma-associated herpesvirus (Wang, Deng et al. 2017; Salimi-Jeda, Badrzadeh et al. 2021). The majority of these viruses induce the transcription of TERT through two main mechanisms. These include the specific activation of TERT expression facilitated by the synthesis of viral oncoproteins, as well as the insertion and rearrangement of oncogenic viral genomes at the TERT gene locus, which occurs through the exploitation of enhancers (Tornesello, Cerasuolo et al. 2022; Rasouli, Dakic et al. 2024).

ALT mechanism and its role in human cancers

In cell types lacking telomerase activity or where its function is suppressed, the elongation of telomere length may occur via a mechanism dependent on HR, referred to as ALT. Within this framework, it is suggested that cells might utilize a telomeric DNA sequence as a template for replication onto the telomere of a different, non-homologous chromosome. Additionally, this mechanism may involve extrachromosomal telomeric DNA, which can be present in either circular or linear forms (Rosso, Jones-Weinert et al. 2023) Telomeric DNA can extend TTAGGG sequences to another region within the same telomere via the formation of loops, or to the telomere of a sister chromatid. This arrangement resembles a replication fork recognized and lengthened by DNA polymerase (Hoang and O'Sullivan 2020). Studies have indicated that ALT occurs in roughly 15–20% of tumors that lack active telomerase and mitigates telomere shortening in mammalian somatic cells when examined under in vitro conditions (Zhao, Wang et al. 2019; Recagni, Bidzinska et al. 2020). The ALT mechanism is predominantly identified in aggressive tumors of mesenchymal origin that are difficult to treat, as well as in a minor fraction of epithelial cancers, including those arising from bone (62%), soft tissues (32%), neuroendocrine systems (40%), peripheral nervous system (PNS; 23%), and central nervous system (CNS; 15%) (Dilley and Greenberg 2015; Apte and Cooper 2017). Significant advancements have been achieved in our comprehension of the ALT phenotype, which is marked by pronounced heterogeneity, variable telomere lengths (Sommer and Royle 2020), and increased occurrences of telomere sister chromatid exchanges (t-SCEs) (Blagoev, Goodwin et al. 2010). Additionally, this phenotype is characterized by a substantial presence of extrachromosomal telomeric repeat DNA (ECTR) (Komosa, Root et al. 2015; Chen, Shen et al. 2017), and the formation of a distinct nuclear structure associated with telomeric DNA, known as ALT-associated promyelocytic leukemia (PML) bodies (APBs) (Armendáriz-Castillo, Hidalgo-Fernández et al. 2022; Gaela, Hsia et al. 2024). ALT lacks a universally accepted definition, and a majority of cancer cells exhibit one or more phenotypic markers, revealing intratumoral heterogeneity characterized by variations in telomere lengths and TMM activity. This activity includes the coexistence of ALT and telomerase functioning within different cellular populations in the same tumor (Gocha, Nuovo et al. 2013; Pezzolo, Pistorio et al. 2015; Recagni, Bidzinska et al. 2020; MacKenzie, Watters et al. 2021). It has been proposed that stricter regulation of telomerase expression in mesenchymal cells, along with a minor fraction of epithelial cells, may compel these cells to adopt ALT as a mechanism for extending telomere length (Kent, Gracias et al. 2019). Biallelic loss-of-function mutations in the histone chaperone DAXX and the chromatin remodeler ATRX have been found to have a significant association with the activation of the ALT pathway, indicating that these proteins could serve as potential candidates for ALT suppression (Heaphy, de Wilde et al. 2011; Valenzuela, Amato et al. 2021; Clatterbuck Soper and Meltzer 2023). Furthermore, neomorphic or gain-of-function missense mutations in histones H3.3 and H3.1, which are known to play a role in histone methylation, have also been linked to increased ALT activity (Chang, Chan et al. 2015; Simeonova and Almouzni 2024). Notably, the tumor suppressor protein p53 is predominantly inactivated in cell lines and tumors exhibiting ALT, despite the fact that mutations in p53 are commonly observed across various human cancers (Chen, Zhang et al. 2022; Gulve, Su et al. 2022; Macha, Koneru et al. 2022). The most significant changes identified concerning ALT are characterized by a marked increase in the expression of TERRA long noncoding RNA (lncRNA), a disturbance in the function of the histone chaperone paralogs ASF1a and ASF1b, as well as a reduction in nucleosomal density accompanied by modifications in histone marks (Episkopou, Draskovic et al. 2014; O'Sullivan, Arnoult et al. 2014). A thorough comprehension of the ALT mechanism holds significant potential for its application in clinical environments, particularly in diagnosing, prognosis, and treating ALT-positive tumors. The diagnosis of ALT is assessed through a non-invasive C-circle (CC) assay, which may be enhanced by the incorporation of quantitative PCR (qPCR) techniques to improve both

