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Chapter 6

Telomeres and Telomerase in Neuroblastoma

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http://dx.doi.org/10.5772/intechopen.69567

Abstract

Telomeres are nucleoprotein structures located at the ends of linear chromosomes. In most human adult normal somatic cells, telomeres shorten after each cellular division. This shortening ultimately leads to senescence and/or apoptosis. By contrast, in most cancer cells, telomerase activation compensates this loss and confers to these cells their infinite cell proliferation potential. Neuroblastoma (NBL) is a malignant tumor of the peripheral sympathetic nervous system and the most frequent extracranial solid tumor of childhood. NBLs are remarkably heterogeneous both at the levels of biology, genetic and clinical courses. Indeed, some of NBLs can regress spontaneously or after a mild treatment, while others are in the high-risk category with poor prognosis. The molecular bases underlying this heterogeneity are poorly understood. MYCN (V-Myc Avian Myelocytomatosis Viral Oncogene Neuroblastoma-derived Homolog) amplification, recognized as strongly associated with unfavorable patient outcome, is found in only 40% of the high-risk disease, indicating the involvement of other mechanisms. Recent observations suggest that telomerase expression and telomere dysfunctions may be one critical step in NBL development. This review provides recent insights on telomeres/telomerase regulation in NBL. Because of their involvement in the tumor cell biology, telomere and telomerase are currently at the core of new drug development.

Keywords: telomerase, telomeres, regulation, therapies, neuroblastoma

1. Introduction

Cancer development is a multistep process requiring genetic and epigenetic events leading to the deregulation of the expression of key genes. Among these genes, telomerase, by its

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action on telomere maintenance, plays a major contribution in carcinogenesis and drug resistance. This enzyme is activated in almost 90% of cancers, including neuroblastoma (NBL).

Neuroblastoma is a malignant tumor of the peripheral sympathetic nervous system and the most common extracranial solid tumor in childhood [1, 2]. NBL is remarkably heterogeneous and displays a wide spectrum of differentiation stages from benign ganglioneuroma and well-differentiated tumors to undifferentiated malignant NBL. NBL is also a heterogeneous disease in terms of outcome and response to treatments: from spontaneous regression to resistance to all known treatments. In about 60% of the cases, NBL is diagnosed as a disseminated high-risk disease (stage 4), and most are diagnosed after 18 months of age. Genomic amplification of *MYCN* (V-Myc avian myelocytomatosis viral oncogene neuroblastoma-derived homolog) has been strongly associated with unfavorable patient outcome in approximately 20% of primary neuroblastoma tumors and approximately 40% of high-risk NBL. This alteration has thus been established as a robust marker for the definition of high-risk NBL. However, that *MYCN* amplification occurs only in 40% of high-risk NBL indicates that other genetic and/or epigenetic alterations play an important role in this disease. Array comparative genomic hybridizations have been widely employed to discover genome abnormalities and evaluate patient's risk. NBL displays several numerical and structural copy number variations such as the loss of 1p, 3p, 9p, 11q, and 14q, and the gain of 1q, 2p, and 17q, which identify high-risk subsets of NBL [3, 4]. That aggressive stage 4 neuroblastoma expressed high levels of telomerase activity, whereas favorable tumors had no or little telomerase expression and activity [5, 6], suggest an important role of this enzyme in the biology of neuroblastoma and its response to chemotherapy. NBL has a very low mutation frequency. The most two mutated genes are *ALK* (anaplastic lymphoma kinase) coding a tyrosine kinase, altered in about 7–8% of all primary tumors and 50% of familial NBL cases [7, 8], and *ATRX* (alpha thalassemia/mental retardation syndrome, X-linked) in about 10% of NBL and generally found in older patients.

Recently, the next-generation sequencing has shown that high-risk NBLs are characterized by defects that in common lead to the activation of telomere maintenance pathways supporting the idea that targeting these pathways will benefit to the patients [9, 10].

Many excellent recent reviews [11, 12] already exist on telomeres and telomerase in many aspects (structure and functions, regulation, and epigenetic control). This paper will therefore briefly review the recent knowledge on this topic, then, it will focus on the mechanisms of telomerase reactivation and telomere length maintenance in NBL, and discuss how these regulatory mechanisms can be targeted or "manipulated" for therapeutic purposes to modify cell fate and anticancer drug response in NBL.

2. Telomeres and telomerase

2.1. Telomeres

Every normal human somatic cell has a molecular clock for dividing, a process discovered by Leonard Hayflick, half a century ago, who observed that diploid cells in culture can divide

only a limited number of times before stopping in a state known as the cellular senescence or the "Hayflick limit" [13]. In eukaryotic organisms, conventional DNA polymerases alone cannot fully replicate the ends of linear chromosomes, called telomeres. Therefore, telomere ends are progressively shortened after each cellular division [14, 15]. This leads to genomic instability and senescence or apoptosis.

Telomeres are specialized nucleoprotein structures made of 10–15 kb of short non-protein-coding repetitive 5′-TTAGGG-3′ DNA sequences. Telomeric DNA is mainly double-stranded, terminating in a single-stranded 3′ G-rich overhang of 150–200 nucleotides (nt) [16, 17]. These double-stranded repeats have one guanosine-rich strand (G-strand) copied by the lagging-strand replication, and one cytosine-rich strand (C-strand) synthesized by leading-strand replication. The telomeric DNAs are bound by shelterin protein complexes consisting of telomeric repeat factors 1 and 2 (TRF1 and TRF2), repressor/activator protein 1 (RAP1), TRF1- and TRF2-interacting nuclear protein 2 (TIN2), tripeptidyl-peptidase 1 (TPP1), and protection of telomeres 1 (POT1) [18, 19]. TRF1 and TRF2 bind to the double-stranded telomere DNA repeats, whereas POT1 binds to the single-stranded G-rich overhang. The three remaining proteins of the shelterin complex act as adaptors to mediate the interactions between the complex constituents: POT1 interacts with TPP1, a ternary complex of other proteins (TINT1/PTOP/PIP1), which interacts in turn with TIN2 that plays a key role in stabilizing the shelterin complex *via* its interaction with TPP1, TRF1, and TRF2. TPP1 also plays a major role in controlling the recruitment of the telomerase to the telomeres. RAP1 binds to TRF2 and acts as a mandatory element to the formation of the t-loop and the protection of the telomeres from the non-homologous end-joining (NHEJ) process. This process is dependent on the ataxia telangiectasia-mutated (ATM) kinase, the ataxia telangiectasia, and Rad3 (ATR) kinase, involved in the repair of the double-stranded and the single-stranded DNA breaks, respectively.

