Review

Mechanisms of telomere maintenance in pediatric brain tumors: Promising targets for therapy – A narrative review

Simone Minasi[1], Francesca Gianno[1,2], Hiba Alzoubi[1,3], Manila Antonelli[1], Felice Giangaspero[1,2], Francesca Romana Buttarelli[1]

[1]Department of Radiological, Oncological and Anatomo-Pathological Sciences, Sapienza University of Rome, Rome, Italy.
[2]Departement of Neuropathology, Mediterranean Neurological Institute Neuromed, Pozzilli, Italy.
[3]Department of Basic Medical Sciences, Faculty of Medicine, Yarmouk University, Irbid, Jordan.

Abstract

Recent advances in genetic and molecular characterization of telomere maintenance mechanisms (TMMs) highlighted their strong relationship with cancer pathogenesis; neoplastic cells rely on two mechanisms to maintain telomere length and escape from replicative senescence: (a) reactivation of telomerase expression and (b) activation of alternative lengthening of telomere (ALT). Our aims are to describe the role of telomere maintenance in the context of recently published literature regarding pediatric brain cancers and to discuss the emerging therapeutic strategies to target telomerase-positive and ALT-positive tumors. In this review, we illustrate the incidence of TMM via telomerase or ALT and discuss the importance of analyzing telomere length and ALT-associated genetic alterations in certain histological/molecular subtypes of pediatric brain tumors, as potential therapeutic biomarkers. Telomerase-dependent TMM is a common mechanism in SHH-medulloblastomas and ependymomas, which could potentially benefit from antitelomerase therapies, while ALT-dependent TMM is more frequently activated in α-thalassemia/mental retardation syndrome X-linked/H3.3-mutated pediatric high-grade gliomas, metastatic medulloblastomas, and choroid plexus tumors, which could potentially be treated with ALT-targeted drugs. Conversely, pediatric low-grade gliomas lack both mechanisms of telomere maintenance, and anti-TMM therapies do not appear to be a promising strategy for these tumors.

Keywords: Alpha-thalassemia/mental retardation syndrome X-linked, alternative lengthening of telomere, antialternative lengthening of telomere therapy, antitelomerase therapy, glioma, H3.3, medulloblastoma, pediatric brain tumors, telomerase reverse transcriptase, telomerase

Introduction

Telomeres are specialized DNA–protein structures, capping the end of each linear chromosome. Human telomeric DNA is constituted by a variable of tandem repeats of double-stranded TTAGGG, ranging from 2 to 30 kb, and by a 3'-G-rich single-stranded overhang (G-tail), with a length of around 150 nt.[1] Telomeres are also capped by six proteins that form the shelterin complex: telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), tripeptidyl peptidase I, protection of telomeres 1, TRF1-interacting nuclear factor 2, and repressor/activator protein 1.[2-4]

Telomeric DNA, along with the shelterin protein complex, maintains the telomere-specific structure, allows discrimination of telomeres from double-stranded DNA breaks, and protects the ends of the chromosomes from degradation, fusion, and recombination for maintaining genomic integrity.[5,6]

Telomere length shortening plays a crucial role in the cellular process of replicative senescence;[7,8] in each cycle of DNA replication, the inability of DNA polymerase complex to replicate the 3'-end of the lagging strand in the linear chromosomes leads to telomere shortening.[9] The ends of chromosomes progressively shorten until the cells reach the maximal number of cell divisions (Hayflick limit), undergoing chromosomal instability, senescence, and apoptosis.[7-10] The

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Address for correspondence: Prof. Felice Giangaspero, Department of Radiological, Oncological and Anatomo-Pathological Sciences, Sapienza University of Rome, Rome, Italy.
E-mail: felice.giangaspero@uniroma1.it

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average telomere length typically ranges from 10 to 15 kb in the human somatic cells and shortens at a rate of around 50–200 bp with each cell division; in the absence of telomere maintenance mechanisms (TMMs), cells divide in an average between 50 and 70 times before the loss of chromosome capping function at telomeres leads to replicative senescence.[11] When a critical telomere length is reached, further shelterin proteins lose their binding sites and telomeric DNA cannot form a protective secondary structure.[12,13]

Cancer cells overcome this limit and escape from replicative senescence by activating a TMM, necessary for unlimited replication and immortalization of the neoplastic cells, preventing genetic instability associated with critical telomere shortening.[1,7,8] Neoplastic cells rely on two mechanisms to elongate telomeres: (a) reactivation of telomerase expression and (b) activation of alternative lengthening of telomere (ALT).[14-20]

The aim of this review was to describe the incidence of TMM via telomerase or ALT in different histological/molecular subtypes of pediatric brain tumors (PBTs), since telomerase-targeted and ALT-targeted therapies are currently tested in preclinical studies and could constitute a promising approach for certain tumor types.

Database Search Strategy

Literature review was electronically performed using PubMed database. The following combinations of keywords were used to initially select the articles to be evaluated: alternative lengthening of telomere; telomerase activation; telomerase target therapy; ALT target therapy, senescence escape, and TMM in pediatric brain tumors. Most of the elected studies (144/191, 75.4% of all references) were published from 2010 to 2020. The older publications from 1985 to 2009 were included in consideration to their relevance in the description of telomerase or ALT mechanisms, of their incidence in cancer, and of the targeted therapies.

Telomere Maintenance Mechanism via Telomerase Reactivation

Human telomerase is a reverse-transcriptase heterodimer formed by a noncoding RNA template (telomerase RNA component), an enzymatic subunit (telomerase reverse transcriptase [TERT]), and a series of auxiliary components.[21] Telomerase is responsible for synthesizing telomeric DNA to compensate the erosion of telomeres during each DNA replication.[22] Telomerase RNA component is necessary as a template for the elongation of telomeres, while TERT catalyzes the process with its reverse-transcriptase activity by adding nucleotides to the chromosome 3'-ends.[21,22]

The expression and activity of telomerase are strictly controlled.[23] Some long-lived cells, as stem cells, germinal cells, and early progenitor cells, require telomere length maintenance for escaping from senescence and allowing unlimited replication; these cells normally use the enzyme telomerase to maintain telomere length.[24] In contrast, the majority of human somatic cells, except lymphocytes and endothelial cells, completely lack telomerase activity.[25]

Neoplastic cells from several tumor types (85%–90%) use the canonical TMM to maintain telomere length by reactivating telomerase expression, allowing them to escape from replicative senescence and apoptosis.[25,26] The main genetic alterations associated with telomerase-dependent TMM in tumor cells are (1) *TERT* promoter (*TERTp*) mutations; (2) amplification of the gene *TERT*; (3) structural rearrangements of regulatory elements; and (4) epigenetic changes of *TERTp*.