sensitivity and specificity (Chen, Dagg et al. 2021). Furthermore, profiling mutations in ALT-related genes such as ATRX, DAXX, and H3.3 can effectively indicate ALT activity in various tumor types (MacKenzie, Watters et al. 2021). Notably, ALT telomeres can be differentiated from their normal counterparts by the presence of unique telomeric repeat variants, such as TCAGGG and TGAGGG, which attract specific factors, including the nuclear receptors COUP-TF2 (NR2F2) and NR2C/F, as well as TF4 and the zinc-finger nucleosome remodeling complex NuRD-ZNF827. These interactions modify telomeric chromatin and promote telomeric recombination. The orphan nuclear receptor NR2C/F, which has been identified as directly interacting with ALT telomeres and playing a pivotal role in the expression of the ALT phenotype, has been shown to facilitate telomere clustering and targeted telomere insertion (TTI) at non-telomeric locations (Marzec, Armenise et al. 2015; Sommer and Royle 2020; Frank, Rademacher et al. 2022). The presence of these telomere-related structures has been shown to create obstacles for the replication machinery. They may act as prevalent fragile sites across the genome, promoting chromosomal rearrangements and contributing to genomic instability in tumor cells exhibiting ALT (Sfeir, Kosiyatrakul et al. 2009; Bosco and de Lange 2012). The prior identification of the depletion of NR2C/F class nuclear receptors and NuRD-ZNF827 has been shown to inhibit the phenotypic characteristics associated with ALT (Conomos, Stutz et al. 2012; Conomos, Reddel et al. 2014). ALT relies on the activity of BLM helicase, which is responsible for resolving these intermediates (Jiang, Zhang et al. 2024). The prognostic implications of ALT activity in human cancers are frequently characterized by inconsistency and complexity. Prior research has indicated that cancers exhibiting ALT are typically associated with unfavorable prognoses, primarily due to the chromosomal instability inherent in these tumors, which may lead to resistance to treatment (Lawlor, Veronese et al. 2019; Recagni, Bidzinska et al. 2020). As a result, individuals with ALT-positive tumors often necessitate targeted therapeutic approaches. Conversely, it has also been observed that certain categories of ALT-positive tumors can correlate with improved prognostic outcomes (Sung, Lim et al. 2020). TERRA lncRNA has been shown to play a significant role in the regulation of telomere dynamics by inhibiting the ribonucleoprotein hnRNPA1 from displacing replication protein A (RPA), a protein that binds to single-stranded DNA, at telomeres during the S-phase of the cell cycle (Flynn, Centore et al. 2011; Oo, Pálfi et al. 2022; Xu, Senanayaka et al. 2024). Notably, the levels of TERRA lncRNA decrease during the G2 phase, which allows RPA to interact with POT1, a protein that specifically recognizes single-stranded telomeric DNA. RPA is essential as part of the ALT pathway, particularly in ALT cells, where its retention is critical for initiating HR that supports recombination and ALT activity (Oliva-Rico and Herrera 2017; Dueva and Iliakis 2020; Lalonde and Chartrand 2020). In ALT-positive tumors, the abnormal persistence of TERRA expression during the G2 phase results in RPA retention at telomeres, which activates the ataxia telangiectasia and Rad-3-related protein (ATR) kinase, a key player in DNA damage response and checkpoint signaling, thereby enhancing recombination and ALT activity (Oakley and Patrick 2010; Liu, Byrne et al. 2023; Agrawal, Lin et al. 2024). Consequently, targeting the ATR signaling pathway with inhibitors presents a promising therapeutic strategy for treating cancers characterized by ALT-positive tumors (Flynn, Cox et al. 2015). Additionally, the heterodimer Hop2-Mnd1 has been identified as crucial for meiotic recombination, as it stimulates RAD51 and Dmc1, processes that are also involved in telomere movement and clustering during ALT recombination (Arnoult and Karlseder 2014; Bugreev, Huang et al. 2014; Tsubouchi 2023; Ngoi, Pilié et al. 2024).

