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TELOMERE BIOLOGY OF CUTANEOUS T-CELL LYMPHOMAS

Sous la direction d'Edith CHEVRET et de Paula SOARES

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Table of Contents

Abbreviations list ..... P: 3

Introduction ..... P: 4

- Telomeres' structural organization and function ..... P: 4
- Shelterin complex ..... P: 6
- Telomerase ..... P: 7
- Telomeres and telomerase in cancer ..... P: 8
  - Cancer cells immortalization ..... P: 8
  - Telomere length-dependent telomerase activity regulation ..... P: 9
  - Telomere length-independent telomerase activity ..... P: 10
  - *hTERT* transcription regulation ..... P: 10
    - *hTERT* transcription activation in hematological malignancies..... P: 13
  - *hTERT* post-transcription regulation ..... P: 14
- TERRA ..... P: 15
- Telomeres and telomerase rationale as therapeutic targets ..... P: 16
- Primary cutaneous T-cell lymphomas ..... P: 17
- CD30+ lymphoproliferative disorders (LPDs) ..... P: 17
- Mycosis fungoïdes (MF) ..... P: 18
- Sézary syndrome (Sz) ..... P: 19
- Telomeres and telomerase in CTCL ..... P: 21

Problematic and objectives ..... P: 22

Telomere length estimation in cancer cells ..... P: 23

Is qPCR alone valid for telomeres length measurement in cancer cells?.... P: 24

Telomerase regulation in cancer cells ..... P: 25

Spotlight on hTERT' complex regulation in cutaneous T-cell lymphomas .. P: 26

Telomere biology players beyond hTERT ……... P: 27
New telomere biology players in CTCL……... P: 28

Therapeutic targeting telomerase in CTCL …… P: 29
A new target for an old drug ……..... P: 31

Conclusion – Perspectives ……..... P: 32
References ……..... P: 35

Abbreviations list

ATF	Activating Transcription Factor
cALCL	Cutaneous anaplastic large cell lymphomas
cDNA	Complementary cDNA
CLL	Chronic lymphocytic leukemia
CTCL	cutaneous T-cell lymphoma
DSS	Disease-specific survival
EF-1α	Human elongation factor-1 α
EFS	Etablissement Français du Sang
GRCh 37	Genomic coordinates are based on build 37
GWAS	genome-wide association
hTERT	telomerase reverse transcriptase
LPDs	CD30+ lymphoproliferative disorders
MACC1	Metastasis-Associated in Colon Cancer Protein 1
MF	Mycosis fungoides
NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ	Immunodeficient mice carry two mutations on the NOD/ShiLtJ genetic background; severe combined immune deficiency (*scid*) and a complete null allele of the IL2 receptor common gamma chain ($IL2rg^{null}$).
POT1	Protection of telomeres 1
RAP1	TERF2 interacting protein
RT domain	Reverse transcriptase domain
Sh	Short airpin
SNP	Single nucleotide polymorphism
Sz	Sézary syndrome
TERRA	Telomeric repeat-containing RNAs
TIN2	TRF1 interacting nuclear factor 2
T-MF	Transformed mycosis fungoïdes
TPP1	Adrenocortical dysplasia protein homolog
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
TSS	Transcriptional start site
WHO-EORTC	World Health Organization and the European Organization for Research and Treatment of Cancer

Introduction

Telomeres' structural organization and function

The ends of linear genomes are comprised of a unique and genetically stable structure termed telomere. Mammalian telomeres are composed of tandem repeats (TTAGGG)n that terminates in a 3' single-stranded G-rich overhang that have a central role in sustaining a diverse array of telomeric functions. Indeed, the G-rich overhang that has approximately 30-500 nucleotides, folds back and invades the double-stranded telomeric helix, forming a lariat-like structure called telomeric loop or T-loop. The overhang pairs with the opposite strand, giving rise to a smaller displacement loop, the D-loop (**Figure 1**). This whole secondary structure is stabilized by the shelterin complex (Griffith, Comeau et al. 1999; di Fagagna, Reaper et al. 2003).

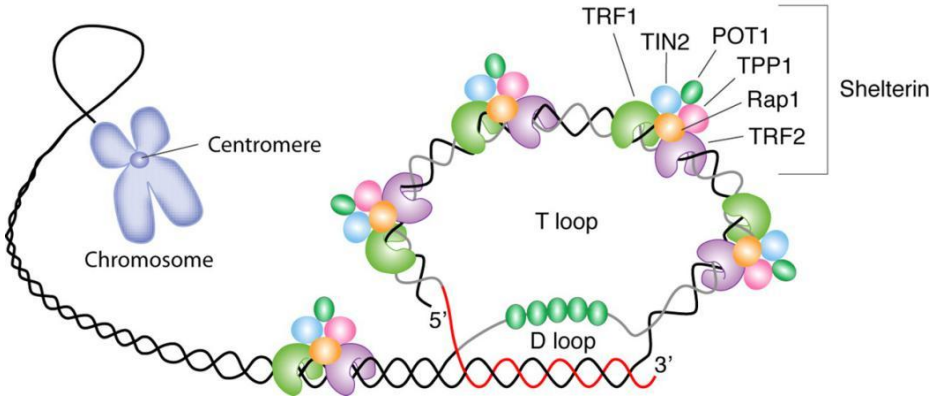

Figure 1 – Schematic representation of telomere structure

Telomeric 3′ end terminates as a single-stranded, G-rich overhang (colored in red). Telomeres are capped by shelterin protein complex that physically shield the DNA. From (Calado and Young 2008).

The T-loop is also stabilized by the G-rich character of telomere 3' single-stranded overhang. Indeed, the 3' overhang takes on a secondary structure formed from the hydrogen bonding of guanine residues in tetrad formations, called G-quadruplex structure (**Figure 2**). This structure blocks telomerase physical access to telomeres, by preventing telomeric DNA linearization (Biffi, Tannahill et al. ; Sfeir ; Oganesian and Karlseder 2009).

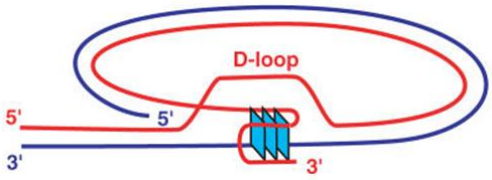

Figure 2 – **G-quadruplex**

Telomeric 3' overhang takes on a secondary structure. Adapted from (Patel, Phan et al. 2007)

Mammalian telomeric DNA is assembled into evenly spaced nucleosomes that are enriched with repressive epigenetic marks that are characteristic of constitutive heterochromatin (**Figure 3**) (Benetti, Garcia-Cao et al. 2007). The heterochromatic state of telomeres is important for proper telomere function, as it modulates telomere length and telomere ability to undergo homologous recombination (Schoeftner and Blasco).

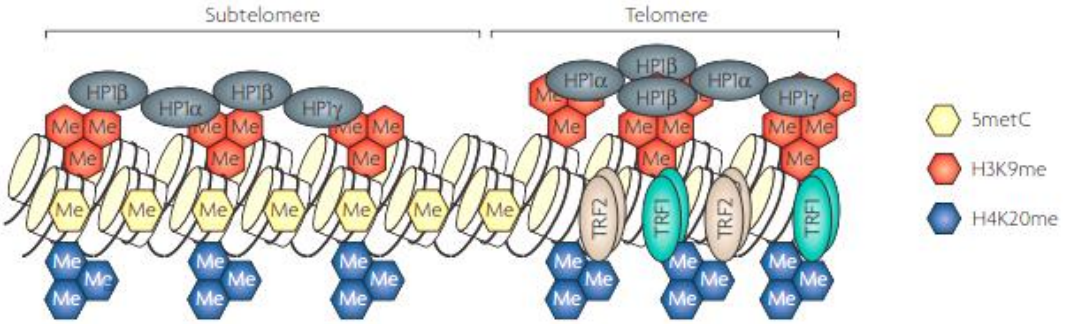

Figure 3 – **Epigenetic modifications at mammalian pericentromeric and telomeric regions**

Normal-length telomeres have features of constitutive heterochromatin, such as subtelomeric DNA hypermethylation (5metC, DNA methylation at 5-methylcytosine - in yellow), hypermethylation of histone H3 at lysine 9 (H3K9 - in red) and histone H4 at lysine 20 (H4K20 - in blue), hypoacetylation of histones H3 and H4, and heterochromatin protein HP1 (grey color) binding at both telomeres and subtelomeres. This suggests that they have a compacted and 'closed' conformation, which is not accessible to telomerase and that represses recombination between telomeric repeats. Adapted from (Blasco 2007)

Telomeres solve two basic problems that are inherent to linear genomes. First, thanks to its specialized structure, they distinguish chromosome ends from DNA double-strand breaks, thereby preventing unwanted DNA-damage signaling and genome instability. Second, they prevent loss of essential genetic information (O'sullivan and Karlseder ; Gümüs-Akay and Tükün 2012). Telomere functions are mainly regulated by shelterin protein complex and telomerase enzyme.

Shelterin complex

Telomeric DNA is bound by the shelterin complex, composed of six proteins: TRF1 (telomeric repeat binding factor 1, also known as TERF1), TRF2 (telomeric repeat binding factor 2, also known as TERF2), RAP1 (TERF2 interacting protein, also known as TERF2IP), TIN2 (TRF1 interacting nuclear factor 2, also known as TINF2), TPP1 (adrenocortical dysplasia protein homolog, also known as ACD) and POT1 (protection of telomeres 1) (De Lange 2005). The exquisite specificity with which shelterin binds to the telomeric DNA is conferred by three of its components: TRF1 and TRF2 bind to the double stranded region of the DNA, whereas POT1 coats the single stranded overhang. The other three shelterin components bind to the telomeres through protein-protein interactions. RAP1 binds TRF2; TPP1 binds POT1; and TIN2 binds TRF1, TRF2 and TPP1 simultaneously (**Figure 4**) (Sfeir ; Palm and de Lange 2008). Most shelterin components are essential to survival of mammalian cells, as its depletion either drives cells into cellular senescence or results in early embryonic lethality (Martinez and Blasco ; Patel, Vasan et al.). Together, shelterin complex protects chromosome ends from activating a DNA damage response, inhibits inappropriate repair mechanisms and maintains telomeric length and structure. However, each protein plays a unique role in telomere homeostasis (**Figure 4**) (Palm and de Lange 2008).

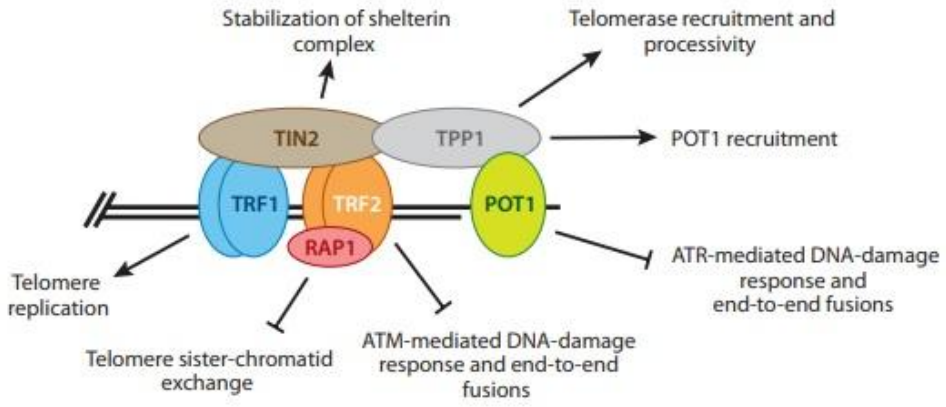

Figure 4 – Shelterin complex organization and individual components' functions

The basic function of each subunit that composes the shelterin complex is indicated. Adapted from (Xu, Li et al.)

The major function of TRF1 is to negatively regulate telomere length by suppressing telomerase activity, as its absence from telomeres results in telomere elongation. TRF1 disruption also significantly increases the levels of fragile telomeres and sister-telomere associations (Zimmermann, Kibe et al. ; Sfeir, Kosiyatrakul et al. 2009). TRF2, on the other hand, facilitates the T-loop formation *in vitro* (Doksani, Wu et al.). TRF2 disruption activates an ataxia telangiectasia mutated protein (ATM) mediated DNA-damage signal, causes end-to-end telomere fusions, and stimulates telomere homologous recombination (Denchi and de Lange 2007). The association between Rap1 and TRF2 is important for repression of this homologous recombination (Sfeir, Kabir et al. ; Sfeir, Kosiyatrakul et al. 2009). POT1 represses possible telomere extension by telomerase and prevents inappropriate activation of the ataxia-telangiectasia and Rad3-related (ATR) DNA damage response and telomere fusions (Guo, Deng et al. 2007; Churikov and Price 2008). TPP1 is thought to be directly involved in telomerase recruitment to telomeres (Wang, Podell et al. 2007). Finally, TIN2 plays an essential role in stabilizing the shelterin complex as it bridges the double stranded and single stranded telomere binding proteins (Takai, Kibe et al.). Thus, shelterin complex shapes and safeguards human telomeres (De Lange 2005).

Telomerase

Telomerase is a specialized reverse transcriptase that uses an RNA template to elongate the telomeres by addition of 5'-TTAGGG-3' repeats to the terminal 3' overhang. Human telomerase enzyme is composed of two core sub-units: a catalytic sub-unit named human telomerase reverse transcriptase (hTERT) and an RNA component named human telomerase RNA component (hTERC or TERC). In addition to these main components, several telomerase-associated proteins are required for proper functioning *in vivo* (**Figure 5**) (Shay, Zou et al. 2001; Cristofari and Lingner 2006). Dyskerin, GAR1, NH2P, and NOP10 bind to hTERC and are responsible for the stability, accumulation, maturation and assembly of telomerase (Cohen, Graham et al. 2007; Vulliamy, Beswick et al. 2008). Various proteins interact directly with hTERT, including the chaperone p23, HSP90, TEP1, Ku, hEST1 and PinX1. These proteins are involved in the regulation of telomerase assembly, post-translational modification, localization, and enzymatic function. Telomerase

associated proteins can vary in different cells and tissue types, providing some specificity for the regulation of telomerase (Ozturk, Li et al.).

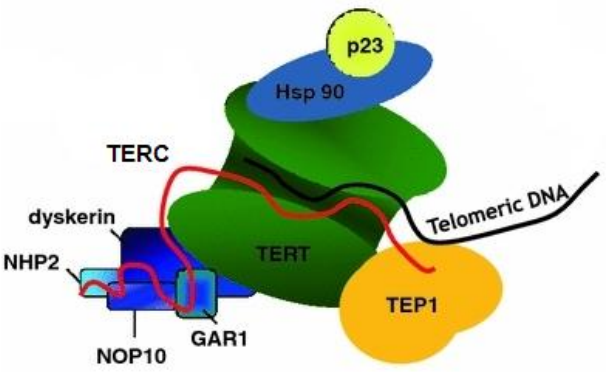

Figure 5 – **Schematic representation of telomerase holoenzyme**

Several telomerase-associated proteins are required for proper functioning of telomerase. Adapted from (Wojtyla, Gladych et al.)

Telomerase activity is undetectable in most normal human somatic cells. However, it is expressed during early development and remains fully active in specific germline/embryonic stem cells (Kim, Piatyszek et al. 1994). In addition, modest levels of telomerase activity are observed in proliferative tissues with high renewal potential such as the bone marrow, skin, gastrointestinal tract and testis as well as in activated lymphocytes (Vaziri, Dragowska et al. 1994; Shay and Bacchetti 1997). Telomerase activity should be strictly regulated to meet proliferative needs of specific cellular functions, while at the same time preserving proliferative barriers against tumorigenesis (Gümüs-Akay and Tükün 2012).

Telomeres and telomerase in cancer

Cancer cells immortalization

A normal cell only undergoes a limited number of cell divisions before entering into a permanent state of growth arrest. Indeed, due to the inherent inability of replication machinery to fully duplicate linear templates, telomeric sequences shorten after every cell division, until they reach the Hayflick limit. At the Hayflick limit one or a few critically shorten telomeres, which lose their capping function and activate DNA damage checkpoints, trigger an irreversible growth arrest known as replicative senescence and coined Mortality stage 1 (M1). Senescence involves p53 and pRb/p16 pathways. Senescence leads to the termination of cell proliferation and it is

considered as an important tumor-suppressive mechanism (Campisi 2005). Cells that can bypass this replicative senescence by inactivation of important cell cycle checkpoint genes (e.g. p53) continue to divide and lose their telomeres further until reaching the crisis stage or Mortality stage 2 (M2) (Shay, Pereira-Smith et al. 1991). The escape from crisis and achievement of cancer cell immortalization occurs through the engagement of a telomere-maintenance mechanism, which allows the maintenance of stable but usually shortened telomere lengths (**Figure 6**). Cancer cells can engage in a DNA recombination pathway, known as alternative lengthening of telomeres (ALT); or can activate/up-regulate the telomerase enzyme. The ALT pathway occurs in only ~10–15% of cancers, whereas telomerase activation occurs in 85–90% of all human cancers (Londono-Vallejo, Der-Sarkissian et al. 2004; Kyo, Takakura et al. 2008).

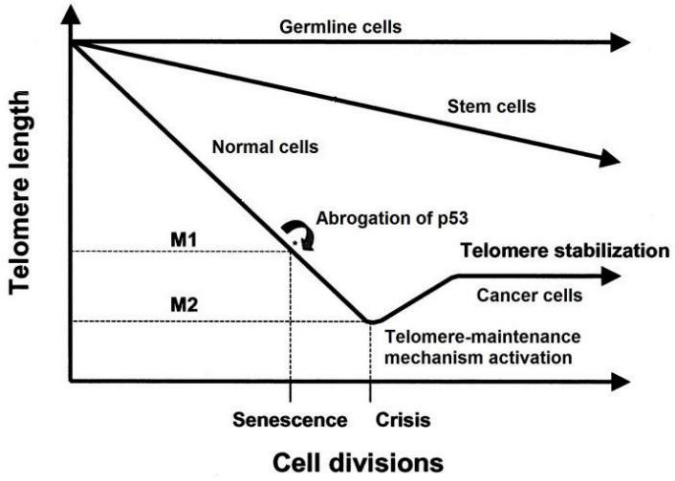

Figure 6 - Two-step hypothesis of cellular senescence and immortalization

M2 is characterized by widespread cell death. Rare surviving cells acquire unlimited proliferative potential and stabilization of telomere length, almost universally by activation of telomerase. Aapted from (Shay and Wright)

Telomere length-dependent telomerase activity regulation

hTERT is the limiting factor for telomerase activity in many cancers (Liu, Snow et al. 2000). Indeed, *hTERT* expression is transcriptionally shut-off in somatic cells, but the other telomerase associated components are constitutively expressed in most mammalian cell types; expression of *hTERT* mRNA in somatic cells is sufficient to reconstitute telomerase activity; and the expression levels of *hTERT* show strong correlation with telomerase activity (Bodnar, Ouellette et al. 1998; Vaziri and

Benchimol 1998). However, telomere length maintenance by telomerase is a complex multistep process that involves a series of molecular events including, transcription, mRNA splicing, maturation, processing and nuclear localization of both hTERC and hTERT subunits, as well as post-translational modifications and correct folding of hTERT, assembly of ribonucleoprotein complex, and accessibility of the holoenzyme to the telomeres (MacNeil, Bensoussan et al.).

Telomere length-independent telomerase activity

In addition to telomere length maintenance, telomerase is also involved in gene expression regulation, DNA damage repair, cell proliferation, apoptosis, WNT/β-catenin and nuclear factor NF-kB signaling pathways, MYC-driven oncogenesis, as well as cell adhesion and migration, and epithelial–mesenchymal transition (Pestana, Vinagre et al.). All these activities of telomerase are considered as its non-canonical functions. They are attributed to *hTERT* and if deregulated, are thought to significantly contribute to the process of oncogenesis.

hTERT transcription regulation

hTERT has a pivotal role in cancer cells, as it is largely implicated in both telomere length-dependent and independent activities. Therefore, understanding how *hTERT* is activated in cancer cells and how it contributes to further progression of the disease continues to be a major area of research.

The expression of *hTERT* is primarily determined by the transcriptional activity of its promoter (Liu, Lai et al. 2004). The *hTERT* promoter has complex regulation dynamics whereby multiple transcriptional regulatory elements play functional roles in different contexts either individually or interactively. Although the *hTERT* promoter does not have typical transcription regulatory elements like TATA and CAAT boxes, it contains recognition sequences for multiple important transcription factors. It contains at least five GC boxes, which are binding sites for the zinc finger transcription factor SP1 as well as two E-boxes that provide binding sites for c-MYC, or MAD1. Several transcription factors bind to the *hTERT* promoter core to activate or repress its transcription. The transcription factors that up-regulate transcription include c-MYC, SP1 and E-twenty-six (ETS) family members. Transcription factors such as p53, MAD, WT1, AP-1 and CTCF have been shown to down-

regulate *hTERT* transcription (**Figure 7**) (Akincilar, Unal et al. 2016). Even if there are a lot of transcription factors involved in telomerase expression regulation, none of them clearly account for the cancer specificity of *hTERT* expression (Gladych, Wojtyla et al.).

Figure 7 - Schematic representation of the hTERT promoter

Binding sites for various transcription factors are shown, including both activators (SP1, c-myc/Max) and repressors (p53, AP1, WT1, Max-Mad1, E2F and CTCF) The transcription start site (TSS) and the translation start site (ATG) are indicated. In dark blue, a CpG island. At -124 and -146 (from the ATG start site) positions, locates the two hotspot *hTERT* promoter mutations. Adapted from (Akincilar, Unal et al. 2016)and (Azouz, Wu et al.)

hTERT has a GC rich core promoter (**Figure 7**), thus the chromatin environment also plays a role in its transcriptional regulation (Guilleret and Benhattar 2004). Methylation of gene promoters is generally known to repress transcription; however, several studies revealed complex methylation patterns for the active/inactive *hTERT* promoter. Indeed, *hTERT* promoter region from −1100 to +150 position, is mostly hypermethylated through specific DNA methyltransferases (DNMTs) in cancer cells, while between −150 and +150 we observe an absence of methylation. *hTERT* partial hypomethylation of its core promoter is required for gene transcription, however, specific hypermethylation around CTCF binding site prevents the binding of this methylation sensitive repressor, which also allows gene transcription (Azouz, Wu et al. ; Zhu, Zhao et al. ; Guilleret and Benhattar 2003). Thus, *Tert* promoter methylation represents a unique model for transcription in which hypermethylation of cytosine islands causes inhibition of *Tert* expression and this differs among different cell types (Avin, Umbricht et al. 2016). It remains unsolved how the interplay between transcription factors and the telomere chromatin milieu controls *hTERT* transcription.

Non-coding mutations within *hTERT* core promoter provided a first definitive mechanism of cancer specific telomerase activation (Vinagre, Almeida et al. 2013). Two hotspot mutations, located at -124 C>T and -146 C>T (from the ATG start site)

(**Figure 7**), generates a new consensus binding site for ETS/TCFs transcription factors, which increases *hTERT* transcription and activity (Horn, Figl et al. 2013; Huang, Hodis et al. 2013). While these non-coding *hTERT* promoter mutations are the most frequent promoter mutations in cancer, the level and frequency varies with cancer types (Leão, Apolónio et al. 2018).

Genome-wide association studies (GWAS) opened the door to understanding associations between common genetic variants and human disease or phenotypes. Numerous GWAS of cancer etiology have identified variants in telomere biology genes as being associated with cancer risk or outcomes. Notably, single nucleotide polymorphisms (SNPs) in the *TERT-CLPTM1L* locus on chromosome 5p15.33 were found associated with multiple cancer types (Baird 2010; Mocellin, Verdi et al. 2012). Specific regions of this locus associate with different cancers, but these variants do not specifically encode deleterious coding alleles in *hTERT*. However, they often appear to be associated with telomere length (Mocellin, Verdi et al. 2012).

hTERT expression is a common feature both of solid and hematological cancers (Shay and Bacchetti 1997). However, the mechanism by which this enzyme is activated may be cancer type specific. *hTERT* promoter mutations represent the most frequent genetic alteration that drives h*TERT* expression and telomerase activation in human cancers. Although this mechanism has been reported in many types of human cancers, such as skin, central nervous system, thyroid, skin, and liver; it is rarely reported in hematological malignancies (Gaspar, Sa et al.).

As *hTERT* is a central piece of tumor initiation and progression, and it is primarily regulated at transcription level, a literature revision on the mechanisms preferentially used by hematological malignancies to activate this gene transcription was done.

hTERT transcription activation in hematological malignancies

genes

MDPI

Review

Telomerase Activation in Hematological Malignancies

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Abstract: Telomerase expression and telomere maintenance are critical for cell proliferation and survival, and they play important roles in development and cancer, including hematological malignancies. Transcriptional regulation of the rate-limiting subunit of human telomerase reverse transcriptase gen (*hTERT)* is a complex process, and unveiling the mechanisms behind its reactivation is an important step for the development of diagnostic and therapeutic applications. Here, we review the main mechanisms of telomerase activation and the associated hematologic malignancies.

Keywords: telomerase activity; hematological malignancy; amplification; epigenetic; polymorphism; mutation; virus

1. Introduction

The ends of all linear genomes are comprised of a unique and genetically stable structure, termed telomere, which preserves genome integrity [1,2]. Due to the "end replication problem", telomeric sequences shorten after every cell division, triggering the activation of DNA damage pathways that result in senescence and cell death. Telomere erosion and replicative senescence/apoptosis, limits the replicative capacity of cells, which is considered an important tumor-suppressive mechanism [3]. Shortening of telomeres may be counteracted by telomerase, an enzyme specialized in the elongation of telomeric ends [4]. Although the major function of telomerase is telomere elongation, accumulating evidence suggests that telomerase also possess telomere independent functions like enhanced survival, chemo-resistance, invasion and metastasis of malignant cells [5–8].

Telomerase consists of two core components: a catalytic subunit, human telomerase reverse transcriptase (hTERT) with reverse transcriptase activity and an RNA component, human telomerase RNA component (hTR), used as a template for the elongation of telomeres. In vivo telomerase activity requires additional components that associate with hTERT and hTR to form the holoenzyme [9]. Somatic cells do not display detectable telomerase activity, with the exception for germ cells, stem cells and some immune cell types with high proliferative needs. However, in such cells, the telomerase activity is only sufficient to delay, but not to completely prevent telomere shortening [10]. In about 85%–90% of all cancer cells, telomerase is reactivated, allowing the cells to circumvent senescence and divide indefinitely [11]. The remaining cancer cells, preserve the telomere length by a non-telomerase mechanism, known as alternative lengthening of telomeres (ALT), which involves the use of a DNA template [12].

