Human telomerase reverse transcriptase regulation by DNA methylation, transcription factor binding and alternative splicing (Review)

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Abstract. The catalytic subunit of telomerase, human telomerase reverse transcriptase (hTERT), plays an essential role in telomere maintenance to oppose cellular senescence and, is highly regulated in normal and cancerous cells. Regulation of hTERT occurs through multiple avenues, including a unique pattern of CpG promoter methylation and alternative splicing. Promoter methylation affects the binding of transcription factors, resulting in changes in expression of the gene. In addition to expression level changes, changes in promoter binding can affect alternative splicing in a cotranscriptional manner. The alternative splicing of hTERT results in either the full length transcript which can form the active telomerase complex with hTR, or numerous inactive isoforms. Both regulation strategies are exploited in cancer to activate telomerase, however, the exact mechanism is unknown. Therefore, unraveling the link between promoter methylation status and alternative splicing for hTERT could expose yet another level of hTERT regulation. In an attempt to provide insight into the cellular control of active telomerase in cancer, this review will discuss our current perspective on CpG methylation of the hTERT promoter region, summarize the different forms of alternatively spliced variants, and examine examples of transcription factor binding that affects splicing.

Contents

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1. Telomeres and telomerase

Each round of DNA replication results in the shortening of DNA strands due to the inability of the replication complex to completely replicate the lagging strand. This process in turn eventually results in genomic instability and loss of genetic information after multiple rounds of replication. To solve this end-replication problem, chromosome ends are capped by telomeres, which consist of six nucleotide repeats and specialized binding proteins that buffer replication losses (1). The repeats can be regenerated by the telomerase enzyme complex, which consists of a non-coding RNA, hTR, serving as the hexamer repeat template, and the catalytic subunit, reverse transcriptase (hTERT). By counteracting the telomere shortening incurred by the DNA replication process, the telomerase complex lengthens telomeres, thereby prolonging cell survival and allowing continued proliferation (2).

2. Telomerase regulation

Telomerase activity is low to absent in somatic cells, though highly expressed in embryonic and stem cells. Telomerase is also upregulated in cancer, as over 90% of human malignancies show telomerase expression, considered an early event in cancer progression (3,4). In cancer, the main mechanism of telomerase activation is through regulation of hTERT transcription, through genetic changes such as mutations altering transcription factor binding, by epigenetic changes such as histone modification and chromatin remodeling or promoter methylation, and by alternative splicing of the transcript (2,5-7). As explored below, a connection between the promoter methylation and the alternative splicing is emerging in the field as a means by which cancer cells turn on hTERT expression, resulting both in active telomerase and telomere elongation.