TMMs as possible therapeutic targets

The distinction between normal and cancer cells is evident in their telomere length and telomerase activity, which has led to the exploration of anti-telomerase therapies and anti-ALT strategies as potential cancer treatments. These approaches aim to progressively shorten the telomeres of cancerous cells, ultimately eliminating both cancer and cancer stem cells, while minimizing damage to healthy cells. This selectivity is attributed to the typically lower telomerase activity, absence of the ALT mechanism, and generally longer telomeres found in normal cells compared to their malignant counterparts (Reddel 2014). An expanding body of research has identified various therapeutic strategies centered around telomerase to accomplish this objective. These strategies encompass antisense oligonucleotides (ASO), agents that mimic telomere uncapping, modulators of expression, immunotherapeutic approaches targeting telomerase, inhibitors of reverse transcriptase, stabilization of G-quadruplex structures, and gene therapy (Jafri, Ansari et al. 2016; Trybek, Kowalik et al. 2020; Ali and Walter 2023). Nevertheless, anti-telomerase therapy approaches are unlikely to demonstrate clinical effectiveness in a significant proportion of cancers, specifically those that are often mesenchymal in origin and rely on ALT mechanisms for telomere maintenance, which accounts for approximately 10–15% of such cases. Therefore, it is imperative to develop ALT-specific targeting strategies to provide therapeutic benefits for patients suffering from these tumors, which typically have a poor prognosis (Zhang, Luo et al.

2022). Notably, studies have indicated that anti-telomerase therapies may inadvertently promote the activation of ALT as a resistance mechanism, while anti-ALT treatments can trigger the reactivation of telomerase. Consequently, there is a pressing need for anti-TMM therapy strategies that utilize selective agents targeting both telomerase and ALT concurrently to achieve optimal clinical outcomes (Hu, Hwang et al. 2012; Dilley and Greenberg 2015). Fig. 2 depicts an overview of strategies aimed at targeting TMMs in immortalized cancer cell lines, and the subsequent sections will provide an in-depth examination of various anti-TMM techniques.

Anti-telomerase therapy approaches

Oligonucleotides have emerged as potential therapeutic agents for the inhibition of telomerase through a variety of mechanisms. These include TERC-targeting oligonucleotides like GRN163L (Imetelstat) and miRNAs, in addition to T-oligonucleotides that are homologous to the 3'-telomeric overhang (Schrank, Khan et al. 2018; Eckburg, Dein et al. 2020). GRN163L characterized as a 13-mer oligonucleotide sequence, functions as a competitive antagonist by binding to the TERC template region. This binding action obstructs the recruitment of TERC to telomeric DNA, thereby preventing it from serving as a template for telomeric sequence synthesis and effectively terminating the primary process of telomerization (Röth, Harley et al. 2010a; Schrank, Khan et al. 2018; Bruedigam, Porter et al. 2024).