It is believed that the limiting factor for telomerase activity is the level of hTERT [13]. Corroborating this idea is the fact that *hTERT* expression is transcriptionally shut-off in somatic cells, but the other telomerase associated components are constitutively expressed in most mammalian cell types; expression of *hTERT* mRNA in somatic cells is sufficient to reconstitute telomerase activity; and the expression levels of *hTERT* show strong correlation with telomerase activity [14–16].

The expression of *hTERT* is primarily determined by the transcriptional activity of the *hTERT* gene promoter. The *hTERT* promoter does not have typical transcription regulatory elements like TATA and CAAT boxes, but it contains a number of binding sites for multiple important transcription factors, which integrate *hTERT* transcriptional responses with many important pathways that are deregulated in various tumor types [17]. Transcriptional factors and signaling pathways frequently activated in tumor cells, like c-Myc, specific protein 1 (SP1), upstream transcription factor 1 (USF1), signal transducer and activator of transcription 3 (STAT3), phosphoinositide 3-kinase (PI3K), and nuclear factor of activated T-cells (NFAT), can positively stimulate *hTERT* promoter expression. In contrast, Mad, histone deacetylases, E2F1, transforming growth factor-β-activated kinase 1 (TAK1), Wilms' tumor 1 (WT1), p53, mothers against decapentaplegic homolog (Smad3), and Menin signaling negatively regulate *hTERT* promoter expression. [18]. Even if there are a lot of transcription factors involved in telomerase expression regulation, none of them clearly account for the cancer specificity of *hTERT* expression [19]. The *hTERT* gene has a GC rich promoter and may therefore be under epigenetic regulation [20,21]. DNA hypomethylation or histone methylation around the transcription start site of the *hTERT* promoter triggers the recruitment of histone acetyltransferase (HAT) activity, allowing *hTERT* transcription [11]. It is also described in childhood brain tumors that hypermethylation in specific CpG sites upstream of *hTERT* transcription start site, results in telomerase expression [22]. There are also exogenous factors influencing *hTERT* transactivation: several viruses or virus proteins that interact with telomerase are known to be involved in tumorigenesis of infected tissues [23]. Since telomerase activity is a hallmark of the immortal cell phenotype, unveiling the mechanism of telomerase reactivation is an important step for the development of diagnostic and therapeutic applications [24]. This review aims to summarize the mechanisms utilized by hematological malignancies to reactivate telomerase expression.

2. Telomeres and Telomerase in Hematologic Malignancies

While *hTERT* expression and telomerase activity are increased in both virus-driven and virus-unrelated lymphoproliferative disorders, telomeres are generally short in virus-unrelated malignancies, and several data suggest that virus-associated tumors and/or pre-neoplastic disorders are characterized by longer telomeres [25–27]. This observation may reflect differences in the timing of hTERT activation and, therefore, telomere length stabilization. Indeed, from a theoretical perspective, shorter or longer telomeres could both contribute to oncogenesis [28]. On one hand, long telomeres suggest an early activation of *hTERT* that may contribute to a delay in replicative senescence and prolonged time to acquire genetic alterations critical for the induction of a fully transformed phenotype [23]. On the other hand, telomere shortening ultimately results in genetic instability and activation of *hTERT* may thus occur as a subsequent step, necessary for the immortalization of cells with acquired oncogenic potential. Accordingly, in adult T-cell leukemia/lymphoma (ATLL), telomerase activity appears as a key event in the development and progression of the disease, whereas in acute myeloid leukemia (AML), in chronic myeloid leukemia (CML) and in B-cell diseases, it was demonstrated that telomerase activity is not required for the initiation of disease, but it is required for its maintenance [29–32]. High telomerase activity is related with progressive disease, worse prognosis, or chemotherapy resistance in the group of hematologic neoplasias [33]. In addition, inhibition of telomerase in leukemia cell lines induces progressive telomere shortening and eventual proliferative arrest or cell death via apoptosis in vitro and in vivo [29,34–36]. The observed functional requirement of telomerase in established hematologic malignancies provides a rationale to therapeutically target telomerase in these diseases.

3. Mechanisms of Telomerase Reactivation in Hematologic Malignancies

Although the transcription factors known to bind to *hTERT* promoter may regulate *hTERT* transcription on specific cell type and physiological conditions, none of them are sufficient on their own to promote immortalization of somatic cells [37].

3.1. hTERT Amplification

The *hTERT* gene is frequently amplified in human tumors, including hematological malignancies [38]. In most cases, the amplified region encompassed most or all of the chromosome 5p region. In several cases, chromosomal break points were mapped to regions close to the *hTERT* promoter, suggesting that chromosomal rearrangements could either relieve the promoter from its stringent repressive epigenetic environment or place it in the proximity of enhancers at different chromosomal sites [39,40]. The telomerase reverse transcriptase-cleft lip and palate transmembrane protein 1-like protein (TERT-CLPTM1L) locus including the gene encoding *hTERT* gene is rarely but recurrently targeted by somatic chromosomal translocations to immunoglobulin heavy (IGH) and non-IG loci in B-cell neoplasms, including acute lymphoblastic leukemia, chronic lymphocytic leukemia, mantle cell lymphoma and splenic marginal zone lymphoma. In addition, tumors bearing chromosomal aberrations involving *hTERT* showed higher TERT transcriptional expression and increased telomerase activity [41,42].

Multiple copies of the *hTERT* gene are unlikely to yield a sufficient amount of the *hTERT* transcript, so the marked activation of *hTERT* transcription during tumorigenesis may be a combination of gene amplifications with other genetic or epigenetic mechanisms [37].

3.2. Epigenetic Regulation of hTERT Gene

Epigenetic modifications imply reversible changes in the genome of cells without any alteration in the DNA sequence. Increasing evidence suggests the epigenetics as an important mechanism involved in cancer initiation, progression, treatment and prognosis. There are three major epigenetic mechanisms that are known to regulate gene transcription in carcinogenesis: modified DNA methylations, histone modifications, and deregulated microRNA (miRNA) expression [43].

The epigenetic plasticity of the *hTERT* gene promoter is a determinant for the control of telomerase activity, and it has been shown that epigenetic modulation may repress *hTERT* transcription in hematological malignancies and may provide an additional level of enzyme regulation [44].

3.2.1. DNA Methylation

There are conflicting studies regarding the correlation between hypermethylation of the *hTERT* promoter, *hTERT* gene expression and telomerase activity [45,46].

Regarding hematological malignancies, in a cohort of childhood acute lymphoblastic leukemia (ALL), it was reported that the tumor samples had a methylated promoter, and that the *hTERT* promoter methylation status defines specific ALL subgroups [47]. In addition, in some T cell lymphomas, *hTERT* expression goes along with *hTERT* promoter hypermethylation [48]. It is believed that the promoter hypermethylation interferes with the binding of inhibitors, such as the CTCF transcription factor, which allows the *hTERT* gene to be transcribed [49]. On the contrary, some cells of the lymphoid system seem to escape methylation-dependent mechanism of *hTERT* regulation. Leukemias and lymphomas, including B cell chronic lymphocytic leukemia (CLL), express high levels of telomerase but exhibit low levels of *hTERT* promoter methylation [50]. Moreover, ALL (HL-60) and Burkitt's lymphoma (Raji) cell lines, were found to have hypomethylated *hTERT* promoters [45,51]. In telomerase-positive B cells, *hTERT* is targeted by paired box 5 (PAX5), a B cell-specific factor, which is sufficient to activate TERT expression [48].

It is believed that the unusual correlations between DNA methylation and expression in cancer cells may, in part, result from the varied methods used to study differing regions of the *hTERT*

promoter. Indeed, it was observed that a small region around the transcription start site has little or no methylation, while there is a densely methylated region 600 base pairs upstream the transcription start site [45]. The core promoter demethylation is required for *hTERT* transcription in tumor cells, while the *hTERT* promoter in many normal cells and tissues is either unmethylated or hypomethylated, indicating that *hTERT* silencing does not require extensive CpG methylation at its promoter [46]. In addition, treatment with DNA methyltransferase inhibitors (DNMTIs), including decitabine (DAC) and azacitidine (AZA), is able to cause a reduction in *hTERT* gene expression and telomerase activity [52–54]. These two types of drugs are the first molecules that have been approved for the treatment of patients with AML and myelodyplastic syndrome (MDS) [55].

3.2.2. Histone Modifications

Another prevalent epigenetic mechanism that affects *hTERT* transcription is histone modification that includes histone acetylation, methylation, phosphorylation and ubiquitination. Histone deacetylation/methylation, in particular, have been reported to be responsible for the repressive status of the *hTERT* promoter [56].

The native chromatin environment is critical for the tight regulation of the *hTERT* gene, as the induction of *hTERT* transcription and telomerase activity in some telomerase-negative cells, was observed upon a treatment with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor [57,58]. The histone acetylation confers an opened chromatin structure, allowing transcription factors to bind to the DNA [59,60]. HDAC inhibitors are agents that have attracted interest due to their ability to induce not only cell differentiation, but also to promote growth arrest, apoptosis and sensitivity to certain chemotherapeutic reagents. Some HDACs were approved by the United States Food and Drug Administration (U.S. FDA) for the treatment of certain hematological diseases [61]. Modest clinical activity has been reported using HDACs as single-agent therapy, but they appear to be synergistic in vitro and improve response rates in vivo when combined with other agents [62]. Regarding hematological malignancies, the functional impact of HDAC inhibitors on *hTERT* transcription impairment was reported. TSA has an antiproliferative and apoptosis inducing effect on the human leukemic cell line U937, associated with the inhibition of *hTERT* expression and telomerase activity [63]. In leukemia cells it was verified that *hTERT* promoter DNA methylation and histone deacetylation status may contribute to the transcriptional repression of the *hTERT* gene and associated cell differentiation induction, observed during all trans retinoic acid (ATRA) treatment [50,64]. Furthermore, it was reported that the epigenetic modification of the distal domain of the *hTERT* promoter, determines the retinoid capacity to repress telomerase in maturation resistant acute promyelocytic leukemia cells during cellular differentiation [44].

Imatinib (IM) is a tyrosine kinase inhibitor selective for the *BCR-ABL* fusion gene, the cytogenetic hallmark of CML [65]. IM administration also inhibits telomerase activity independently of its effect on the BCR-ABL protein, and is mainly caused by hTERT post-translational modifications caused by the downregulation of various members of the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) pathway [66,67]. IM resistance is a major problem in therapy and disease relapse of CML. Recent studies have shown that by targeting telomerase expression using a dominant negative form of hTERT, or by the treatment with HDAC inhibitors, the risk of IM resistance may be reduced and the IM induced apoptosis in leukemia cells may be enhanced [68,69].

Histone methylation also plays a role in *hTERT* regulation. SMYD3, a histone methyltransferase (HMTase), is capable to induce *hTERT* transcription and telomerase activity in normal human fibroblasts and cancer cell lines through histone H3 K4 trimethylation [70]. H3-K4 methylation may function as a critical licensing element for transcription factors, such as c-Myc, through which the trans-activation of the *hTERT* gene is initiated [71].

3.2.3. MicroRNAs (miRNAs)

MiRNAs are a family of 19–24 nucleotide non-protein-coding RNA molecules that regulate the stability and translation of target mRNAs [72]. Various studies have revealed that loss- or gain-of-function of specific miRNAs contributes to cellular transformation and tumorigenesis, including hematological diseases [73,74]. The main mechanism that underlies the aberrant miRNA expression is that they are frequently localized in fragile sites prone to translocations and cancer-associated genomic regions (CAGRs) such as minimal regions of loss of heterozygosity (LOH), minimal regions of amplification and common breakpoint regions [75,76]. Many studies propose miRNAs as novel biomarkers and predictors of treatment response of hematological malignancies due to the specific miRNA signatures that allows for discrimination between different subtypes of leukemia and lymphoma with a greater degree of accuracy compared to traditional gene expression analysis [73,77,78].

Besides the effort to identify and catalog aberrantly expressed miRNAs in disease, very little is known about the functional consequences of miRNA dysregulation. It has been demonstrated that miRNAs are implicated in the regulation of *hTERT* expression through a regulatory network which is interconnected with other pathways also involved in tumor development. The miRNAs that target the *hTERT* 3′UTR have been identified as tumorigenesis inhibitors, and, consequently, are commonly found downregulated in many types of cancer [79]. The functional impact of miRNAs in B-cell ALL was reported with the restoration of miR-196b expression, which led to significant down-regulation of c-myc and its effector genes, including *hTERT*, suggesting a tumor suppressor function role for miR-196b [80].

Among all the factors regulating telomerase expression and activity, a few polymorphisms and mutations were also identified.

4. TERT Polymorphisms

The presence of single nucleotide variants (SNVs) in the *hTERT* locus, including the promoter and downstream introns, may be an alternative or additional mechanism influencing enzyme's expression and activity. The genetic variability in the *hTERT* genomic region may affect telomerase function and can modulate telomere length and contribute to the development of cancer as well as the outcome of chemotherapy [81,82]. There are some *TERT* promoter polymorphisms associated with increased risk of developing hematological diseases, and even suggested as prognostic markers of survival [83,84].

Regarding *hTERT* transcription activation, the *TERT* promoter region SNP rs2735940 was associated with risk of childhood ALL. The rs2735940 T allele increased the levels of the *TERT* mRNA compared with the C allele [84]. The role of the mechanism of *hTERT* regulation was also demonstrated via MNS16A polymorphism in patients with Non-Hodgkin Lymphoma (NHL) [85]. The polymorphic element MNS16A, located downstream of exon 16 of the *TERT* gene and upstream of the putative promoter region of an antisense *TERT* transcript, has promoter activity depending on the number of tandem repeats. Carriers of the MNS16A short (S) allele display higher telomerase activity than the long/long (LL) genotype carriers. In fact, longer alleles at MNS16A exhibit stronger promoter activity compared to the shorter alleles, but this leads to increased expression of antisense *TERT* mRNA with a conceivable, or at least partial, silencing of the sense telomerase transcript [86].

5. *hTERT* Subunit Mutations

Clinically, loss-of-function mutations in *hTERT* or *hTR* might increase the risk of chemotherapy resistance and predisposal to specific human diseases, like bone marrow failure or dyskeratosis congenita and acquired aplastic anemia, diseases that predispose to MDS and AML [87–89]. These clinical observations suggest that telomerase deficiency may contribute to the development of hematopoietic malignancy. Although most cancer cells express telomerase to maintain their proliferative capacity,

telomerase deficiency may lead to telomere attrition, hypothesized as a molecular mechanism that promotes genomic instability and predisposal to cancer development including leukemia [90,91].

Analysis of MDS/AML cases, secondary to bone marrow failure or dyskeratosis congenita allowed the establishment of an association between *hTERT* mutations and poor prognosis [92,93]. In addition, screening of de novo cases of MDS and AML for telomerase mutations have reported the existence of loss-of-function and non-synonymous mutations in the *hTERT* gene implicated as risk factors for AML [94,95].

6. TERT Promoter Mutations

Recently, two hot spot mutations in the *TERT* promoter, (−124 G > A and −146 G > A, C > T on the opposite strand) were reported in several different solid tumors [96,97]. These mutations have strong clinical implications with worse prognosis and poor survival and may represent a novel therapeutic target in solid tumors [96–99]. However, hematological malignancies are not reported to be subjects for somatic promoter mutations in the *TERT* gene [83,94,97,100].

7. Virus-Driven Lymphoid Malignancies

Viruses involved in lymphomagenesis may directly up-regulate telomerase expression and activity [23,101,102].

7.1. HTLV-1-Associated Lymphomas

Infection with the human T-lymphotropic virus type I (HTLV-I) is associated with the development of an aggressive form of T-cell leukemia known as adult T-cell leukemia/lymphoma (ATLL) [103]. High telomerase activity is associated with disease progression, so reactivation of telomerase seems to be a critical event in the development and progression of ATLL [27,104]. HTLV-I virus can directly induce endogenous telomerase up-regulation through the viral protein Tax that plays a central role in the modulation of *hTERT* expression [105]. Indeed, Tax represses *hTERT* promoter in proliferating cells, while it activates it in quiescent cells, allowing the cell cycle progression [106]. Notably, in ATLL cells, Tax expression is very low-to-undetectable, yet these cells retain strong telomerase activity. This suggests that alternative/additional mechanisms, independent of Tax protein, may induce *hTERT* expression and telomerase activity. Additionally, the viral protein HTLV-1 basic leucine zipper (HBZ) expressed in ATLL cells increasese transcriptional activity of JunD, an AP-1 protein, while, HBZ in association with JunD activates the *hTERT* promoter [107]. Furthermore, it has also been shown that interleukin-2 (IL-2) signaling was associated with a PI3K-dependent transcriptional up-regulation of the *hTERT* promoter in HTLV-1 transformed cells. Activation of the PI3K pathway mediates cytoplasmic sequester of the WT1 protein, a strong hTERT promoter suppressor [108].

7.2. EBV-Associated Lymphoproliferative Disorders

Epstein-Barr virus (EBV) is involved in the pathogenesis of several lymphoproliferative disorders, including Burkitt's and Hodgkin's lymphomas, post-transplant lymphoproliferations, and a subset of T/natural killer (NK) cell lymphomas [109]. Among EBV latency gene products, latent membrane protein 1 (LMP-1) is considered the strongest oncoprotein, being essential for immortalization of B cells. A crucial prerequisite for EBV-driven transformation is the induction of latent EBV genes and the down-regulation of lytic EBV gene expression, concomitantly with the induction of *hTERT* expression and activity [110]. *LMP-1* activates *TERT* at the transcriptional level via NF-kB and (Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinases) MAPK/ERK1/2 pathways [111].

8. Conclusions

The regulation of the *hTERT* gene is a very complex process. The repressed *hTERT* promoter can be activated by multiple mechanisms in different hematological malignancies. In addition

to a variety of transcription factors that bind and promote *hTERT* transcription/inhibition, the chromatin environment and nucleosomal conformation appear to be among the major mechanisms that tightly regulate the *hTERT* gene in the majority of hematological malignancies. Through epigenetic modulation, the *hTERT* locus is able to adopt a decondensed state, and then allow the binding of sequence-specific transcription factors that will activate its transcription. Chromosomal translocations of the *hTERT* locus may, in fact, be an important mechanism of telomerase activation, as it allows the escape of the promoter from its native repressive chromatin environment. Amplification of the *hTERT* gene was reported in hematological malignancies, and combined with other genetic or epigenetic mechanisms, results in a marked activation of *hTERT* transcription during tumorigenesis. In virus-driven lymphoid malignancies, the *hTERT* promoter may also be activated directly by viral proteins.

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hTERT post-transcription regulation

The *hTERT* gene is 42 kilobases (kb) long and contains 16 exons. The reverse transcriptase domain is coded by exons 5–6–7–8–9 (**Figure 8**) (Akincilar, Unal et al. 2016).

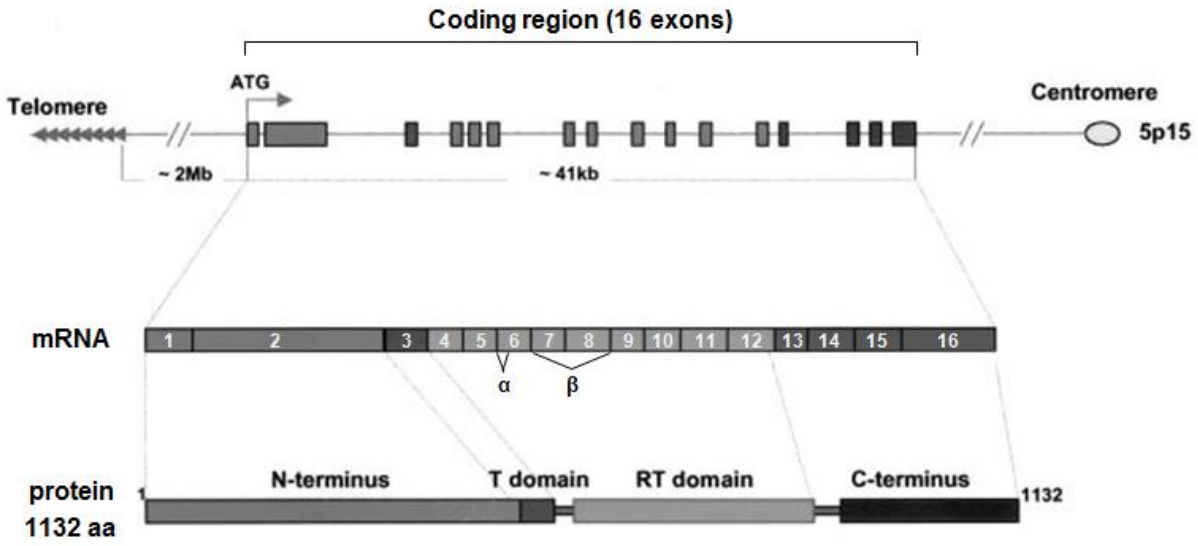

Figure 8 - Schematic representation of *hTERT* gene organization

The human hTERT gene consists of 16 exons and 15 introns located on the short arm of chromosome 5 (5p15.33), approximately 2 Mb away from the telomere. It is transcribed towards the centromere. The specific telomerase domain (T domain), reverse transcriptase domain (RT domain), and the C-terminal region of the hTERT protein are indicated. The two most studied splicing sites at the RT domain α and β sites are also indicated. Adapted from (Cong, Wright et al. 2002)

hTERT is subjected to alternative splicing and its modulation is detected in multiple types of cancers, where cancer cells utilize an alternative splicing switch that results in discernible isoform signatures (Bollmann). This switching is not random and also seen in tissue development (Ulaner, Hu et al. 2001). *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 (Liu, Wang et al. 2017). Alternative splicing yielding inactive and/or inhibitory forms of *hTERT* allows for downregulation of telomerase activity without complete repression of transcription. Over twenty different isoforms of *hTERT* have so far been reported, with the most common being deletions in the reverse transcriptase domain, such as the α, β and α/β deletion (**Figure 8**). 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 (Avin, Umbricht et al. 2016). All known isoforms result in an inactive telomerase complex. While normal cells express mainly inactive *hTERT* isoforms, a splicing switch occurs in cancer cells, resulting in production of the full length active transcript (Khosravi-Maharlooei, Jaberipour et al.).

TERRA

For a long time, telomeres and the adjacent subtelomeric region were considered to be transcriptionally silent. However, it was demonstrated that mammalian telomeres are in fact transcribed by RNA polymerase II, and result in telomeric repeat-containing RNA (TERRA) ranging from 100 nucleotides to 9 kilobases (**Figure 9**) (Azzalin, Reichenbach et al. 2007; Schoeftner and Blasco 2008).

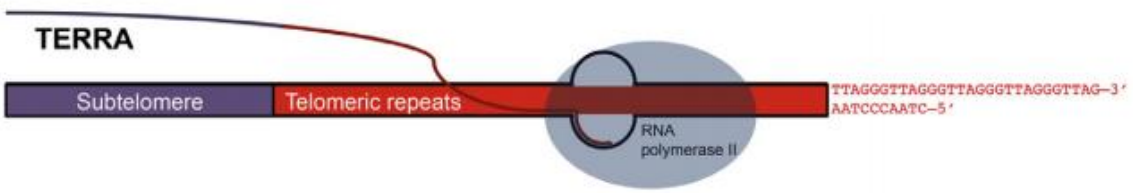

Figure 9 - Schematic representation of TERRA transcription.

TERRA synthesis, which is mainly mediated by RNA polymerase II, derives from the subtelomeric region (purple) and extends towards the end of the chromosome into the telomeric repeats (red). Adapted from (Feretzaki and Lingner)

TERRA transcription is regulated by the heterochromatic state of telomeres and subtelomeric regions (Schoeftner and Blasco 2008; Nergadze, Farnung et al. 2009). Interestingly, emerging evidences indicated that TERRA plays a key role in the regulation of heterochromatic state of telomeres, as it promotes deposition of repressive heterochromatic marks to chromosome ends. TERRA molecules interacts with shelterin components TRF1 and TRF2, which is thought to be a link to TERRA localization to telomeres, sustaining the enzymatic activities of TERRA binding factors at telomeres (Deng, Norseen et al. 2009). TERRA sequences are complementary to the RNA sub-unit of telomerase. So, through direct basepairing with hTERC, TERRA can act as inhibitors of telomerase activity. However some reports suggest that TERRA can act as a positive regulator of telomerase (Bettin, Oss Pegorar et al.). TERRA expression and localization must be tightly regulated. On

one hand TERRA transcripts can help to protect telomeres from activation of DNA damage responses (DDR), on the other hand, high levels of TERRA transcripts can negatively impact telomeric DNA replication or fuel DDR at chromosome ends during telomere dysfunction (Azzalin, Reichenbach et al. 2007).

Telomeres and telomerase rationale as therapeutic targets

Telomere biology plays a critical and complex role in the initiation and progression of cancer. Although telomere dysfunction resulting from replicative attrition constrains tumor growth by engaging DNA-damage signaling pathways, it can also promote tumorigenesis. Expression of telomerase enzyme enables telomere-length homeostasis and allows tumor cells to escape the antiproliferative barrier posed by short telomeres.

Due to its over-expression in the majority of cancers, and minimal or nonexistent expression in most somatic cells, telomerase is a unique cancer biomarker. Thus, telomerase and other telomere components are attractive targets for developing effective therapeutics against cancer (Ivancich, Schrank et al. ; Jafri, Ansari et al.). Several therapeutic strategies have been developed to treat cancer. Antisense oligonucleotides complementary to *hTERC* template region (Imetelstat, GRN163L), is by far the most promising telomerase inhibitor (Bryan, Rice et al.). However, despite its great *in vitro* and *in vivo* potential, clinical trials showed toxicity and limited efficacy (Chiappori, Kolevska et al.). Therapies based on telomerase inhibitors require a long period of treatment to induce cell death. Another strategy to inhibit telomerase is the use of G-quadruplex stabilizers (Merle, Evrard et al.). Although telomeres engage in such secondary structure, G-quadruplex may form anywhere in the genome. This therapeutic strategy has very little effect on telomere length and can induce off-target effects (Bernal and Tusell). Another approach is targeting shelterin telomere capping. The expression of mutant hTERC templates generates erroneous newly synthesised telomeric-strands which prevent shelterin binding and protection. Telomere uncapping through shelterin modification precipitates telomere dysfunction and fast cell growth inhibition (Li, Rosenberg et al. 2004). Inhibitors targeting telomere and telomerase assembly, as well as T-oligo (DNA oligonucleotide homologous to the telomere 3′ overhang region, which causes cytotoxic effects by inducing DDR) were developed (Cruz, Wojdyla et al.).