Due to the tandem organization of the G-rich telomeric DNA, the telomeres can form specialized four-stranded helical structures that involve Hoogsten-type base pairing between four guanines, named G-quadruplex (or G4) [20]. Alternatively, the G-strand overhang is also involved in the formation of the t-loop in which it invades the double-stranded region [21]. It has been hypothesized that those structures, which are not mutually exclusive, are able, by sequestering the 3′ end, to prevent the extension of the telomeres by telomerase.

Besides their role of capping chromosomes and protecting them from being recognized as DNA breaks [22], telomeres ensure proper chromosome segregation during mitosis [23] as well as transcriptional silencing of genes located close to them. Indeed, telomere shortening can alter gene expression by a process named telomere position effect (TPE) [24]. This process leads to the reversible silencing of genes near the telomere and thus is dependent on telomere length. In yeast, TPE can repress genes located up to 20 kb from the end [25, 26]. Recently, using a Hi-C (chromosome capture followed by high-throughput-sequencing) technique, three genes located at three different subtelomeric ends (1p, 6p, and 12p) were reported to have their expression altered with telomere length: *ISG15* (interferon-stimulated gene 15kD), *DSP* (Desmoplakin), and *C1S* (complement component 1s subcomplement). This phenomenon occurs through chromosomal looping between the loci of these genes and their respective telomere ends [27, 28]. Therefore, many loci may be regulated by telomere length.

This observation provides a new potential mechanism by which telomere shortening could contribute to the aging process and cancer development.

Two mechanisms of telomere maintenance have been identified in humans: the telomerase-mediated maintenance observed in 90% of cancers and, in the remaining 10%, the alternative lengthening of telomeres (ALT), which depends on homologous recombination [29, 30].

2.2. Telomerase: a ribonucleoprotein complex with multiple functions

Elizabeth Blackburn and her graduate student Carol Greider (2009 Nobel Prize in Physiology or Medicine) who worked on the ciliated protozoan *Tetrahymena thermophila* identified the enzyme responsible for the telomeric repeat synthesis [31]. This ribonucleoprotein (RNP) enzyme maintains telomere length by adding repetitive sequences to chromosome ends, slowing down telomere attrition [32].

Telomerase is an RNA-dependent DNA polymerase that plays a key role in carcinogenesis. By synthesizing telomeric DNA at the termini of chromosomes and stabilizing telomere lengths, it overcomes the senescence barrier due to the progressive telomere shortening associated with cell divisions [33]. Normal human somatic cells have very low or undetectable telomerase activity. By contrast, this activity has been detected in a wide range of human cancers (85–90%), in stem cells and adult germline tissues [34, 35]. By its action on telomeres, this enzyme confers to cancer cells their infinite cell proliferation potential and controls cell survival [36–38]. Telomerase is believed to be a significant target in cancer therapy since its upregulation appears to be a feature of malignant cells.

The human telomerase is a ribonucleoprotein enzyme (127 kDa) composed of at least two components, a catalytic subunit, telomerase reverse transcriptase (hTERT), and a template RNA component (hTR) (**Figure 1**).

hTR is a non-polyadenylated 451-nt long non-coding RNA containing eight conserved regions (CR1–CR8) that acts mainly as a template for the synthesis of the telomeric DNA. hTR binds hTERT *via* a template-pseudoknot domain (CR1/CR2) and a stem-loop domain (CR4/CR5) located in the middle of this RNA structure, which interacts with the DNA-binding domain of hTERT (**Figure 1**). hTERT protein functions as a dimer in the telomerase complex. Additional proteins are also required for a functional telomerase complex *in vivo*: small nucleolar RNPs, NHP2 (non-histone protein 2), NOP10 (nucleolar protein 10), GAR1, shelterin, and the ATPases Pontin and Reptin [39, 40]. The shelterin complex aids at the stabilization of the 3′ end at the telomeres. The appropriate stabilization of hTR and its proper interaction with hTERT involves the recruitment of dyskerin (DKC1), an RNA-binding protein, facilitated by the ATPases Reptin and Pontin [41]. The assembly of telomerase occurs in the nucleus in the Cajal bodies, and its localization at telomeres requires TCAB1 (Telomerase CAjal Body protein 1) and TPP1 proteins [42, 43].

Loss-of-function mutations of either *hTERT* or *hTR* are associated with pathologies such as aplastic anemia, pulmonary fibrosis, and dyskeratosis congenital, diseases characterized by stem cell depletion, deficiency in tissue regeneration, and tissue atrophy [44].

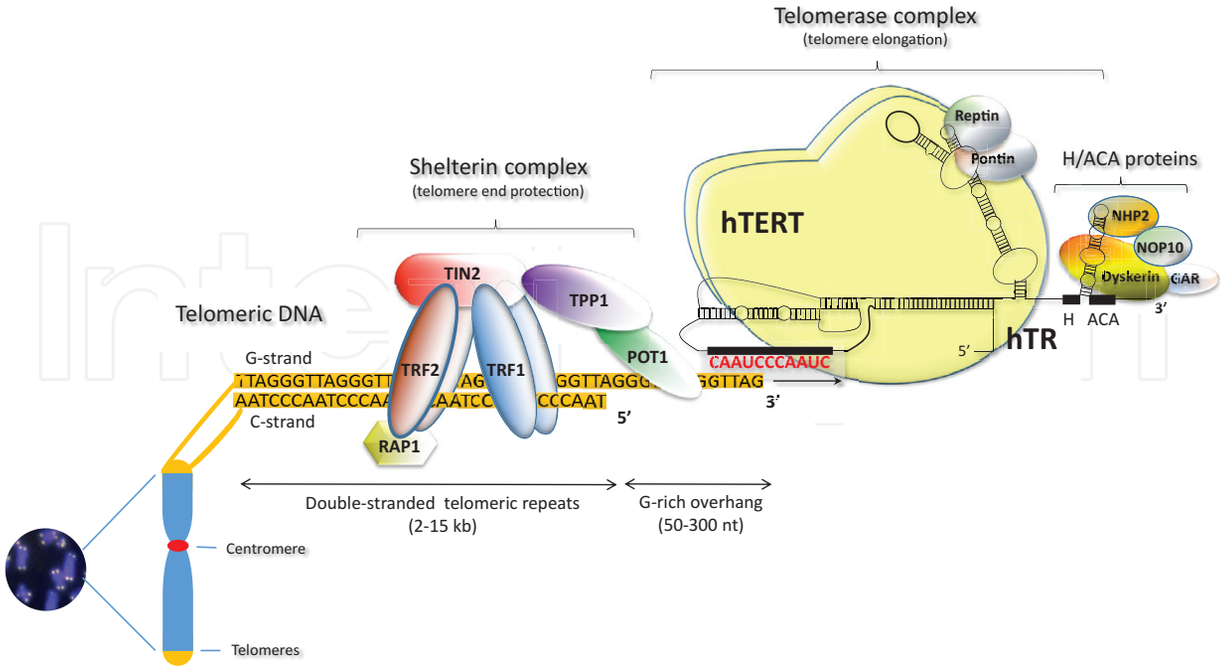

Figure 1. The human telomere and telomerase: the human telomerase is composed at least of telomerase reverse transcriptase (hTERT), telomerase RNA component (hTR), and accessory proteins that are members of the H/ACA small nucleolar ribonucleoprotein family: dyskerin; NHP2 (non-histone protein 2); NOP10 (nucleolar protein 10); GAR1 ribonucleoprotein.