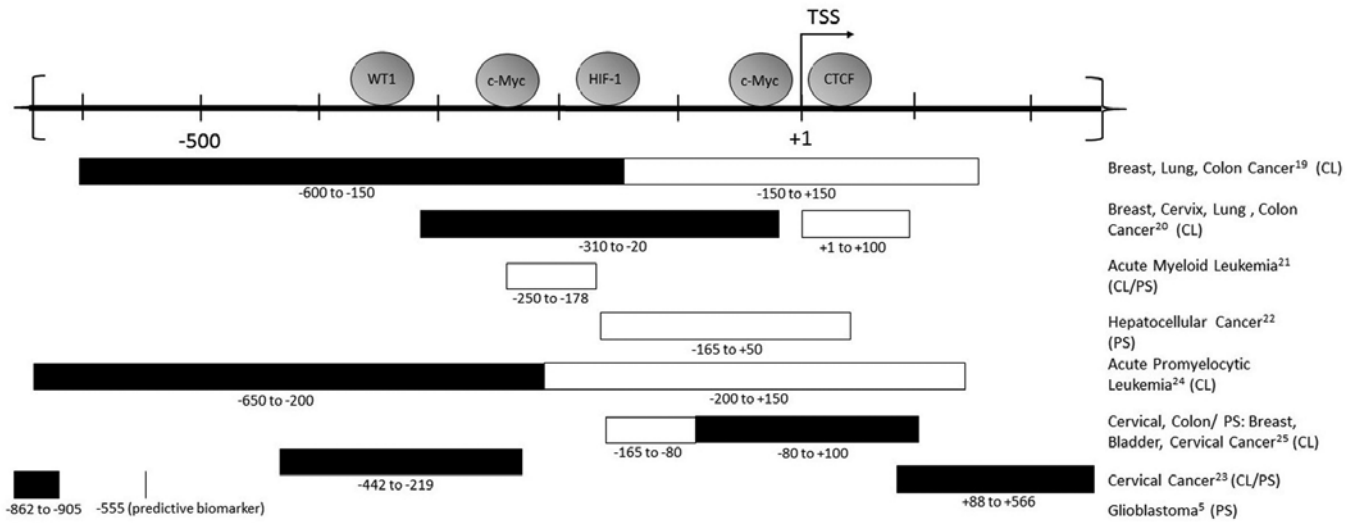

Figure 1. hTERT promoter exhibits a distinct methylation pattern in cancer cells positive for telomerase activity. hTERT promoter is -1876 to +335 relative to the transcription start site (TSS), with section -600 to +200 depicted above. Transcription factors (WT1, c-Myc, HIF-1, CTCF) and their relative binding sites at the promoter are shown. The methylation status of the hTERT promoter in several cell lines (CL) and/or patient blood or tumor samples (PS) are shown, with black boxes indicating methylated CpG sites, and white boxes indicating unmethylated CpG sites. In glioblastoma the CpG site at -555 is methylated.

3. Gene regulation by methylation

In higher eukaryotes, DNA methylation at CpG sites in and around gene promoter regions controls and regulates gene expression. CpG methylation is directed by DNA methyltransferases (DNMTs) which methylate the fifth carbon of the pyrimidine ring of cytosine (8). The principal methyltransferase, DNMT1, adds methyl groups during DNA replication and *de novo* methylates DNA in cancer (9). Many CpG sites are clustered into CpG islands that are typically 1,000 base pairs long and have high GC content. Approximately 80% of CpG sites are methylated in mammals, largely in intergenic regions known as heterochromatin, while most sites in promoters and first exons remain unmethylated (10,11). The promoter and transcription start site (TSS) tends to be unmethylated in actively transcribed genes, since methylated DNA is associated with gene silencing through both interference of transcription factor binding and, by affecting chromatin architecture (12). Importantly, aberrant DNA methylation is a hallmark of cancer cells, and tends to occur early in cancer development (13). In cancer, characteristic changes in methylation patterns involve both genome-wide CpG hypomethylation, which occurs predominantly in intergenic regions, and hypermethylation of CpG islands at promoters. Promoter hypermethylation may result in silencing of tumor suppressors, and promoter hypomethylation can result in activation of proto-oncogenes (14). Intergenic hypomethylation may also lead to expression of dormant non-coding RNA species and otherwise suppressed genetic elements transcribed from normally silent regions of the genome (15-17).

4. hTERT promoter methylation in cancer

The hTERT promoter is located in a 4 kb CpG island -1800 to +2200 (relative to TSS), and has a GC content of 70% (18). The precise pattern of promoter methylation that results in activation of hTERT in cancer is still under investigation. However, a methylation pattern does emerge from many studies of the promoter, including extensive bisulfite sequencing of telomerase positive cancer cell lines. The promoter region of the actively transcribed hTERT is demethylated at the TSS [-200 to +100], while the promoter region further upstream of the TSS [-650 to -200] is hypermethylated (19). Studies examining specific sections of the hTERT promoter corroborate this in a myriad of cancer cell lines as well as hematological malignancies and solid tumor types, as depicted in Fig. 1 (5,19-26). Its methylation status is also considered a biomarker; in pediatric brain tumors, one methylated CpG site in the promoter (cg11625005) is used as a reliable marker for tumor progression and prognosis (5).