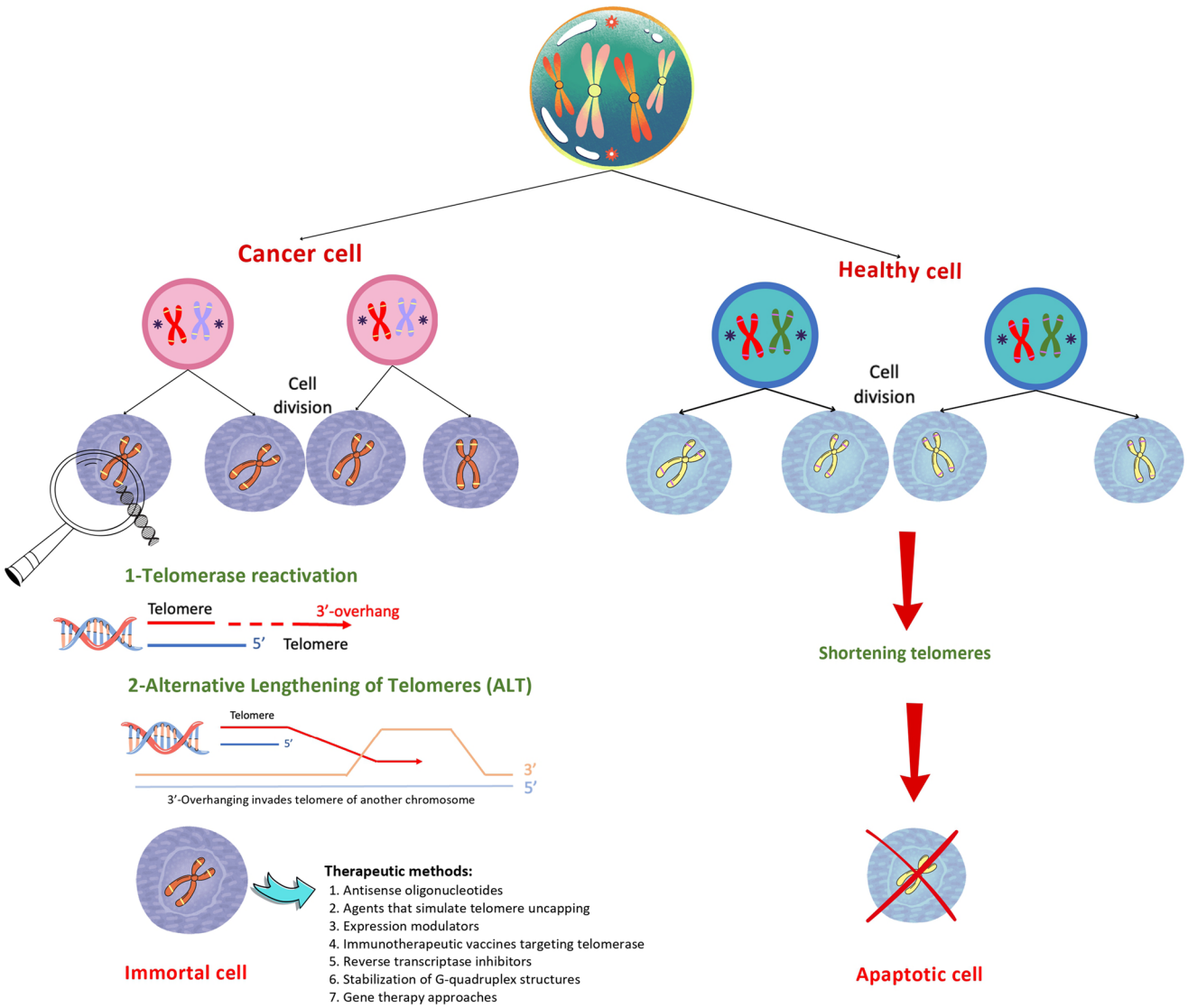

Fig. 2 Schematic illustration of two distinct mechanisms by which telomeres are maintained in cancerous cells, along with the corresponding therapeutic approaches aimed at targeting these mechanisms

Although GRN163L demonstrates potential antitumor efficacy, it has been shown to exhibit toxic side effects when used as monotherapy across various human malignancies (Thompson, Drissi et al. 2013; Frink, Peyton et al. 2016; Wang, Hu et al. 2018). Notably, this agent is associated with significant hematological toxicity, particularly manifesting as neutropenia and thrombocytopenia, which considerably restricts its application in clinical practice (Ratain, Kelsey et al. 2010; Thompson, Drissi et al. 2013; Chiappori, Kolevska et al. 2015; Salloum, Hummel et al. 2016). It has also been observed that certain varieties of tumor cells treated with GRN163L exhibit signs of accelerated aging and undergo apoptosis over an extended period (Shammas, Koley et al. 2008; Röth, Harley et al. 2010b; Burchett, Yan et al. 2014). The application of GRN163L as a viable anti-tumor therapeutic agent is hindered by significant adverse effects on mesenchymal stem cells. These effects encompass notable changes in cellular morphology, a reduction in adhesion capabilities, and an interruption of the cell cycle, particularly manifesting as a G1 phase arrest (Joseph, Tressler et al. 2010; Tokcaer-Keskin, Dikmen et al. 2010; Nitta, Go et al. 2012; Schrank, Khan et al. 2018) GRN163L has been reported to exhibit limited efficacy when administered as a monotherapy for tumor cells, alongside significant adverse effects on both hematopoietic and mesenchymal stem cells. However, promising outcomes have been observed when GRN163L is utilized in conjunction with other molecularly targeted therapies or employed to enhance the sensitivity of tumor cells to radiation treatment (Koziel and Herbert 2015; Burchett, Etekpo et al. 2017; Wu, Zhang et al. 2017; Hu, Huang et al. 2019). T-oligos, which are analogous to the 3'-telomeric overhang, represent a novel category of telomere-targeted therapeutic strategies. These oligonucleotides accumulate within the nucleus and are recognized due to their homology with telomeric sequences. They compete with conventional telomeric DNA, resulting in the disruption of the typical telomere architecture through the action of distinct shelterin complex proteins. Furthermore, T-oligos activate the ataxia telangiectasia mutated (ATM) signaling pathway and induce cytotoxic effects by exposing the telomere overhang. This exposure subsequently triggers cellular responses to DNA damage and initiates the DNA damage response (DDR) mechanism (Crees, Girard et al. 2014; Chhabra, Wojdyla et al. 2018; Schrank, Khan et al. 2018; Eckburg, Dein et al. 2020). The introduction of T-oligos into clinical trials faces certain challenges, primarily due to their susceptibility to nuclease-mediated degradation and a lack of comprehensive understanding regarding their mechanisms of action. Nevertheless, T-oligos have exhibited significant anti-tumor effects when utilized in combination therapy approaches, as well as enhancing the sensitivity of cancer cells to radiation treatment (Sarkar and Faller 2011; Pitman, Wojdyla et al. 2013; Wojdyla, Stone et al. 2014).