Besides its canonical role, accumulated evidence indicates that telomerase elicits other functions in several essential cell-signaling pathways, including apoptosis, differentiation, DNA damage responses, and regulation of gene expression [45–50]. Even though these functions appear independent of telomerase activity, it is not excluded that some transient effect at telomeres can affect chromatin structure and gene expression. One example of these non-canonical functions of hTERT is the demonstration that hTERT binds NF-κB p65 subunit and regulates some of its target genes such as *IL (Interleukin)-6*, *IL-8*, *TNF (tumor necrosis factor) α*, and matrix metallo proteinases (*MMPs*) [51, 52]. In turn, NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) can activate the expression of hTERT by binding to its promoter indicating a feed-forward loop between telomerase and NF-κB [53]. hTERT protein can also function as a transcriptional modulator of the Wnt (wingless-related integration site)/β-catenin-signaling pathway [54]. Indeed, hTERT and β-catenin co-associate at the Wnt/β-catenin target gene promoters by forming a complex with the ATPase subunit of SWI/SNF (switch/sucrose non-fermentable) chromatin-remodeling complex, BRG1 (Brahma-related gene 1). Interestingly both wild-type and catalytically inactive hTERT led to the reactivation of Wnt/β-catenin target genes suggesting that this function is independent of its conventional function at telomeres. In turn, the Wnt/β-catenin pathway regulates the expression of hTERT in embryonic stem cells and in cancer cells through the recruitment of β-catenin to the promoter of *hTERT* indicating, in this case also, a positive feed-forward loop between telomerase and β-catenin [55]. Furthermore, functioning as an RNA-dependent RNA polymerase, hTERT has been implicated in the production of small-interfering (si) RNAs in a Dicer-dependent

manner and thereby is involved in posttranscriptional gene silencing [56]. This activity of hTERT occurs through the interaction of hTERT protein with BRG1 and nucleostemin [57, 58]. For example, hTERT, through this activity, mediates the production of endogenous siRNAs using the RNA component of the mitochondrial RNA-processing endonuclease (RMRP) as a template.

Recently, it has been shown that BRG1 plays an essential role in maintaining the proliferation and viability of NBL cells. Interestingly, BRG1 is consistently upregulated in several NBL cell lines and in advanced stages of NBLs. Furthermore, high BRG1 levels have been correlated with poor patient outcome [59]. Therefore, BRG1 inhibition could be a possible new line of treatment for high-risk NBL patients. In view of these observations, the relationship between BRG1 and hTERT in NBL should be investigated.

2.3. Human hTERT regulation

Given the key role of telomerase in malignant transformation and tumor progression, great efforts have been deployed to unravel the mechanisms underlying telomerase activation.

2.3.1. hTERT gene and its promoter

The *hTERT* gene is 42-kb (kilobases) long with 16 exons [60] located in humans on the short arm of chromosome 5 (5p15.33) [61], more than 2 Mb away from telomeres. The reverse transcriptase domain is coded by exons 5–9. Differential splicing of *hTERT* mRNA has been demonstrated during embryonal development in various tissues. So far, 22 isoforms have been described resulting from alternative splicing [62, 63]; however, only the full-length isoform, which retains the reverse transcriptase activity, is able to elongate telomeres [64, 65]. Variants that lack the reverse transcriptase domain could affect telomerase activity by acting as competitive inhibitors as reported for the α-variant [66] or may have by themselves telomere-independent activities [62, 67, 68]. However, these experiments should be interpreted cautiously because they are based on overexpression conditions that are far beyond the physiological conditions.

Telomerase activity is generally well correlated with *hTERT* expression indicating that *hTERT* is a key regulator of this enzyme. *hTERT* gene is mostly regulated at the transcriptional level. This regulation is complex and includes multiple levels [69, 70].

The *hTERT* promoter does not have typical transcription-regulatory elements as TATA or CAAT boxes but is GC-rich. The core promoter harbors at least five GC boxes and two E-boxes (enhancer boxes with the canonical sequence of 5′-CACGTG-3′), which are sites for Sp1 (specificity protein 1), and c-Myc-binding, respectively, as well as multiple other transcription factor-binding sites involved in *hTERT* gene transcription: E-26 (ETS) family members, E2F, AP1 (activator protein 1), p53, p21, HIF (hypoxia-inducible factor), NF-kB, β-catenin, CTCF (CCCTC-binding factor), WT1 (Wilms' tumor 1), and MZF2 (myeloid zinc finger 2) [71] (**Figure 2**).

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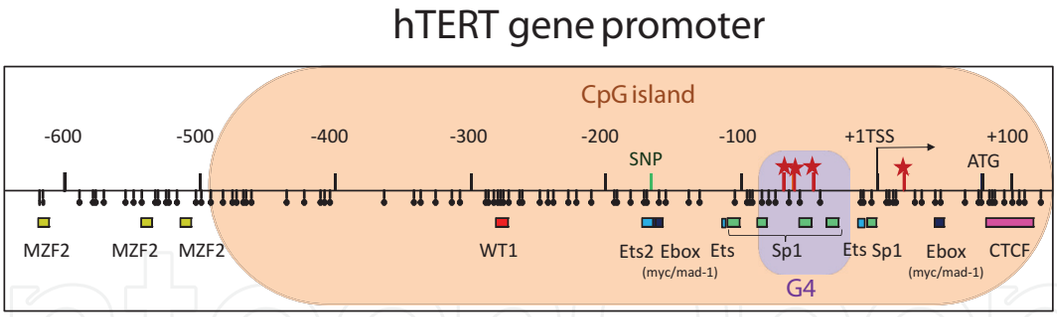

Figure 2. Schematic representation of the *hTERT* promoter: binding sites for various transcription factors are shown. The transcription start site (TSS, +1) and the translation start site (ATG, +78) are indicated. Stars indicate hotspot promoter mutations. The rs2853669 polymorphism (SNP) is shown in the ETS2-binding site. CTCF: CTCF factor-binding site; E-Box: Myc/Mad-family factor-binding site; Sp1: binding site for the SP1 transcription factor; ETS: ETS-domain site; WT1: binding site for the Wilms' tumor 1 transcription factor; MZF2: binding site for the myeloid zinc finger 2 transcription factor. Vertical tick marks with dark circles indicate the location of CpG dinucleotides. G4 indicates the localization of the G-quadruplex structure that can be adopted by *hTERT* promoter.