TERTp hotspot mutations are located − 124 bp (C228T) and − 146 bp (C250T) upstream of the transcriptional start site ATG and are the most common alterations related to telomerase up-regulation, harbored by a wide spectrum of human tumors including melanomas (67%–85%), brain tumors (28%–84% glioblastoma and 19%–42% medulloblastoma), hepatocellular carcinomas (24%–59%), bladder cancers (~50%), thyroid cancers (~30%), and cutaneous squamous cell carcinomas (~50%).[14,27]

TERT amplification is a rare alteration that correlates with telomerase reactivation in cancer. Barthel *et al*.,[14] in a cohort including 6835 patients and covering 31 tumor types, showed the presence of amplification in a total of only 4% of tumors. *TERT* can be also activated by structural rearrangements (~3%), leading to repositioning of enhancer elements that activate *TERT* transcription, more frequently found in neuroblastoma.[14,28] Finally, *TERTp* methylation provided an additional regulatory mechanism for *TERT* expression.[14] In particular, the hypermethylation of *TERTp* on a specific region rich in CpG sites – 600 bp upstream of the transcription start site, called UTSS region, was found to be associated with telomerase upregulation.[29,30] This opposite association between *TERTp* methylation and increased *TERT* expression may result from loss of CTCF binding, a transcriptional repressor reported to specifically bind to the unmethylated *TERTp* DNA.[14,31]

Moreover, the activity of telomerase is also regulated by the shelterin complex and the telomeric repeat containing RNA (TERRA), which is transcribed by RNA polymerase II from subtelomeric and telomeric DNA.[2,32,33] The main alterations on *TERTp* associated with telomerase upregulation in cancer are shown in Figure 1.

Telomere Maintenance Mechanism via Alternative Lengthening of Telomere

In cancer cells which do not reactivate telomerase, the maintenance of telomere length is achieved via ALT, a telomerase-independent, recombination-based mechanism. ALT mechanism was originally shown in the immortalized cell lines and subsequently found in several types of human cancers.[15,16] Compared to telomerase reactivation, a lower

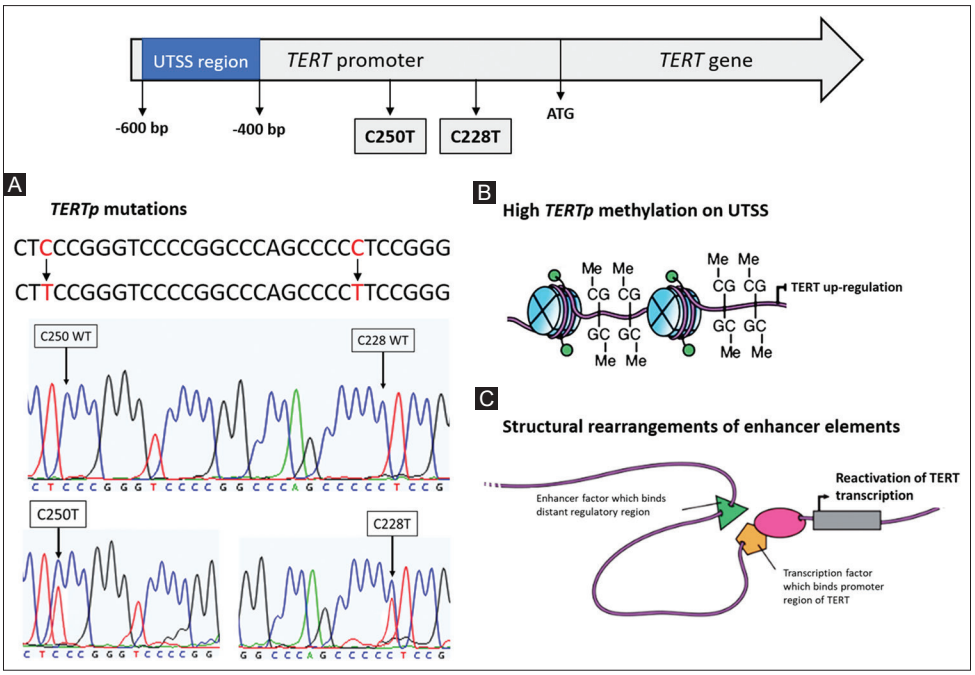

Figure 1: Schematic representation of telomerase reactivation by *TERTp* mutations, *TERTp* methylation and rearrangements of enhancer elements. (A) *TERT* promoter region on chromosome 5 with the DNA sequence of the hotspot mutations C250T and C228T, evidencing the presence of double peaks in chromatograms. (B) Methylation of *TERTp* on CpG sites of UTSS region, which are hypermethylated in tumors that upregulate telomerase. (C) Structural rearrangements of regulatory elements distant from *TERT* locus, which reposition of enhancer factors that activate *TERT* transcription. TERT: Telomerase reverse transcriptase

proportion of cancers (10%–15% overall) activate ALT to maintain telomere length and escape from senescence.[14,17-19]

The prevalence of ALT in cancers is not uniform across the different tumor types; Heaphy *et al*.[18] have systematically analyzed the presence of ALT phenotype in 6110 primary tumors from 94 different cancer subtypes by telomere-specific fluorescence *in situ* hybridization, showing ALT activation in 3.73% (228/6110) of all tumors, arising from the bladder, cervix, endometrium, esophagus, kidney, liver, central nervous system (CNS), and lung. At present, several studies showed that among TMM-positive cancers, the majority of ALT-activated tumors arise from neuroendocrine systems (e.g., pancreatic neuroendocrine tumor), mesenchymal and neuroectodermal cells including bone (e.g., osteosarcoma), soft tissues (e.g., leiomyosarcoma), and peripheral nervous system and CNS (e.g., glioblastoma), while ALT has rarely been reported in epithelial malignancies.[14,17-19,34-36] ALT was also found in pediatric cancers; a recent study analyzed the presence of ALT in 653 pediatric patients with 23 cancer types from the Pediatric Cancer Genome Project, highlighting the activation of ALT in 28.7% of solid tumors, 10.5% of brain tumors (mostly high-grade glioma), and 4.3% of hematological cancers.[20]