The third category of oligonucleotide-based therapies includes exogenous small interfering RNAs (siRNAs) and endogenous miRNAs that act as tumor suppressors. These molecules play a crucial role in inhibiting cellular processes such as proliferation, migration, invasion, and metastasis by regulating the expression of hTERT at the post-transcriptional level. This regulation is achieved through their interaction with the RNA-induced silencing complex (RISC), which effectively silences target genes (Zhang, Xiao et al. 2015; Zhou, Fei et al. 2016; Guzman, Sanders et al. 2018; Zhang, Chen et al. 2023). However, oncogenic miRNAs, including miR-21, have been shown to facilitate the transformation of tumor cells by enhancing the expression of TERT. This mechanism presents potential targets for anti-oncogenic miRNA antisense therapy, which aims to inhibit their function (Yang et al. 2015; Nguyen and Chang 2017; Rhim, Baek et al. 2022). In addition to TERT, the TERC gene and its corresponding mRNA are also susceptible to modulation by both oncogenic and tumor-suppressive miRNA therapies (Eckburg, Dein et al. 2020). Moreover, studies have revealed that miRNAs can affect TERT expression by altering upstream signaling pathways, thereby offering additional avenues for therapeutic intervention (Wang, Sun et al. 2012; Ohira, Naohiro et al. 2015; Farooqi, Mansoor et al. 2018; Eckburg, Dein et al. 2020). Short hairpin RNA (shRNA) can be introduced into the nucleus through an expression vector, leading to sustained expression and more prolonged gene silencing compared to siRNA. A significant challenge associated with siRNA-based therapeutic approaches is their limited biological stability and transient silencing capability, as approximately 99% of duplex siRNAs are degraded within 48 h post-delivery into cells (Hu, Zhong et al. 2020). Research has shown that plasmid-based delivery systems for shRNA targeting either TERT or TERC can effectively reduce telomere length and inhibit the growth of cancer cells (Chen, Gu et al. 2017).

Anti-telomerase immunotherapy is another therapeutic approach mediated by immune cells. Vaccines that target telomerase can enhance the sensitivity and activation of $CD8^+$ and $CD4^+$ T lymphocytes against cancer cells that present TERT peptides as surface antigens via human leukocyte antigen (HLA) classes I and II. This process ultimately results in the generation of telomerase-specific cytotoxic T lymphocytes (CTLs), which are instrumental in the destruction of TERT-positive malignant cells (Mizukoshi and Kaneko 2019; Berei, Eckburg et al. 2020; Ellingsen, Mangsbo et al. 2021; Yan, Lin et al. 2023). Numerous TERT-targeted peptide vaccines have been developed to date, with GV1001 being the most advanced among them. This particular vaccine has shown remarkable efficacy and

