

# Telomere Abnormalities Regulate Neuroblastoma

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Telomere maintenance is a powerful prognostic marker of HRNB thereby representing an attractive target for the development of novel therapeutic treatments.

telomere maintenance

TERT

ATRX

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ALT

neuroblastoma

## 1. Introduction

Neuroblastoma (NB) is the most common extracranial malignant solid tumor in infancy and is derived from cells of the embryonal neuronal crest [1][2]. It is a very heterogeneous disease in terms of outcomes and response treatments, from spontaneous regression (~50% of infants) to widely disseminated tumors that are frequently resistant to multimodal treatments [3][4][5]. Despite multimodal therapeutic treatments, the majority of relapsed high-risk neuroblastoma (HRNB) patients still succumb to the disease. The genomic amplification of MYCN was strongly associated with unfavorable patient outcomes in approximately 20% of primary NB tumors and 40% of HRNB. However, since the amplification of MYCN only occurs in 40% of HRNB, other genetic and/or epigenetic alterations may play an important role in the remaining 60% of this disease subtype. In addition to MYCN amplification, other segmental chromosomal aberrations, including 1p deletion, 11q deletion, and 17q gain, have been detected in HRNB. The gain of chromosome 17q and the loss of chromosome 1p were observed in one-half and one-third of NB cases, respectively, and are associated with an adverse patient outcome [4][5][6][7]. The loss of 11q is also observed in about one-third of NB tumors and is a marker of poor prognosis. In recent years, several massive parallel sequencing studies identified MYCN, TERT, and  $\alpha$ -thalassemia /mental retardation syndrome X-linked (ATRX) aberrations as frequent and mutually exclusive drivers in HRNB [8][9][10]. Due to these three frequent genomic alterations, which converge to activate telomere maintenance mechanisms (TMM) [8][9][11], telomeres and TMM have emerged as pivotal attributes and indicators of poor prognosis for HRNB, with longer telomere length being associated with a worse prognosis [12][13][14]. In contrast, the absence of any detectable TMM was associated with spontaneous regression and excellent survival [13], supporting the beneficial effects of targeting TMM pathways for patients [8][9][15].

Clinical therapeutic strategies in NB are established on the basis of risk classification. Patients with low-risk disease spontaneously regress or undergo surgical resection, but chemotherapy is performed when a residual tumor is found or when surgical removal is difficult. For the intermediate-risk group, treatment options include chemotherapy for the progressive disease after surgery and/or relapse, an emergency irradiation for the cases with neurological symptoms. HRNB, on the other hand, is very difficult to treat and requires multimodal therapy to

achieve the current survival rate of slightly less than 50%. HRNB is currently treated with a number of DNA-damaging agents, anti-GD2 antibodies, immunity checkpoint inhibitors targeting PD-L1, 13-cis-RA, ALK inhibitors, and CAR-T therapy [5]. Despite significant advances in the field of NB therapy, HRNB continues to have poor prognosis. There are no available clinical agents to effectively target TMM in NB.

## 2. Telomere Maintenance by Telomerase Activation

Telomerase is a functional ribonucleoprotein enzyme complex that is responsible for maintaining telomere lengths by synthesizing telomeric DNA repeats at the 3' ends of linear chromosomes, thereby compensating for telomere attrition during each round of DNA replication [16]. It is a reverse transcriptase that consists of two essential components: a functional catalytic protein subunit called human telomerase reverse transcriptase (hTERT) encoded by the TERT gene, and an essential RNA component known as human telomerase RNA (hTERC or hTR), encoded by the TERC gene. The limiting factor for telomerase activity is TERT expression, which is detectable in more than 90% of human malignancies [17].