As telomerase exerts additional functions, other than telomere maintenance in tumor biology, these non-canonical functions of *hTERT* could provide novel therapeutic targets. Hence, inhibitors of tankyrase (which has an important role in telomere homeostasis, mitotic spindle formation and WNT/β-catenin signaling patway) and HSP90 (involved in signal transduction, intracellular transport and protein degradation) have been explored to selectively kill cancer cells (Cruz, Wojdyla et al.).

Primary cutaneous T-cell lymphomas

Primary cutaneous lymphomas (PCL) are defined as malignant lymphoprolifetarions presenting in the skin with no evidence of extracutaneous disease at the time of diagnosis. PCL often have a completely different clinical behavior and prognosis from histologically similar systemic lymphomas, which may involve the skin secondarily, and therefore require different types of treatment. PCL include a heterogeneous group of cutaneous T-cell lymphomas (CTCL) and cutaneous B-cell lymphomas (CBCL). In the Western world, CTCL constitute approximately 75% -80% of all primary cutaneous lymphomas, and CBCL 20% -25% (Willemze, Jaffe et al. 2005). In the last decade the World Health Organization–European Organization for Research and Treatment of Cancer (WHO-EORTC) consensus classification has served as a gold standard for the diagnosis and classification of CTCL, which include entities with indolent, intermediate, and aggressive clinical behavior. (Willemze, Jaffe et al. 2005; Willemze, Cerroni et al. 2019). The most common subtypes, comprising 95% of CTCL, include CD30+ lymphoproliferative disorders (LPDs), mycosis fungoïdes (MF), and Sézary syndrome (Sz) [Sidiropoulos, 2015 #64].

CD30+ lymphoproliferative disorders (LPDs)

LPDs are the second most common group of CTCL, accounting for approximately 25% of all CTCL. This group includes primary cutaneous anaplastic large cell lymphoma (C-ALCL), lymphomatoid papulosis (LyP), and borderline cases. Because of the overlapping histologic and phenotypic features, clinical presentation and clinical course are used as decisive criteria to differentiate between LyP and C-ALCL. C-ALCL presents as solitary, grouped or, uncommonly, multifocal nodules and tumors (**Figure 10**). Cutaneous relapses are common, but extracutaneous

dissemination occurs in only 10-15% of patients. LyP is characterized by a chronic course of recurrent, self-healing papulononecrotic or nodular skin lesions (**Figure 10**). The histologic picture of LyP is extremely variable and may resemble different types of CTCL. Recognition of these different types of LyP is important to avoid misdiagnosis of other often more aggressive types of CTCL, but has no therapeutic or prognostic implications. LPDs prognosis is usually favorable with a 5 year DSS greater than 95% (Willemze, Jaffe et al. 2005; Willemze, Cerroni et al. 2019).

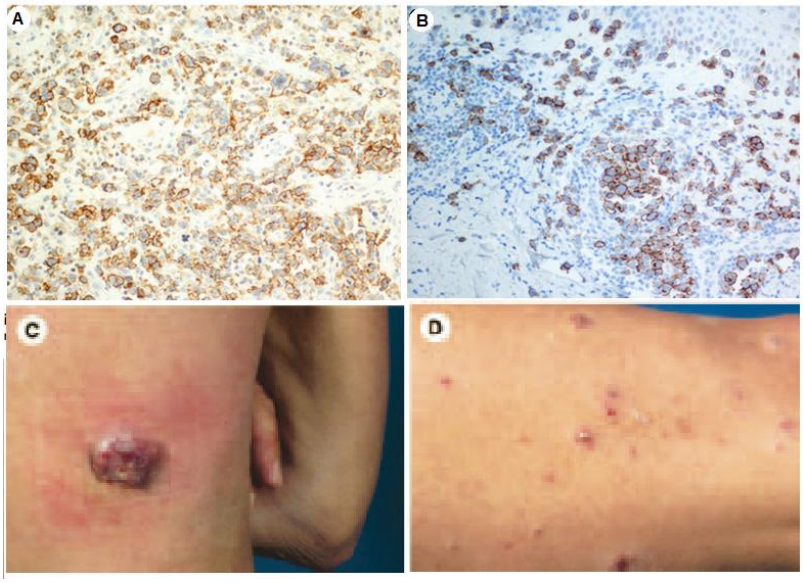

Figure 10 - CD30+ lymphoproliferative disorders (LPDs)

A and B panels: histologic pictures that can be found both in C-ALC and in LyP. The final diagnosis depends on the clinical presentation. In combination with the solitary tumor of the patient shown in panel C, the definite diagnosis will be C-ALC; in combination with recurrent, self-healing papulonecrotic skin lesions (D), the final diagnosis is LyP. Adapted from (Sidiropoulos, Martinez-Escala et al.) (Willemze, Jaffe et al. 2005)

Mycosis fungoïdes (MF)

MF is the most common type of CTCL and accounts for 60% of CTCL. It is an epidermotropic CTCL characterized by the proliferation of small to medium sized T lymphocytes with cerebriform nuclei, and present typically an indolent clinical course with slow progression, from patches to more infiltrated plaques and eventually to tumors (**Figure 11**). In some patients, lymph nodes and visceral organs may become involved in the later stages of the disease (Willemze, Jaffe et al. 2005).

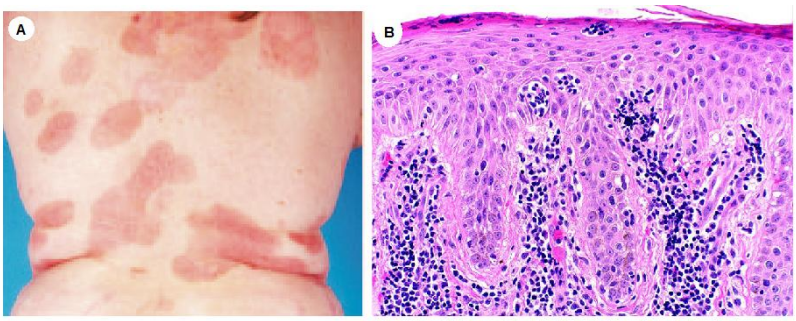

Figure 11 - Mycosis fungoides (MF)

(A) Typical patches and plaques on the trunk. (B) Infiltration of atypical T cells into the epidermis with formation of Pautrier microabscess. From (Kim, Hess et al. 2005; Willemze, Jaffe et al. 2005)

The prognosis of patients with MF is dependent on stage, and in particular the type and extent of skin lesions and the presence of extracutaneous disease. The 5 year disease-specific survival (DSS) is of 88% (Willemze, Cerroni et al. 2019; Willemze, Welting et al. 2019). Large cell transformation within skin or node biopsies may occur (**Figure 12**). Transformed mycosis fungoides (T-MF) is defined by the existence of more than 25% of infiltrating atypical T-cells or clusters of large cells with nuclei that are more than 4 times the normal size, and is often associated with aggressive clinical course (Talpur, Sui et al. ; Vergier, de Muret et al. 2000).

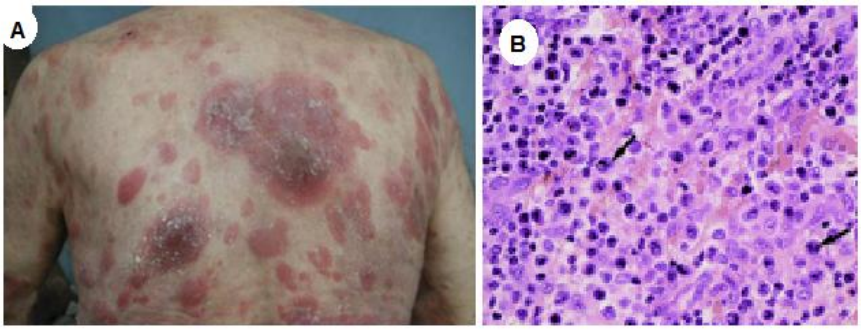

Figure 12 – Transformed mycosis fungoides (T-MF)

(A) Clinical presentation of a patient with generalized patches, plaques, and tumors. (B) Histology of a lesional skin biopsy with > 25% of infiltrating atypical T-Cells (black arrows). Adapted from (Talpur, Sui et al.) and (Vergier, de Muret et al. 2000)

Sézary syndrome (Sz)

Sz is a rare leukemic type of CTCL, traditionally defined by the triad of pruritic erythroderma, generalized lymphadenopathy and the presence of clonally related

neoplastic T cells with cerebriform nuclei (Sezary cells) in the skin, lymph nodes and peripheral blood (Willemze, Jaffe et al. 2005) (**Figure 13**).

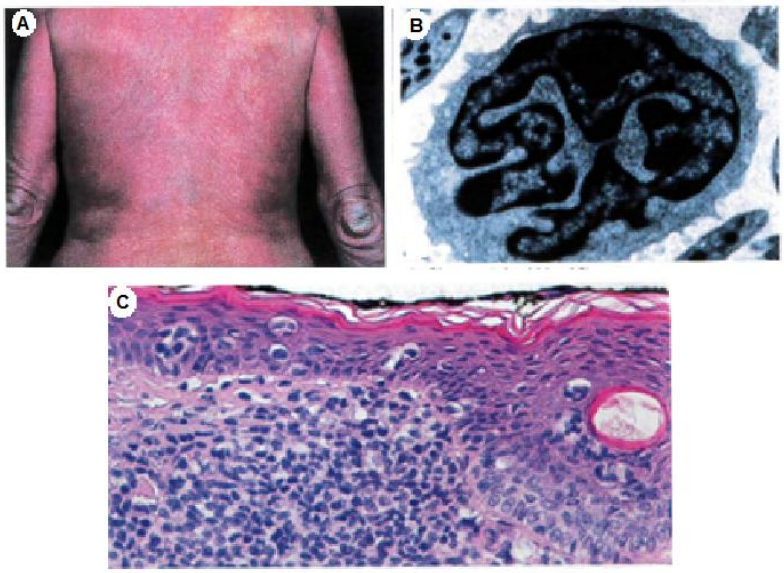

Figure 13 - Sézary syndrome (Sz)

(A) Generalized skin disease with erythroderma. (B) Morphology of a Sézary cell, by ultrastructural examination.(C) Skin infiltrated with epidermotropic infiltrates of atypical, cerebriform lymphocytes. Adapted from (Prince, Whittaker et al. 2009; Swerdlow 2017)

The histologic features of Sz may be similar to those in MF. However, the superficial perivascular infiltrates may be sparse, epidermotropism may be minimal or absent, and in as many as one-third of biopsies from patients with otherwise classic Sz the histologic picture may be aspecific. Since both clinical and histopathological presentation may be non-specific, demonstration of peripheral blood involvement is crucial for the diagnosis of Sz. Criteria for blood involvement include in addition to demonstration of clonally related neoplastic T-cells in skin and peripheral blood, either an absolute Sezary cell count of more than 1000/µL, or an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio ≥ 10, CD4+/CD7- cells ≥ 30% or CD4+/CD26- cells ≥ 40%. Genetic alterations in Sz are diverse and complex. Recent large-scale genomic studies showed alterations in genes involved in T-cell activation, cell cycle regulation, DNA damage repair, chromatin remodeling, NF-kB activation and JAK-STAT signaling (Willemze, Jaffe et al. 2005). These studies have not only contributed to new insights in the molecular pathogenesis of Sz, but also provided new therapeutic targets, which are currently tested in clinical trials (Wilcox ; Willemze,

Cerroni et al. 2019). The prognosis is generally poor, with a median survival between 2 and 4 years, and a 5 year DSS is of 36% (Willemze, Cerroni et al. 2019).

The absence of molecular biomarkers, similar to those available for other hematological malignancies, did not allowed the development of applicable tools for patient risk stratification and has also hindered the development of true targeted therapies for CTCL. Currently available drug therapies, when effective, simply control the disease and the only curative option is stem cell transplantation (Willemze, Hodak et al.).

Telomeres and telomerase in CTCL

Telomere length and telomerase activity were studied in CTCL. Samples from patients with Sz, T-MF and c-ALCL were analyzed in parallel with corresponding cell lines to evaluate the importance of telomere length telomerase activity as target candidates for diagnostic and therapeutic purposes. Compared with controls, short telomeres were observed in aggressive CTCL subtypes (Sz and T-MF) and were restricted to neoplastic cells in Sz. While no genomic alteration of the *hTERT* locus was observed in patients' tumor cells, telomerase activity was detected. Furthermore, telomerase inhibition rapidly impeded *in vitro* cell proliferation and led to cell death, while telomerase overexpression stimulated *in vitro* proliferation and clonogenicity properties and favored tumor expansion in immunodeficient mice. These results indicate that, besides maintenance of telomere length, telomerase exerts additional functions in CTCL. Therefore, targeting these functions might represent an attractive therapeutic strategy, especially in aggressive CTCL (Chevret, Andrique et al.).

Problematic and objectives

The lack of insight into the key molecular targets underlying CTCL etiology and the risk for disease progression has hindered the development of true targeted therapies for CTCL.

Despite inter and intra patient heterogeneity (Chevret and Merlio ; Willemze, Jaffe et al. 2005), there is a common characteristic to the majority of CTCL, regardless of their aggressiveness: *hTERT* expression (Chevret, Andrique et al.). *hTERT* expression along with short telomeres, as well as POT1 mutations, were found associated with CTCL aggressiveness which indicates that telomere biology plays a role in CTCL lymphomagenesis (Chevret, Andrique et al. ; Pinzaru, Hom et al.). Therefore, we aimed to elucidate the molecular mechanisms regulating telomere biology in CTCL, which may allow the identification of biomarkers related to disease progression and/or aggressivenessthat may ultimately conduct to the identification of new therapeutic targets for CTCL.

We specifically focused on understanding how *hTERT* is reactivated and how its regulation contributes to further progression of CTCL. We also assessed the involvement of TERRA in CTCL lymphomagenesis; and we tested the value of a known compound as an anti-telomerase drug.

Thus, we:

1) investigated mechanisms known to be involved in *hTERT* transcription activation, in order to unveil the mechanism underlying telomerase expression in CTCL cells;

2) investigated *hTERT* mRNA alternative splicing as a regulating mechanism implicated on telomerase non-canonical functions;

3) characterized TERRA transcript profile of different CTCL sub-types in order to access the involvement of this molecules in CTCL lymphomagenesis;

4) and investigated the potential of *hTERT* as a therapeutic target of a known statin, already used in clinic, to try to find possible therapeutics to CTCL.

Telomere length estimation in cancer cells

Normal human cells progressively lose telomeres with each cell division until a few short telomeres become uncapped leading to a growth arrest known as replicative senescence (Shay and Wright 2005). Upon specific genetic and epigenetic alterations, as well as the engagement on a telomere length maintenance mechanism, human cells may thus bypass replicative senescence and continue to proliferate indefinitely, triggering tumorigenesis (Shay and Wright). Hence, telomere length is not only at the basis of cellular aging but also cancer, as well as other metabolic and inflammatory diseases (Henriques and Ferreira ; Savage).

The increased utility of telomere length assessment as a risk factor for cancer development emphasized the importance of human populations' telomere length measurement (Barrett, Iles et al. ; Samassekou, Gadji et al.). Thus, there is a need of reliable methods to accurately measure telomere length which will allow the establishment of association between telomere length and human disease.

Cancer cells accumulate genetic and chromosomal abnormalities and we do not always have access to a lot of cells or genetic material to work with. Hence, the validation of techniques that allows precise telomere length measurement from cancer cells, with small quantities of DNA is needed.

Is qPCR alone valid for telomeres length measurement in cancer cells?

Cancer Medicine
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Is qPCR alone valid for telomeres length measurement in cancer cells?

Journal:	*Cancer Medicine*
Manuscript ID	Draft
Wiley - Manuscript type:	Original Research
Search Terms:	cancer biology, lymphoma, biomarkers, molecular genetics
Abstract:	Background: Telomere shortening is linked to a range of different human diseases, hence reliable measurement methods are needed to uncover such associations. Among the plethora of telomere length measurement methods, qPCR is reported as easy to conduct, and the most cost-effective and suitable approach to study low DNA quantity samples. Methods: Cancer cells' telomere length was evaluated by relative and absolute qPCR methods. Results: Robust and reproducible telomere length measurements were optimized taking in account a careful reference gene selection and by knowing the cancer cells ploidy. We correlated qPCR results with "gold standard" results from TRF measurements. Conclusions: Based on our data, we provide guidance and recommendations for accurate telomere length measurement by qPCR in cancer cells. Furthermore, our data emphasize the requirement of samples with both, high DNA quality and high tumor cells representation.

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Contents:

Abstract	**4**
Introduction	**5**
Material and Methods	**7**
Results	**12**
CTCL cells cytogenetic analysis	**12**
CTCL cells telomere length	**12**
Relative and absolute telomere length measurements	**12**
DNA sample quality	**13**
Sample' tumor cell percentage	**14**
Discussion	**14**
References	**17**

Table 1 and Table 2

Supplementary Table 1

Figures 1 to 4

Supplementary Figure 1

In this original article we aimed to validate the applicability of qPCR when assessing telomere length in cancer cells, taking advantage of our expertise on telomere homeostasis investigation in primary cutaneous T cell lymphomas

For Review Only

Abstract

Background: Telomere shortening is linked to a range of different human diseases, hence reliable measurement methods are needed to uncover such associations. Among the plethora of telomere length measurement methods, qPCR is reported as easy to conduct, and a cost-effective approach to study samples with low DNA amounts.

Methods: Cancer cells' telomere length was evaluated by relative and absolute qPCR methods.

Results: Robust and reproducible telomere length measurements were optimized taking in account a careful reference gene selection and by knowing the cancer cells ploidy. qPCR data were compared to "gold standard" measurement from Terminal Restriction Fragment (TRF).

Conclusions: Our study provides guidance and recommendations for accurate telomere length measurement by qPCR in cancer cells. Furthermore, our data emphasize the requirement of samples with both, high DNA quality and high tumor cells representation.

Keywords: cancer, tumor, telomere length, qPCR, southern blot

1) text: 21 pages (including title page, abstract, main text, references, and figure legends); 2) Tables: 3 pages (including 2 Tables and 1 supplementary Table); and 3) Figures: 5 pages (including 4 Figures and 1 supplementary Figure)

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Introduction

Telomeres are highly conserved repetitive (TTAGGGn) DNA–protein structures located at the ends of eukaryotic chromosomes [1, 2]. They have important functions on chromosomal stability and replication [3]. Due to the “end replication problem” telomeric sequences shorten after every cell division, leading to replicative senescence, cell cycle arrest or apoptosis [4, 5]. Telomere progressive shortening can potentially induce genetic instability and neoplastic transformation and may be counteracted by an enzyme specialized in the elongation of telomeric ends, telomerase [6]. This enzyme is silenced in most somatic cells and expressed in about 90% of cancer cells [7]. The remaining 10% of cancers activate an alternative telomere length mechanism known as ALT [8]. The re-expression of telomerase allows cells to circumvent senescence and to achieve immortalization by maintaining functional telomeres [9]. As protectors of chromosome ends, telomeres are involved in the pathogenesis and clinical progression of human diseases, including cancer and a number of metabolic and inflammatory diseases [10-12]. Considering the role of telomere length in biological homeostasis, there has been a growing interest in measuring telomere length accurately and efficiently [13, 14].

A wide range of methods have been developed to measure telomere length, such as terminal restriction fragment (TRF) analysis by Southern blot, quantitative polymerase chain reaction (qPCR) amplification of telomere repeats relative to a single copy gene, and fluorescent *in situ* hybridization (FISH) to quantify telomere repeats in individual cells (interphase-FISH and flow-FISH) or in individual chromosomes (metaphase-FISH). The advantages and drawbacks of each method have been discussed in many reviews [15-19]. TRF analysis was the first technique developed for telomere length measurement, and is often considered as the “gold standard” for

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all other techniques. In this procedure, genomic DNA is exhaustively digested by a cocktail of restriction enzymes, resulting in short genomic fragments and longer uncut telomeres. Telomere fragments are then resolved by agarose gel electrophoresis and detected by Southern blot using a labeled telomere probe. The average telomere length is determined by quantification of the intensity of labeled telomere DNA smear, compared to a DNA ladder with known fragment sizes in kilobases (kb). TRF analysis requires large amounts of DNA (0.5 to 10 µg) and has a maximum detection threshold of around 20 kb because of the resolutive nature of agarose gel electrophoresis [20, 21]. Nowadays, qPCR is the most commonly used method for assessing telomere length. qPCR is low-cost, not very time consuming, is amenable to a high-throughput format and, unlike TRF assay, it can be performed with small quantities of DNA (less than 100 ng), which are substantial advantages when studying cancer cells [22, 23]. In this procedure, telomere length is quantified by comparing the amplification of the telomere product (T) to the amplification of a single-copy gene (S). The T/S ratio yields a value that is proportional to average telomere length, allowing the determination of relative telomere length [24-27]. Nevertheless, to obtain accurate, precise and reproducible data, several factors should be considered [28, 29]. In this work, we aimed to compare and validate the applicability of qPCR when assessing telomere length in cancer cells, taking advantage of our expertise on telomere homeostasis investigation in primary cutaneous T cell lymphomas (CTCL). CTCL are a heterogeneous group of extranodal non-Hodgkin's lymphomas in which we previously reported that telomere shortening was associated with disease aggressiveness [30].

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Material and Methods

Cell Lines

Five CTCL cell lines were analyzed in this study. Three cutaneous anaplastic large cell lymphoma (c-ALCL): Mac1, Mac2A and Mac2B (DSMZ), one transformed mycosis fungoïdes (T-MF): MyLa, kindly provided by Dr K. Kaltoft (Aarhus, Denmark) and one Sézary syndrome (Sz): HuT78 (ATCC). They were cultured as suspension cells in Roswell Park Memorial Institute Medium (RPMI) 1640 media (Gibco) supplemented with 100U/mL penicillin, 100µg/mL streptomycin (Gibco) and 10% foetal bovine serum (Eurobio), except for HuT78 cells, which were supplemented with 20% foetal bovine serum. All cell lines were maintained at 37ºC with 5% CO2 and regularly tested for Mycoplasma contamination.

Patients and healthy donors

Sz patients (n = 10, 51 ≤ age ≤ 86, mean age 71), were selected from the dermatology department of University Hospital Center (CHU) of Bordeaux, diagnosed according to the criteria of the World Health Organization and the European Organization for Research and Treatment of Cancer (WHO-EORTC) [31]. Healthy donors (n = 21, 52 ≤ age ≤ 97, mean age 68) were recruited from both Etablissement Français du Sang (EFS), and CHU of Bordeaux, France. Peripheral blood mononuclear cells from Sz patients and healthy donors were isolated by PANCOLL® density gradient centrifugation (PAN-Biotech). Each patient gave written consent.

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Conventional cytogenetics

MyLa, HuT78, Mac1, Mac2A and Mac2B cells in the logarithmic growth phase were incubated with Colcemid (Gibco). Cells were harvested and fixed according to the standard cytogenetic methods (KCl hypotonic treatment and ethanol-acetic acid fix Normapur 3:1 ratio). Fixed cells were spread on Superfrost glass slides (Thermo Scientific). Metaphases were treated for R-banding and then scanned on AxioImager Z1 (Zeiss) using Metafer software (MetaSystems). For each cell line, five to ten metaphases were analyzed using Ikaros karyotyping software (Metasystems). Karyotypes were assessed by a cytogeneticist and chromosomal formulas were written according to International System for Human Cytogenetic (ISCN) 2016 nomenclature.

Multicolor Fluorescence in situ Hybridization (mFISH)

mFISH karyotype was carried out in accordance with supplier's instructions using 24XCyte kit (MetaSystems) on cell lines and patient cells metaphase spreads. Cytogenetic preparations were performed as previously described [32]. For each sample, nearly 20 metaphases were analyzed by means of ISIS software for mFISH (MetaSystems). Chromosome abnormalities were defined according to ISCN 2016 recommendations.

DNA Extraction

Genomic DNA was extracted, by a salt precipitation method adapted from Roylance *et al* [33]. Briefly, about 3 to $5x10^6$ cells were washed with PBS. The pellets were resolved in nuclei lysis buffer (10 mM Tri-HCl/pH 8.2, 2 mM EDTA, 400 mM NaCl) completed with 0.1% Nonidet P-40, 1/10 RNAse A (10mg/ml) and a proteinase K

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buffer solution (2mg/ml proteinase K, 2mM EDTA, 1% SDS), prepared freshly prior to use. Suspensions were incubated overnight at 56°C. The DNA was precipitated with ethanol and then resolved in DNase-RNase free distilled water. DNA concentration was measured by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) and its quality was further analyzed by classic agarose gel electrophoresis. The extracted material was maintained at 4ºC during quality assessment and qPCR analysis, otherwise it was stored at -20°C.

Terminal Restriction Fragment telomere length measurement

Telomere measurement was carried out following the protocol of TeloTAGGG Telomere Length Assay Kit (Roche). Briefly, 1.5 µg of DNA was digested with HinfI and RsaI enzymes. Digested samples were run on agarose gel and the telomere fragments were then transferred to a nylon membrane Hybond-N+ (Amersham). DNA was fixed and a DIG-labeled telomeric probe was hybridized to the membrane. After a series of stringent washes and incubation with the secondary anti-DIG antibody, the telomeric DNA was detected by chemiluminescent imaging (ImageQuant LAS 4010, GE Healthcare). Images were analyzed using ImageJ software (IJ 1.46r). Telomere content was calculated by the equation: TRF mean=$\Sigma OD_i/\Sigma(OD_i/L_i)$, where OD_i is the chemiluminescent signal and L_i is the length of the TRF fragment at position i.

qPCR relative telomere length measurement

Telomere length was calculated by a standard quantitative qPCR assay as previously reported [30]. The normalizing control gene used was Kallikrein Related Peptidase 3 (KLK3), located at 19q13.33. Target DNA was added to a reaction containing the pair of primers (telomere or KLK3) and Takyon™ No Rox SYBR® MasterMix dTTP Blue

(Eurogentec), according to the manufacturer's instructions. PCR experiments were carried out on a Stratagene Mx3005P system (Agilent Technologies) and analyzed with MxPro 4.01 QPCR software Stratagene (Agilent Technologies).

Primer sequences for both telomeres and KLK3 were the follow:

Telc 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'

Telg 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3' [24].

KLK3-forward 5'-AGGCTGGGGCAGCATTGAAC-3'

KLK3-reverse 5'-CACCTTCTGAGGGTGAACTTG-3'.