importance while maintaining a favorable safety profile, particularly in terms of its impact on bone marrow function (Mizukoshi and Kaneko 2019; Jo, Kim et al. 2024). The peptide vaccine GV1001 is characterized as an HLA-II-restricted peptide, which elicits robust responses from both $CD4^+$ and $CD8^+$ T cells, as well as the activation of CTLs across various types of malignancies (Staff, Mozaffari et al. 2014; Kim, Cho et al. 2022; Kim, Kim et al. 2022b). TERT-based peptide vaccines have been shown to provoke significant immune responses in T-type cells, notably including GX301 and Vx-001 (UV1). The GX301vaccine comprises four peptides derived from TERT and is classified as a multi-peptide vaccine, exhibiting greater efficacy compared to single-peptide alternatives (Fenoglio, Traverso et al. 2013; Fenoglio, Parodi et al. 2015; Kailashiya, Sharma et al. 2017; Dosset, Castro et al. 2020; Negrini, De Palma et al. 2020). Notably, these vaccines elicit distinct immune responses that vary according to the type of tumor. For instance, GV1001 has demonstrated superior effectiveness in treating pancreatic cancer, non-small cell lung carcinoma (NSCLC), and melanoma, while GX301 has shown marked efficacy in prostate and kidney cancers. Additionally, UV1 has proven to be particularly effective in both prostate cancer and NSCLC (Kailashiya, Sharma et al. 2017; Bajaj, Kumar et al. 2020; Negrini, De Palma et al. 2020; Relitti, Saraswati et al. 2020; Ellingsen, Mangsbo et al. 2021). In recent developments, DNA vaccines have emerged alongside peptide vaccines, which are designed to elicit immune responses in T cells through the introduction of the TERT gene sequence or a plasmid that encodes the TERT peptide into antigen-presenting cells (APCs). Notably, the two vaccines belonging to this category are phTERT and INVAC-1 (Yan, Pankhong et al. 2013; Thalmensi, Pliquet et al. 2016; Melssen and Slingluff 2017; Negrini, De Palma et al. 2020). Additionally, immunotherapy also employs dendritic cells (DCs), which are recognized as the most potent APCs, to stimulate immune responses. A notable example of a DC-based cancer vaccine is GRNVAC1, which elicits a robust polyclonal immune response that has demonstrated efficacy, safety, and tolerability across a range of cancer types (DiPersio, Collins Jr et al. 2009; Khoury, Collins Jr et al. 2010; Relitti, Saraswati et al. 2020; Yu, Sun et al. 2022). Another innovative DC-based vaccine involves the transfection of TERT mRNA, enabling the presentation of TERT-associated antigens to T cells; this approach is referred to as the TAPCells vaccine (Salazar-Onfray, Pereda et al. 2013; Galati and Zanotta 2018; Zhang, Tang et al. 2023).

The enzyme reverse transcriptase has been found to exhibit activity in a significant number of tumor cells, as well as in cells that are infected with retroviruses. Consequently, inhibitors targeting this enzyme may play a vital role in the therapeutic management of both neoplastic cells and virally infected cells. In prior research, various nucleoside analogs have been recognized as effective inhibitors and antagonists of telomerase, demonstrating irreversible inhibition. Notable examples of these compounds include azidothymidine (AZT), acyclovir, and penciclovir, all of which have shown to be instrumental in cancer therapy, particularly when utilized in combination treatments (Gomez, Armando et al. 2016; Fang, Hu et al. 2017; Wang, Zhou et al. 2017). A distinct category of reverse transcriptase inhibitors, referred to as small molecule inhibitors, includes compounds like BIBR1532 acid, which exert their effects by non-competitively binding to the active site of TERT, thereby specifically inhibiting the activity of telomerase (Altamura, Degli Uberti et al. 2020). One approach to indirectly suppress telomerase activity involves the stabilization of G-quadruplex structures, which effectively prevents the single-stranded telomere overhang from unfolding and being detected by TERC. Among the various compounds studied for their ability to bind to and stabilize G-quadruplexes in the context of cancer therapy, Telomestatin, BRACO-1910, and RHPS4 have garnered significant attention (Bryan 2020; El-Khoury et al. 2023). In addition, novel steroid derivatives, specifically malouetine, and steroid FG have been identified as effective stabilizers of G-quadruplexes, as well as inducers of telomere uncapping. These compounds exhibit a nonplanar and nonaromatic configuration, distinguishing them from previously characterized G-quadruplex ligands (Xu, Di Antonio et al. 2017; Eitsuka, Nakagawa et al. 2018; Awadasseid, Ma et al. 2021; Zegers, Peters et al. 2023).