5. Transcription factor regulation by methylation of hTERT promoter

It is known that the hTERT methylation pattern plays a major role in transcription factor binding, which in turn alters the expression of hTERT. This is demonstrated by experimental evidence pointing to a 'minimal promoter' corresponding to transcription factor binding sites, e.g. c-Myc for hTERT at -258 to -78, that must be unmethylated in order for hTERT expression to occur (27). Methylation also plays a significant role further upstream in the hTERT promoter where many repressor binding sites are located. These sites are hypermethylated in cancer to prevent binding of repressors such as the Wilms' tumor protein (WT1) and the transcriptional repressor CCCTC-binding factor (CTCF) that binds to CpG rich regions at the TSS (21,25,27-29). Perturbing the methylation status of the promoter with 5aza-2'-deoxycytidine (DAC), which globally reduces DNA methylation by DNMT inhibition, results in reduced levels of hTERT transcription, which may be due to demethylation of the repressor binding sites (30).

c-Myc. Hypomethylation at the minimal promoter of hTERT allows c-Myc, a key positive regulator of hTERT expression,

to bind 242 bases upstream of the TSS at the E-box. Studies have shown that c-Myc binding is methylation-sensitive and that binding is greatly reduced or absent when the site is methylated (31). This control of hTERT expression by E-box methylation is also seen in human embryonic teratocarcinoma cells. The undifferentiated embryonic cells have higher expression of hTERT and telomerase activity with hypomethylation of the minimal promoter. Conversely, during the process of differentiation of these cells there is a significant increase in methylation at the E-box, resulting in inactivation of hTERT expression. This mode of repression of hTERT can be reversed by treatment of late differentiating cells with DAC, highlighting the importance of c-Myc binding and regulation by methylation (27).

WT1. In contrast to the minimal promoter being hypomethylated to allow c-Myc binding, the promoter region further upstream, corresponding to the binding sites of multiple repressors, is hypermethylated in cancer. For example, the repressor WT1, with its binding site from -358 to -349, is known to suppress hTERT transcription (27). This is supported by a study in clear cell renal cell carcinoma, in which WT1 is overexpressed, and direct binding of WT1 to the promoter results in repression of hTERT (32). WT1 binding is also known to be methylation sensitive, with binding interference assays showing reduced binding when one or more methylated bases are present in the binding sequence (33). The WT1 binding site of hTERT exhibits increased CpG methylation in cancer, resulting in blocking the repressive effects of the factor for hTERT expression (20,34).

CTCF. Similar to WT1, as a repressor of hTERT, CTCF binds adjacent to the transcription start site, near the beginning of exon one (+4 to +39), and near the beginning of exon two (+422 to +440). Studies have shown that CTCF binding at the first site represses hTERT transcription and that this binding is blocked in cancer (35). Blocking is established by methylation as CTCF's binding affinity is inversely correlated with the degree of methylation; CTCF is unable to bind fully methylated DNA. Cancer cells have aberrant methylation in the first CTCF binding site, typified by HPV-transformed cervical cancer cells showing increased methylation and activated hTERT expression (23). Conversely, in colon cancer cell lines, downregulating DNTM1, and the resultant demethylation of the CTCF binding site, causes increased CTCF binding and repression of hTERT (36,37). Furthermore, in breast cancer cells, hTERT transcript levels increase with a concomitant decrease in cellular apoptosis when CTCF is downregulated by siRNA (38).

6. Role of transcription factor binding on splicing

While it is known that transcription factors binding to the promoter regulate gene expression, it is becoming evident that such binding also affects splicing. Proteins with a *cis* regulatory role in both transcription and splicing have been demonstrated in multiple genes. The regulation is believed to act through the kinetic coupling model, where the rate of transcription elongation affects alternative splicing (39,40). Experiments assessing alternative splicing in a promoter swapping system have established promoter specificity in controlling alternative splicing (41). This splicing role is consistent with splicing occurring co-transcriptionally as splicing factor assembly arises during transcription. As detailed below, manipulation of splicing through transcription factors can be accomplished either by the factor directly influencing the spliceosome or, by recruitment of additional factors (42,43).