Telomere (2 cycles of 95°C for 20 sec and 49°C for 20 sec, followed by 30 cycles of 95°C for 20 sec and 60°C for 20 sec, with signal acquisition) and KLK3 (40 cycles of 95°C for 20 sec and 60°C for 20 sec, with signal acquisition) reactions were run in separate 96 well plates.

Data were collected from triplicate reactions for each sample (cell lines, patients and healthy donors) and further from three independent biological experiments for cell lines. Triplicate values were accepted when the standard deviation of Ct was below 0.5 among replicates. Results were expressed as $2^{-\Delta Ct}$, which allows quantifying the cycle number difference between the telomeres and KLK3.

qPCR absolute telomere length measurement

Telomere length was calculated by means of Absolute Human Telomere Length Quantification qPCR Assay Kit. (CliniSciences, France). The kit provided a primer solution for telomere amplification and another one that recognizes and amplifies a 100 base pair region on human chromosome 17. This last primer solution was used as single copy reference (SCR). Target DNAs were added to a reaction containing the pair of primers (telomere or SCR) and FastStart Essential DNA Green Master

(Roche), according to the manufacturer's instructions. PCR experiments were carried out on a Stratagene Mx3005P system (Agilent Technologies) and analyzed with MxPro 4.01 QPCR software Stratagene (Agilent Technologies). Telomere and SCR reactions were run in the same 96 well plate and followed the same qPCR program setup (initial denaturation step at 95ºC for 10 min, followed by 32 cycles of 95°C for 20 sec, 52ºC for 20 sec and 72°C for 42 sec, with signal acquisition).

Data were collected from duplicate reactions for each sample (cell lines, patients and healthy donors), and from three independent biological experiments for cell lines. Duplicate values were accepted when the standard deviation of Ct was below 0.5 among replicates. The provided reference genomic DNA sample with known telomere length in kilobases served as reference to calculate samples' telomere length ($2^{-\Delta\Delta Ct}$). The final result represents the average telomere length per chromosome.

Statistical analysis

Statistical analyses were performed on GraphPad Prism (version 5.01) and included the calculation of mean, standard deviation of the mean, and *P* values by paired Mann-Whitney test (nonparametric *T*-test). Correlations between different telomere length measurement methods were calculated using Pearsons Correlation and R^2 coefficient of correlation and *P* values were reported. Data obtained with cells from one sample were considered as one experiment (*n*). The significance level was set as $P = 0.05$.

Results

CTCL cells cytogenetic analysis

Cytogenetic investigation consisted of analyzing the karyotype and mFISH for all cell lines (MyLa, HuT78, Mac1, Mac2A and Mac2B). HuT78 cell line was hypertriploid (77 to 81 chromosomes), all others cell lines were near-diploid. MyLa had 47 to 49 chromosomes, Mac1 had 45 to 47, Mac2A had 45 to 46 and Mac2B had 44 to 45. Full chromosomal formulas are available in **Supplementary table 1**. For Sz patients, the complex karyotype was determined by mFISH. All Sz patients (1 to 9) were near-diploid, except patient 10 which was triploid.

CTCL cells telomere length

Relative and absolute telomere length measurements

We measured the relative and the absolute telomere length of CTCL cells. The relative telomere length was assessed by means of a standard qPCR method (**Figure 1A.**). By this method, HuT78 and Mac1 were the cell lines presenting the shortest telomeres, followed by Mac2A and Mac2B, while MyLa presented the longest telomeres. We were not able to measure the telomere length of one Sz patient with this method since we never succeeded to amplify neither the reference gene nor the telomeres (**Figure 1A.**).

The absolute telomere length was assessed by qPCR and by measuring the TRF length means. These two methodologies were applied only on cell lines due to the huge amounts of DNA required for TRF analysis (**Figure 1B.**). qPCR absolute telomere lengths were calculated considering cell ploidy: the average telomere length per chromosome was calculated dividing the cell average telomere length by the

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number of cell chromosomes (**Table 1.**). With this absolute qPCR method we succeeded to calculate the telomere length of all Sz patients (**Figure 1B.**). We also came to the observation that Mac1 and HuT78 presented the shortest telomeres, followed by Mac2B and Mac2A. MyLa was the cell line with the longest telomeres among all cell lines studied (**Figure 1B.** and **Table 1.**). The TRF analysis allowed us to observe that Mac2B and Mac2A were the cell lines with the shortest telomere lengths, close followed by Mac1 and HuT78. MyLa was the cell line presenting the longest telomeres (**Figure 1B.**). The mean cell lines' telomere length estimated by qPCR (4.369±0.144 kb) was similar to that estimated by TRF (5.297 kb), $P = 0.7904$ (**Figure 1B.** and **Table 1.**).

Cell lines' telomere length results estimated by TRF, strongly correlated with results from relative (**Figure 2A. a**) and absolute (**Figure 2A. b**) qPCR approaches (R^2 = 0.9763, P = 0.0016 and R^2 = 0.8890, P = 0.0163, respectively). Cell lines' telomere length estimation by qPCR-based assays (**Figure 2A. c**), strongly correlated with each other (R^2 = 0.9616, P = 0.0032). Sz patients' telomere length estimations by relative and absolute qPCR approaches (**Figure 2B.**) strongly correlated with each other (R^2 = 0.8568, P = 0.0003).

DNA sample quality

When analyzing Sz patients' telomere length, we observed the occurrence of an "outlier" far from patients' average telomere length (**Figure 3A. a**). We verified by agarose gel electrophoresis that it was due to DNA degradation (**Figure 3A. b**). Thus, this patient sample was excluded from this study. This was further analyzed in 2 cell lines (**Figure 3B.**), one with short telomeres and another with long telomeres: following heat DNA degradation (**Figure 3B. a**), their telomere lengths significantly

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increased (**Figure 3B. b**). We compared the KLK3 (reference gene) and telomeres Ct values of both cell lines. We observed that the most remarkable difference between undegraded and degraded DNA was at the level of KLK3 gene Ct. Indeed, KLK3 gene Ct value increased in degraded DNA (**Table 2**).

Sample' tumor cell percentage

We observed that the telomere length of our Sz patient cohort (**Figure 4A.**) were significantly shorter when compared with that of healthy lymphocytes (P = 0.0238). We then compared the telomere lengths based on samples' tumor cell percentage (**Figure 4B.** and **4C.**). We observed that samples with more than 50% of tumor cells (**Figure 4B**.) had significantly shorter telomeres that those of healthy lymphocytes (P = 0.0374), while telomere length of samples with less than 50% of tumor cells (**Figure 4C**.) was not statistically different from those of lymphocytes from healthy donors (P = 0.1719).

Discussion

In the present study we intended to evaluate and compare methods to ascertain telomere length in clinical samples using as a model Sézary syndrome disease, an aggressive CTCL subtype. We also aimed to identify putative factors interfering with an accurate evaluation.

We used a qPCR commercial kit to measure the absolute telomere length of CTCL cells. As a commercial kit, it is assured to render results with high rehabilitee, sensitivity and reproducibility, and to reduce intra and inter-assays discrepancies [34]. Furthermore, it allows obtaining telomere length in absolute kilobases, otherwise only possible by TRF analysis. As TFR requires large DNA quantities, it cannot be applied

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in studies where large amounts of cells or genetic material are not available, which is often the case when studying cancer cells [27].

The main hurdle to use qPCR-based techniques on cancer cells relies on the selection of an appropriate reference gene [27, 29]. Ploidy abnormalities and chromosome rearrangements are commonly associated with cancer development, making it very likely to select a reference gene that is amplified or lost [35]. Cytogenetic data allowed us to investigate chromosome 17 status of CTCL cells, since the kit uses a 100 base pair-long region on this chromosome as a reference. By cytogenetic data, we guaranteed (under the resolution limit, around 5MB), the selection of a stable reference gene for qPCR relative telomere length measurement, and we verified that the single copy gene reference proposed by the qPCR kit is suitable for CTCL absolute telomere length measurement. Karyotype information was furthermore essential to complement the advantages of telomere qPCR, as cell ploidy allowed the correct calculation of the average telomere length per chromosome (**Table 1.**). This is particularly important because when studying cancer cells, the single telomere length measurement by itself has no biological meaning if not compared to the telomere length of a representative healthy population. So, the correct telomere length calculation is extremely important to assess and discover associations between telomere length and a certain disease. In this work, the vast majority of CTCL cell lines and Sz patients studied were diploid or near-diploid, so the ploidy did not influence telomere length result. However, for HuT78 cell line and patient 10 that presented a near-triploid and a triploid karyotype respectively, the ploidy correction factor influenced telomere length measurement (**Table 1.**).

Regarding telomere length results obtained with the different measurement methods (**Figure 1.**), the qPCR-based results, which specifically measures telomere

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sequences, are concordant with each other (**Figure 2.A c** and **2B.**). TRF analysis, on the other hand, measures the telomeres including their subtelomeric region, which generally overestimates telomere length around 1kb [18]. Indeed, mean cell lines' telomere length estimated by TRF (5.297 kb) is around 1kb greater than that estimated by qPCR (4.369±0.144 kb) (**Figure 1B.**).

Another crucial aspect of telomere length measurement is DNA quality. It is established that one of the primary requests for qPCR based techniques in general, and for telomere-qPCR in particular, is the use of DNA of high quality [29]. Indeed, DNA degradation strongly influences telomere length measurements (**Figure 3.**). Upon DNA degradation, we observed that the most remarkable difference, between uncompromised DNA and degraded DNA, occured at the level of KLK3 gene Ct (our reference gene) (**Table 2.**). The number of cycles to obtain a detectable log-linear phase of amplification increased upon DNA degradation, which means that we obtained less KLK3 product amplification in degraded samples. Consequently, as the telomere amplification did not significantly change, the ratio telomere/KLK3 decreased and this translated into longer telomeres (**Table 2.** and **Figure 3B. b**). This is in contradiction with TRF method, where DNA degradation produces a bias toward shorter lengths [19]. Thus, we emphasized the importance of regularly check samples' DNA quality.

Finally, we reinforced the impact of analyzing samples with high percentage of tumor cells, as it can influence telomere length evaluation relatively to healthy lymphocytes (**Figure 4.**). On one hand, samples with more than 50% of tumor cells presented significantly shorter telomere lengths, compared to healthy lymphocytes. On the other hand, samples with less than 50% of tumor cells presented telomeres with no statistical difference from healthy lymphocytes. This corroborated our previous

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observations that short telomere length is a characteristic of Sz tumor cells and that the surrounding non-tumor cells present longer telomeres [30]. Therefore, the analysis of samples with high tumor cells proportion will grant more precise results providing a way to accurately distinguish unhealthy from healthy population. We further assured that the telomere length of Sz patients was not due to their advanced ages (**Supplementary Figure 1.**). Hence, we discriminated between natural telomere shortening and a pathological decrease, which is a hallmark of Sz cells.

In conclusion, the increased utility of telomere length assessment as a biomarker of cancer cells emphasized the importance of accurate telomere length estimation. qPCR is the most commonly used method for telomere length measurement as it is a very advantageous tool, yet several critical factors should be taken into consideration to guarantee an accurate telomere length calculation in cancer cells.

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Figure legends

Figure 1. CTCL cells' telomere length. (A) Relative telomere length measurement by a standard relative qPCR assay. (B) Absolute telomere length measurement by qPCR (a) and by TRF (a and b). The mean cell lines' telomere length estimated by qPCR (4.369±0.144 kb) was similar to that estimated by TRF (5.297 kb), $P = 0.7904$ (a).

Arbitrary units (A.U.); Cutaneous T-cell lymphoma (CTCL); Kilobases (kb); Non statistically significant (n.s.) Quantitative real-Time polymerase chain reaction (qPCR); Sézary (Sz); Terminal restriction fragment (TRF)

Figure 2. CTCL cells' telomere length assays correlation. (A) Correlation on CTCL cell lines: a) telomere length results estimated by TRF strongly correlated with results from relative qPCR ($R^2 = 0.9763$, $P = 0.0016$) b) telomere length results estimated by TRF strongly correlated with results from absolute qPCR ($R^2 = 0.8890$, $P = 0.0163$) and c) telomere length estimation by qPCR-based assays strongly correlated with each other ($R^2 = 0.9616$, $P = 0.0032$). (B) Correlation on Sz patients: telomere length estimations by relative and absolute qPCR approaches strongly correlated with each other ($R^2 = 0.8568$, $P = 0.0003$).

Arbitrary units (A.U.); Cutaneous T-cell lymphoma (CTCL); Kilobases (kb); Quantitative real-Time polymerase chain reaction (qPCR); Sézary (Sz); Terminal restriction fragment (TRF)

For Review Only

Figure 3. Influence of DNA quality in telomere length measurement. (A. a) Sézary (Sz) patients' relative qPCR telomere length measurement and (A. b) patient samples 1,2,3,4 and 5 DNA quality analysis by agarose gel eletrophoresis. (B. a) DNA heat degradation of two cell lines (one with short telomeres and another with long telomeres), confirmed by agarose gel eletrophoresis and (B. b) their relative qPCR telomere length measurement. Telomere length of both cell lines significantely increased following DNA degradation (P = 0.0001 for short telomere cell line and P = 0.0037 for long telomere cell line).

Arbitrary units (A.U.); Sézary (Sz); **P < 0.01; ***P < 0.001

Figure 4. Influence of samples' tumor cell percentage on telomere length comparition with healthy donors. (A.) Telomere length of Sz patients' samples not sorted were significantly shorter when compared with that of healthy lymphocytes (P = 0.0238). (B.) Telomere length of Sz patients' samples with more than 50% of tumor cells have significantly shorter telomeres that those of healthy lymphocytes (P = 0.0374). (C.) Telomere length of Sz patients' samples with less than 50% of tumor cells was not statistically different from those of lymphocytes from healthy donors (P = 0.1719).

Kilobases (kb); Non statistically significant (n.s.); Sézary (Sz); *P < 0.05

Supplementary Figure 1. Age influence on telomere length of healthy lymphocytes and Sz patients. Sz patients qPCR absolute telomere length comparition with aged-mached healthy lymphocytes. Telomere length of Sz patient

For Review Only

samples (mean 4.247±0.140) were shorter than healthy aged-matched lymphocytes (mean 5.712±0.200) P = 0.0238. This confirmed our previously reported data, on another Sz patient cohort assessed with another telomere length estimation method [30]. Kilobases (kb); Sézary (Sz)

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Table 1 – CTCL cells' absolute telomere length estimated by absolute qPCR

	Diploid telomere length (kb)	Ploidy	Corrected telomere length (kb)
Cell lines			
Mac1	1.063±0.035	Near-diploid	**1.063±0.035**
Mac2A	3.965±0.131	Near-diploid	**3.965±0.131**
Mac2B	3.192±0.105	Near-diploid	**3.263±0.108**
MyLa	13.013±0.430	Near-diploid	**12.471±0.412**
HuT78	1.858±0.061	Hypertriploid	**1.082±0.036**
Mean			**4.369±0.144**
Sz patients			
1	2.819±0.093	Near-diploid	**2.819±0.093**
2	3.656±0.121	Near-diploid	**3.656±0.121**
3	5.559±0.183	Near-diploid	**5.559±0.183**
4	5.392±0.178	Near-diploid	**5.392±0.178**
5	2.930±0.097	Near-diploid	**2.930±0.097**
6	4.623±0.153	Near-diploid	**4.623±0.153**
7	2.077±0.069	Near-diploid	**2.077±0.069**
8	8.226±0.272	Near-diploid	**7.883±0.260**
9	3.387±0.112	Near-diploid	**3.462±0.114**
10	3.804±0.126	Triploid	**2.536±0.084**
Mean			**4.094±0.135**

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Table 2 – Ct values for KLK3 and Telomeres of two cell lines following heat degradation

Cell lines		Ct (KLK3)	Ct average	Ct (Telomeres)	Ct average	$2^{(-\Delta Ct)}$
Short telomere	not heated	24.00	23.95	24.05	24.09	0.90
		23.89		24.13		
	heated	26.87	26.83	23.38	23.40	10.82
		26.79		23.41		
Long telomere	not heated	22.07	22.14	16.18	16.09	66.16
		22.20		16		
	heated	24.06	24.17	15.33	15.26	483.36
		24.28		15.18		

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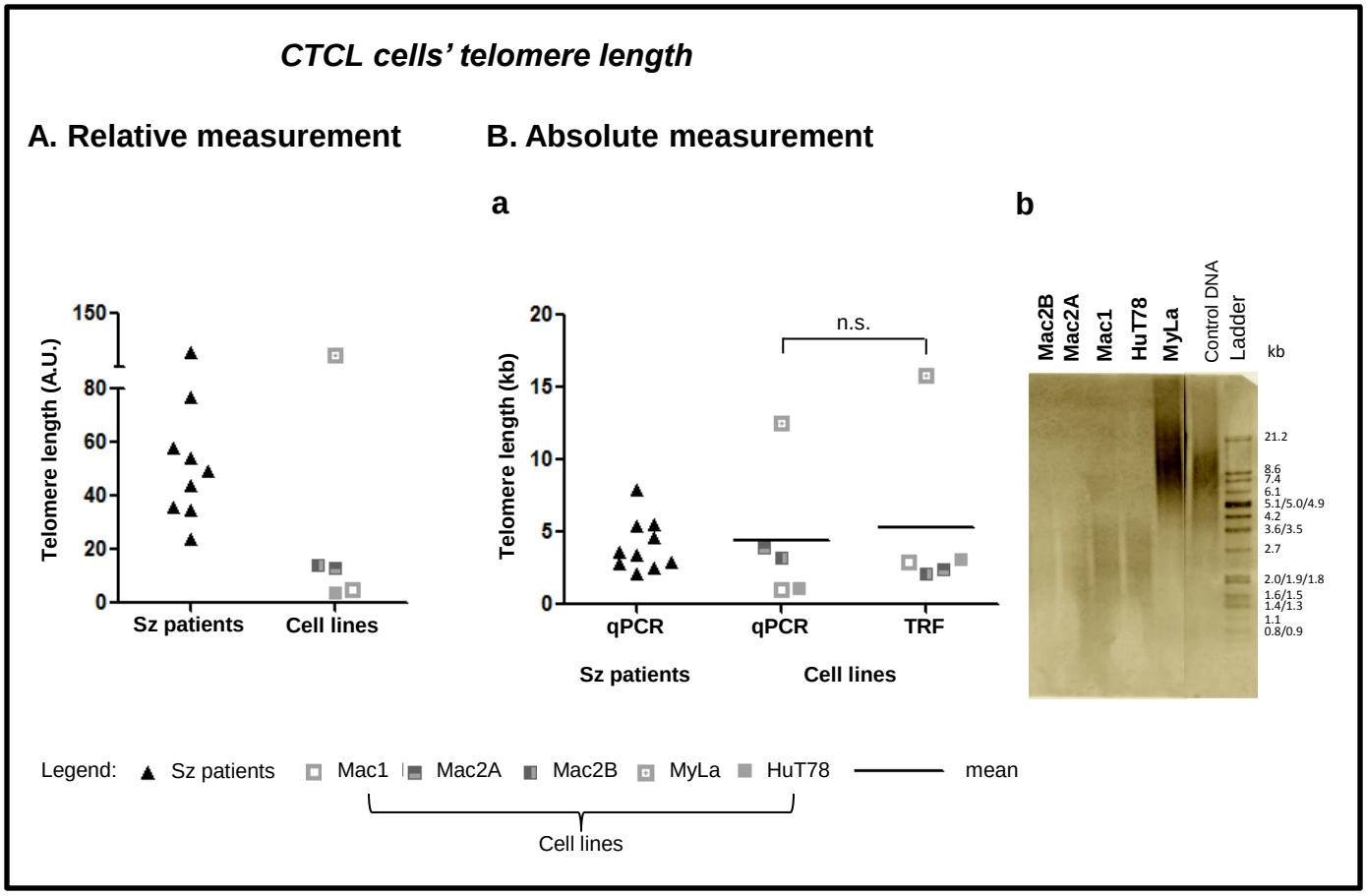

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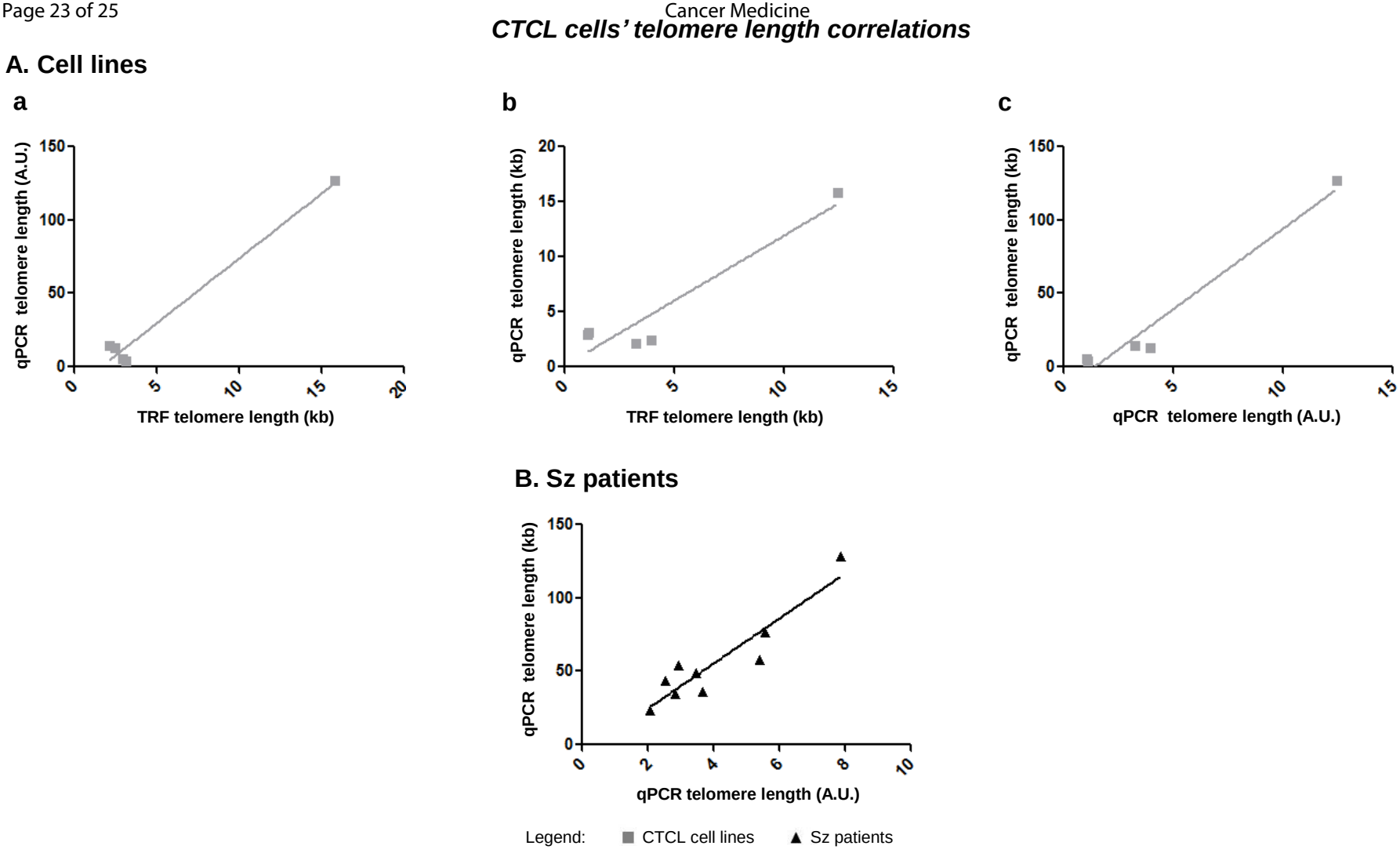

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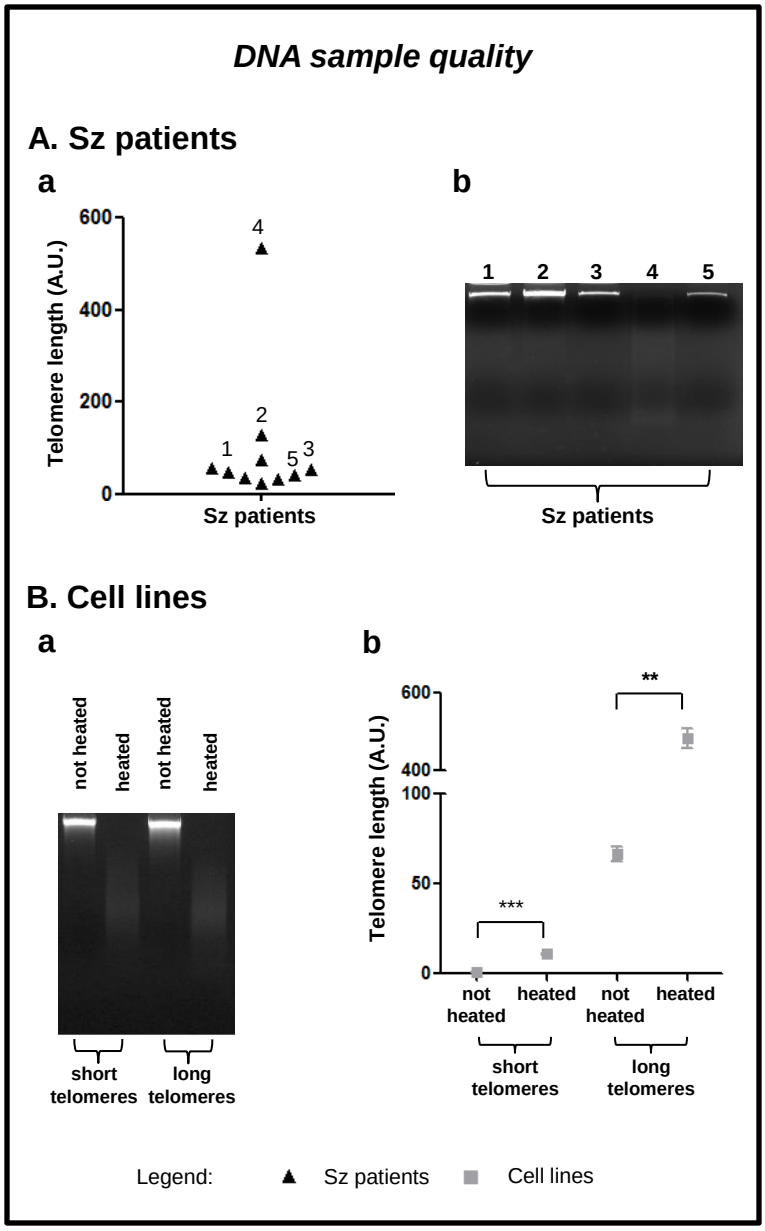