2.3.2. Transcriptional regulators of hTERT

The factors that bind *hTERT* promoter were characterized as transcriptional activators or transcriptional repressors or can play both roles depending on the cell type and the cellular context. Different factors can sometimes compete for binding to the same site or cooperate for binding to adjacent sites on *hTERT* promoter. Most of the studies concern the effect of transcription factors interacting with the core region of *hTERT* promoter, spanning from −180 bp (base pair) upstream to +1 bp downstream of the transcription start site. However, transcription factors interacting with a more distant region can also play an important role. Their action can be modulated by epigenetic modifications (see below). Therefore, the transcriptional level of *hTERT* results from a complex regulatory network of all these factors. These latter observations can explain the contradictory results that are reported in the literature. Given the complexity of *hTERT* regulation, it is difficult to integrate all the information. In addition, many factors indirectly regulate *hTERT* transcription through their interaction with other signaling pathways. All these processes ensure a tight and coordinated control of the *hTERT* gene in order to silence it in most human somatic adult cells. This control is lost in most malignant cells.

Recent articles have already reviewed exhaustively the roles of specific regulatory factors of *hTERT* [33, 71, 72]. Here, we have selected the most important ones, those whose binding has been demonstrated by *in vivo* assays, and also those that could be involved in the physiopathology of neuroblastomas.

2.3.2.1. c-Myc/Max/Mad-1

c-Myc and its dimerization partner Max (Myc-associated factor X) bind to regulatory elements called E-boxes and recruits histone acetyltransferases in order to activate the transcription of various genes, including *hTERT* [73]. c-Myc binds to two canonical E-box sequences

(5′-CACGTC-3′) found in the core promoter of *hTERT* (at −165 and +44 bp from the transcription start site). This binding leads to the upregulation of *hTERT* gene, and the increase in telomerase activity [74, 75]. However, c-Myc alone is not always sufficient to upregulate the expression of *hTERT* suggesting the requirement of additional factors. For example, the cooperation between Sp1 and c-Myc has been demonstrated to upregulate *hTERT* expression in human foreskin keratinocytes transduced by E6 [76]. Recently, it has been reported that c-Myc can exert a dual role on *hTERT* promoter. Indeed, besides its action as a transcriptional activator of *hTERT*, c-Myc can also maintain its promoter in a repressive state [77]. This latter action is independent of the two E-boxes.

Numerous factors thereby are able to indirectly upregulate *hTERT* expression through their action on c-Myc expression: transforming growth factor (TGF)-β/Smad signaling, estrogen, Aurora-A, Survivin, Leptin, mitogen-activated protein kinase (MAPK)/PI3K (phosphoinositol-3-kinase)-signaling pathway, MMP9, and Sirtuin 1 (SIRT1). Conversely, many factors are able to repress *hTERT* expression by counteracting the c-Myc expression or activity. The most important one is Mad-1 (Max dimerization protein 1), a potent antagonist of c-Myc, which acts as a direct competitor for dimerization with Max and binding on the E-boxes. Other factors are also known to act on c-Myc expression and/or inhibit its binding to *hTERT* promoter: breast cancer 1 (BRCA1), p27KIP21, HIF-1alpha, and so on.

Despite the strong evidence of the action of c-Myc as a transcriptional activator of *hTERT*, several studies reported the lack of correlation between c-Myc expression and hTERT mRNA levels [78, 79]. However, in these studies, the direct binding of c-Myc on *hTERT* promoter has not been investigated.

NBL cells generally do not express c-Myc but N-MYC, a protein belonging to the same family. c-Myc and N-MYC are encoded by different genes but have similar structures and domains. As c-Myc, N-MYC protein was shown to be recruited to the *hTERT* promoter and to activate it in NBL [80]. However, it is not known whether N-MYC can always functionally replace c-Myc in *hTERT* regulation. Moreover, hTERT has been shown to regulate c-Myc protein stability by interacting directly with c-Myc suggesting the existence of a feed-forward loop. In addition, in c-Myc-driven lymphoma, hTERT can also be recruited to c-Myc target promoters [81]. Such a crosstalk between hTERT and N-MYC protein has not been reported in NBL yet.

2.3.2.2. Specificity protein 1 (Sp1)

Sp1 is a transcription factor that binds to GC-box motifs in the promoter of *hTERT*. It activates *hTERT* gene expression in telomerase-positive cells but suppresses it in telomerase-negative one [82]. Sp1 may work in cooperation with c-Myc to upregulate *hTERT* expression. However, in cooperation with Sp3, another GC-box binding protein, Sp1 can also suppress the expression of *hTERT*.

It is important to note that this GC-rich region of *hTERT* promoter is able to form a tandem G-quadruplex structure [83]. The formation and stabilization of these structures that involves at least three of the five Sp1-binding sites could, therefore, interfere with *hTERT* transcriptional regulation.

2.3.2.3. Nuclear factor κ-light-chain-enhancer of activated B cells (NF-kB)

NF-kB is a transcription factor complex playing a role in telomerase expression and activity either directly through its binding on *hTERT* promoter or indirectly through the modulation of the expression of transcription factors known to affect *hTERT* expression. The binding site of NF-kB on *hTERT* promoter is located 600 bp upstream of the ATG translation start codon. However, recently, another pathway for the activation of *hTERT* by this complex has been proposed. This new mechanism involves a hotspot *hTERT* promoter mutation (see below).

2.3.2.4. Upstream stimulatory factor (USF) proteins

As c-Myc, USF proteins bind directly to E-box motifs on the core promoter of *hTERT*. They play both activating and repressing roles in the regulation of *hTERT* gene expression depending on the cell context.

2.3.2.5. CCCTC-binding factor

CTCF transcription factor binds at the beginning of exon 1 (+4 to +39 bp) and near the beginning of exon 2 (+422 to +440 bp) relative to the ATG in *hTERT* promoter. It is a repressor of hTERT transcription. CTCF is unable to bind to methylated DNA and therefore its binding is dependent on the degree of DNA methylation [84].