Direct splicing role of WT1 with U2AF and RBM4. WT1, a negative regulator of hTERT, promotes splicing of multiple transcripts by both interacting directly with several splicing factors and through incorporation into spliceosomes. In the nucleus, the regulator co-localizes with the splicing machinery, specifically with U2AF and RBM4. The U2AF heterodimer is essential for binding upstream of the splice site and helps the U2 snRNA anneal at the branch point. Interaction with WT1 affects U2AF binding and therefore splice site selection (44). Also in splice site selection, RBM4 influences alternative splicing during selection of the 5' exon splice site, and interacts with WT1 in nuclear speckles, thought to be compartments for spliceosome assembly (45). Recruitment of these splicing factors by WT1 highlights the link between the transcription factor binding and spliceosome assembly.

Indirect splicing role of WT1 with SRPK1. While WT1 interacts with the splicing machinery as discussed above, it also plays an indirect role in splicing by transcriptionally repressing SRPK1, a splicing factor kinase. As shown in acute myeloid leukemia, where it is often overexpressed, WT1 causes alternative exon usage events commonly seen in the disease (46). Conversely, when WT1 is knocked down in hematopoietic progenitor cells, vascular endothelial growth factor (VEGF) exhibits an atypical splicing pattern (47). In studying the relationship between WT1 and VEGF, an important growth factor in cancer, loss of WT1 results in increased abundance of isoform VEGF-a120, while transfection of WT1 results in exon inclusion and loss of the 120 isoform. This demonstrates that WT1 is essential for controlling the splicing of VEGF-a (48). Studies of the VEGF-b isoform have also shown alteration of splicing patterns with WT1 manipulation. Transfection of various cell lines with WT1 results in an increase of VEGF165b. Furthermore, inclusion of the WT1 KTS sequence, which plays a role in RNA binding, shows no effect on splicing, while a single nucleotide change that alters DNA binding of WT1 changes the splicing of VEGF. Thus, splicing correlates with the ability of the transcription factor WT1 to stably bind DNA, rather than WT1 acting post-transcriptionally (49).

Splicing role of HIF-1 and hypoxia on hTERT. Hypoxia is known to enhance hTERT expression through activation of the hTERT promoter in stem cells as well as in cancer (50,51). In hypoxic tumor conditions, HIF-1 is overexpressed and activates the hTERT promoter by binding at two sites between -165 and +51. If the HIF-1 binding sites are mutated, hTERT promoter activity decreases even in normoxic conditions and under hypoxic conditions, the HIF-1 induced overexpression of hTERT is lost. It has been shown that in hypoxia, cell survival is increased through maintenance of an undifferentiated state, and this correlates with increased hTERT expression and telomerase activity compared to normoxic conditions (51,52).