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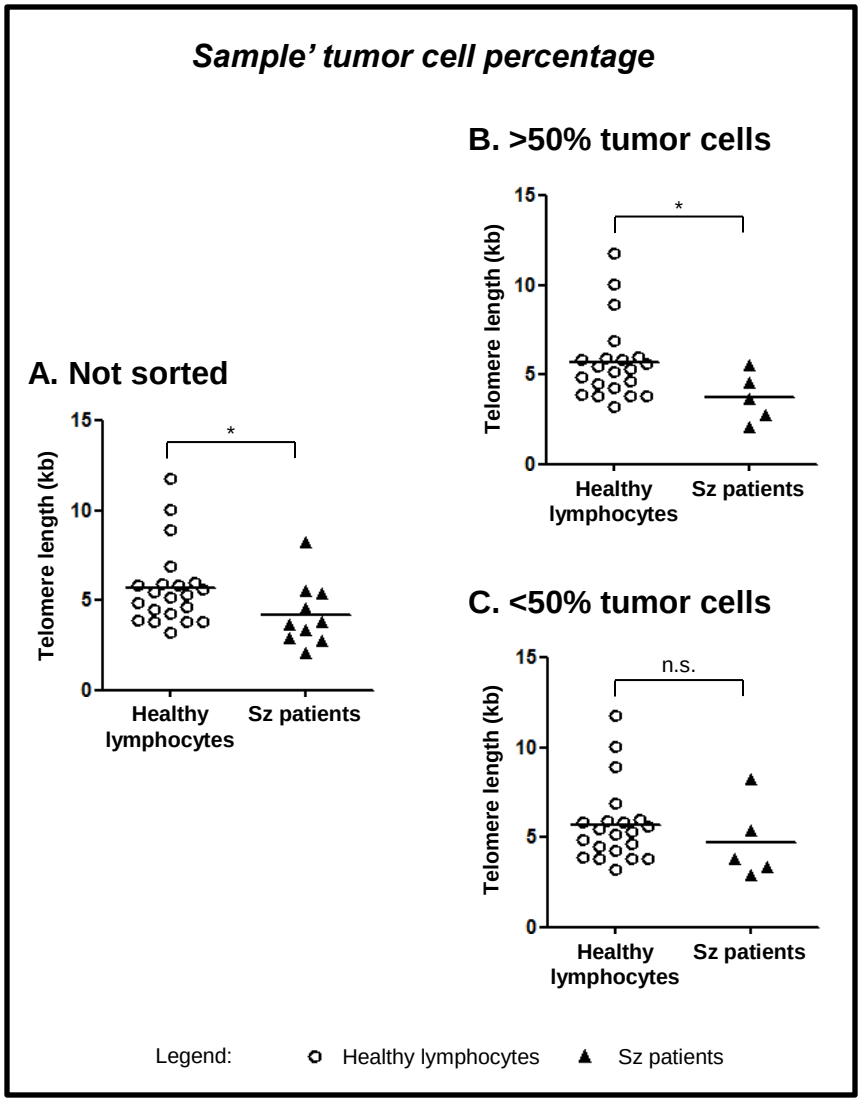
Arbitrary units (A.U.); Sézary (Sz); **P < 0.01; ***P < 0.001



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Kilobases (kb); Non statistically significant (n.s.); Sézary (Sz); *P < 0.05

Telomerase regulation in cancer cells

Telomerase is a reverse transcriptase that adds telomeric repeats to chromosomal ends. In most normal human somatic cells, telomerase is repressed and telomeres progressively shorten, leading to limited proliferative life-span. Telomerase reactivation is associated with development and progression of malignant tumors and its catalytic component, hTERT, is the key regulator of telomerase activity (Shay and Wright 2011). Telomerase activity is primarily determined at transcriptional level, through the regulation of *hTERT* promoter activation (Cifuentes-Rojas and Shippen). hTERT transcription can be activated via multiple genetic mechanisms including h*TERT* promoter mutations (Vinagre, Almeida et al. 2013). Beside its canonical role in catalyzing synthesis of telomeric DNA, telomerase has other non-canonical roles in cell proliferation, adhesion, migration, genome stability and protection against apoptosis [ref]. Telomerase canonical and non-canonical functions may be regulated by *hTERT* single nucleotide polymorphisms (SNPs) and *hTERT* post-transcriptional regulation, through alternative splicing mechanism (Bollmann).

Telomerase expression is a characteristic of cutaneous T-cell lymphomas (CTCL), and along with short telomeres is associated with disease aggressiveness (Chevret, Andrique et al.). Since there is no information on the mechanism by which telomerase is activated nor on how its telomere length-dependent and independent activities are regulated, we decided to investigate such mechanisms in CTCL. We intended to unveil *hTERT* regulating mechanisms which may allow the identification of potential molecular biomarkers and/or therapeutic targets for CTCL.

Spotlight on hTERT' complex regulation in cutaneous T-cell lymphomas

Title: Spotlight on *hTERT*' complex regulation in cutaneous T-cell lymphomas

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Key Points: CTCL, Telomerase, hTERT, promoter mutations, SNP, splicing variants

Abstract

As a major cancer hallmark, there is a sustained interest in understand telomerase contribution to cancer cells' abilities in order to therapeutically target this enzyme. This is particularly relevant in primary cutaneous T-cell lymphomas (CTCL), a malignancy known to have telomerase dysregulation. We investigated mechanisms involved in telomerase transcriptional activation and activity regulation in a Franco-Portuguese cohort of 94 CTCL patients, as well as 8 cell lines, and compare them with 101 healthy controls. We showed that, not only polymorphisms located at *hTERT* (human telomerase reverse transcriptase) promoter (rs2735940 and rs2853672) but also at gene coding region (rs2853676) could influence CTCL risk, and that *hTERT* promoter mutations even if rarely, occur at -146 position from the ATG start site. Our results sustained that post-transcriptional regulation of *hTERT* plays a crucial role in CTCL. Sézary patients present a specific pattern of *hTERT* splicing variants, different from healthy controls, which not only correlate with the characteristic shorter telomere length of Sézary cells, but may also explain the delayed apoptosis and the low proliferation index observed in this disease. Indeed, we manipulated with shRNAs, *hTERT* splicing transciptome in aggressive CTCL cell lines, which allowed us to observe that each pattern of *hTERT* variants had a specific biological consequence. As so, α+β- transcripts seems to protect cells from cell death, while α-β+ in a specific context seems to induce it. Moreover, we hypothesize that α-β- transcripts has an indirect role in telomerase activity regulation.

Introduction

Telomere erosion either due to oxidative damage or replicative senescence limits cells' replicative capacity, which is considered as an important tumor-suppressive mechanism [1]. Telomerase is a ribonucleoprotein complex composed by the human telomerase reverse transcriptase (hTERT) subunit with reverse transcriptase (RT) activity, associated with the human telomerase RNA component (hTR) used as a template for telomere elongation, and additional proteins [2]. Although numerous factors are involved in telomerase activity, *hTERT* is its limiting factor in many cancers [3]. Furthermore, *hTERT* is implicated in tumor formation and progression, since its expression is determinant to cell immortalization and resistance to senescence and apoptosis [4-6]. These telomere-independent functions are considered as telomerase non-canonical functions [7]. Thus, regarding hTERT pivotal role in cancer cells, its expression is highly regulated and complex [8, 9].

Non-coding mutations within *hTERT* core promoter provided a first definitive mechanism of cancer specific telomerase (re)activation (Figure 1) [10, 11]. Two hotspot mutations, located at -124 C>T and -146 C>T (from the ATG start site), generates a new consensus binding site for ETS/TCFs transcription factors, which increases *hTERT* transcription and activity two to four times [12]. This mechanism have strong clinical implications conferring worse prognosis and poor survival in many cancers such as bladder, liver, thyroid (follicular cell-derived tumors), skin and central nervous system [13-15]. At the post-transcriptional level, *hTERT* pre-mRNA is subject to alternative splicing, which generates a proteome diversity with different biological functions. To date, around 20 transcript variants have been identified, including both nucleotide insertions and deletions [16, 17].

Cells exhibiting telomerase activity co-express, at significant levels, different *hTERT* transcripts [18]. The two most studied *hTERT* alternative splicing events occur within the telomerase RT domain at α and β sites (Figure 2). The α site originates from a 36 long base pair (bp) inframe deletion on exon 6 which removes most of RT motif A from the coding sequence (Figure 2). The β site encompasses a 183 bp long deletion from exons 7 and 8 that generates a truncated protein lacking the RT motifs B to E as well as the C-terminal part of hTERT [19]. Splicing at α and β sites can occur separately or in combination, generating either α+β+, α-β+, α+β- and α-β- transcripts (Figure 2). Only the full-length RT domain *hTERT* transcript, α+β+, exhibit telomerase activity [20]. α-β+ variant is a negative regulator of telomerase activity and α+β- protein as it conserves the RNA-binding motif, can also act as a negative regulator of telomerase activity together with its capacity to protect cancer cells from cell death by apoptosis [19, 21, 22]. No speficic function of α-β- variant has been assigned. Genetic variants of *hTERT* were also found to have a crucial role in the risk and prognosis of human cancers. Indeed, based on genome-wide association studies (GWAS), single nucleotide polymorphisms (SNPs) on hTERT-locus (5p15.33) have been consistently associated with increased risk for developing various types of cancers, such as lung, pancreatic, breast, bladder, ovarian, prostate, and testicular germ cell cancers as well as glioma, melanoma, and non-melanoma skin cancers [15, 23]. *hTERT* SNPs may locate either within its promoter, or in intronic and exonic regions. Among all *hTERT* SNPs found associated with human cancers, rs2735940 promoter genetic variation (Figure 1) could influence *hTERT* expression by affecting promoter transcription activity and telomere length [24, 25]. rs2853669 SNP that also locates at *hTERT* promoter (Figure 1) was reported to affect telomerase activity and telomere length [26, 27]. Indeed, rs2853669 variant disrupts a putative binding site

for Ets/TCF transcription factors and several lines of evidence suggest that it modifies the prognostic value of *hTERT* promoter mutations [28, 29]. rs2853672 and rs2853676, two SNPs located at intron 2 (Figure 2) were found associated with telomere length [30, 31]. Finally, rs10069690 SNP locates at intron 4 (Figure 2) creates an alternative splice donor site leading to the expression of an alternative *hTERT* splicing variant (INS1b). INS1b is a negative regulator of telomerase activity that affects telomere length [32].

Primary cutaneous T-cell lymphomas (CTCL) are a heterogeneous group of non-Hodgkin lymphomas presenting in the skin with no evidence of extracutaneous disease at the time of diagnosis [Willemze, 2019 #57]. The most common subtypes, comprising 90% of CTCL, include mycosis fungoides (MF) (which represents half of CTCL cases), Sézary syndrome (Sz) and CD30+ lymphoproliferative disorders (LPDs) [Sidiropoulos, 2015 #64]. Patients with MF typically experience an indolent disease course from patch/plaque-stage disease to cutaneous tumors and a 5 year disease-specific survival (DSS) of 88% [Willemze, 2005 #77][Willemze, 2019 #57]. However, a minority undergo a process of large-cell transformation (transformed mycosis fungoides, T-MF), which often heralds more aggressive disease with the cancer spreading to lymph nodes and/or internal organs [Agar, 2010 #79][Vural, 2018 #93]. Sz is a rare aggressive leukemic type of CTCL, traditionally defined by the triad of pruritic erythroderma, generalized lymphadenopathy, and clonally related neoplastic T cells with cerebriform nuclei (Sézary cells) in the skin, lymph nodes, and peripheral blood. The 5 year DSS is of 36% [Willemze, 2019 #57]. LPDs, comprise a spectrum of conditions with similar histologic and molecular features, but different clinical presentations. They include lymphomatoid papulosis (LyP) and primary

cutaneous anaplastic large cell lymphomas (cALCL), both with favorable prognosis and a 5 year DSS greater than 95% [Sauder, 2017 #152][Willemze, 2019 #57].

Telomerase expression and telomere length deregulation are common features of hematological diseases, including CTCL [33-35]. We previously demonstrated that telomerase is expressed in different CTCL subtypes (cALCL, T-MF and Sz), but short telomeres are a hallmark of the aggressive T-MF and Sz subtypes, and restricted to neoplastic cells in Sz. Furthermore, besides telomere length maintenance, telomerase exerts additional functions in CTCL [35].

In this work we aimed to assess the role of mechanisms of *hTERT* expression/activity in CTCL lymphomagenesis and to take advantage of our cancer model to deepen knowledge on the regulation of telomerase functions that can be operational in other neoplasias. Our results could be valuable in future therapeutic approaches against telomerase in cancer cells.

Methods

Patients and healthy controls. Tumors classified according to the criteria of the World Health Organization and the European Organization for Research and Treatment of Cancer (WHO-EORTC) [Willemze, 2019 #67] were retrieved from French and Portuguese institutions. 61 peripheral blood and DNA samples were retrieved from the dermatology department of University Hospital Center (CHU) of Bordeaux (France) as well as 33 representative formalin-fixed paraffin-embedded (FFPE) samples from the pathology archives of Instituto Português de Oncologia de Lisboa (IPO-L) and Centro Hospitalar Vila Nova de Gaia/Espinho (Portugal). A total of 94 patient samples were analyzed (29 ≤ age ≤ 87, mean age 65), including 22 LPDs (14 cALCL and 8 LyP), 39 MF (24 classic MF and 15 T-MF) and 33 Sz. Patient

samples were compared with peripheral blood from 101 healthy donors (24 ≤ age ≤ 85, mean age 60) recruited from the Etablissement Français du Sang (EFS), and CHU of Bordeaux, France. Peripheral blood mononuclear cells from patients and healthy donors were isolated by PANCOLL® density gradient centrifugation (PAN-Biotech). Each patient gave written consent. All the procedures described in this study were in accordance with national and institutional ethical standards and previously approved by Local Ethical Review Committees.

Cell lines. Experiments were performed on eight cell lines. Four cALCL: Mac1, Mac2A, Mac2B (DSMZ - German Collection of Microorganisms and Cell Cultures) and FEPD (Prof. G. Delsol, Toulouse, France), one T-MF: My-La (Dr K. Kaltoft, Aarhus, Denmark), and one Sz: Hut78 (ATCC). We also included two Sz cell lines established at our laboratory (L1 and L2), derived from two different clones from a Sz patient [36]. A T-cell lymphoblastic leukemia cell line, 1301 (Sigma-Aldrich), was used as a positive control for *hTERT* splicing variants amplification. Cell lines were cultured as previously described [35, 36].

Nucleic acid isolation. Genomic DNA was isolated using a salt extraction procedure [35]. Total RNA was isolated by means of TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Both DNA and RNA concentrations were measured by NanoDrop 2000 spectrophotometer (Thermo Scientific) and their quality were further analyzed by classic agarose gel electrophoresis and on Agilent 2200 TapeStation system (Agilent Technologies), respectively. DNA was stored at -20°C and RNA was stored at -80°C, until further genetic analysis.

Telomere length measurement. Telomere length was estimated from genomic DNA samples by means of quantitative polymerase chain reaction (qPCR), using the Absolute Human Telomere Length Quantification qPCR Assay Kit (CliniSciences), with precautions [*Ropio et al*, submitted] see section Telomere length estimation in cancer cells. Result represents the average telomere length per chromosome.

Telomerase activity measurement. Telomerase activity was assessed by means of TRAP assay kit (TRAPeze RT telomerase detection kit, Chemicon), according to manufacturer's instructions with some modifications [35].

***hTERT* promoter mutations detection.** The -146:C>T and -124:C>T *hTERT* promoter mutations were screened by PCR followed by direct Sanger sequencing [Vinagre, 2013 #26]. Primers used are available in Supplemental table 1.

hTERT SNPs genotyping. SNPs were identified either by allele-specific PCR or by using TaqMan probes. *hTERT* SNPs rs2735940, rs2853672, rs2853676 and rs10069690 were genotyped by allele-specific PCR. For this approach, two forward or two reverse primers were designed (Supplemental table 1). The 3′ base of each primer matched only one of the biallelic SNP bases to be evaluated and an additional mismatch was added. A common reverse/forward primer was designed downstream/upstream of the polymorphic site. Allele-specific reactions were analyzed in DNA samples, with each individual forward and reverse primer sets and Takyon™ No Rox SYBR® MasterMix dTTP Blue (Eurogentec), according to the manufacturer's instructions. All SNP amplification reactions followed the same qPCR program setup: initial denaturation step at 95ºC for 3 min, followed by 40 cycles of

95°C for 20 sec and 60ºC for 20 sec, with signal acquisition. *hTERT* rs2853669 polymorphism was analyzed by means of TaqMan SNP genotyping assay (Life Technologies) in an ABI Prism 7500 Fast system (Life Technologies). Some of cell lines analyzed were derived from the same patient (Mac1/Mac2A/Mac2B and L1/L2) with each group presenting the same allele combination.

Total *hTERT* and RT domain α and β splicing variants expression estimation. Total RNA was reverse transcribed using SuperScript II reverse transcriptase kit (Invitrogen), following manufacturer's instructions. Complementary DNA (cDNA) was amplified, using specific primers (Supplemental Table 1) and Takyon^{TM} No Rox SYBR® MasterMix dTTP Blue (Eurogentec), according to the manufacturer's instructions. TATA box binding protein (TBP) was used as normalizing control gene. Amplifications were carried out on a Stratagene Mx3005P system (Agilent Technologies) and analyzed with MxPro 4.01 QPCR software Stratagene (Agilent Technologies). *hTERT* splicing variants were amplified as follow: initial denaturation step at 95ºC for 3 min, followed by 45 cycles of 95°C for 20 sec and 60ºC for 60 sec with signal acquisition. Results are expressed as $2^{-\Delta Ct}$. 1301 cell line was used as a positive control and its dissociation curves were used as reference (Supplemental Figure 1).

Lentiviral sh *hTERT* β splicing variants construction and production. Constructs shβ+, shβ- and shScramble (non-targeting) used as control, were cloned into *pLKO.1-Tomato* (Addgene) vector at AgeI/*Eco*RI sites. shRNA primer sequences are available at Supplemental table 2. Lentiviral vector construction maps are available in

Supplemental Figure 2. Lentiviral vectors were used to transfect HEK293T cells at Bordeaux University Vectorology platform, to induce viral production.

Lentiviral titration and cell transduction. Both MyLa and HuT78 were infected with a dilution series of shβ+, shβ- and shControl lentiviral supernatants to determine the titer for each production. After 72h of infection, the percentage of transduced cells was assessed by flow cytometry and standard curves were generated. MyLa and HuT78 cells were then transducted with the volume of virus needed to obtain 33% of transduced cells. After 10 days, positively transduced cells were selected by flow cytometry on FACS Aria III sorter (BD Biosciences).

Cell proliferation analysis. Cell proliferation was measured by direct cell-counting in a hemocytometer (KOVA). 2×10^5 cells per well were seeded into 12-well plates and counted after 3, 6 and 10 days. The experiment was performed in quadruplicate.

Apoptosis/Necrotic Cell Detection. Apoptotic/necrotic cell proportion was measured using annexin VePE antibody (BD Biosciences), according to manufacturer's recommendations, and Hoechst 33342 (H3570; Molecular Probes, Eugene, OR) was added 5 minutes before sample acquisition. Apoptosis and necrosis were analyzed on FACS Canto II cytometer (BD Biosciences) and quantified using FlowJo software (FlowJo®). The experiment was performed in quadruplicate.

Anchorage-independent cell growth analysis. MyLa and HuT78 transduced with shβ+, shβ- and their respective controls were put in soft agar in 6 well plates and tested for their anchorage-independent cell growth capacities based on the formation

of cell colonies. The soft agar procedure was previously described [35]. The experiment was performed in quadruplicate.

Statistical analysis

RT domain α and β splicing variants statistical analyses were performed on GraphPad Prism software (version 5.01). Data from patients were collected from triplicate reactions from each sample. Data from cell lines were collected from triplicate reactions from two independent biological experiments. Results were presented as mean ± standard deviation. Paired Mann-Whitney test (nonparametric *T*-test) was used to compare transcript expression levels between patients and controls and to compare MyLa and HuT78 transduced cell lines with their respective controls. The significance level was set as $p = 0.05$.

Genotype frequencies for the *hTERT* SNPs were obtained using SPSS 23 (IBM SPSS Statistics). Compliance of alleles with the Hardy-Weinberg equilibrium was measured at the level of the control population using a $\chi 2$ test (level of significance set to p-value <0.05). Comparison of genotype frequencies between groups was assessed by unconditional logistic regression (level of significance set to p-value <0.05) with SPSS 23. Odds ratios (OR) with respective confidence intervals (95% CI) were calculated considering the genotypic and the dominant models of inheritance.

Results

1. *hTERT* could be a CTCL risk predictor: rs2735940, rs2853672 and rs2853676 *hTERT* SNPs are associated with CTCL risk

Two *hTERT* promoter SNPs, rs2735940 T>C and rs2853669 T>C, along with three *hTERT* intronic SNPs, rs2853672 G>T, rs2853676 G>A and rs10069690 C>T (Figure 1) were genotyped in 101 healthy controls, as well as in 66 CTCL patients and 8 cell lines (Table 1).

The distribution of all SNP genotypes in healthy control group were in accordance with Hardy-Weinberg equilibrium: rs2735940 T>C ($\chi2=0.06$, $P>0.05$), rs2853669 T>C ($\chi2=0.3$, $P>0.05$), rs2853672 G>T ($\chi2=0.3$, $P>0.05$), rs2853676 G>A ($\chi2=0.3$, $P>0.05$) and rs10069690 C>T ($\chi2=1.73$, $P>0.05$).

Genotyping of rs2735940 revealed that this *hTERT* promoter SNP impact CTCL risk. Indeed, rs2735940 TC and CC genotypes were significantly more prevalent in patients than in controls (OR (95%CI) =3.00, $p = 0.010$ and OR (95%CI) =3.79, $p = 0.011$, respectively). Thus, CTCL risk was significantly increased in rs2735940 C allele carriers (adjusted OR (95%CI) =3.20, $p = 0.004$) (Table 1). rs2853669 *hTERT* promoter SNP genotyping, on the other hand, demonstrated that genotypes distribution was not different between patients and controls (Table 1). Hence, no impact on CTCL risk was observed. Genotyping of *hTERT* coding region revealed that CTCL risk was significantly impacted by rs2853672 and rs2853676 SNPs (Table 1). Thus, while for rs2853672 G>T the T allele enhanced the risk for CTCL by two times (OR (95%CI) =2.18, $p = 0.039$), for rs2853676 G>A the minor allele A was associated with lower risk for CTCL (OR (95%CI) =0.46, $p = 0.028$) (Table 1). Genotyping of rs10069690 C>T revealed a decrease in the prevalence of TT

genotype in CTCL patients (OR (95%CI) = 0.14 (0.017-1.21), *p* = 0.074), although not statically significant.

For cell lines, a specific combination of these five polymorphisms were determined (Supplemental Table 3).

2. *hTERT* transcription regulation in CTCL: *hTERT* promoter mutations are a rare event in CTCLs

The occurrence of the two hotspot *hTERT* promoter mutations, -124 C>T and -146 C>T (from the ATG start site) (Figure 1), were analyzed in CTCL cell lines. Out of the 8 cell lines analyzed, only one (MyLa, a T-MF cell line) harbored the -146 C>T mutation (Table 2) and the mutation was in homozygosity (data not show). This encouraged us to retrospectively investigate these mutations in a cohort of 8 patients with a history of T-MF, along with 18 LPDs, 24 classic MF and 17 Sz patients. Among our Franco-Portuguese CTCL cohort, only one Sz patient harbored the -146 C>T mutation, which represented 5.9% of Sz cases and 1.5% of all CTCL patients (Table 2).

3. *hTERT* post-transcription regulation in CTCL

3.1 Aggressive CTCL presents a specific pattern of *hTERT* splicing variants

The expression of telomerase RT domain α and β spliced variants (Figure 2) was analyzed in CTCL cell lines, Sz patients and healthy controls. Furthermore, we analyzed blood patient samples with less and more than 50% of Sézary cells (samples with low and high tumor content, respectively) (Figure 3).

In healthy controls, among the possible four *hTERT* splice combinations, only α+β+ and α+β- transcripts were detected (Figure 3A), with α+β- being the most abundantly

expressed (Figure 3B). All *hTERT* variants were detected in Sz patients, but only two of them (2/10, 20%) expressed concomitantly all four transcripts. There was a high disparity in the pattern of *hTERT* variants presented by each Sz patient. α+β- was the variant most expressed by Sz patients (>85%), except for patient 9, which presented α+β+ as its *hTERT* variant most expressed (Supplemental figure 3). Sz patients with low tumor content expressed a pattern of *hTERT* splicing variants similar to controls, except for α-β+ transcript that was significantly more expressed in patients. On the other hand, patients with high tumor content expressed higher levels of *hTERT* spliced transcripts compared to healthy controls (Figure 3A). Concordantly, patients with high tumor content exhibited a drastic change in this pattern (Figure 3B). Comparatively to controls, Sz patients with high tumor content presented an increase of *hTERT* β+ transcripts in detriment of *hTERT* β- transcripts. Thus, we observed a 12 fold and a 5 fold increase of α+β+ and α-β+ expression, and a 38% decrease of α+β- percentage (Figure 3B).

All seven CTCL cell lines analyzed expressed the four *hTERT* spliced transcripts, except Mac1 that did not express α-β- transcript (Figure 3A). There was also a disparity in the pattern of *hTERT* spliced variants presented by each cell line (Supplemental figure 3). On average, α+β+ accounted for the majority of *hTERT* transcripts (46.8%), followed closely by α+β- (44%). The α-β+ and α-β- accounted for the smallest proportions of transcripts (6.8% and 2.3%, respectively) (Figure 3B).