The ultimate strategy involving telomere-based therapies pertains to the anticancer potential of gene therapy, wherein the TERT gene, typically a supportive element in approximately 85% of cancer cells that demonstrate TERT overexpression, is transformed into a detrimental adversary through the activation of gene expression driven by the TERT promoter (Hong and Yun 2019). The human TERT promoter presents a noteworthy advancement in cancer gene therapy, as it demonstrates the ability to target a wide range of malignancies, unlike many earlier cancer-specific promoters that were limited to selectively addressing only a narrow spectrum of cancers in a tissue-specific context. This broad applicability of the TERT promoter in therapeutic applications holds significant promise for the development of gene therapies that can effectively engage with the majority of cancer types (Quazi 2022). The limited expression of transgenes driven by the human TERT promoter in normal somatic cells and bone marrow progenitor cells results in negligible acute and chronic toxicity. A prevalent approach in TERT promoter-driven gene therapy involves the expression of therapeutic genes, which may encompass anticancer transgenes, miRNAs, or components of the

CRISPR/Cas9 system (Li, Tan et al. 2011; Watanabe, Ueki et al. 2011; Xiong, Sun et al. 2012; Higashi, Hazama et al. 2014; Liu et al. 2016; Huang, Zhuang et al. 2017; Hong and Yun 2019; Balon, Sheriff et al. 2022). The forefront of anticancer gene therapy has been marked by the innovative use of transgenes that facilitate the direct elimination of cancer cells. This approach includes the delivery of suicide genes, which encode enzymes responsible for converting prodrugs into active therapeutic agents, through adenovirus-mediated systems (Zeng, Zhang et al. 2024). Additionally, the incorporation of proapoptotic genes has also been explored as a means to induce programmed cell death in malignant cells (Rubis, Holysz et al. 2013). The utilization of TERT promoter-driven and CRISPR/Cas9-based genetic circuits presents a novel strategy for the targeted silencing of specific oncogenes in various cancer types. A notable instance of this therapeutic methodology involved a lentiviral delivery system designed to express a guide RNA targeting HRAS, alongside a human TERT promoter-driven GAL4 and UAS-activated Cas9 nuclease (referred to as HRAS-LV). This system demonstrated a significant enhancement in the silencing of HRAS in bladder cancer-derived cell lines (Huang, Zhuang et al. 2017). Moreover, the human TERT promoter can produce oncolytic adenoviruses that are specific to cancer by enhancing the expression of critical replicative genes, including the E1A gene (Zhou, Ma et al. 2021). The predominant optimization within this therapeutic framework involved the creation of various modified forms of human TERT (mTERT) that facilitate the replication of oncolytic adenoviruses. This was achieved by incorporating additional binding sites for oncogenic transcription factors, such as Sp1 and c-Myc, located upstream of the promoter region. Furthermore, a hybrid cancer-specific promoter was engineered by merging the promoters of E2F and mTERT, supplemented with multiple hypoxia response elements (HRE), resulting in two unique hypoxia-responsive and cancer-specific promoters, designated as HEmT and HmTE. Additionally, an advanced hybrid cancer-specific mTERT promoter was developed by integrating six copies of HRE and five copies of c-Myc binding sites upstream of mTERT, culminating in the formation of H5CmTERT (Kim, Kim et al. 2003; Li, Hong et al. 2018; Oh, Hong et al. 2018). The mTERT promoters have been identified as effective enhancers of transgene expression levels. As a result, oncolytic adenoviruses that utilize mTERT promoter-driven replication exhibit enhanced potency and prolonged antitumor effects. Furthermore, the CRISPR/Cas9 technology presents a viable approach for rectifying prevalent mutations in the human TERT promoter, thereby paving the way for personalized therapeutic strategies. A notable example includes a C > T single-nucleotide mutation found in the proximal promoter region of TERT, which is frequently observed in various cancers at positions − 124 and − 146, adjacent to the ATG codon of TERT. This mutation is associated with an increase in the transcriptional activity of the altered TERT promoters (Liu, Yuan et al. 2016; Balon, Sheriff et al. 2022) The application of CRISPR/Cas9 to correct these mutations has yielded significant outcomes in urothelial cancer cell lines (Xi, Schmidt et al. 2015).