The co-existence of telomerase and ALT pathways was previously found in various tumor types; however, at present, it has not yet been clear whether the activation of telomerase and ALT can co-exist within the same cell or within different heterogeneous cell subpopulations in a tumor, and whether switching between the two mechanisms is possible.[37-39]

Alternative lengthening of telomere mechanism

ALT mechanism is dependent on the activation of a homologous recombination DNA-repair mechanism to maintain the telomere length.[40-42]

The first study in yeast showed the existence of two distinct recombination pathways to maintain telomere length in the absence of telomerase, a RAD51-dependent and -independent mechanism.[43] Subsequently, studies on the human neoplastic cells revealed that TMM via ALT is mediated by a pathway of break-induced replication[44,45] Dilley *et al*.[40] evidenced the direct implication of three DNA-repair and recombination proteins (POLD3, PCNA, and RAD52) in the human mechanism of ALT. As recently reported by Zhang *et al*.,[46] ALT occurs through two distinct break-induced replication-like mechanisms; one recombination mechanism requires RAD52 for maintaining telomeres, while RAD52-knockout cells use a mechanism dependent on POLD3 and POLD4, demonstrating the bifurcated framework and dynamic nature of ALT.

However, although several hallmarks, mutations, and de-regulations involved in the ALT pathway have been discovered, the molecular mechanism remains still elusive.

Hallmarks of Alternative Lengthening of Telomere

TMM via ALT exhibits several hallmarks: (1) long and heterogeneous telomere length,[18,35,42] (2) presence of ALT-associated promyelocytic leukemia nuclear bodies (APBs),[36,42,47] (3) presence and accumulation of extrachromosomal telomere repeats, with generation of high

levels of C-rich circular telomeric DNA repeats (C-circles),[48-52] and (4) elevated level of telomere sister chromatid exchanges[49,51-53]

Previous works showed that telomere length distribution in the ALT-positive cells is highly heterogeneous and ranges from less than 3 kb to more than 50 kb, differently from telomerase-positive cells in which all telomeres typically have a similar length of around 10 kb.[18,19,35,50] Tumors with ALT pathway exhibit different telomere length distribution within individual cells and across tumor cell populations.[34,37] Previous studies also revealed the presence of telomeric clusters around the promyelocytic leukemia bodies, forming structures named APBs in ALT-activated cells;[36] APBs contain proteins that are known to function in DNA-repair and recombination processes, suggesting that APBs could provide a recombinogenic microenvironment to promote ALT.[42,45-47] Moreover, a recent work identified a new marker of ALT: the upregulation of the long noncoding RNA TERRA; it has been shown that mammalian cell lines harboring active ALT have higher TERRA levels compared with telomerase-positive cells.[54] However, the role of TERRA in the activation of ALT has not yet been fully clarified.[55]

The most common and reliable methods for detecting the activation of ALT in the neoplastic cells are the evaluation of telomere length by quantitative fluorescence *in situ* hybridization,[16,18,35] the C-circle assay,[51] and the APBs assay.[42,56] However, a systematic comparison from different methods of ALT detection is still challenging due to differences in laboratory techniques, data configuration, and normalization.[34,38,57]

Alternative lengthening of telomere-associated genetic alterations

It has been shown that ALT is frequently associated with loss-of-function mutations in the chromatin remodeling genes, α-thalassemia/mental retardation syndrome X-linked (*ATRX*) and death domain-associated protein (*DAXX*).[18,35,58] Moreover, mutations in other genes, such as *H3F3A* coding for the histone H3.3, *SMARCAL1*, and *IDH1*, have been described in ALT-positive cells, suggesting their involvement in the ALT development.[59-62]

ATRX is an ATP-dependent helicase, part of the SWI/SNF family; it has been shown that ATRX, and its partner DAXX, function together as a chromatin remodeling complex that loads the histone variant H3.3 into telomeric and other repetitive heterochromatic regions.[63,64] The depletion of ATRX in the murine cells leads to the loss of the histone H3.3 at telomeres, creating a more open chromatin environment accessible to recombination proteins.[65] Therefore, it has been shown a strong association between ATRX loss and ALT in several neoplastic cell lines and tumor types.[18,35,58,66]

ATRX inactivation can be driven not only by point mutations, insertion or deletion of bases, large deletions, but also by genetic alterations not detected by direct DNA sequence analysis such as promoter silencing mutations; these alterations are not localized to any specific domain of the protein and are correlated with the loss of its nuclear expression, detectable by immunohistochemistry.[35,58,62]

Functional inactivation of DAXX was found to be associated with ALT, less frequently compared to ATRX and only in few cancer types; in particular, *DAXX* mutations characterized ALT-positive pancreatic neuroendocrine tumors (22%) and were mutually exclusive with *ATRX*.[35,67] However, the role of DAXX in ALT process is still poorly understood.

Recently, as described below, mutations on the gene *H3F3A* have been identified to play a significant role in the pathogenesis of pediatric high-grade brain tumors and seem to be linked with the ALT phenotype.[62,68-71] Moreover, a recent study in ALT-positive glioblastomas evidenced that mutations on *SMARCAL1*, a member of the SWI/SNF family chromatin remodeling proteins, were associated with ALT, suggesting the *SMARCAL1*-inactivating alterations as a novel genetic mechanism of ALT.[59,60]

The main hallmarks and genetic alterations involved in the ALT activation are shown in Figure 2.