3.2. Modulation of RT domain splicing transcriptome affects *hTERT* expression and telomerase canonical function

RT domain *hTERT* splicing variants expression were modulated by shRNAs in MyLa and HuT78, two cell lines representative of aggressive subtypes of CTCL (T-MF and

Sz, respectively) (Figure 4). The effects produced by each shRNAs were different from each cell line, in accordance with their different origins (Figure 4A). Comparing to MyLa controls, the shβ+, induced an increase of α+β- percentage (81.4% vs 68.6%) and a decrease of α+β+ percentage (16.1% vs 29%). In HuT78, comparing to controls, the shβ+ induced a slightly increase of *hTERT* β+ variants percentage. Most remarkably, we observed a 1.8 fold increase of α-β+ variant. The shβ- on MyLa cells did not affect overall β+/β- transcripts proportions, comparatively to controls. However it induced a 1.7 fold increase of α-β+ percentage. On HuT78 cells, shβ- induced a decrease of 6.5% of α+β+ and an increase of 19% of α-β+ variants percentages compared to controls (Figure 4A). The shβ+ on MyLa cells induced a significant decrease of total *hTERT* expression (p = 0.0152) (Figure 4C) but had no impact on telomerase activity (Figure 4D), comparing to controls. On HuT78 cells, sh β+ induced a significant decrease of total *hTERT* expression (p = 0.0032) (Figure 4C) and an increase in telomerase activity (Figure 4D), comparing to controls. The shβ- induced in MyLa cells a significant decrease of total *hTERT* expression (p = 0.0182) (Figure 4C) as well as of telomerase activity (Figure 4D), comparing to controls. On HuT78 cells, the shβ- induced a significant decrease of total *hTERT* expression (p = 0.0014) (Figure 4C), and an increase of telomerase activity (Figure 4D).

3.3. Modulation of RT domain splicing transcriptome affects telomerase non-canonical functions

The impact of RT domain *hTERT* splicing variants expression modulation on cell proliferation, cell death and cell anchorage-independent growth capacities were evaluated in MyLa and HuT78 cells (Figure 5). Compared to controls, we observed

an increase of cell proliferation capacities in MyLa cells after shβ+ transduction, while no impact was observed upon shβ- transduction (Figure 5A.1). Flow cytometry analysis revealed in shβ+ MyLa cells an increase of cell viability caused by a decrease in apoptosis (p = 0.0286) and necrosis (p = 0.0591). In shβ- MyLa cells a decrease in cell viability was observed, due to a significant increase of cell death by apoptosis (p = 0.0286) (Figure 5B.2). Furthermore, shβ+ MyLa cells *in vitro* tumorigenic capacities were similar to the control, contrasting to the drastic decrease observed on shβ- MyLa cells (p = 0.0047) (Figure 5C.3).

In HuT78 cells, shβ+ lentivirus vector induced a decrease in cell proliferation capacities while shβ- transduction had no impact (Figure 5A.4). Concordantly, in shβ+ HuT78 cells we observed a decrease of cell viability, associated with a significant increase of cell death, both by apoptosis (p = 0.0286) and necrosis (p = 0.0286). On shβ- HuT78 cells, we observed a significant increase of cell death by necrosis (p = 0.0286) (Figure 5B.5). Tumorigenic capacity of HuT78 were totally abolished upon transduction with shRNAs (p = 0.0082) (Figure 5C.6).

Discussion

As a major cancer hallmark, there is a sustained interest in understand telomerase contribution to cancer cells' abilities in order to target telomerase for cancer treatment [Jafri, 2016 #160][Allegra, 2017 #161]. This is particularly relevant in CTCL, a malignancy known to have telomerase deregulation [Chevret, #53]. An increasing number of studies suggest that single nucleotide polymorphisms within *hTERT* could influence the susceptibility to human cancers [Baird, 2010 #33][Mocellin, 2012 #162]. In this work, we showed that not only polymorphisms located at *hTERT* promoter but also at gene coding region could influence CTCL risk.

While the occurrence of somatic mutations on *hTERT* promoter are a frequent mechanism regulating *hTERT* transcription and activity in solid cancers, they have been rarely observed in hematological malignancies [Mosrati, 2015 #178][Yan, 2013 #179][Vinagre, 2013 #26]. *hTERT* promoter mutations can be present in CTCL. While no *hTERT* promoter mutations had been reported in CTCL, such alteration was found in a single Sz patient (1/17; 5.9%) and in one out of eight cell lines (Table 2). Interestingly, both the patient sample and the cell line were found mutated at the same position (-146), and corresponded to aggressive CTCL subtypes. Nevertheless, the mutated cells (patient and cell line) did not express higher levels of *hTERT*, comparing with other CTCL cells (data not shown). This is in accordance with the literature that states that *hTERT* overexpression due to -146 C>T mutation is less marked than the -124 C>T mutation.

Aside telomerase canonical function on telomere elongation, telomerase also possesses other non-canonical functions largely implicated in cancer initiation and progression, including in CTCL diseases [Liu, 2016 #163][35]. *hTERT* is the major determinant for telomerase activity, thus its transcription as well as its activity are highly regulated [Ramlee, 2016 #165][Ropio, 2016 #171]. Unveil the molecular basis of *hTERT* transcriptional activation and activity regulation is primordial in order to target telomerase in cancer treatment. In CTCL, *hTERT* is regulated at post-transcription level, as it is subjected to mRNA splicing and our results sustained that each pattern of *hTERT* variants has a specific biological consequence (Figure 5). In our cancer model, we observed that the α+β- variant proportion affected cell growth and apoptosis in the same way as in a breast cancer model [Listerman, 2013 #31]. As so, the increase of α+β- proportion induced a decrease of cell death by apoptosis and conferred a cell growth advantage (shβ+ in T-MF cells). The inverse happened

when α+β- proportions decreased (shβ+ in Sz cells). We further observed that in the absence of variations of α+β- proportions (shβ- in T-MF cells and shβ- in Sz cells), it was the variation of α-β+ that influenced cell death. Indeed, while there were no differences in α+β- proportions, the increase of α-β+ induced an increase of cell death. So, α+β- seems to protect cells from cell death, while α-β+ in a specific context seems to induce it. We also showed that Sz patients with high tumor content present a specific pattern of *hTERT* splicing variants, different from healthy controls, while Sz patients with low tumor content presented a pattern no different from controls (Figure 3B). These results, not only correlate with shorter telomeres characteristic of Sézary cells, but they may also explain the delay in apoptosis and the low proliferation index observed in this disease [35][*Ropio et al*, submitted][37]. Interestingly, the fact that transduced HuT78 cells did not express α-β- variant, allowed us to decipher a putative indirect role for this variant in telomerase activity regulation. Even in the absence of this variant, α+β- and α-β+ variants maintained the previous observed effects on cell death and proliferation. However, the described roles of α-β+ and α+β- as negative regulators of telomerase activity seemed to be impaired. Indeed, in the absence of α-β-, an increase of telomerase activity was observed despite the increase of its negative regulator. Consequently, we hypothesize that α-β- has an indirect role in telomerase activity regulation. We speculate that its presence is somehow required so α-β+ and/or α+β- could regulate telomerase activity. In acute myeloid leukemia it was reported that low risk patients can be identified by a high expression of α-β-*hTERT* variant, which strengthens our assumption on α-β- role [Calvello, 2018 #180]. Furthermore, our own observations from HuT78 and L1 wild type cells, reinforce our hypothesis. Indeed, these two Sz cell lines presented the same *hTERT* splicing variants pattern, except for α-β- variant

proportion (Supplemental Figure 3B). L1 presented a higher α-β- proportion than HuT78 (7.8% vs 1.2%) associated with a significantly lower telomerase activity compared to HuT78 (Supplemental Figure 4).

Modulation of RT domain splicing transcriptome on CTCL cells, allowed us to understand telomerase activity regulation and to infer on *hTERT* spliced variants functions. Our results support *hTERT* variants roles already described by others, reinforce the overlap of functions between α-β+ and α+β-, and propose an indirect regulator role for α-β-.

Telomerase activity is indeed tightly regulated as its own splicing variants are thought to regulate each other's functions. This work identified a possible genetic predisposition to CTCL based on hTERT SNPs and the presence of hTERT promoter mutation in a Sz patient. Furthermore, our results underscore the biological functions of *hTERT* splice transcripts in non canonical effect of TERT in CTCL tumorigenesis since inhibition of the different isoforms resulted in apoptosis and necrosis that also support that telomerase may be a therapeutic target in CTCL.

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Authorship Contributions

JR performed the experiments and collected data helped by MPC for cell culture and flow cytometry analysis; RB, AP and JV for *hTERT* promoter mutations and SNP analysis; JF, AC and YI for lentiviral construction and TRAP assay. DC contributed to primers design. MBB, JPM, MSS, JC and MC contributed to patient recruitment and selection. CD and PB did the SNP statistical analysis. JR, MPC, JPM, MSS, PS and EC analyzed and interpreted the data. JR, PS and EC prepared and wrote the manuscript. JPM, MSS, PS and EC edited. All authors commented on and revised the manuscript.

Conflict of Interest Disclosures

The authors declare no conflict of interest.

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Tables

Table 1. Genotypic frequencies of rs10069690 C>T, rs2853676 G>A, rs2853672 G>T, rs2853669 T>C and rs2735940 T>C *hTERT* polymorphisms in CTCL cell lines, CTCL patients and healthy control.

Locus/genotype	Cell lines n (%)	Controls n (%)	Patients n (%)	OR (95% CI)*	*p-value*
rs2735940	n = 8	n = 101	n = 66		
TT	2 (25)	40 (39.6)	11 (16.7)	1.00 [a]	
TC	6 (75)	48 (47.5)	39 (59.1)	3.00 (1.31-6.89)	**0.010**
CC	0	13 (12.9)	16 (24.2)	3.79 (1.35-10.6)	**0.011**
Dominant model (C carrier vs TT[b])		61 (60.4) / 11 (39.6)	55 (83.3) / 11 (16.7)	3.20 (1.44-7.08)	**0.004**
rs2853669	n = 8	n = 96	n = 66		
TT	0	40 (41.7)	28 (42.4)	1.00[a]	
TC	8 (100)	42 (43.7)	29 (43.9)	0.84 (0.41-1.75)	0.649
CC	0	14 (14.6)	9 (13.6)	0.80 (0.29-2.21)	0.660
Dominant model (C carrier vs TT[b])		56 (58.3) / 40 (41.7)	38 (57.6) / 28 (42.4)	0.83 (0.42-1.64)	0.595
rs2853672	n = 8	n = 101	n = 66		
GG	2 (25)	38 (37.6)	16 (24.2)	1.00[a]	
GT	6 (75)	50 (49.5)	38 (57.6)	2.04 (0.94-4.44)	0.069
TT	0	13 (12.9)	12 (18.2)	2.67 (0.94-7.62)	0.063
Dominant model (T carrier vs GG[b])		63 (62.4) / 38 (37.6)	50 (75.8) / 16 (24.2)	2.18 (1.04-4.58)	**0.039**
rs2853676	n = 8	n = 101	n = 66		
GG	4 (50)	47 (46.5)	40 (40.6)	1.00 [a]	
AG	3 (37.5)	42 (41.6)	26 (39.4)	0.59 (0.29-1.19)	0.141
AA	1 (12.5)	12 (11.9)	0	0.00	0.999
Dominant model (A carrier vs GG[b])		54 (53.5) / 47 (46.5)	26 (39.4) / 40 (60.6)	0.46 (0.23-0.92)	**0.028**
rs10069690	n = 8	n = 101	n = 66		
CC	0	50 (49.5)	36 (54.5)	1.00[a]	
CT	8 (100)	38 (37.6)	29 (43.9)	1.28 (0.64-2.57)	0.479
TT	0	13 (12.9)	1 (1.5)	0.14 (0.017-1.21)	0.074
Dominant model (*T carrier vs CC[b]*)		51 (50.5) / 50 (49.5)	30 (45.5) / 36 (54.5)	1.02 (0.52-1.98)	0.963

[a] Reference value [b] Reference genotype

Table 2. *hTERT* promoter mutations analysis in CTCL cells

	Mutation rate (%)	Mutation
Patients	1/67 (1.5%)	
LPDs	0/18 (0%)	-
cALCL	0/10 (0%)	-
LyP	0/8 (0%)	-
MF	0/32 (0%)	-
MF	0/24 (0%)	-
T-MF	0/8 (0%)	-
Sz	1/17 (5.9%)	-146 C > T
Cell lines	1/8 (12.5%)	
c-ALCL	0/4 (0%)	-
T-MF	1/1 (100%)	-146 C > T
Sz	0/3 (0%)	-

hTERT promoter hotspot mutations (−124 bp and −146 bp upstream the ATG transcriptional start site, (TSS) analyzed in CTCL patients and cell lines. LPDs : CD30+ lymphoproliferative disorders. cALCL: cutaneous anaplastic large cell lymphomas. LyP: lymphomatoid papulosis. MF: mycosis fungoides. T-MF: transformed mycosis fungoides. Sz: Sézary syndrome

Supplemental Table 1. Primer sequences (5' to 3') and annealing temperature used for hTERT gene investigations: promoter mutations screening, SNPs genotyping, total mRNA expression, splicing variants expression

Primers	Primer sequence	Annealing (°C)
***hTERT* promoter mutations**		
Fw pmutTERT	5'-CAGCGCTGCCTGAAACTC-3'	61.5
Rv pmutTERT	5'-GTCCTGCCCCTTCACCTT-3'	
hTERT* SNPs		
Fw (T) rs2735940	5'-GGATTTCTAGAAGAGCGGCC**T**-3'	
Fw (C) rs2735940	5'-GGATTTCTAGAAGAGCGGCC**C**-3'	60
Rv rs2735940	5'-TATGGAGCTAGCATTTGAACAG-3'	
Fw rs2853672	5'-AGGGTGCCTGCAGGTTACCTA-3'	
Rv (G) rs2853672	5'-CATATTGGCTGACCACGTACA**C**-3'	60
Rv (T) rs2853672	5'-CATATTGGCTGACCACGTACA**A**-3'	
Fw rs2853676	5'-TCGCCCCCTCACATGGATTG-3'	
Rv (G) rs2853676	5'-GAGGGAAGTCTGACGAATGC**C**-3'	60
Rv (A) rs2853676	5'-GAGGGAAGTCTGACGAATGC**T**-3'	
Fw rs10069690	5'-ACGGCTCCTGCACCCCAC-3'	
Rv (C) rs10069690	5'-ACACGGGATCCTCATGACA**C**-3'	60
Rv (T) rs10069690	5'-ACACGGGATCCTCATGACA**T**-3'	
Total hTERT (Figure 2)		
a) Fw hTERT	5'-GCATTGGAATCAGACAGCAC-3'	60
b) Rv hTERT	5'-CCACGACGTAGTCCATGTTC-3'	
hTERT splicing variants (Figure 2)		
c) Fw hTERT α+	5'-TGTACTTTGTCAAGGTGGATGTG-3'	
d) Fw hTERT α-	5'-CTGAGCTGTACTTTGTCAAGGAC-3'	60
e) Rv hTERT β+	5'-GTACGGCTGGAGGTCTGTCAA-3'	
f) Rv hTERT β-	5'-GGCACTGGACGTAGGACGTGG-3'	
Normalizing gene		
Fw TBP	5'-CACGAACCACGGCACTGATT-3'	60
Rv TBP	5'-TTTTCTTGCTGCCAGTCTGGA-3'	

***In bold**, the specific SNP mismatch. Underlined, the common sequence mismatch added to each allele specific primer

Supplemental Table 2. Primer sequences (5' to 3') used for lentiviral short hairpin (sh) RNA vector cloning

Primers	Primer sequence
sh *hTERT* β+	
Fw **sh β+**	5'-CCGGGCATCAGGGGCAAGTCCTACGCTCGAGCGTAGGACTTGCCCCTGATGCTTTTTG-3'
Rv **sh β+**	5'-AATTCAAAAAGCATCAGGGGCAAGTCCTACGCTCGAGCGTAGGACTTGCCCCTGATGC-3'
sh *hTERT* β-	
Fw **sh β+**	5'-CCGGCAAGAGCCACGTCCTACGTCCCTCGAGGGACGTAGGACGTGGCTCTTGTTTTTG-3'
Rv **sh β+**	5'-AATTCAAAAACAAGAGCCACGTCCTACGTCCCTCGAGGGACGTAGGACGTGGCTCTTG-3'
sh Scramble	
Fw **sh control**	5'-CGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3'
Rv **sh control**	5'-AATTAAAAACAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG-3'

Supplemental Table 3. Genotyping results of rs2735940 T>C, rs2853669 T>C, rs2853672 G>T, rs2853676 G>A and rs10069690 C>T *hTERT* polymorphisms in CTCL cell lines

		Locus/genotype				
Pathology	**Cell lines**	**rs2735940**	**rs2853669**	**rs2853672**	**rs2853676**	**rs10069690**
c-ALCL	**FEPD**	TT	TC	GG	GG	CT
	Mac1	TC	TC	GT	AG	CT
	Mac2A	TC	TC	GT	AG	CT
	Mac2B	TC	TC	GT	AG	CT
T-MF	**MyLa**	TT	TC	GG	AA	CT
Sz	**HuT78**	TC	TC	GT	GG	CT
	L1	TC	TC	GT	GG	CT
	L2	TC	TC	GT	GG	CT

cALCL: cutaneous anaplastic large cell lymphomas. T-MF: transformed mycosis fungoides. Sz: Sézary syndrome

Figure Legends

Figure 1. Telomerase expression mechanisms in cancer. Telomerase activity detection in cancer cells is closely related to acquired expression of *hTERT* gene located at the short arm of chromosome 5. *hTERT* transcription activation may be due to (A) germline genetic variations and (B) promoter hotspot mutations. (A) *hTERT* germline genetic variations both in the promoter and in the gene coding region were found associated with cancer risk. (B) *hTERT* promoter hotspot mutations (−124 bp and −146 bp upstream the ATG transcriptional start site, TSS) create binding sites for ETS transcription factors, which increases gene transcription and activity. Genomic coordinates are based on build 37 (GRCh 37, hg19/Human).

Figure 2. Schematic representation of *hTERT* RT domain post-transcription events. Known hTERT RT domain protein motifs are shown (1, 2, A, B, C, D and E). The two main alternative splicing sites in the RT domain of *hTERT* are the α splice site in exon 6, which produces a 36-bp in frame deletion; and the β splice site in exons 7 and 8, which results in a 182-bp deletion producing a nonsense mutation that truncates the protein. Four possible combinations of *hTERT* alternative splicing are possible: α+β+, α-β+ (β+ variants), α+β- and α-β- (β- variants). a) and b) positions correspond to the primer pair used to amplify total *hTERT*. c), d), e) and f) positions of the primers used to amplify the *hTERT* alternative splicing variants.

Figure 3. *hTERT* RT domain splicing variants in CTCL cells. Healthy controls, Sz patients (with low and high tumor content) and cell lines representative of different subtypes of CTCL were analyzed. A) Relative expressions of α+β+, α-β+, α+β- and

α-β- transcripts. A.1) Controls expressed low levels of α+β+ transcript. Both groups of Sz patients expressed higher levels of α+β+ transcript than controls, even if the difference was not observed significantly different from the controls. Cell lines expressed scatter amounts of α+β+ transcript. A.2) α-β+ was not detected in controls. Both groups of Sz patients expressed significantly higher amounts of α-β+ transcript than controls. All cell lines expressed similar α-β+ transcript amount, except Mac1 that expressed a higher level than the others cell lines. A.3) α+β- was highly expressed in controls. The expression of α+β- transcript in low tumor content Sz patients was no different from controls while, it was significantly higher in high tumor content Sz patients. All cell lines expressed the same α-β+ transcript amount, except Mac2B that expressed higher levels than the others cell lines. A.4) α-β- transcript was not detected in controls. The α-β- transcript in Sz patients was not observed statistically different from controls. Cell lines expressed scatter amounts of α-β- transcript. B) *hTERT* splice variants pattern. In controls, 97% of *hTERT* transcripts were α+β- variant and the remaining 3% were α+β+. While the pattern from low tumor content Sz patients were not different from controls, high tumor content Sz patients presented a drastically different pattern of *hTERT* splice variants, with a clear increase in the proportion of β+ variants. In cell lines α+β+ accounted for the majority of hTERT transcripts (46.8%), followed closely by α+β- (44%). The α-β+ and α-β- accounted for the smallest proportions of transcripts (6.8% and 2.3%, respectively).

Figure 4. Modulation of *hTERT* RT domain splicing transcriptome. MyLa and HuT78 were transduced with shRNAs (controls in white, shβ+ in blue and shβ- in red) in order to modulate the expression of RT domain hTERT splicing variants. A) One

example of MyLa and HuT78 cells selected by flow cytometry after lentiviral transduction. B) *hTERT* splice variants pattern. Lentiviral transduction induced remarkable changes in MyLa cells *hTERT* splice variants pattern while modest changes were observed in HuT78 cells *hTERT* splice variants pattern. C) hTERT total expression was found statistically affected by lentiviral transduction in both cell lines. D) Telomerase activity was found affected in both cell lines, except for shβ+ MyLa cells.

Figure 5. *in vitro* effect of *hTERT* RT domain splicing transcriptome modulation. A) Cell proliferation capacities estimated by direct cell counting. A.1) MyLa cells proliferation capacities increased after shβ+ transduction. A.4). shβ+ transduction decreased HuT78 cells proliferation capacities. B) Cell death by apoptosis/necrosis, assessed by flow cytometry. B.2) On MyLa cells, Shβ+ transduction reduced cell death both by apoptosis (significantly) and necrosis (not satistically significant), wheras, shβ- transduction significantly increased cell death only by apoptosis. B.5) On HuT78 cells, shβ+ transduction increased significantly cell death both by apoptosis and necrosis. On shβ- HuT78 cells, only cell death by necrosis was found significantly affected. C) Anchorage-independent cell growth analysis by soft agar assay. C.3) shβ- MyLa cells' anchorage-independent cell growth capacities were severely decreased. Panels a and b show colony images of both MyLa control and shβ- MyLa cells. C.6) shRNA impaired anchorage-independent cell growth capacities in HuT78 cells.

Supplemental Figure 1. *hTERT* RT domain α and β spliced variants in 1301 cell line. 1301 cell line was used as a positive control for amplification by RTqPCR of the

four *hTERT* splice transcripts. A) Amplification plots of α+β+ (blue), α-β+ (green), α+β- (red) and α-β- (gray) transcripts, and B) corresponding dissociation curves. C) PCR products were run in a 2% agarose gel to confirm the attended weights of amplified products α+β+ (202 pb), α-β+ (189 pb), α+β- (172 pb) and α-β- (159 pb). D) *hTERT* splice variants pattern. In 1301, α+β+ was the variant most expressed, accounting for 78% of *hTERT* transcripts. α+β- accounted for 11.2% of transcripts, α-β+ 9.9% and α-β- 0.9%.

Supplemental Figure 2. Maps of pLKO.1 vectors containing *hTERT*β+ and *hTERT*β- shRNA inserts. Constructs shβ+, shβ- cloned into *pLKO.1-Tomato* vector.

Supplemental Figure 3. *hTERT* splice variants pattern of CTCL cells. A) Individual Sézary patients either with low tumor content (case1 to cas 5) or with high tumor content (case 6 to case10) revealed a high disparity in hTERT splice variants pattern. B) Cell lines presented a disparity in hTERT splice variants pattern. For Mac2A, Mac2B and L2, α+β+ was the variant most expressed, while for the others cell lines α+β- was the variant the most expressed. For all cell lines α-β- was the variant less expressed.

Supplemental Figure 4. Sz CTCL cell lines' telomerase activity. Telomerase activity was assessed by TRAP on two Sz cell lines: HuT78 and L1.

Supplemental Figure 5. B) Relative expressions of α+β+, α-β+, α+β- and α-β- transcripts. B.1) Apart from shβ- HuT78 cells, α+β+ expression was observed affected by lentiviral transduction (statistically significant or not). B.2) α-β+ transcript

was found affected in shβ+ MyLa and HuT78 cells, even if no statistically difference was noted. B.3) α+β- transcript expression was found statistically affected in shβ- MyLa cells and in shβ+ HuT78 cells. B.4) α-β- transcript was observed drastically affected in MyLa cells while it was not detected in HuT78 transduced cells.

Telomerase expression mechanisms in cancer

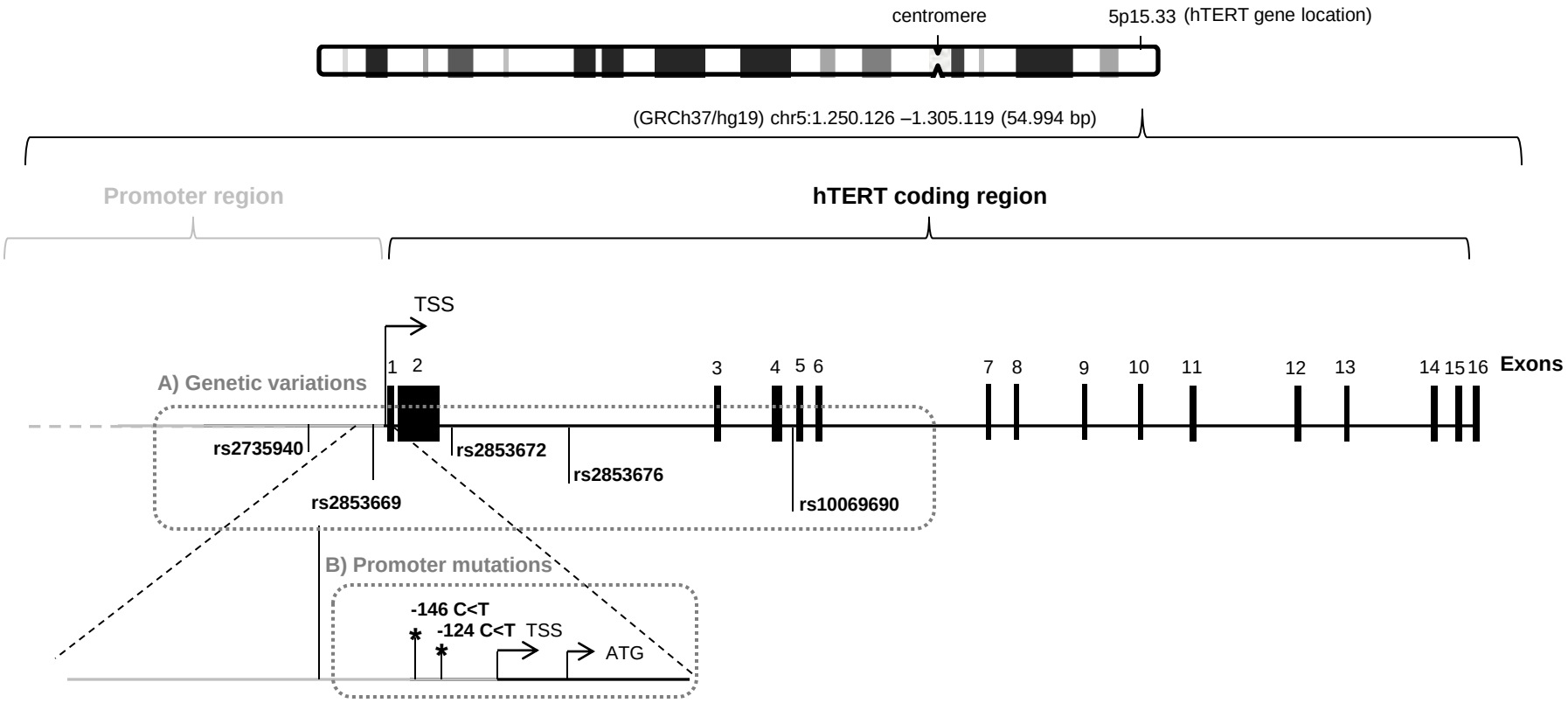


Figure 1.