Normal human somatic cells exhibit very low or undetectable telomerase activity. In contrast, the overexpression of TERT was previously identified in 73% of all cancers by systemic analysis of TERT gene amplification in the Cancer Genome Atlas (TCGA) cohort including 6835 patients and covering 31 tumor types [18]. Tumor cells from several tumor types normally reactivate telomerase expression to maintain telomere lengths, thereby escaping senescence or apoptosis and promoting unlimited replication [17][19][20]. The up-regulation or reactivation of telomerase is a critical feature in the development of about 85% of human cancers [21], and is correlated with poor prognosis [14][22][23][24]. High telomerase expression levels define a large group of HRNB with increased invasiveness and poor prognosis [25], similar to MYCN -amplified tumors [26]. Telomerase may be activated by the induction of TERT , which may occur through at least two pathways: MYCN amplification and genomic rearrangements around TERT , including TERT promoter mutations, TERT structural variants ( TERT SVs ), and epigenetic modifications through TERT promoter methylation [8][9][14][27]. Immunofluorescence of 102 primary NB revealed the variable expression of TERT in 99 cases, 31 and 68 of which showed high and low TERT expression levels, respectively. High TERT expression levels were identified as a robust predictor of event-free-survival (EFS) and OS in NB patients, irrespective of age [28]. A recent study demonstrated that telomerase-activation-associated alterations occurred in approximately one-third of primary NB, were associated with poor patient survival, and were an independent prognostic marker in the multivariable analysis of TERT rearrangements in 457 pretreatment NB [27]. Approximately 10%–20% of untreated tumors harbored TERT rearrangements and the amplification of MYCN , respectively, both of which induced the expression of TERT , which is consistent with previous findings [9]. In addition, TERT mRNA levels were elevated in approximately 4% of untreated tumors that lacked genomic aberrations in TERT or MYCN . NB with TERT rearrangements, MYCN amplification, or high TERT expression without these alterations were associated with unfavorable prognostic variables and adverse patient outcomes [8][9][27].

MYCN amplification is a powerful prognostic indicator for HRNB that is associated with high telomerase activity and the upregulation of TERT [9][29]. Since MYC-binding sites have been detected in the TERT promoter region, the

overexpression of MYCN appears to promote telomere stabilization via telomerase activation through a transcriptional increase in the expression of TERT [30][31][32]. In a large cohort of 379 NB tumors, the amplification of MYCN and TERT rearrangements was strongly associated with elevated TERT mRNA levels [13]. Moreover, Peifer et al. [9] reported the strong upregulation of TERT expression in MYCN -amplified tumors, whereas HRNB without TERT or MYCN alterations had low TERT mRNA levels and lacked telomerase activity and the activation of the ALT pathway.

TERT structural variations involving rearrangements of the TERT gene are associated with the strong induction of TERT expression, suggesting that these alterations regularly contribute to TMM and the immortalization of tumors. Similar to other studies, Koneru et al. [15] identified TERT SV in approximately 20% of HRNB patients, and all TERT SVs tumors had highly upregulated TERT mRNA. The activation of TERT in HRNB was not limited to tumors with TERT SVs or the amplification of MYCN because some tumors without these alterations expressed TERT as highly as TERT SVs and MYCN -amplified tumors [13][15]. In addition, TERT expression levels in some MYCN -amplified tumors were as low as those in ALT tumors. MYCN overexpression studies revealed that MYCN upregulated TERT expression via an intact and nonrepressed TERT promoter, but was not sufficient to overcome TERT repression in telomerase-negative and TERT SVs -positive cell lines, indicating an unidentified mechanism for the activation of TERT in NB. RNA-seq of TERT expression and the C-circle assay to detect ALT showed that 12%–26% of HRNB tumors (including some MYCN -amplified tumors) had low TERT expression levels and lacked ALT activation, and these patients had significantly better OS.

### 3. Telomere Maintenance by ALT

In the absence of telomerase activity, tumor cells maintain functional telomeres by utilizing an alternative route of TMM, namely, ALT. A lower proportion of human tumors (approximately 10%–15%) adopt ALT over telomerase reactivation to potentiate their replicative immortality [33][34][35][36]. Although ALT occurs in common tumors, it is the most prevalent in tumors of mesenchymal origin, including those arising from bone, soft tissue, neuroendocrine systems, and the peripheral and central nervous systems, and is generally associated with a poor prognosis [36][37][38]. Approximately 25%–30% of NB employ the ALT pathway, which is generally associated with unfavorable NB in older children without MYCN amplification and independent of telomerase activation status [8][9][15]. These tumors have a very poor clinical outcome. Another study that used telomere length as a marker of ALT indicated that this mechanism may occur in up to 59% of NB tumors [28].

The coexistence of ALT and telomerase was reported in various tumor types [39][40][41]. Some antitelomerase-based treatments demonstrated the ability of some tumor cells to escape death and switch from telomerase to ALT [39][42][43]. However, the mechanisms underlying possible switching or the co-existence of telomerase and ALT within the same cell or different heterogeneous cell subpopulations in a tumor remain unclear. ALT patient tumors, cell lines, and PDXs in NB expressed very low TERT mRNA levels, indicating that ALT and telomerase activation occur in a mutually exclusive manner [15] rather than coexist. In accordance with these findings, the recent profiling of ALT-positive NB tumors showed minimal to no TERT mRNA expression linked to low telomerase activity, resulting from epigenetic silencing of the TERT locus by H3K27me3 [44]. In contrast, Pezzolo et al. [28] suggested that the

coexpression of the ALT mechanism and TERT, observed in 60% of analyzed NB tumors, plays a major role in NB tumor progression. Moreover, the coexistence of cancer-cell subpopulations with different telomere lengths within NB correlated with a poor clinical outcome and disease progression in NB patients.