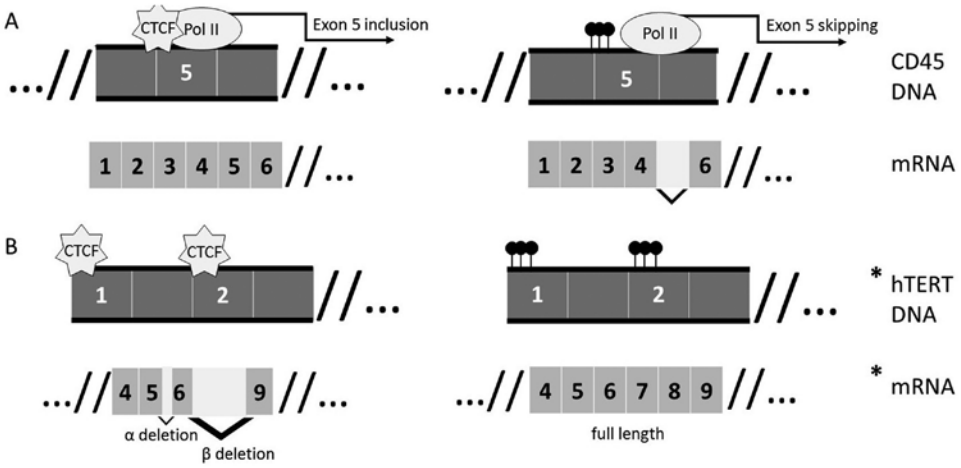

Figure 2. Intragenic binding of CTCF affects transcript splicing. (A) The well-established mechanism of exon 5 inclusion in CD45. Binding of exon 5 by CTCF to its unmethylated binding site causes pausing of polymerase II and inclusion of exon 5 in the final mRNA. When the site is methylated, CTCF cannot bind and the final transcript skips exon 5. (B) Hypothetical (*) mechanism of long range CTCF interactions influencing hTERT splicing. Common exon deletions in hTERT splice variants with the CTCF binding sites at exon 1 and exon 2. CTCF binding at exon 1 is known to affect hTERT expression, while its potential role in splicing is unknown.

Furthermore, the splicing pattern of hTERT is quite variable at differing levels of oxygenation. Overall, the hTERT splicing pattern is altered in hypoxia, with an increased expression of the full length active form, while the β deletion and α/β double deletion splice variants remain unchanged. Additionally, blocking α and β splicing results in spontaneous human embryonic stem cell differentiation (50). In hypoxia, HIF-1 increases the association of RNA Pol II with transcription initiation factors, resulting in more effective and efficient transcription. It has been proposed that modulation of hTERT transcription by HIF-1 in hypoxia controls splicing by affecting Pol II rates of transcription and enhancing expression of the active full length transcript (53).

Direct splicing role of CTCF intragenic binding with Pol II pausing. The direct role of CTCF modulating alternative splicing in a co-transcriptional manner has recently emerged. Shukla and colleagues (56) showed that CTCF binding at actively transcribed DNA causes RNA polymerase II pausing, resulting in recognition of weak splice site signals and therefore inclusion of 'weak' exons in CD45. They also demonstrated that the pausing was methylation dependent; when CTCF cannot bind the methylated site, weak exons are not included in the final spliced transcript (Fig. 2A) (55,56). Multiple additional genes have been shown to have promoter-proximal CTCF binding (100 bases downstream of the TSS) that results in longer Pol II pausing compared to genes without promoter-proximal binding sites. This effect is dependent on the binding site position relative to the TSS, with less pausing associated with sites at greater distance from the TSS (57). RNA polymerase II pausing due to CTCF results in decreased processivity and contributes to promoter proximal pausing, thereby directly modulating the dynamics of transcription and splicing (57,58).

Indirect splicing role of CTCF by facilitation of long range interactions. CTCF is well known for its architectural role in establishing boundaries based on topologically associating domains (TADs) that link distant enhancers with promoters and other regulatory sequences. This chromatin looping by CTCF allows for architectural reorganization resulting in co-location of factors to influence transcription. Indeed, approximately 15% of CTCF binding sites are located in promoters, while over 40% of CTCF binding sites are located in the 5'UTR, introns, and other intragenic regions. For example, the murine Myb locus has a CTCF binding site in the first intron, which loops to the promoter during differentiation, and a second CTCF site in a regulatory element further upstream. This CTCF interaction allows for juxtaposition of necessary transcription factors to regulate Pol II and modulate expression of Myb. After differentiation, the expression of the gene is halted due to CTCF architecture reorganization. This highlights the ability of CTCF to regulate initiation of transcription as well as elongation and Pol II pausing during transcription by looping together promoter, upstream enhancer elements and intronic sequences. Influencing the elongation of Pol II, as well as the juxtaposition of regulatory factors, are known to be important for splicing, as discussed above regarding the elongation rate of Pol II and CD45 splicing (59,60).