Telomere Maintenance Mechanisms in Pediatric Brain Tumors

CNS tumors are the most common solid tumors affecting childhood (0–19 years) and the principle cause of cancer-related death in the pediatric age (0–14 years).[71-74] PBTs account for around 15%–20% of all neoplastic disease in children and comprise multiple separate pathological entities with different survival, symptoms, and localizations.[71,72]

In the past, PBTs were diagnosed and graded according to the histological criteria. During the last years, the extensive use of high-throughput molecular, genetic, and epigenetic profiling techniques largely increased the knowledge on the origin and biological features of these pediatric neoplasms; recent updates in the genetic/molecular characterization of PBTs have shown a substantial heterogeneity even among PBTs with the same histological classification, such as molecular subgroups of medulloblastoma,[75-77] glioma,[69,71,78] and ependymoma.[79-81] Consequently, adult brain tumors and PBTs have been re-classified, combining histologic and molecular data, with significant clinical correlations in terms of anatomical location and prognosis.[71,82]

In recent years, several studies have also shown that gliomas in children differ fundamentally from those in adults, regarding genetic and epigenetic profiles, associated driver mutations, radiological features, anatomical distribution, and clinical outcome, leading to the separation of several PBTs from the adult counterparts.[69-71,83,84] In line with these findings, the incidence of TMMs in adult and pediatric cancers has

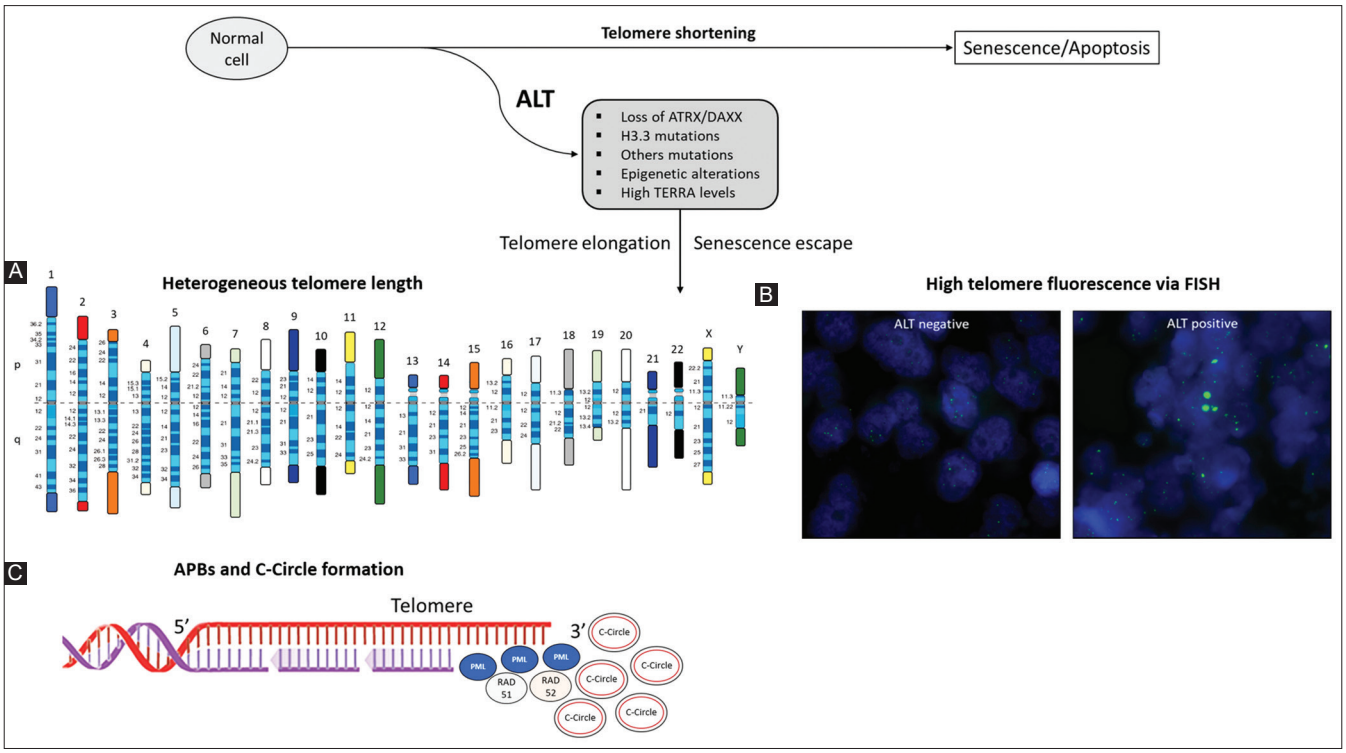

Figure 2: Schematic representation of telomere maintenance triggered by ALT. Image summarizes the main alterations associated with ALT (in the box) and shows the hallmarks of ALT, including long and heterogeneous telomere length (A), ultrabright telomeric signals in ALT-positive tumor analyzed via Q-FISH, compared with ALT-negative sample (B), formation of APBs and extrachromosomal telomere C-circles (C). ALT: Alternative lengthening of telomere, APBs: ALT-associated promyelocytic leukemia nuclear bodies, Q-FISH: Quantitative fluorescence *in situ* hybridization. (B) Reprinted from Minasi *et al*.[107]

been found substantially different, confirming that adult and pediatric tumors should be considered separately also regarding the activation of TMMs.[14,20,34,85]

All previous reported incidences of TMMs triggered by telomerase or ALT in various histological/molecular subtypes of pediatric brain tumors are summarized in Table 1.

Pediatric high-grade glioma

Diffuse high-grade gliomas account for ~11% of all CNS tumors in children.[69,71,72] Different from gliomas in adults, which tend to be restricted to the cerebral hemispheres, pediatric high-grade gliomas (pHGGs) can arise throughout the CNS, with around 50% of cases occurring in midline locations.[69,83] *IDH1*/2 mutations and *TERTp* mutations are key molecular alterations for adult patients but are relatively rare in pediatric gliomas, which are characterized by mutations in the gene *H3F3A* encoding the histone H3.3.[20,66,69,86,87]

The two hotspot variants H3.3-K27M and H3.3-G34R/V define different pHGG molecular subgroups;[69] a novel diagnostic entity entitled "*diffuse midline glioma (DMG) H3-K27M-mutant*" has been introduced in the WHO classification of CNS tumors,[71] while the clinicopathological significance of H3.3-G34R/V has not been completely elucidated but will probably constitute an own biological entity in the upcoming classification.[78]