Schematic representation of *hTERT* RT domain post-transcription events

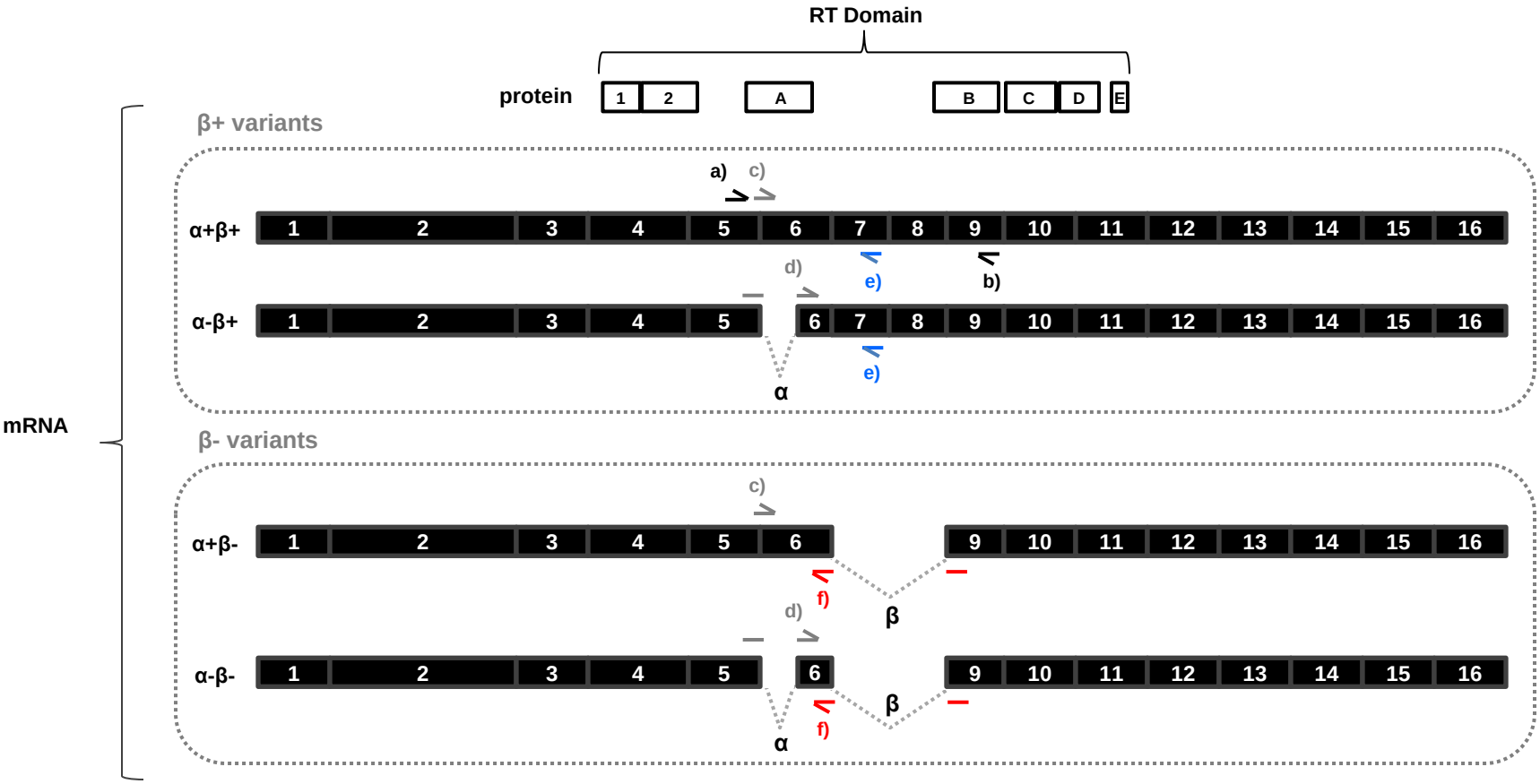


Figure 2.

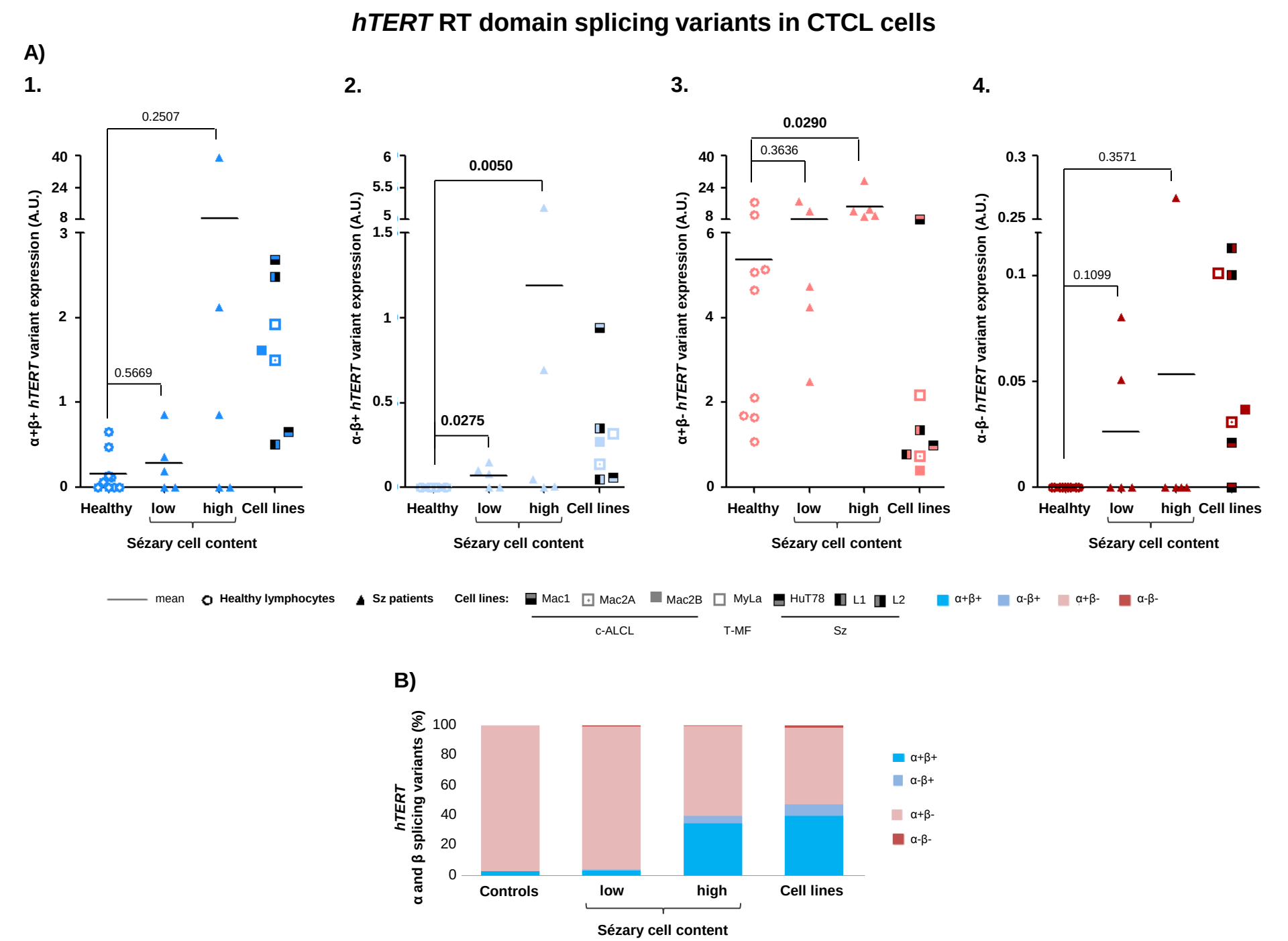

hTERT RT domain splicing variants in CTCL cells
A)
1.
2.
3.
4.
α+β+ hTERT variant expression (A.U.)
α-β+ hTERT variant expression (A.U.)
α+β- hTERT variant expression (A.U.)
α-β- hTERT variant expression (A.U.)
0.2507
0.5669
0.0050
0.0275
0.0290
0.3636
0.3571
0.1099
Healthy
Healhty
low
high
Cell lines
Sézary cell content
mean
Healthy lymphocytes
Sz patients
Cell lines:
Mac1
Mac2A
Mac2B
MyLa
HuT78
L1
L2
c-ALCL
T-MF
Sz
α+β+
α-β+
α+β-
α-β-
B)
hTERT α and β splicing variants (%)
Controls
low
high
Cell lines
Sézary cell content

Figure 3.

Modulation of *hTERT* RT domain splicing transcriptome

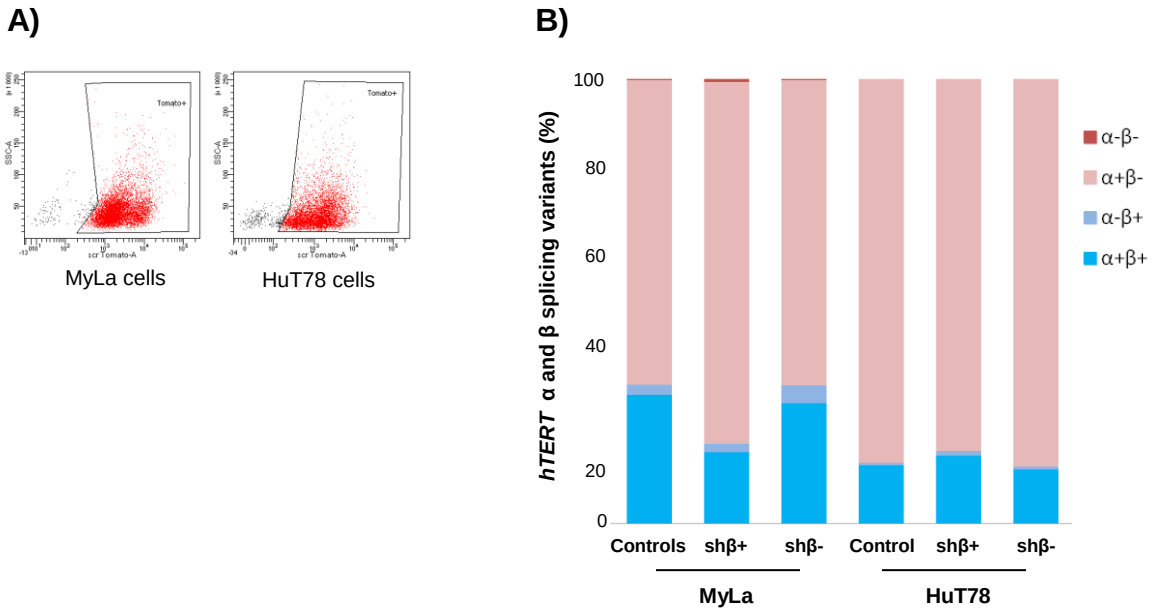


Figure 4.

in vitro effect of *hTERT* RT domain splicing transcriptome modulation

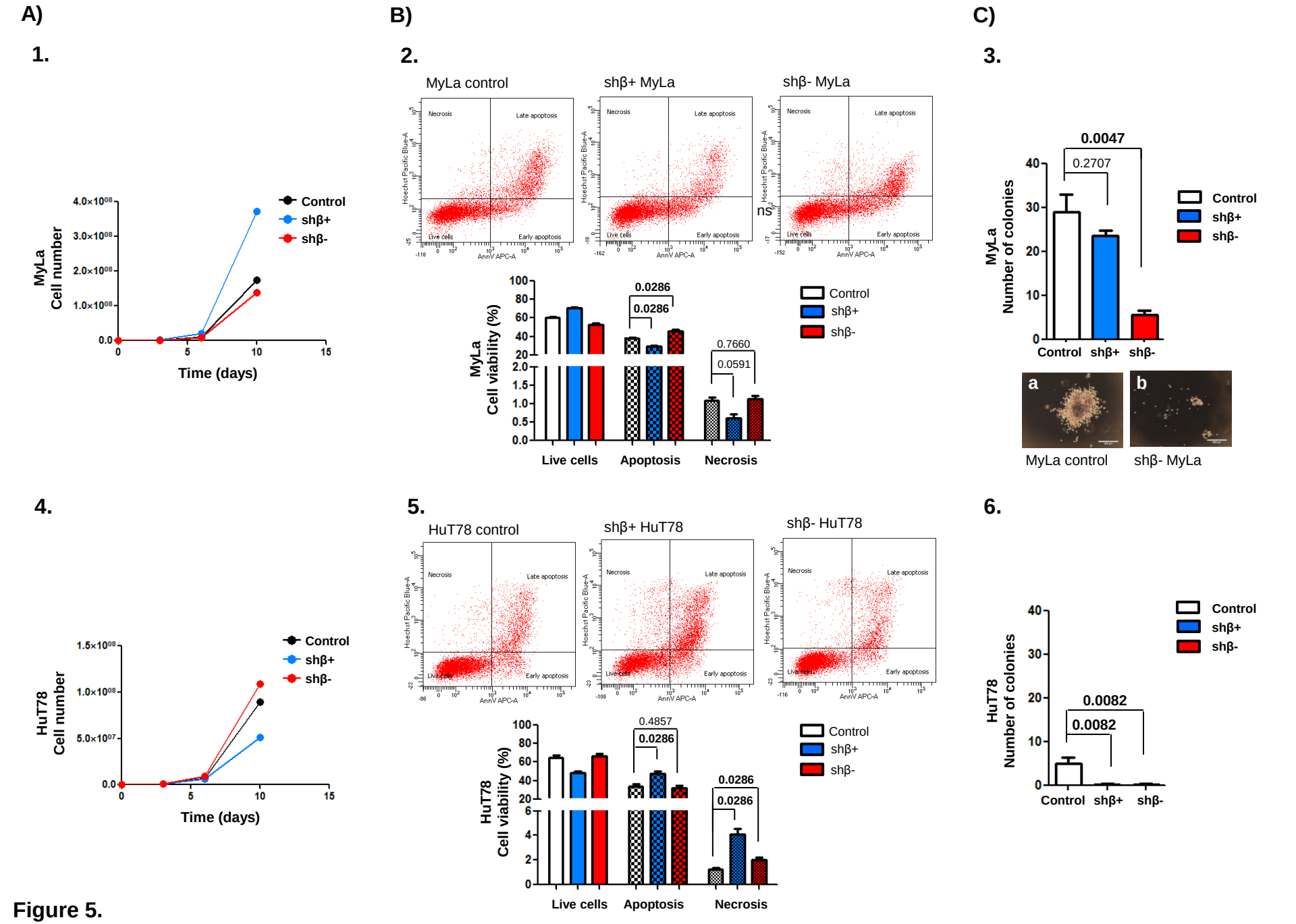
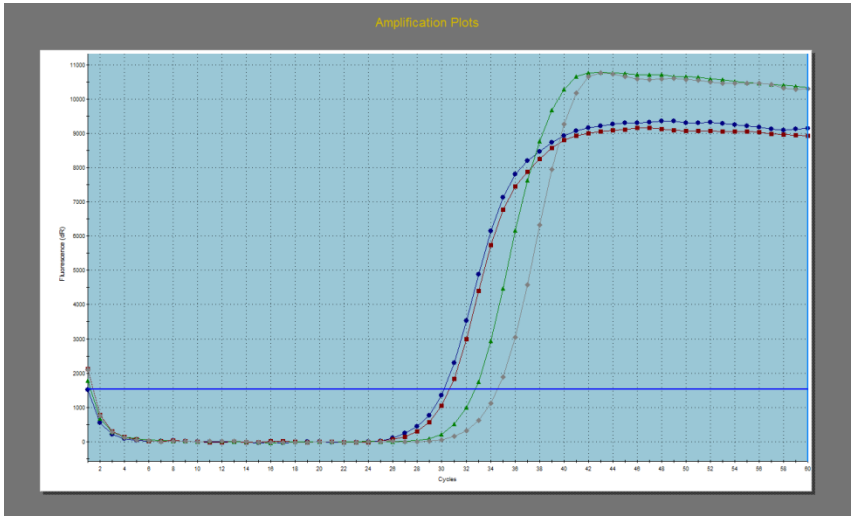


Figure 5.

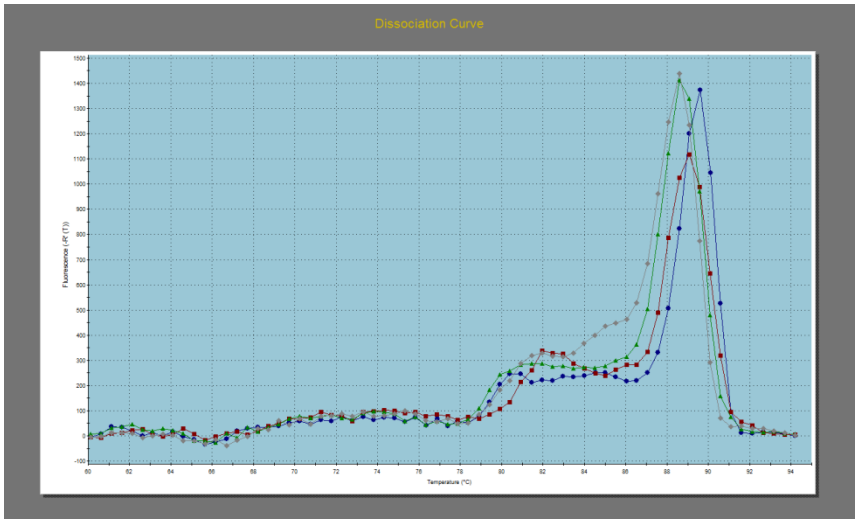
Supplemental Figures

hTERT RT domain α and β spliced variants in 1301 cell line

A.

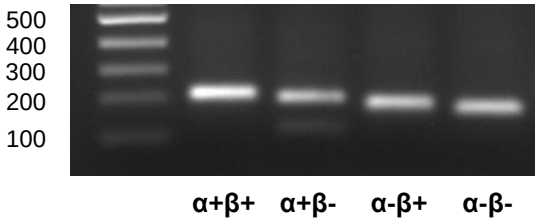


B.

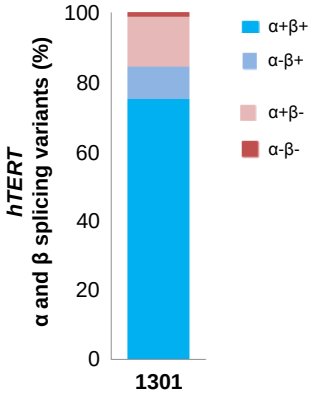


α+β+ α-β+ α+β- α-β-

C.



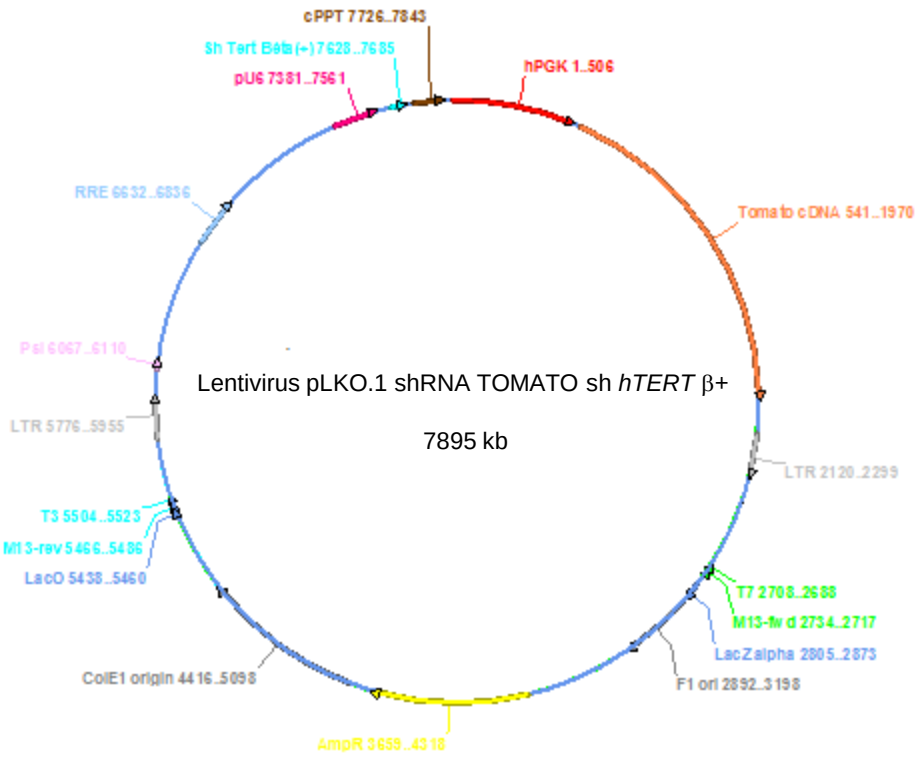
D.



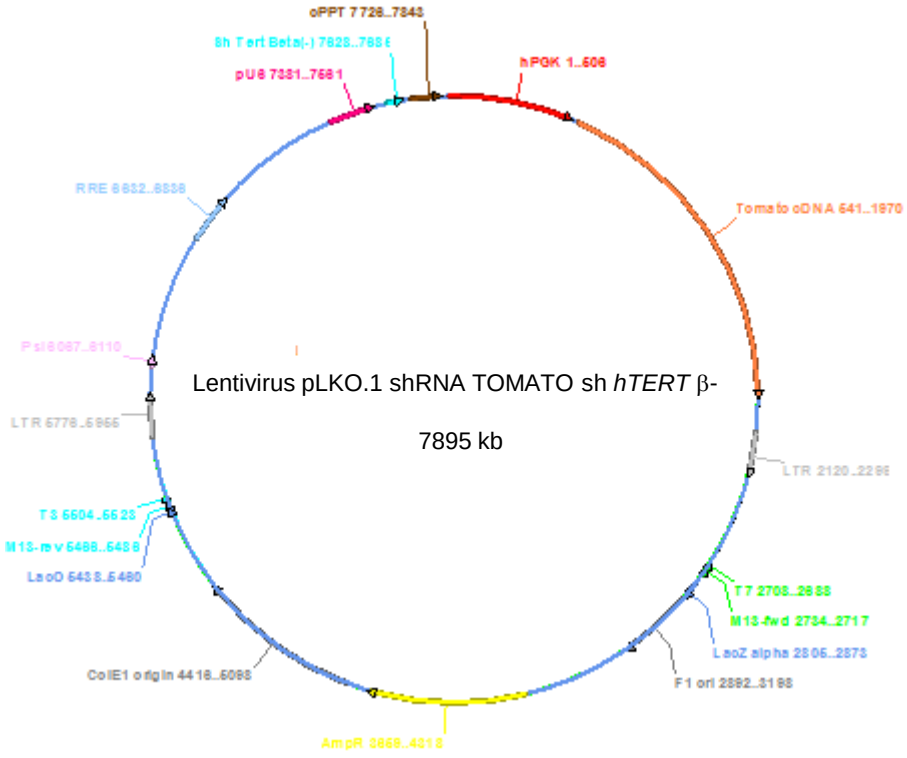
Supplemental Figure 1.

Maps of pLKO.1 vectors containing *hTERT*β+ and *hTERT*β- shRNA inserts

A.

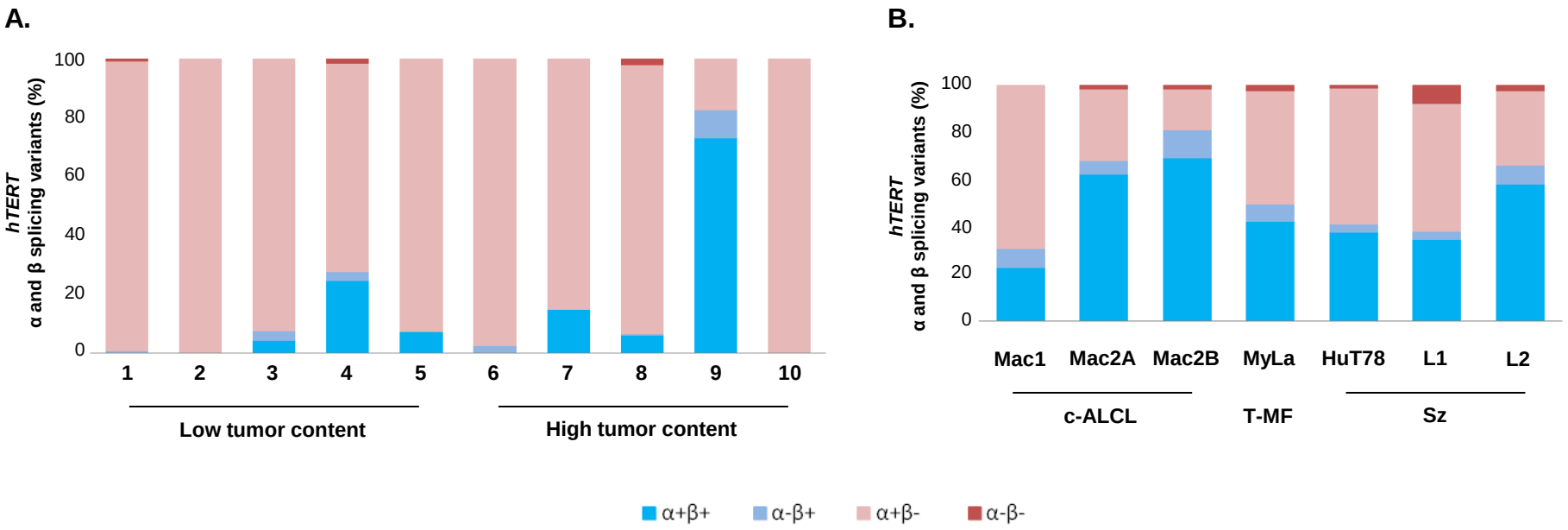


B.