ALT is a homology-directed recombination-dependent replication pathway that utilizes telomeric DNA as a template for elongation. Although the molecular mechanisms by which ALT telomere maintenance occurs remain unclear, previous findings indicated that ALT telomeres were prone to replication stress and that double-strand breaks caused by replication fork collapse may give rise to break-induced telomere synthesis, resulting in long tract telomere extensions of up to 70 kb [17][36][45][46][47]. A recent study suggested that ALT is a bifurcated pathway involving RAD52-dependent and RAD52-independent mechanisms mediated by break-induced DNA replication [48], very similar to that observed at double-strand breaks in yeast [49].

In addition to a lack of reliance on telomerase, ALT is characterized by a number of markers, including heterogeneous telomere length [50][51], a high level of telomere-sister chromatid exchange [52], a specialized telomeric nuclear structure called ALT-associated promyelocytic leukemia protein bodies (APBs) [53], and the presence of extrachromosomal telomeric DNA repeats in the form of partial double-stranded circles, termed C-circles [54][55]. Increased replicative stress and telomeric DNA damage-induced foci, a potential driver of the generation of ALT, are frequently observed in ALT-positive cells and regarded as a hallmark of ALT [56][57][58]. Recent studies identified two new possible markers of ALT: mitotic DNA synthesis [45][47] and the upregulation of long noncoding telomeric repeat-containing RNA (also called TERRA) [59]. A previous study [44] screened for ALT in primary and relapsed NB (n = 760), characterized its features using multiomics profiling, and suggested that ALT-positive tumors are clinically and molecularly distinct. ALT is clinically associated with a prolonged disease course and poor outcome. One of its molecular features is mutated ATRX and/or reduced protein abundance, heterochromatic telomeric chromatin, a slow proliferative capacity, and the frequent formation of neotelomeres.

## 4. ALT and ATRX Genetic Alterations

Although details on the ALT mechanism remain unclear, different factors may be involved in its activation during cell immortalization and cancer development. Loss-of-function (LoF) genetic alterations in the chromatin remodeling genes ATRX and DAXX (death domain-associated protein) were associated with ALT in multiple malignancies [17][60][61][62][63][64]. Moreover, ALT is less commonly associated with mutations in TP53, IDH, H3.3 G34R/V, H3F3A, and SMARCL1 [65][66][67][68][69][70][71]. LoF mutations in ATRX are the most common genetic lesions in NB [72][73]. Although frequently screened for, DAXX mutations are rare in NB. Approximately 50% of ALT NB are associated with somatic alterations in ATRX [8][9][15][74][44]. ALT was significantly more frequent in ATRX - mutant NB than that in ATRX wild-type tumors [89.5% (17/19) vs. 22.2% (4/18), p < 0.0001] [10]. ALT was examined in a cohort of 149 NB [74], and detected in approximately 25% (36/49) of tumors on the basis of the C-circle assay. It was not present in MYCN -amplified tumors and correlated with poor outcomes in NB. The whole-exome and - genome sequencing of 13 ALT-positive tumors revealed that 8 had an ATRX mutation, 1 had a DAXX mutation, and 1 had a TP53 mutation, whereas 4 were the wild types for ATRX and DAXX. ALT tumors also had significantly higher relative telomere content than that of ALT-negative tumors (p < 0.0001), irrespective of age because ALT

NB patients were significantly older at diagnosis than those with ALT-negative NB ( $p < 0.0001$ ). Roderwieser et al. [27] assessed APBs, a hallmark of ALT-positive tumors, in 273 out of 457 NB. ALT activation was detected in 49 out of 273 cases (17.9%) and associated with unfavorable prognostic variables and adverse outcomes. APBs were mainly observed in the tumors of patients aged 18 months or older at diagnosis (29.6%) and predominantly in stage 4 tumors (25.2%). In contrast, APBs occurred mutually exclusively with MYCN amplification and TERT rearrangements. In addition, genomic alterations in ATRX were identified in 8 out of 109 evaluable cases (7.3%), all of which were positive for APB, whereas no ATRX mutations were detected in 12 additional APB-positive cases, and the genes involved in ALT were unknown in the remaining cases. Koneru et al. recently performed the C-circle assay as an ALT biomarker; 25 of 107 HRNB tumors (23.4%) were positive for ALT and genomic alterations in ATRX were detected in 13 out of 23 ALT tumors (57%). The remaining ALT tumors lacked ATRX genomic alterations as well as other known ALT-associated genes ( DAXX , H3F3A , or SMARCAL1 ) [15]. Moreover, non-ALT tumors (C-circle-negative) lacked ATRX genomic alterations, which were confirmed to be the wild type for ATRX using Sanger sequencing. ALT activation without ATRX genomic alterations was also observed in NB cell lines and PDXs. The ALT cell line SK-N-FI [22][27] and two ALT PDXs (COG-N-589x and COG-N-620x) were wild-type ATRX that expressed the ATRX protein, indicating that ALT occurs in NB with and without ATRX genomic alterations.