Interestingly, pHGGs are frequently mutated in *ATRX*, which incorporate the histone H3.3 into telomeres;[18,35,68,69,88,89] in particular, hemispheric pHGGs with H3.3-G34R/V showed a significant overlap with *ATRX* mutations.[62,68,35,70,88]

As previously described, telomerase-dependent TMM is generally triggered by *TERTp* mutations (C228T and C250T) and hypermethylation,[14,20,29,30] while ALT-dependent TMM activation is mediated by the deregulation of ATRX/DAXX/H3.3 complex.[18,35,58,66] TMM via telomerase-dependent mechanism seems to be uncommon in pHGGs, given the low frequency of *TERTp* mutations (1.5%–11%) and the absence of telomerase reactivation,[20,66,87] while the presence of ALT in pHGGs was associated with loss of ATRX, highlighting a significantly increased prevalence of ALT in pediatric glioblastoma (44%).[18] Subsequently, given that pediatric gliomas frequently harbored key genetic alterations on *ATRX* and *H3F3A*, several studies showed that pHGGs activated ALT mechanism with high incidence (19.2%–53%).[20,68-70,90,91]

Furthermore, tumors previously referred as diffuse intrinsic pontine gliomas; a class of high-grade glial tumors of the brainstem significantly enriched in H3.3-K27M (60%–80%) and H3.1 (HIST3H1B)-K27M mutations (20%) is now included in the newly defined entity DMG.[71,92-94] Two independent studies showed the presence of ALT activation in 3/11 (27.2%) and 9/48 (18.8%) DMGs, respectively, not

Table 1: Incidence of telomere maintenance mechanisms triggered by telomerase and alternative lengthening of telomere in different types of pediatric brain tumors, analyzed in several cohorts from different studies

Tumor type	Telomerase-dependent TMM (%)	ALT-dependent TMM (%)
pHGG	11% *TERTp* mutation[66]	44%[18]
	3% *TERTp* mutation[87]	22%[90]
	0%[20]	53% all, 27.2% in DMG[68]
	73.3% in DMG[68]	19.2%[69]
	2% *TERTp* mutation in DMG[95]	18.8% in DMG[90]
pLGG	0%[100]	0%[100]
		1.19%[90]
		2.6%[20]
MDB	85.7%[100]	7%[18]
	20.8% *TERTp* mutation, mainly SHH[66]	2.2%[90]
	21% *TERTp* mutation, mainly SHH[106]	
	18.2% *TERTp* mutation in metastatic MDB[107]	32% in metastatic MDB[107]
EPD	64%[116]	0%[116]
	2.7% *TERTp* mutation[66]	0%[90]
	0% *TERTp* mutation[87]	
	0% *TERTp* mutation[117]	0%[117]
Other rare pediatric CNS tumors	0% *TERTp* mutation[66]	0% AT/RT[90]
	0% *TERTp* mutation[87]	22.6% choroid plexus tumors[90]

AT/RT: Atypical teratoid/rhabdoid tumor, ALT: Alternative lengthening of telomere, CNS: Central nervous system, DMG: Diffuse midline glioma, EPD: Ependymoma, MDB: Medulloblastoma, pHGG: Pediatric high-grade glioma, pLGG: Pediatric low-grade glioma, TMM: Telomere maintenance mechanism, SHH: Sonic Hedgehog molecular subgroup

always associated with histone mutations.[68,90] Moreover, high telomerase RNA component and TERT expression were identified in 11/15 (73.3%) DMG samples, and given the high incidence of telomerase expression, authors suggested that telomerase inhibition may be a promising therapeutic approach.[68] However, in strong contrast with this publication, other studies evidenced a very low frequency of *TERTp* mutations in DMGs (~2%).[69,95]

At present, it is clear that a subpopulation of pHGGs activates ALT, while the reactivation of telomerase is rare; in the future, a promising therapeutic approach for these ALT-positive patients could be represented by the novel ALT-targeted drugs.

Pediatric low-grade glioma

Pediatric low-grade gliomas (pLGGs), defined as WHO grade I and II neoplasms, constitute approximately 30% of all PBTs and are a heterogeneous group of tumors with different histological changes, including pilocytic astrocytoma (~16% of all CNS pediatric tumors), pilomyxoid astrocytoma, diffuse grade II astrocytoma, ganglioglioma, and pleomorphic xanthoastrocytoma.[71,96] The survival rates of pediatric patients with LGGs are good, with 5-year survival around 75% overall and 10-year survival over 90% for patients with complete resection of tumor.[74]

Several genetic/molecular alterations were reported in pLGGs; in particular, de-regulation of factors involved in the signaling pathway of mitogen-activated protein kinase, including BRAF mutation or KIAA1549:BRAF fusion, FGFR1 mutation or structural rearrangement, NF1 mutation, NTRK-family fusions, and alterations in MYB or MYBL1.[96-98] The development of drugs which specifically target BRAF, FGFR1, and NTRK has led to the possibility of further therapeutic options for these neoplasms, using specific inhibitors currently in progress of clinical trials.[96,99]

Only few studies explored the role of TMMs in pLGGs. Tabori *et al*.[100] first analyzed telomerase reactivation and ALT in a cohort of pLGGs; authors never found telomerase activity (0/11). Moreover, 0/45 pLGGs in this study were positive for ALT.[100] Authors showed that pLGGS lack any mechanism of telomere maintenance, induced both by telomerase and ALT, suggesting that senescence triggered by telomere shortening could play a key role in pLGG evolution and could explain the spontaneous growth arrest and regression that occasionally characterizes pLGGs.[100] Subsequently, other publications observed ALT in only 1/84 (1.19%) and 1/38 (2.6%) pLGGs, respectively, confirming that TMM via ALT is almost absent in pLGG.[20,90]

Medulloblastoma

Medulloblastoma is a grade IV tumor and represents a heterogeneous class of embryonal tumors, mostly localized in the cerebellum; it is the second most common PBT, accounting for approximately 20% of all primary CNS tumors in children.[71,101]