ALT-specific targeting strategies

Numerous studies have established that therapies targeting telomerase are unlikely to demonstrate clinical effectiveness in cancers characterized by ALT (Temime-Smaali, Guittat et al. 2009; Wu, Chen et al. 2019; Awadasseid, Ma et al. 2021). Thus, effective treatment strategies for patients with tumors that exhibit ALT must be specifically designed to target this mechanism, particularly given the often poor prognoses associated with such tumors. It has been suggested that therapies based on telomerase, including telomerase inhibitors, may prompt tumor cells to adopt ALT as a means of resistance. Consequently, the use of ALT inhibitors in conjunction with telomerase inhibitors could be beneficial for both telomerase-positive tumors and those that are initially ALT-positive. However, it is important to note that telomerase reactivation can be a resistance strategy in tumors undergoing treatment with ALT inhibitors. Therefore, it is essential to prescribe both telomerase and ALT inhibitors, regardless of the TMM employed by the tumor (Gao and Pickett 2022). Notably, ALT cells typically possess significantly shorter telomeres compared to telomerase-positive cells, and the complete disruption of the ALT mechanism can lead to severe telomere uncapping, which in turn triggers a cascade of detrimental effects, including damage signals, toxic chromosomal fusions, cellular senescence, apoptosis, and increased genomic instability, ultimately resulting in cell death (Kaul, Cesare et al. 2011; Ali and Walter 2023) Recent studies have identified key characteristics of ALT cells, highlighting alterations in the chromatin landscape surrounding telomeres, the upregulation of TERRA lncRNA, enhanced activation of the ATR signaling pathway, increased interactions with nuclear receptors, telomere repositioning driven by HR, and recombination events occurring between non-sister chromatids. These identified features present significant potential for the development of therapies targeting ALT mechanisms (Sohn, Goralsky et al. 2023). In particular, the inhibition of ATR emerges as a promising strategy for treating cancers that exhibit a high frequency of ALT, as it is believed to enhance the sensitivity of ALT-dependent cells to therapeutic interventions (Episkopou, Draskovic et al. 2014). As mentioned earlier, APBs have been recognized as pivotal elements in the formation of structural platforms

and the mediation of telomere recombination within ALT cells (Draskovic, Arnoult et al. 2009; Marchesini et al. 2016; Zhang, Zhao et al. 2020; Zhang, Genois et al. 2021). The overexpression of Sp100, a constituent of PML bodies, has been shown to interfere with APBs, leading to the sequestration of the Mre11-Rad50-Nbs1 (MRN) complex, which ultimately results in the suppression of ALT activity (Jiang, Zhong et al. 2005; Deeg, Chung et al. 2016). Notably, the inhibition of MRN complex formation or the depletion of its components also contributes to the reduction of ALT activity (Kavitha et al. 2010; Lamarche, Orazio et al. 2010; Bian, Meng et al. 2019). Given that ALT can be viewed as a distinct and aberrant variant of HR, targeting this HR pathway prevalent in ALT cells may offer a strategic approach to selectively eliminate these cells (Dilley and Greenberg 2015). Additionally, inhibitors of PCNA and BLM have shown potential as effective therapeutic agents against ALT tumor cells (Punchihewa, Inoue et al. 2012; Nguyen, Dexheimer et al. 2013; Pan, Drosopoulos et al. 2017). It has been emphasized that therapeutic strategies aimed at either diminishing or excessively activating ALT recombination could leverage the inherent vulnerabilities of ALT-positive tumors, facilitating targeted cell death (Dilley and Greenberg 2015). In conclusion, nearly all methodologies employed for telomerase inhibition apply to ALT inhibition, encompassing antisense oligonucleotides, miRNA and siRNA targeting, small molecule therapies, and a diverse array of gene therapy techniques.

Conclusion

The widespread presence of TMMs and the specific involvement of mechanisms exclusive to cancer cells renders these pathways attractive targets for therapeutic intervention. Nevertheless, despite the extensive and expanding knowledge in this domain that has led to numerous strategies for anti-telomerase and ALT-based cancer treatments, several uncertainties and contradictions persist. These include the need for more refined TERT-based therapeutic strategies and fundamental unresolved questions regarding the ALT mechanism. Addressing these inquiries not only enhances our understanding of the biological processes underlying cancer progression but also paves the way for the development of innovative cancer therapies that could offer greater efficacy and specificity, ultimately curtailing tumor growth and enhancing patient outcomes.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Competing interests All authors claim an absence of financial interests and an absence of conflicts of interest.

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