In a cohort [44] of primary and relapsed NB, 9.2% (66/720) and 47.5% (19/40) of tumors, respectively, were classified as ALT-positive, indicating the strong enrichment of this molecular subgroup in relapsed cases irrespective of the INSS stage. The activation of ALT was mutually exclusive to TERT rearrangements and 55% of ALT-positive tumors had LoF mutations in ATRX , including single nucleotide variants, large deletions, and focal intragenic ATRX duplications. Mutations in the ATRX complex members DAXX and H3F3A were extremely rare in this cohort. In contrast, somatic mutations in TP53 pathway genes ( TP53 , CREBBP , ATM , ATR , CDKN2A , and MDM2 ) were significantly enriched ( $p = 0.01$ ) in ALT-positive tumors. ALT-positive tumors had the highest prevalence of CDK4 amplifications, higher CCND1 mRNA expression levels, copy number losses in POLD3 and ATM, mutations in Synaptic nuclear envelope protein 1, and deletions in receptor-type tyrosine-protein phosphatase delta. Canonical activating RAS pathway mutations ( HRAS , NRAS , KRAS , BRAF , RAF1 , CDK4 , CCND1 , and NF1 ) were significantly more frequent in relapsed ALT-positive NB ( $p = 0.0013$ ), supporting the specific impact of RAS pathway mutations on relapsed ALT-positive tumors. Previous findings also revealed that patients with TERT or ALT activation and harboring alterations in the RAS / TP53 cellular pathway were very high-risk cases that were prone to relapse and had a very poor clinical outcome [13]. In addition, integrating proteomic profiling identified reduced ATRX protein levels as a biomarker of ALT-positive NB that is independent of mRNA levels and ATRX mutations [44]. Decreased ATRX protein levels despite an unchanged abundance of mRNA may be attributed to the reduced translation and/or increased degradation of ATRX in ALT-positive tumors, which may result from the downregulation of the DAXX protein. Consistent with this finding, the knockdown of DAXX decreased ATRX protein levels in NB cells. Interestingly, DAXX protein levels were significantly reduced in ALT-positive ATRX wild-type tumors, while mRNA levels did not significantly differ and no recurrent mutation patterns in DAXX were observed, which is consistent with previous findings [13]. Reduced DAXX protein levels impair the

assembly of the ATRX/DAXX complex, which then induces the degradation of orphan ATRX protein molecules. Hence, further studies are needed to elucidate the mechanisms underlying ATRX/DAXX complex reductions.

Age is a powerful indicator of the clinical outcome of NB [75]. A pan-NB analysis of 702 NB samples by Brady et al. [76] revealed that MYCN and TERT alterations were enriched in younger patients (median age of 2.3 and 3.8 years, respectively), while ATRX was more common in older children (median age of 5.6 years). This group also showed age-associated mutual exclusivity between ATRX and TERT and between ATRX and MYCN, indicating the susceptibility of different ages to specific oncogenic events.

The loss of ATRX functions was recently shown to not only be mutually exclusive to the amplification of MYCN, but also incompatible with the overexpression of the MYCN protein due to the detrimental accumulation of RS. Zeineldin et al. [10] recently demonstrated that the knockout of ATRX decreased colony formation in MYCN -amplified NB cell lines, while no changes were observed in MYCN wild-type NB cell lines. Correspondingly, the induction of MYCN expression in ATRX -mutant NB cells and U2OS cells (ATRX mutation) resulted in the significant loss of viability. Electron microscopy studies showed that a concomitant ATRX mutation and MYCN amplification resulted in mitochondrial disruption. Therefore, the synthetic lethality of MYCN amplification and ATRX mutation warrants further study as a novel therapeutic strategy for the treatment of NB patients.

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