Medulloblastomas comprise four molecular-defined subgroups (WNT, SHH, Group 3, and Group 4), associated with different cells of origin, specific genetic landscapes, copy number alterations, methylation profiles, and different clinical outcome of patients.[76,77,102] The outcome of WNT subgroup is excellent, with a 5-year survival rate over 95%,

while Group 3 patients exhibit the worst survival (45%–60%); Group 4 and SHH are characterized by intermediate overall survival (75%–80%), depending on the histology, presence of metastases, and molecular alterations as *MYC* and *MYCN* amplification.[77,102-104] Moreover, each of these molecular subgroups is characterized by intertumoral heterogeneity, comprising different genetic subtypes.[76,105]

Several studies have investigated the incidence of TMMs in medulloblastomas and the presence of telomerase- and ALT-associated molecular alterations in these heterogeneous tumors. The first study by Tabori *et al*.[100] observed the telomerase activity in 6/7 medulloblastomas (85.7%). Moreover, a later publication found the presence of *TERTp* mutations in 19/91 medulloblastomas (20.8%), associated with older patients.[66] Subsequently, other studies confirmed the presence of *TERTp* mutations in approximately 18%–21% of medulloblastomas, highlighting that the highest incidence of *TERTp* mutation was observed in adult patients of SHH group, while Group 3 and Group 4 medulloblastomas harbored this alteration in <5% of cases.[76,87,106]

Moreover, medulloblastomas can often present leptomeningeal dissemination, and approximately 30% of pediatric medulloblastomas occur with metastasis at the onset.[102,104] A recent study showed that medulloblastomas with metastasis at the onset harbored *TERTp* mutations in 18.2% of samples belong to all molecular subgroups, suggesting that TMM-induced by telomerase reactivation is not restricted to SHH variant in metastatic patients.[107]

It has been shown that alterations affecting the ATRX/DAXX/H3.3 complex are uncommon in medulloblastomas; *DAXX* and *H3F3A* mutations were never observed,[35,89,107] while *ATRX* mutations were extremely rare (1.5%).[35,108,109] Regarding the presence of ALT, 55 medulloblastomas were analyzed and ALT was observed in 7% of tumors, mostly associated with anaplastic histology (18%).[18] Later, ALT activation was found in 3/137 (2.19%) medulloblastomas, suggesting that ALT does not have a primary role.[90] Moreover, another study showed a higher incidence of ALT (32.1%) in metastatic medulloblastomas, highlighting that it could be a common process in medulloblastomas with metastatic spread at diagnosis.[107]

At present, a subpopulation of SHH adult medulloblastomas seems to frequently activate telomerase to maintain the telomere length, while ALT appears to be rare in medulloblastomas but more frequent in metastatic ones. Further studies in larger series of patients will be needed, to better understand the role of TMMs in medulloblastomas.

Pediatric ependymoma

Ependymomas arise from ependymal cells, which form the lining of ventricles in the brain and the central canal of spinal cord and produce cerebrospinal fluid; these tumors account for 6%–10% of cancers in children.[73,110,111] Approximately 90% of ependymomas in children occur intracranially, in the posterior fossa or within supratentorial compartments.[72,80] The clinical outcome of pediatric patients with ependymomas is highly variable; the 10-year overall survival of ependymomas is approximately 60%, with spinal ependymomas associated with better prognosis compared to intracranial.[72,112] Histologically, ependymomas are composed by different subtypes and are classified as subependymoma and myxopapillary ependymoma (grade I), which are almost exclusively in adults, classic ependymoma (grade II), and anaplastic ependymoma (grade III); however, histological criteria showed poor predictive value.[71,81,111]

During the last years, several studies showed that ependymomas are characterized by heterogeneous genetic mutations, different epigenetic profiles, and copy number alterations.[71,79-81,113] In particular, two distinct molecular subgroups of posterior fossa ependymomas (PFA and PFB) have been defined; PFA affects predominantly infants and is associated with genetic alterations of *VEGF*, *PDGFR*, and mitogen-activated protein kinase pathway, while PFB affects predominantly older children and adults, occurs less frequently, and is associated with better prognosis.[79,114] Moreover, it has been shown that the fusion C11orf95-RELA on chromosome 11 represents a key genetic alteration in supratentorial ependymomas, harbored by more than 70% of cases.[71,81,114]

Regarding the activation of TMMs, Tabori *et al*.[115] first highlighted a correlation between pediatric ependymomas and telomerase activity. Then, another study, using TRAP assay, found that 23/36 (64%) ependymomas activated telomerase to maintain the telomere length; all these samples were negative for *TERTp* mutations but exhibited a strong association with *TERTp* hypermethylation.[116] Other studies confirmed very low frequency or even absence of *TERTp* mutations in pediatric ependymomas, suggesting that telomerase reactivation could be triggered by *TERTp* hypermethylation or other alterations.[66,87,117]

Moreover, 76 pediatric ependymomas were analyzed by telomere fluorescence *in situ* hybridization and C-circle assay, and none of these cases showed the presence of ALT or the loss of ATRX nuclear expression.[116] Authors evidenced that telomerase-dependent mechanism appears to be frequently activated in pediatric ependymomas, while ALT-dependent mechanism is absent, suggesting the use of telomerase inhibitors as a promising therapeutic strategy for these tumors.[116] Subsequently, other studies confirmed the absence of ALT in pediatric ependymomas.[90,117]

Further studies will be necessary to elucidate the impact of telomerase reactivation in ependymomas; however, these data suggest that telomerase-dependent TMM may represent a key mechanism for senescence escape in pediatric ependymomas.

Other rare pediatric central nervous system tumors

Other rarer CNS tumors in children comprise pediatric meningiomas, rare embryonal tumors, pineoblastomas, germinal tumors, choroid plexus tumors, and craniopharyngiomas (<5% of frequency each).[71,118]