2.3.2.6. Wilms, tumor protein 1

WT1 is described as a repressor of *hTERT*. Its binding site lies from −358 to −349 bp on *hTERT* promoter [85]. However, similar to CTCF, the binding of WT1 is known to be methylation-sensitive. This observation was further supported by Azouz et al. whose experiments, in an acute promyelocytic leukemia (APL) cell model, showed that hypermethylation of a distal domain of *hTERT* promoter induced by retinoic treatment prevented WT1 binding and therefore, the subsequent *hTERT* gene repression [86]. A recent study reports an inverse correlation between *WT1* expression and *MYCN* amplification and expression. Moreover, a high expression of WT1 has been associated with a poor outcome only for patients showing non-*MYCN*-amplified tumors [87]. In a cohort of 67 primary NBL tumors, no significant correlation between WT1 and hTERT expression was found ($p = 0.056$), whereas, considering only stage 4 tumors ($n = 51$), there was a significant higher WT1 expression in patients with a low hTERT expression ($p = 0.033$).[1]

2.3.3. hTERT promoter mutations

Recently, hotspot promoter recurrent mutations were identified first in sporadic and familiar malignant melanoma [88, 89]. These mutations, which cause an adenine-to-cytosine (A>C) mutation or a cytosine-to-thymine (C>T) transition at chromosome 5: 1,295,161, 1,295,228, and

[1] E. Ségal-Bendirdjian et al., unpublished results.

1,295,250 (−57, −124, and −146 bp upstream of the ATG translation start codon), are named −57A>C (or A161C), −124C>T (or C228T), and −146C>T (or C250T), respectively. From there, the *hTERT* promoter mutations have been identified in various types of cancers with different frequencies [90]. The two main mutations, −124C>T and −146 C>T, have been suggested to be oncogenic drivers on the basis of experimental data showing that (1) their introduction into the *hTERT* promoter reporter could significantly enhance the promoter activity [91]; (2) the creation of these mutations by genome editing in *hTERT* promoter of human pluripotent stem cells is sufficient to inhibit the repression of *hTERT* gene transcription normally observed in wild-type *hTERT* promoter-bearing stem cells after induction of differentiation [92]; and (3) tumors carrying *hTERT* promoter mutations were frequently observed to express higher levels of *hTERT* mRNA and telomerase activity compared with those carrying a wild-type promoter. The frequency of the −124C>T mutation is generally higher than the frequency of the −146C>T mutations (80 vs. 20%). Other less common mutations were also detected in the *hTERT* promoter such as the CC>TT mutations at −124/−125 (Chr.5: 1,295,228–1,295,229) and −138/−139 (Chr.5: 1,295,242–1,295,243) positions. Note that these mutations occur in the G-rich region of the promoter known to form G-quadruplex structures [83]; therefore they can abrogate their negative effect on transcription by changing their stability and/or altering their recognition.

hTERT promoter mutation rates vary significantly from undetectable to 85% among studied human cancer types. The mutations occur most frequently in bladder, thyroid, hepatocellular cancers, malignant glioblastoma, and melanoma [93–96], while they are rarely present in hematological malignancies, prostate, gastrointestinal, breast, and lung cancers [90, 97, 98]. That a high frequency of *hTERT* promoter mutations was found in a multitude of advanced cancers suggests their key role in the reactivation of telomerase activity.

Mechanistically, −124C>T or −146C>T mutation generates an 11-base nucleotide stretch (5′-CCCCTTCCGGGG-3′), which contains a consensus-binding site (GGAA in reverse complement) for ETS family transcription factors [99]. It was shown that the multimeric GA-binding protein (GABP), an ETS family transcription factor, was specifically recruited to the mutant rather than wild-type *hTERT* promoter in different cancer cells. This recruitment is associated with an enhanced enrichment of active chromatin leading to the opening of chromatin, an increased recruitment of PolII, and upregulation of *hTERT* expression and activity [91, 100]. This effect is further enhanced by the activation of the non-canonical NF-kB-signaling pathway. Indeed, the recruitment of the p52 subunit of NF-kB to the C250T site facilitates the stimulation of *hTERT* transcription.

Genome-wide association studies revealed the presence of single-nucleotide polymorphisms (SNPs) within the *hTERT* locus that were associated with increased risks in a variety of cancers. For example, rs2736100, located in intron 2, was associated with various types of cancer as glioma and colorectal cancer [101, 102]. Another SNP, rs2853669, found at *hTERT* promoter upstream of the first E-box, disrupts an ETS2-binding site [103, 104] and also hampers c-Myc binding to the adjacent E-box. The presence of this SNP can modify the effects of *hTERT* promoter mutations [105].

http://dx.doi.org/10.5772/intechopen.69567

While *hTERT* promoter mutations have been identified in a broad range of cancers, not all cancers possess these mutations suggesting that other mechanisms contribute to hTERT reactivation.

2.3.4. Epigenetic regulation of hTERT transcription

The *hTERT* promoter is located in a 4-kb CpG island from −1800 to +2200 bp (relative to the TSS). Besides the involvement of transcription factors, epigenetic control and overall chromatin structure at *hTERT* promoter add another layer of hTERT regulation. It is well established that DNA methylation, histone acetylation, and methylation are also involved in the regulation of *hTERT* transcription even though the precise role and the molecular mechanisms are not well understood and even contradictory due to the different cellular models studied and the various methods used to analyze these epigenetic modifications.

Considering the methylation pattern categorized in different cell lines, it is possible to narrow the promoter to only two regions: one methylated, sometimes hypermethylated (from −650 to −200 bp from ATG) and one unmethylated or only slightly methylated (from −200 to +100 bp) [86, 106–110].

It is known that DNA methylation at gene promoter plays a major role in transcription factor binding. For example, hypomethylation at the *hTERT* core promoter may allow the binding of c-Myc to the E-boxes. Some reports, including ours, suggest that DNA methylation of *hTERT* promoter might have a key role in *hTERT* expression, but in a way opposite to what has been proposed so far. The hypothesis is that DNA methylation at *hTERT* promoter can contribute to prevent the binding of repressors and could account for high *hTERT* expression. This has been demonstrated for two transcription factors known to repress *hTERT*: CTCF whose binding site is located in exon 1 [71, 111, 112] and WT1 whose binding site is located in the distal promoter [86].

Besides DNA methylation, histones contribute to chromatin organization. Modifications can occur to their amino acid tails: methylation and acetylation are the most common. In general, methylation of histone 3 at its lysine 4 (H3K4) and hyperacetylation of histones are signs of hypo- or unmethylated DNA and active transcription gene. On the contrary, methylation of lysine 9 and 27 of histone 3 (H3K9 and H3K27, respectively) and hypoacetylation of histones are signs of hypermethylated DNA, so inactive transcription gene [110].