7. Telomerase regulation by alternative splicing in hTERT

While in many genes transcription factor binding has been shown to play a role in splicing, the mechanism of hTERT splicing is not as clearly defined. hTERT splicing plays a crucial role in dictating the activity of telomerase, since only the full-length transcript is catalytically active in the telomerase ribonucleoprotein complex. Furthermore, many cancers show changes in hTERT splicing patterns.

hTERT splicing switch in cancer. Modulation of alternative splicing is detected in multiple types of cancers, where cancer cells utilize an alternative splicing switch that results in discernible isoform signatures (61). This switching is non-random and also seen in tissue development, including the hTERT splice switch, well characterized in kidney develop-

ment and various cancer cell types (62-74). This mode of hTERT regulation has been proposed to be necessary due to the difficulty to completely cease transcription, since even very small amounts of transcript may have significant cellular effects, given the limited number of cellular targets, i.e., 92 telomeres in the normal human cell (2). Cancer cells with active telomerase have an average of 20 hTERT transcripts per cell resulting in 100-500 active telomerase complexes (75). Alternative splicing yielding inactive and/or inhibitory forms of hTERT allows for downregulation of telomerase activity without complete repression of transcription.

Mechanism of hTERT alternative splicing. Studies on the mechanisms of alternative splicing of hTERT so far have revealed regulation primarily by long range interactions, not at nearby splice sites as seen in other genes. One proposed mechanism is via variable number tandem repeats (VNTR), that are located in intronic regions over 1 kb from the splice site, regulating splicing of the β deletion form. The mechanism of VNTR control over splicing is not understood, but could involve recruitment of RNA binding factors to the repeats. These RNA binding factors would then interact and form a specific landscape with other proteins conducive to spliceosome recruitment (2,75). The mechanism for the dependence on VNTR for splicing of the β deletion needs further examination, as do additional splicing mechanisms for the other isoforms.

Over twenty different isoforms of hTERT have so far been reported, with the most common being various deletions in the reverse transcriptase domain, such as the α deletion, β deletion and α/β deletion shown in Fig. 2B (76). Other important isoforms have been identified, such as intron 2 and 14 retention in lung and colon cancer as well as exclusion of exon 2 in normal cells (77,78). All known isoforms result in an inactive telomerase complex. For example, the α deletion is a dominant negative variant while the β deletion results in a truncated protein targeted for nonsense-mediated decay (75). While normal cells express mainly inactive hTERT isoforms, a splicing switch occurs in cancer cells, resulting in production of the full length active transcript (79). The cause of the splicing switch is unknown, but is likely due to changes of binding factors, possibly similar to changes to long range interactions through VNTR in the case of the β deletion or, as stated above, from the ability of certain transcription factors to bind and affect transcription elongation rates.

8. Concluding remarks

Human telomerase reverse transcriptase expression has many facets of regulation, including promoter methylation and alternative splicing, as outlined above. These two methods of regulation can become intertwined by the co-transcriptional nature of mammalian pre-mRNA splicing. This allows transcription factors bound to the gene, including the promoter, to influence alternative splicing of the transcript. Understanding the regulation of hTERT is crucial to understanding telomerase activity in normal cells as well as in cancer. Elucidating the mechanism of cellular control of hTERT transcription will further our knowledge of the intricate instructions directing activation of this essential genome maintenance machinery.

Study of hTERT transcription is a unique opportunity to understand the control of a low abundance transcript with a prominent role in cancer. Insight into how cancer commandeers the regulatory control, be it through methylation or through the splicing machinery, to produce full length hTERT and resulting in active telomerase would be highly beneficial clinically. Currently, therapeutic telomerase inhibitors are not well tolerated by patients. The therapy must be continually administered for multiple replication cycles to have an effect, resulting in the need for extended periods of treatment, which can be challenging due to significant drug toxicity. Promising anticancer therapeutics such as imetelstat, an antisense oligonucleotide binding to hTR, target the active telomerase complex, or consist of small molecule inhibitors inhibiting active TERT binding to the RNA (80). Alternatively, therapies inhibiting TERT from being activated before the telomerase complex forms, by altered expression or splicing, could provide a new mechanistic avenue for effective anticancer therapeutics.

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