Meningiomas are infrequent in childhood, accounting for 1%–4% of all pediatric CNS tumors, compared to 20% of all adult CNS tumors; pediatric meningiomas are histologically atypical (grade II) or anaplastic (grade III) and are often associated with chromosome 22 alterations and *NF2* gene deletion.[119,120] In addition to medulloblastoma, other rarer embryonal tumors of the CNS comprise "embryonal tumor with multilayered rosettes (ETMR), C19MC-altered," recently described as tumor entity in the WHO, and atypical teratoid/rhabdoid tumor (AT/RT).[71] ETMR (grade IV) typically occurs in children under 3 years, it is associated with poor prognosis, and it is characterized by LIN28 expression and the 19q13.42 locus amplification, which contains a cluster of microRNAs (C19MC).[71,121] AT/RT (grade IV) accounts for 1%–2% of PBTs, it more often occurs in children under the age of 1 year, and it is characterized by 22q deletion and loss of INI1/SMARCB1/BAF47 expression.[71,73,122] Pineoblastoma at raw 20 and 22 (grade IV) is a malignant tumor of the pineal region that represents around 1% of all pediatric CNS tumors; pinealoblastomas show an aggressive clinical behavior, often harbor germline mutations on *RB1* and *DICER*, and are often associated with congenital retinoblastoma.[71,123,124] Germinal tumors are a heterogeneous group of cancers that account for 3%–4% of PBTs, histologically divided into germinomas and nongerminomas; genetically, these tumors are often associated with *KIT*, *KRAS*, and *NRAS* mutations.[71,125,126] Choroid plexus tumors (grade I–III) are around 2% of all PBTs and often occur in patients under 2 years; they arise from neuroectoderm and can be associated with *TP53* germline mutations.[127,128] Craniopharyngioma (grade I) can occur in pediatric age in two histological subtypes, adamantinomatous and papillary, and the incidence in children is 3%–5%; the most common genetic alterations associated with craniopharyngiomas are *CTNNB1* mutation, frequent in adamantinomas, and BRAFV600E mutation, frequent in adults.[71,118,129]

Given the rarity of these PBTs, only few studies analyzed their association with TMMs induced by telomerase or ALT. Two independent publications showed the absence of *TERTp* mutations in meningiomas, craniopharyngiomas, embryonal tumors, and germinal tumors, suggesting the lack of telomerase reactivation in these types of cancer.[66,87]

Moreover, all these tumor entities do not seem to be associated with alterations on ATRX/DAXX/H3.3 complex; regarding ALT activation, a single study found 0/29 AT/RT ALT-positive cases, while choroid plexus tumors exhibited the presence of ALT in 7/31 (22.6%) samples.[90] However, at present, there are insufficient amount of data regarding the incidence of TMMs in all these pediatric rare neoplastic entities, and further studies are necessary.

Therapies against Telomere Maintenance Mechanisms

There are several promising novel anticancer therapies that target TMM induced by telomerase or ALT with good potential for clinical applications. The immortalization of neoplastic cells, with the activation of one TMM, is an almost universal hallmark of cancers, whereas normal cells are not able to prevent telomere shortening.[1,7,8,10] For this reason, TMMs and related factors involved in the telomerase or ALT appear to be ideal targets for the development of anticancer therapies, potentially applicable to many cancers.

As described below, several therapeutic strategies have been used to inhibit telomerase, as oligonucleotide and small molecules;[130,131] moreover, multiple ALT-targeted drugs have been tested, including recombination inhibitors, histone deacetylase inhibitors, or G-quadruplex (G4) stabilizer.[132,133]

However, due to the complexity of these mechanisms,[46] the possible co-existence of telomerase/ALT pathways within the same cell or the same tumor,[37,39] the ability of tumors to switch from a TMM to another one,[34] and the difficulty in identifying telomerase/ALT inhibitors which specifically block telomere elongation *in vivo*,[130,133-135] the optimal design of a TMM-targeted therapy remain yet unclear and additional researches are needed.

Antitelomerase therapies

As previously described, the telomerase complex can be upregulated in the neoplastic cells, and it is considered a good target for anticancer therapy because most somatic cells do not have telomerase activity;[25] the selective inactivation of telomerase in tumors appear to be ideal to kill the neoplastic cells without influencing most normal cells.[136] Thus, there are many telomerase-based therapies in the clinical development and under investigation.[130,131,136] Current telomerase-targeted therapies include (1) telomerase inhibitors, as oligonucleotides and small molecules, (2) immunotherapeutic approaches, as vaccines, (3) telomerase-directed gene therapy, and (4) phytochemicals.[131]

Several studies showed that antisense oligonucleotides can inhibit telomerase, inducing telomere shortening and senescence in the neoplastic cell lines.[137-139] The most widely developed and successful is the thio-phosphoramidate oligonucleotide inhibitor called Imetelstat or GRN163 L (Geron Corporation, Menlo Park, CA, USA), which binds the RNA template, blocking telomerase activity.[137,140] Randomized preclinical phase II studies utilizing Imetelstat have been conducted on many cancer types, such as nonsmall cell lung cancer, breast cancer, and glioblastoma; interestingly, it has been shown an effective inhibition of telomerase, with a reduction of tumorigenicity and invasiveness, without significant short-term side effects, suggesting the possible use of Imetelstat as combinatory therapy.[141-143] However, the long-term effects of this treatment have not yet fully investigated, in particular, the effects on normal cells that transiently express telomerase, such as germ cells, lymphocytes, and endothelial cells; moreover, this therapeutic approach has proved too toxic in children with recurrent brain tumors, due to patients commonly developing severe hematological long-term side effects.[144]

Recently, another telomere target therapy was validated in preclinical studies, which is based on the incorporation of 6-thio-2'-deoxyguanosine (6-thio-dG), a telomerase substrate precursor nucleoside, into telomeres by telomerase.[145] *In vivo* studies showed that 6-thio-dG incorporation created DNA damage and induced cell death in cancer cell lines, constituting a promising strategy for telomerase-positive pediatric CNS tumors.[145]

Another approach is the use of small molecule telomerase inhibitors, as epigallocatechin-3-gallate and BIBR1532;[135,146] however, at present, all these molecules have demonstrated limited improvement in the prognosis of patients.[141,147]

Another strategy aimed at blocking telomerase-related immortality is the immunotherapeutic approach, using the active site of telomerase as a target to develop vaccines.[148] The vaccine mechanism is based on the use of peptides generated by the degradation of TERT, which are presented on the cell surface via the MHC pathway, triggering the response of cytotoxic T-lymphocytes that recognize and kill the peptide-presenting cells.[149] Several clinical studies have evaluated the use of TERT immunotherapy combined with chemotherapy as anticancer approach with variable results in many tumor types, such as glioblastoma, nonsmall lung cancer, melanoma, pancreatic cancer, and prostate cancer.[150-157] At present, the most promising TERT vaccine is represented by the GV1001, an MHC II peptide used for the treatment of solid cancers, showing an improvement of survival in patients with pancreatic tumors.[158,159] The telomerase-related vaccines have shown acceptable safety and tolerability, and some of them have generated an immune response in a proportion of patients;[151,152,158,160] however, the limited success in many clinical studies seem to be dependent on the development of self-tolerance and the effects of immunosuppressive tumor microenvironment on T-cells response.[130,157]