2.4. Human *TR* regulation

As *hTERT* is generally expressed only in telomerase-positive cells and its ectopic introduction alone can immortalize normal human cells, hTERT has been regarded as the limiting component of telomerase activity and much of the research has thus focused on the regulation of hTERT gene. However, even though *hTR* is ubiquitously expressed, evidence supports the notion that *hTR* can be also limiting for telomerase activity and telomere maintenance [113]. Indeed, the gene encoding *hTR*, a single-copy gene located on chromosome 3 at 3q26.3 [114], is highly regulated. A number of transcription-binding sites have been validated by either

electrophoretic mobility gel gift assays, promoter reporters, and chromatin immunoprecipitation (ChIP) experiments, including Sp1, Sp3, and NF-Y [115, 116]. Furthermore, different signaling pathways have also been implicated in *hTR* transcription as JNK pathway.

3. Telomeres and telomerase in neuroblastoma

3.1. Telomerase as a biological marker and predictive factor in neuroblastoma

Several distinguishable groups in NBL have been identified based on their telomere biology and telomerase activation suggesting that telomerase expression may be one critical step in the development of neuroblastoma [5]. High telomerase activity allowing the maintenance of telomere length has been previously reported to correlate with advanced stages of the disease and with poor prognosis [5, 117–121]. By contrast, tumors without detectable telomerase activity showed favorable outcomes and some tumors regressed or matured [122]. This phenomenon of spontaneous regression led to propose a specific pattern of the metastatic disease called stage 4S. Children with stage 4S were restricted to infants aged less than 12 months at diagnosis, had generally small primary tumors with dissemination limited to the liver and skin and minimum bone marrow involvement [123]. The mechanisms involved in this regression remain to be elucidated. The expression of the alternate splice variants of *hTERT* could constitute a negative regulatory mechanism of telomerase at the posttranscriptional level and might account for the favorable evolution of these tumors [119, 124]. Therefore, full-length *hTERT* expression and telomerase activity, due to its strong correlation with the biological behavior of neuroblastoma tumors, may prove to be a good indicator of malignancy, in particular in 4S neuroblastoma. This may have consequences in the therapeutic strategies that can be adopted for these patients. As this form presents similar features as classic stage 4, it can be therefore treated as a high-risk group although in these cases, less therapeutic intensity could be given.

Several mechanisms have been proposed to explain the phenomenon of spontaneous regression. These include the neurotrophin receptor signaling when deprivation in nerve growth factor occurs, immune-mediated killing by anti-neural antibodies in patients, epigenetic regulation of gene expression through DNA methylation, histone modifications or chromatin remodeling, and finally telomere shortening and consequently apoptosis. Indeed, most of the tumor samples from 4S NBL have low telomerase activity or short telomeres [5]. This mechanism is further supported by Samy et al. who showed that a neuroblastoma cell line transfected by a dominant-negative form of human telomerase was more prone to apoptosis and had reduced tumorigenicity in a mouse xenograft model compared to untransfected neuroblastoma cells [125].

A correlation between *hTR* expression in primary NBLs, stage of disease, and survival [126, 127] has also been reported demonstrating a potential role for *hTR* as a biomarker even though most of the studies focused on *hTERT* expression. However, *hTR* expression does not always correlate with telomerase activity. This can be explained by the complexity of the different molecular mechanisms involved in telomerase regulation.

Even though the main role of telomerase is to maintain telomere length in tumors, non-canonical functions could also promote tumor growth and contribute to poor prognosis in primary NBLs (Wnt signaling, DNA repair, genomic instability, apoptosis, and escape from oncogene-induced senescence) [117].

Using a novel approach of three-dimensional (3D) telomere quantitative fluorescence *in situ* hybridization on 74 NBL tissue samples, a recent report demonstrates a possible classification of NBLs based on the level of telomere dysfunction, telomere length, and nuclear organization. Telomere dysfunctions were associated with unfavorable tumor characteristics, including *MYCN* amplification, and poor prognosis [128].

3.2. Potential mechanisms of hTERT activation and/or telomere maintenance mechanisms in neuroblastoma

The mechanisms by which telomerase activity is activated in high-stage NBL remain elusive. However, recent studies have shed some light on this important question. Although some controversies may remain, hTERT expression upregulation may occur through at least two pathways: *MYCN* amplification and genomic rearrangements around *hTERT*. Alternatively, ALT pathway can be activated in the absence of telomerase activation providing a mean for tumor cells to stabilize their telomeres as a necessary requirement for the immortalization and progression of the tumors.

3.2.1. MYCN amplification

MYCN amplification is the best characterized genetic marker and a powerful prognostic indicator of high-risk neuroblastoma [3]. *MYCN*-amplified tumors usually exhibit high telomerase activity and expression [118]. As developed earlier, c-Myc-binding sites are present on *hTERT* gene promoter and it is now well demonstrated that this factor, alone, or in cooperation with other transcription factors, determines the activity of *hTERT* promoter. N-MYC and c-Myc proteins are highly homologous. As c-Myc, N-MYC heterodimerizes with Max at consensus E-box sequences, therefore, *MYCN* overexpression could promote telomere stabilization through a transcriptional increase of *hTERT* gene expression associated with an increase in telomerase activity even though only one study reported such a direct interaction by ChIP in NBL [80]. An inverse correlation of *MYCN* and *c-Myc* expression was found in NBL subtypes [129]. As mentioned earlier, whether N-MYC protein can replace all c-Myc functions for *hTERT* regulation is still an unanswered question. Several lines of evidence suggest that MYC family members have separate physiological functions. The interplay between N-MYC and hTERT in NBL needs, thus, to be further investigated.

A recent study shows that *DKC1* gene promoter is targeted by both c-Myc and N-MYC and that high DKC1 expression is an independent prognostic indicator for adverse clinical outcome in NBL. *DKC1* gene encodes the RNA-binding protein dyskerin, a core component of the telomerase holoenzyme. This new function of DKC1 in NBL appears to be telomerase-independent [130].

3.2.2. hTERT promoter mutations

Although hotspot mutations in *hTERT* promoter driving telomerase activity are frequently described in neural crest-derived tumors such as melanoma [88, 89] and in a variety of other neuronal tumors including medulloblastoma and glioma [90, 131, 132], no *hTERT* promoter mutations have been detected in a large series (n = 131) of primary neuroblastoma [133], in line with previous studies performed on a smaller number of patients [90, 134]. However, these mutations were searched only in the core promoter of *hTERT*; it is not excluded that mutations may exist in more distant regulatory elements. The existence of a given SNP should also be searched.

3.2.3. hTERT gains

Chromosome 5p is often amplified in NBL, and focal *hTERT* gains were recently detected in several stage 4 primary NBLs. *hTERT* gains were more frequently detected in *MYCN* non-amplified cases, suggesting that *hTERT* gain performs a function similar to *MYCN* amplification [135, 136]. As N-MYC protein can bind and activate *hTERT* promoter, it is likely that *hTERT* gains are selected in order to increase hTERT expression in the absence of *MYCN* amplification.

3.2.4. hTERT rearrangements

Recent whole-genome sequencing of primary neuroblastoma, performed by two independent groups, discovered recurrent genomic rearrangements in a 70-kb region proximal to *hTERT* locus on the chromosome 5p15.33 [9, 10].

Indeed, in the first study [10], the authors were searching for structural alterations that might occur in high-risk NBL and, analyzing 56 tumors, they identified 4 locations exhibiting clustered breakpoints. These are related to *MYCN* amplifications, *ATRX* deletions, copy number gains of chromosome 17q, and rearrangements located at chromosome 5p15.33 proximal to *hTERT* gene. *hTERT* rearrangements occurred in 21% of tumors and included balanced translocations, copy number gains, high-level amplifications, and chromothripsis [137]. Chromothripsis corresponds to a massive and localized genome rearrangement [138], affecting high-risk tumors. Chromothripsis occurs in 2–3% of human cancers and, in a whole-genome-sequencing study, it occurs in 18% of high-risk NBL [137]. In an extended case series (n = 217), the authors showed that *hTERT* rearrangements are associated with a poor patient outcome and occur in mutually exclusive fashion with *MYCN* amplification and *ATRX* mutations (see below). They do not affect directly the *hTERT* gene or its promoter but they are all associated with an increase in *hTERT* transcription and telomerase activity as well as genes present in its vicinity (*SLC6A18* and *SLC6A19*) [139]. ChIP sequencing of *hTERT*-rearranged tumors indicated next to the breakpoints the presence of histone modifications known to mark active promoters (H3K4me3 and H3K27ac) and transcription elongation (H3K36me3), whereas in cells lacking *hTERT* alterations the repressive mark H3K27me3 was identified. Therefore, the structural rearrangement occurring at 5p15.33 results in a massive chromatin remodeling of this genomic region. The biological effect is the repositioning of regulatory elements very close

to *hTERT* locus that could be responsible for the high induction of *hTERT* expression (up to 90 times that of normal cells) in these tumors.

In a similar whole-sequencing study [9] screening 108 NBLs, structural rearrangements of *hTERT* associated with hTERT overexpression were identified in 23% of cases. In *hTERT*-rearranged NBLs, a significant increase in telomere length has been demonstrated compared to non-rearranged NBLs. Both studies describe *hTERT* rearrangement as the second most frequent genetic defect in high-risk NBL after *MYCN* alteration. It is important to note that in those cases the promoter and coding regions of *hTERT* gene remain non-altered.

These results have been major advances in our understanding of NBL genetic and biology placing telomere biology at the core of this pathology.

3.2.5. Small nucleolar ribonucleoproteins (snoRNPs)

A recent study reported that the expression of proteins involved in the formation and stabilization of snoRNP complex (including DKC1, GAR1, and NHP2 proteins) is elevated in high-risk NBL and associated with poor prognosis. Furthermore, this study shows a positive correlation between DKC1 expression and telomerase activity. This increase is associated with an increase of *hTR* expression. Therefore, in NBL, upregulation of snoRNPs may contribute to telomere maintenance and stabilization [140].

3.2.6. ALT and neuroblastoma: ATRX (alpha thalassemia/mental retardation syndrome, X-linked) mutations

Telomere length does not necessarily correlate with telomerase activity [141]. Recently, it has been reported that some neuroblastomas (generally associated with unfavorable NBL in older children without *MYCN* amplification and regardless of telomerase activation status) preserve their telomere length in the absence of telomerase through telomere-binding proteins and alternative lengthening of telomeres, a process based on DNA repair/homologous recombination pathways [141–143]. ALT tumors represent 10–20% of NBL. These tumors have a very poor outcome. Phenotypically, ALT cells present long and heterogeneous telomere lengths [144], ALT-associated promyelocytic leukemia (PML) nuclear bodies [145], and abundant extra-chromosomal telomeric repeats [146]. A recent whole-genome-sequencing study identifies, in most ALT NBL cases, loss-of-function mutations in *ATRX* [137, 147, 148]. *ATRX* maps to the X chromosome and encodes a SWI/SNF chromatin-remodeling ATP-dependent helicase. ATRX regulates chromatin structure at both centromeric heterochromatin and telomeric regions [149]. In vitro analyses demonstrate its binding to GC-rich sequences and to G-quadruplexes [150]. Loss of ATRX functions may allow the destabilization of repressive heterochromatin at telomeres. NBLs with *ATRX* mutations show longer telomeres. *ATRX* mutations include in-frame deletions, missense, nonsense, and frameshift single-nucleotide variations. They are predominantly observed in adolescent and young adult patients (44% in patients older than 12 years, whereas no mutation was detected in infants <18 months of age [148]) and are frequently associated with chemo-resistance. ATRX protein establishes a functional interaction with DAXX (death domain-associated protein). At telomeres, ATRX and

DAXX proteins cooperate to deposit the histone H3 variants, H3.3, to maintain chromosome stability [151]. Loss of function of *ATRX*/*DAXX* is also associated with ALT activation [152] and poor overall survival among older patients. As mentioned earlier, *ATRX* mutations were mutually exclusive from *MYCN* amplification. However, how *ATRX* mutations lead to NBL progression is still an unanswered question.

3.2.7. ARID1A and ARID1B

Next-generation sequencing, genome-wide rearrangements analyses, and targeted analysis of specific genomic loci of 71 NBL patients identified mutations in chromatin-remodeling complexes encoded by *ARID1A*/*1B* (AT-rich interaction domain 1A/1B) genes in 11% of cases with decreased survival of patients [147]. Both proteins are subunits of the SWI/SNF transcriptional complex. They have emerged as tumor-suppressor genes and thereby when mutated can drive NBL tumorigenesis. However, additional studies will be required to elucidate the role of these proteins in the initiation or progression of NBL.

In conclusion, it is important to note that through either *MYCN* amplification, *hTERT* rearrangements, or *ATRX*/*DAXX* mutations, most high-risk NBLs have activated mechanisms, all of which are involved in the maintenance of telomere length and contribute to the tumor progression. This relationship highlights the major role of telomere/telomerase biology in NBL. These three mechanisms identify therefore distinct groups of NBL patients at very high risk with poor outcome [139]. That low-risk tumors lack such alterations support the notion that these kinds of tumors are more prone to spontaneously regress.

Altogether, these observations provide new important mechanisms that could be targeted in new therapeutic strategies to treat the most aggressive forms of neuroblastoma.