Finally, a variety of substances derived from plants (phytochemicals) have demonstrated to partially inhibit telomerase activity and have been used as anticancer strategies, including allicin, sulforaphane, curcumin, and genistein.[161-165] Moreover, numerous drugs have been demonstrated to have additional effects on telomerase activity, as tyrosine kinase inhibitors, PI3K-Akt-mTOR pathway inhibitors (e.g., rapamycin), DNA methylation inhibitors, and temozolomide.[166-171]

Despite multiple and extensive studies, at present, only one telomerase inhibitor (Imetelstat) is under evaluation in a phase 3 clinical trial, and one vaccine (GV1001) has been approved by the FDA for immunotherapy of pancreatic cancer.

Antialternative lengthening of telomere therapies

ALT is a potential therapeutic target in cancers lacking telomerase activity. Recently, several studies identified multiple factors involved in the ALT activation associated with different types of cancer, which could be used as potential targets for therapy. Two recent reviews summarized a list of potential ALT-targeted drugs, such as inhibitors of recombination factors (ATR, RAD52, SET domain bifurcated 1 protein [SETDB1], and FANCM), histone deacetylase inhibitors, G4 stabilizer, inhibitors of APBs formation (SUMO E3 ligase), and other strategies to restore the proper functioning of the ATRX/DAXX/H3.3 complex.[132,133]

ALT is mediated by a pathway of break-induced replication dependent on ATM, ATR, RFC, and PCNA.[40] The inhibition of ATR disrupts ALT and triggers chromosome fragmentation and apoptosis in a panel of cancer cell lines, suggesting a promising approach in the treatment of ALT-activating tumors.[132,172] The recent evidence regarding the implication of RAD52 in the break-induced replication mechanism and ALT activation has suggested the use of this protein as potential target for future treatments.[40,46,132] Another ALT-specific target of interest is FANCM, recently found to be essential for proliferation of ALT cells;[173] previous studies showed that the inhibition of FANCM is extremely toxic for ALT-positive cells, suggesting the possibility of development of FANCM inhibitors.[174,175] Moreover, another publication highlighted that the formation of the chromatin environment on subtelomeric DNA which triggers ALT is mediated by H3K9 methyltransferase activity of SETDB1, and the loss of this enzyme leads to the reduced recruitment of ALT-related factors, suggesting the use of SETDB1 inhibitors as a future therapy.[176]

Another anti-ALT strategy is the use of histone deacetylase inhibitors, which could be used to selectively inhibit ALT by blocking the histone deacetylation complex that promotes recombination on telomeric DNA;[177] however, it is still unclear whether histone deacetylase inhibitors could be effective enough to induce ALT-positive cells death, without side effects in the nonneoplastic cells.[178] Another approach tested to inhibit ALT is the use of APBs formation inhibitors, as SUMO E3 ligases.[179]

Moreover, one of the most promising approaches is the possibility to target a specific secondary structure of telomeres called G4; these DNA G4 are noncanonical four-stranded helical structures rich in guanine.[180] Interestingly, it has been shown that G4 ligands inhibit both telomerase and ALT pathway, inducing senescence and apoptosis.[181-183] At present, two of these G4 stabilizers reached the stage of clinical trial: CX-3543 (at phase 2) for several tumors, including neuroendocrine tumors, carcinoid tumors, and lymphoma, and CX-5461 (at phase 1) for patients with BRCA1/2-deficient tumors.[183] However, the application of these ligands in ALT-positive tumors is still under study, due to the low selectivity of G4 ligands which could cause unexpected side effects.[184]

Finally, one of the most recent and exciting opportunities for immunotherapy is the use of oncolytic viruses against neoplastic cells with loss of ATRX/DAXX. As previously described, ATRX loss has been directly associated with ALT in several tumor types.[18,35,58,62-64,66] It has been shown that ATRX and DAXX have also a role in the innate viral immune

response, protecting cells from viral infection, and several viruses (e.g., adenovirus, Epstein–Barr virus, and herpes simplex virus) use a protective mechanism to repress ATRX/DAXX-mediated response.[132,185-188] During the early stage of infection, these viruses have been shown to express a protein called ICP0 that induces the blockage of ATRX/DAXX and the degradation of promyelocytic leukemia bodies;[185,189] in line with these findings, ICP0-null viruses are not able to infect and replicate inside ATRX-positive cells.[186] Therefore, the use of ICP0-null oncolytic viruses to directly kill ALT-positive neoplastic cells with ATRX loss, without side effects on non-neoplastic cells that normally express ATRX, could constitute a promising therapy.[132,186,190]

However, it is necessary to highlight that, at present, there are no data regarding the use of an ALT-targeted therapy in human patients that progressed beyond phase 2 in clinical trials.[133]

Conclusions

ALT is a common mechanism in ATRX/H3.3-mutated pHGGs, choroid plexus tumors, and medulloblastomas with metastatic spread at diagnosis; ALT-targeted therapies could represent a promising future strategy to treat these patients, probably combined with radio-and chemotherapy. Telomerase-dependent TMM appears to be a common mechanism in SHH-medulloblastomas, associated with older patients and *TERTp* mutations, and in pediatric ependymomas, rarely associated with *TERTp* mutations while more frequently induced by *TERTp* hypermethylation or other alterations; these patients could potentially benefit in the future from antitelomerase therapies. Telomerase and ALT mechanisms are almost absent in LGGs, pediatric meningiomas, rare embryonal tumors (ETRM and AT/RT, pineoblastomas), germinal tumors, and craniopharyngiomas.

Further studies are necessary to better elucidate other genetic and epigenetic alterations associated with these mechanisms, to identify the best reproducible and reliable methods for TMMs detection in clinical practice, and to evaluate the effects of targeted therapies.

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Conflicts of interest

There are no conflicts of interest.

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