4. Telomerase, a target for cancer therapeutics

That, on one hand, both *MYCN* amplification and 5p15.33 rearrangement targeting *hTERT* locus lead to an increase in telomerase activity and subsequent telomere lengthening, and on the other hand ALT pathway is activated in tumors lacking *hTERT* or *MYCN* alterations indicate that the fate of high-risk NBL is largely dependent on telomerase/telomere biology. These new findings may help to improve tumor diagnostics and prognosis but also supports the development of novel therapeutic strategies targeting telomere/telomerase to treat the most aggressive form of this disease. Different molecules were shown to target telomerase by different mechanisms such as nucleoside analogs and reverse transcriptase inhibitors (zidovudine, stavudine, and tenofir), synthetic non-nucleoside inhibitors as BIBR1532 (2-[(E)-3 naphtalen-2-yl-but-2enoylamino]-benzoic acid), natural compounds (EGCG, MST-132), G-quadruplex stabilizers (telomestatin, BRACO-19), and molecular chaperone inhibitors affecting hTERT assembly (Hsp90 inhibitors). However, these compounds generally lack specificity and have adverse effects, and for now, no clinically validated drug targeting telomerase has been successfully developed. GRN163L (imetelstat), a phosphoramidate oligonucleotide targeting the template region of hTR, has undergone clinical trials by Geron Corporation (Menlo Park,

CA, USA) [153–155]. However, the phase II study has been discontinued in breast cancers and non-small-cell lung carcinoma because no significant improvement in median progression-free survival was demonstrated. Moreover, hematological toxicity has been observed. However, these side effects have been used to propose this drug in hematological diseases. A phase II study evaluating the activity of imetelstat in patients with essential thrombocythemia or polycythemia vera is in progress. However, the actual mechanism of action of this molecule is still to be determined. For now, no clinical trials have been performed on NBL.

Regarding NBL, perhaps future attempts could be to target specifically N-MYC protein in patients who have NBL with *MYCN* amplification. However, the lack of specificity of the strategies targeting directly transcription factors is still a major concern. Bromodomain and extra terminal (BET) inhibitors, as JQ1 to specifically downregulate *MYCN* expression, are at the preclinical stage of evaluation [156]. Small molecules have been identified to inhibit c-Myc/Max interaction as well as decrease c-Myc protein levels and inhibit cell growth [157]. It has been shown that these molecules also interfere with N-MYC/Max interaction resulting in cell cycle arrest in *MYCN*-overexpressing NBL cell lines [158]. The consequences on hTERT expression have not been investigated yet.

The recent identification of *ATRX* mutations associated with their consequences on telomere structure in a specific group of high-risk NBL patients suggests that G-quadruplex stabilizers could be a potential therapeutic strategy to partially reverse the effects of *ATRX* mutations.

Telomerase expression was proposed as a selectively targetable mechanism for retinoids and specifically all *trans* retinoic acid (ATRA), an already clinically relevant drug used to stimulate differentiation of APL. Indeed, in a APL cellular model, it has been shown that retinoids can induce transcriptional repression of *hTERT* gene not only in differentiation of sensitive cells but also in cells resistant to ATRA-induced differentiation [159–161]. As the mechanism of *hTERT* repression occurs at the level of gene transcription, all the functions of telomerase can thus be targeted. Therefore, it is worth considering this antitelomerase property of retinoids in combination with more conventional therapies to target NBL. A recent study reporting the efficacy of a combination therapy, using retinoids and epigenetic modulators, in reducing NBL cell growth supports this idea [162].

5. Conclusions

Several potential chemotherapy strategies based on telomerase and telomere biology have been developed and explored by pharmaceutical and biotechnology companies [163]. However, in spite of ever-growing knowledge on telomere and telomerase biology, a number of questions remain to be answered as most of these numerous strategies are not yet clinically available because of a weak efficiency and/or a high toxicity. Therefore, to develop agents that will be effective, we need a sharper picture of how the enzyme functions and how we can manage to target specifically and destabilize telomeres in cancer cells. Epigenetic therapies aimed at counteracting the genetic alterations (mutations) are emerging alternatives against aggressive tumors.

Telomerase regulation is highly complex, involving the interplay between numerous biological and molecular processes. Despite the extensive studies that have been already done, a lot more is necessary to unravel the mechanisms underlying the switching off/on of *hTERT* gene during cell differentiation and cell transformation. However, progress in this direction is hampered by the absence of standardized methods to measure hTERT expression and telomerase activity and the lack of suitable tools in the study of telomere/telomerase biology.

First, telomerase repeated amplification protocol (TRAP) assay is a rather artificial assay to quantify telomerase activity; it is based on a quantitative real-time polymerase chain reaction (PCR) method that measures only the capacity of the telomerase reverse transcriptase to elongate artificial telomeric substrates without giving any measurement of the other functions of this protein. To date, no assay exists to evaluate the non-conventional functions of hTERT.

Second, *hTERT* expression is generally quantified using also a quantitative real-time PCR; however, in most cases it is not clearly known which *hTERT* isoform (full-length or specific splice variants) is detected. This would explain why, sometimes, comparison between telomerase activity and *hTERT* transcripts yielded contradictory results. Indeed, generally exhaustive measurements using different primer sets for the detection of various *hTERT* isoforms are not usually done. The detection and quantification of splice variants can be of interest for clinical outcome and prognosis. Moreover, further studies are also required to define precisely the functions of these variants.

Third, *hTR* is rarely quantified; however, it is also a limiting factor in telomere homeostasis.

Finally, due to the low expression of telomerase even in cancer cells, the detection of telomerase by western blot or fluorescence is puzzling. In addition, commercially available anti-hTERT antibodies are still a problem with specificity [164]. Because of these limitations, several published studies used overexpressed hTERT protein (generally tagged). However, the unusually high concentration of the protein due to the overexpression could alter the dynamic and the localization of the protein compared to the endogenous protein leading to misinterpretations.

Since the first paper published in 1995 [5], very few scientific advances have been done on a potential involvement of telomere/telomerase in NBL biology. However, the recent findings highlighting the role of telomere/telomerase biology in high-risk NBL will definitely impact the research in this pathology as well as in other cancers and help to develop new therapeutic strategies.

Acknowledgements

The work of the authors was supported by grants from the French National Institute of Health and Medical Research (INSERM), the National Center for Scientific Research (CNRS), the Ligue Nationale contre le Cancer, the Fondation de France (n° 201300038226), the French National Research Agency (ANR), Gefluc, Agence Universitaire de la Francophonie (AUF), Hubert Curien Partnership (PHC-CEDRE), and Ministère de l'Enseignement Supérieur et de la Recherche (MESR). Yann Blondin is acknowledged for his help in preparing **Figure 1**.

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