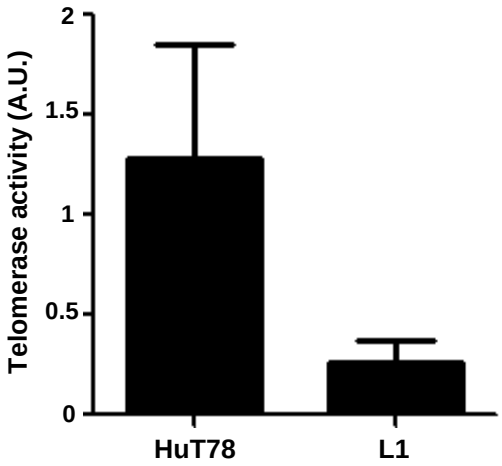
Supplemental Figure 2.

hTERT splicing variants pattern of CTCL cells

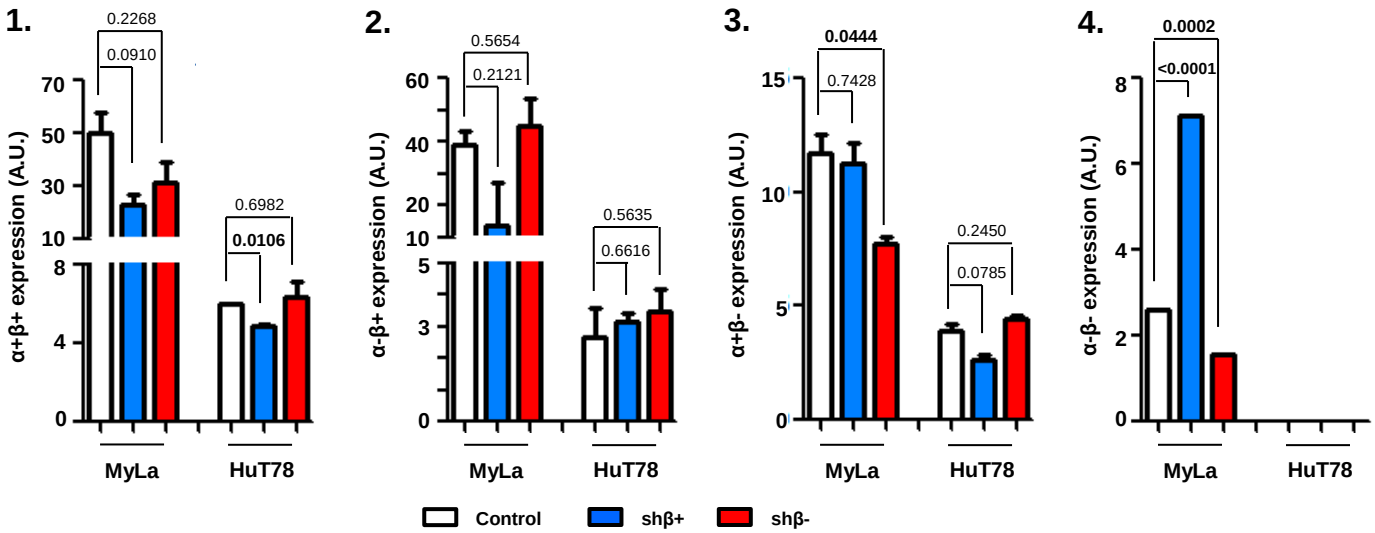


Supplemental Figure 3.

Sz CTCL cell lines' telomerase activity



Supplemental Figure 4.



Supplemental Figure 5.

Telomere biology players beyond hTERT

Telomere regulation is largely mediated by the interaction network that surrounds them (Luo, Dai et al.). Besides telomerase, other factors play important roles in telomere regulation, as when they are deregulated they participate in tumor initiation and/or progression (Kalan and Loayza ; Patel, Vasan et al. ; Sarek, Marzec et al.). Telomeric DNA is bound by a six protein complex, called shelterin (TRF1, TRF2, TIN2, TPP1, POT1 and RAP1). This complex shapes and safeguards human telomeres from unwanted DNA damage repair (De Lange 2005). Altered expression of shelterin proteins was found in various hematological malignancies, which relates with progression or chemotherapy resistance in these diseases (Wang, Xiao et al.). Furthermore, POT1, the first member of shelterin complex to be found mutated in human cancers, is a frequently mutated gene in chronic lymphocytic leukemia (CLL) and was also found mutated in a cohort of CTCL patients (Pinzaru, Hom et al. ; Ramsay, Quesada et al.). CTCL POT1 mutations stimulate telomere elongation and telomere dysfunction, indicating that the compromised function of the shelterin complex can lead to tumorigenesis independent of the classic telomere erosion mechanism (Pinzaru, Hom et al.).

In addition to telomere DNA and telomeric proteins, another factor in the telomere interaction network is RNA. Telomeres are transcribed from many chromosome ends into telomeric repeat-containing RNAs (TERRA), which are long non-coding RNAs that locate afterwards at telomeres (Azzalin, Reichenbach et al. 2007; Schoeftner and Blasco 2008). TERRA levels are strongly reduced in immortalized telomerase-positive cell lines compared with primary cells or cells engaged in ALT and have been implicated in regulation of telomerase activity, telomere length and associated heterochromatization (Bettin, Oss Pegorar et al.).

Since TERRA participates in the fine regulation of cell biology and there is no information on TERRA status on CTCL, a disease with telomere biology deregulation, we evaluated the level of some TERRA transcripts in CTCL cell lines and in Sz patients and compare it with healthy controls.

TERRA opens a new field in the understanding of telomeric functions and related diseases, and may provide valuable insight in telomere biology of CTCL.

New telomere biology players in CTCL

New telomere biology players in CTCL

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Material and methods

Patients and healthy donors

Sz patients (n = 3) were selected from the dermatology department of University Hospital Center (CHU) of Bordeaux, France. Healthy donors (n = 6) were recruited from the Etablissement Français du Sang (EFS), France. Peripheral blood mononuclear cells from patients and healthy donors were isolated by PANCOLL® density gradient centrifugation (PAN-Biotech).

Cell Lines

Experiments were performed on six CTCL cell lines. Three C-ALCL: Mac1, Mac2A, Mac2B (DSMZ), one T-MF: My-La (Dr K. Kaltoft, Aarhus, Denmark), and one Sz: Hut78 (ATCC). We also included one Sz cell line established at our laboratory: L2, derived from a Sz patient clone [*Poglio et al*, submitted]. U20S (ATCC), an osteosarcoma cell line and HeLa (ATCC), a cervical cancer cell line, were used as positive controls for TERRA transcripts amplification. CTCL cell lines were cultured as previously described (*Ropio et al*, submitted, see section Telomere length estimation in cancer cells)[1]. U2OS and HeLa were cultured in DMEM (Gibco), supplemented with 100U/mL penicillin (Gibco), 100µg/mL streptomycin (Gibco) and 10% foetal bovine serum (Eurobio). All cell lines were maintained at 37ºC with 5% CO2 and regularly tested for Mycoplasma contamination.

TERRA transcript quantification

Total RNA was isolated as previously reported [*Ropio et al*, manuscript in preparation, see section Telomerase regulation in cancer cells]. All RNAs were treated with DNAse Max^{TM} kit (Quiagen), according to the manufacturer's instructions and Feretzaki & Lingner and Arnoult, *et al* recommendations [2, 3]. 3µg of ARN was reverse transcribed with SuperScript II reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions and using specific anti-sense primers for each target. Complementary DNA (cDNA) was amplified by quantitative reverse-transcription PCR (qRT-PCR), following manufacturer's instructions, using Takyon^{TM} No Rox SYBR® MasterMix dTTP Blue (Eurogentec) and specific primers. Human

elongation factor-1 *α* (EF-1*α*) was used as normalizing control gene. Sequences of all primers used are available in **Supplemental Table 1.** The chromosomal locations of TERRA analyzed are showed in **Figure 1**. Amplifications were carried out on a Stratagene Mx3005P system (Agilent Technologies) and analyzed with MxPro 4.01 QPCR software Stratagene (Agilent Technologies). Results were expressed as $2^{-\Delta Ct}$.

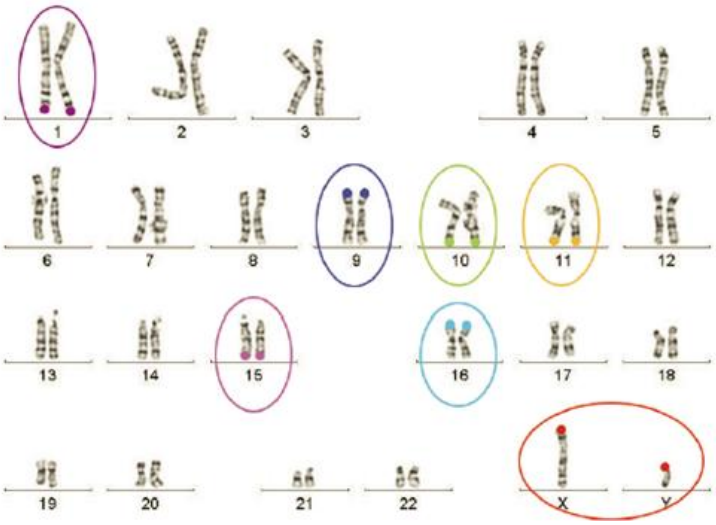


Figure 1 – Telomeric regions analyzed for TERRA

Telomeric regions of chromosomes 1q, 9p, 10q, 11q, 15q, 16p and XpYp are circled and colored. We investigated the telomeric repeat-containing RNAs (TERRA) of the identified telomeres.

hTERT overexpression

Telomerase overexpression was performed as previously reported [4]. My-La cells were seeded into 6-well plates and then transduced either with a lentiviral vector containing *hTERT* complementary DNA or a lentiviral vector containing DsRed2 (used as control).

Statistics

Statistical analysis was performed with GraphPad Prism 5.01. RT-qPCR data were collected from triplicate reactions from one biological experiment. Results were presented as mean ± standard deviation. Paired Mann-Whitney test (nonparametric *T*-test) was used to compare variables. The significance level was set as $p = 0.05$.

Results

1. Validation of TERRA transcript quantification

A RT-qPCR protocol [2, 3] for quantification of TERRA transcripts was validated in HeLa and U2OS cell lines (**Figure 2**).

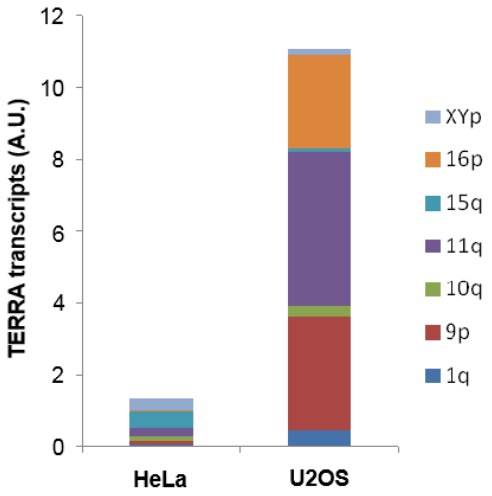


Figure 2 – HeLa and U2OS TERRA transcripts quantification

We confirmed that these cell lines expressed different levels of TERRA transcripts. HeLa express low levels of TERRA transcripts while U2OS expressed high levels (**Figure 2**). This observation confirmed what is described in the literature [2].

As we mastered this RT-qPCR approach to TERRA transcript quantification, we were able to analyze them in CTCL cells.

2. TERRA in CTCL

2.1 CTCL present sub-type specific TERRA profile and aggressive subtypes express low TERRA levels

The expression of TERRA transcripts were quantified in healthy controls, in Sz patients, and in cell lines representative of different sub-types of CTCL: C-ALCL, T-MF and Sz (**Figure 3**).

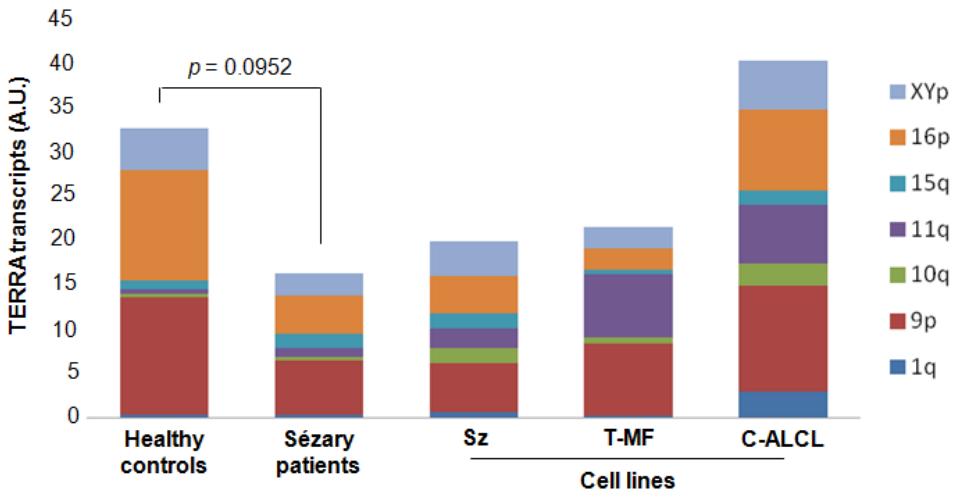


Figure 3 – CTCL TERRA profile

TERRA profile of transcripts from chromosome ends 1q, 9p, 10q, 11q, 15q, 16p and XpYp

Regarding the total amount of TERRA transcripts, Sz patients presented lower levels than controls (p = 0.0952). C-ALCL cell lines, representative of CTCL sub-types with indolent behavior, presented levels similar to controls. T-MF and Sz cell lines, representative of CTCL with aggressive behavior, presented lower levels than both C-ALCL cell lines and healthy controls. The three sub-types of CTCL presented different profiles of TERRA transcripts, and furthermore, each profile was different from the profile presented by controls. It is also worth to notice that the profile presented by Sz patients is similar to that of Sz cell lines.

TERRA 9p, 16p and XpYp were the TERRA most expressed by healthy controls. While these TERRA were also the most expressed by Sz patients, their levels of expression were different comparing to controls (**Supplemental Figure 1**). Indeed, Sz patients presented significantly decreased expression of TERRA 9p (p = 0.0476) and 16p (p = 0.0160), as well as TERRA XpYp, although not significantly (p = 0.2433). For the other TERRA analyzed, no statistical difference from controls were observed (**Supplemental Figure 1**). In CTCL cell lines (as in controls) among the most expressed TERRA were TERRA 9p, 16p and XpYp. However, TERRA 11q was highly expressed in CTCL cell lines, while its expression in healthy controls is very low.

2.2 hTERT induces TERRA transcription

The expression of TERRA transcripts were quantified in MyLa cells overexpressing *hTERT* and in control MyLa cells (**Figure 4**).

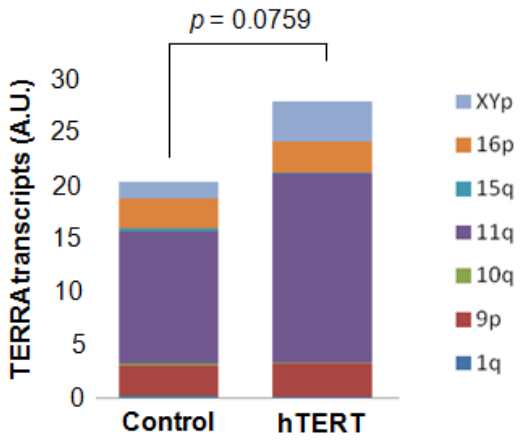


Figure 4 – Effect of *hTERT* overexpression on TERRA expression in MyLa cells

TERRA profile of transcripts from chromosome ends 1q, 9p, 10q, 11q, 15q, 16p and XpYp

hTERT MyLa cells expressed higher levels of total TERRA transcripts (p = 0.0759) than control MyLa. We remarked a significant increase of TERRA 11q (p = 0.0271) and XpYp, although not significant (p = 0.0919). Comparing to MyLa controls, hTERT MyLa cells expressed significant less amounts of TERRA 15q (p = 0.0023). The transcript level of the remaining TERRA analyzed, 1q (p = 0.6859), 9p (p = 0.6284), 10q (p = 0.3324), and 16p (p = 0.8674) was not impacted by *hTERT* overexpression.

Discussion and perspectives

In this work we began to unveil the involvement of long non-coding RNA molecules in CTCL lymphomagenesis. We demonstrated that telomeric chromosomes of CTCL cells are transcribed and that TERRA arises from multiple telomeric chromosomes In our study similar observations were obtained for healthy controls. Here we studied TERRA from chromosome ends 1q, 9p, 10q, 11q, 15q, 16p and XpYp. The global level of TERRA molecules allowed us to discriminate between indolent and aggressive CTCL subtypes, as comparing to healthy controls, indolent CTCL (C-ALCL) expressed similar amount, while aggressive subtypes (T-MF and Sz)

expressed lower amounts. This observations both from cell lines and from Sz patients, correlates with our previous findings on CTCL telomere length, where we reported that indolent CTCL subtypes have telomere length similar to controls, while aggressive subtypes have shorter telomeres [4].

CTCL cells' TERRA profile, besides allowing distinguishing between different subtypes, also allowed us to identify a possible CTCL biomarker, TERRA 11q. Indeed, TERRA 11q is weekly expressed by healthy controls, while its expression is increased in CTCL cells. This profile also allowed us to observe that the decrease of TERRA amounts is mostly due to decreases of TERRA 9p and 16p, so we believe that these TERRA could be involved in telomere shortening. On the other hand, when we overexpressed hTERT on MyLa cells, we induced an increase of TERRA 11q, as well as cell's telomere length (data not shown). Thus, TERRA 11q could be involved in telomere lengthening in CTCL.

In conclusion, our results support that TERRA molecules are involved in CTCL lymphomagenesis and that they may play a regulator role in telomere length maintenance.

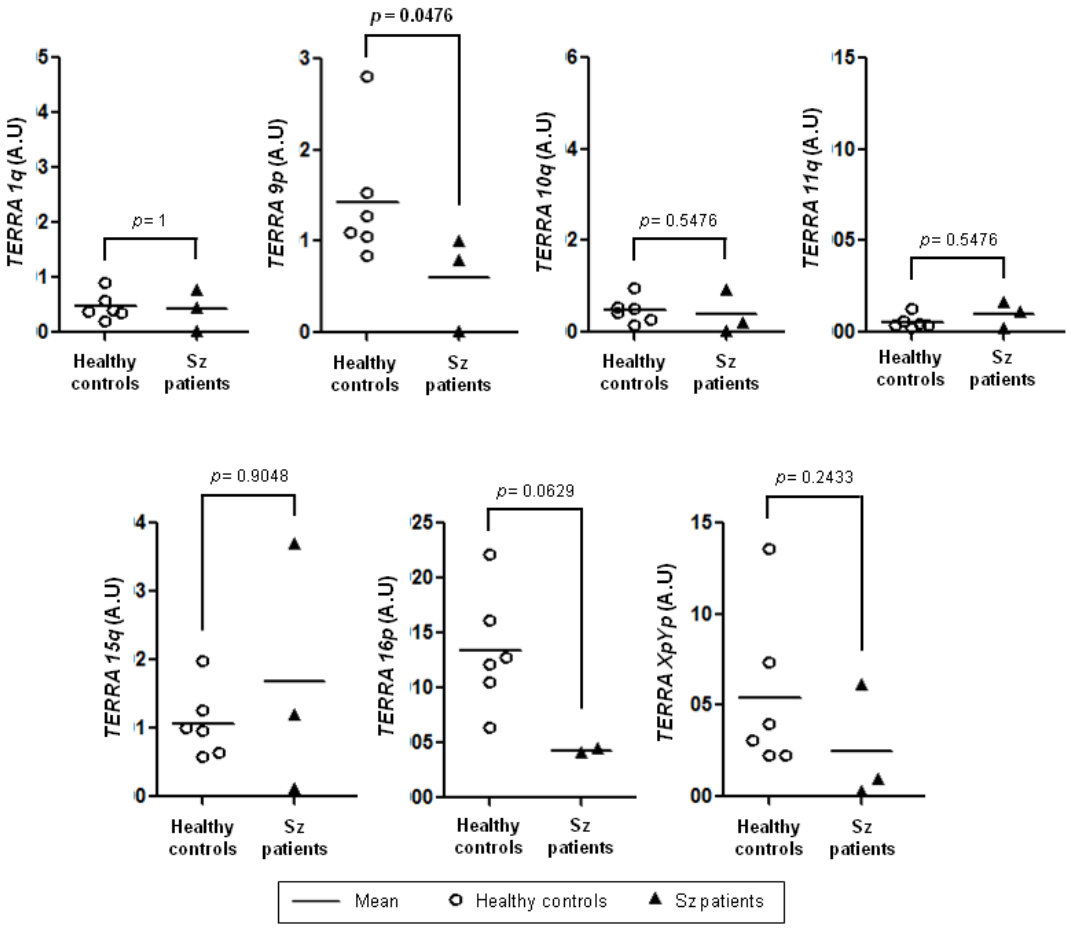
All these results must be confirmed in a larger patient cohort, and it should be interesting to explore the role of TERRA 9q, 11q and 16p in CTCL as well their potential as aggressive CTCL biomarkers.

Apart the report of POT1 mutations in 10% of CTCL, no information on Shelterin protein status is available for CTCL [5]. Thus, as deregulation of this protein complex is a common characteristic of hematological malignancies the characterization of shelterin protein status would provide further insight in telomere biology of CTCL [6]

Supplemental data

Supplemental Table 1 – Primer sequences used to TERRA and shelterin protein amplifications

Primers	Primer sequence
TERRA 1q [2]	
Fw	5'-GCATTCCTAATGCACACATGAC-3'
Rv	5'-ACCCTAACCCGAACCCTA-3'
TERRA 9p [2]	
Fw	5'-GAGATTCTCCCAAGGCAAGG-3'
Rv	5'-ACATGAGGAATGTGGGTGTTAT-3'
TERRA 10q [2]	
Fw	5'-ATGCACACATGACACCCTAAA-3'
Rv	5'-TACCCGAACCTGAACCCTAA-3'
TERRA 11q [3]	
Fw	5'-CTGATTATTCAGGGCTGCAAA-3'
Rv	5'-GCCGCATCGACGGTGAATAA-3'
TERRA 15q [7]	
Fw	5'-CAGCGAGATTCTCCCAAGCTAAG-3'
Rv	5'-AACCCTAACCACATGAGCAACG-3'
TERRA 16p [3]	
Fw	5'-TGTGTTTCAACGCTGCAACTG-3'
Rv	5'-AGTTAGAACGGTTCAGTGTG-3'
TERRA Xp-Yp [7]	
Fw	5'-GCAAAGAGTGAAAGAACGAAGCTT-3'
Rv	5'-CCCTCTGAAAGTGGACCAATCA-3'
EF1α	
Fw	5'-CTGGAGCCAAGTGCTAACATG-3'
Rv	5'-CCGGGTTTGAGAACACCAGT-3'



Supplemental Figure 1 –Sz patients TERRA transcripts

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Therapeutic targeting telomerase in CTCL

Transcriptional activity of *hTERT* promoter is a key point to telomerase expression and activity regulation (Liu, Snow et al. 2000). A number of factors have been identified to directly or indirectly regulate *hTERT* promoter, including cellular transcriptional activators (c-Myc, NF-kB, STAT proteins and Estrogen receptor, etc.) as well as repressors, most of which comprise tumor suppressor gene products, such as p53, WT1, and Menin (Ramlee, Wang et al. 2016; Ropio, Merlio et al. 2016). Some transcription factors, depending on cellular context, have dual roles, which is the case of Sp1 and AP-1 (Ramlee, Wang et al. 2016). Sp1 is known to activate *hTERT* gene expression in telomerase-positive cells but suppress it in telomerase-negative ones (Kyo, Takakura et al. 2000; Knight, Cotter et al. 2001). In telomerase-positive cells, particularly cancer cells, Sp1 may activate *hTERT* expression on its own or together with specific co-activators. For example, Sp1 may work cooperatively with c-Myc and bind their respective response elements in *hTERT* proximal promoter to upregulate *hTERT* transcription (Kyo, Takakura et al. 2000). In addition to c-Myc other factors and molecules have been reported to work together with Sp1 both to activate and repress Sp1-mediated *hTERT* gene (Ramlee, Wang et al. 2016). Epigenetic environment is important in Sp1-mediated regulation of *hTERT* gene. Indeed, in telomerase-negative human somatic cells, Sp1 binding to *hTERT* promoter recruits HDAC proteins to this region, allowing deacetylation of histone subunits which leads to *hTERT* silencing (Cheng, Zhao et al.). AP-1 is a transcription factor complex which consists of components belonging to the c-Jun, c-Fos, Activating Transcription Factor (ATF) and J Domain-Containing Protein (JDP) families. It was initially reported that AP-1 transcription factors repress *hTERT* expression, as the overexpression of its components (c-Fos and c-Jun or c-Fos and JunD) strongly represses *hTERT* promoter activity (Takakura, Kyo et al. 2005). On the other hand, ectopic expression of the viral protein HBZ and JunD was shown to activate *hTERT* promoter (Kuhlmann, Villaudy et al. 2007).

Lovastatin is a cholesterol-lowering and potential anti-neoplastic agent commonly used in clinic. This drug was recently reported to restrict cell migration, thereby intervening in crucial metastatic capabilities in colorectal cancer. It was demonstrated that lovastatin acts, in part, by impairing the binding of c-Jun and Sp1 transcription

factors to Metastasis-Associated in Colon Cancer Protein 1 (MACC1) promoter, thereby inhibiting its transcription (Juneja, Kobelt et al.).

Since there is no information on the effect of lovastatin in CTCL, we particularly investigated the effects of this agent on *hTERT* expression and on its telomere-independent functions. We intended to test this known compound as an anti-telomerase drug, thereby broadening its therapeutic value in oncology.

A new target for an old drug

A new target for an old drug

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Material and methods

Cell lines

Experiments were performed on two aggressive cutaneous T-cell lymphoma (CTCL) cell lines: MyLa, representative of transformed mycosis fungoïdes (T-MF) and HuT78, a Sézary syndrome (Sz) cell line. Cells were cultured as previously described [1] and regularly tested for Mycoplasma contamination.

hTERT overexpression

Telomerase overexpression on MyLa and HuT78 cells was performed as previously reported [1]. We produced hTERT MyLa cells and scramble MyLa (control), as well as hTERT HuT78 cells and scramble HuT78 (control) (**Figure 1**).

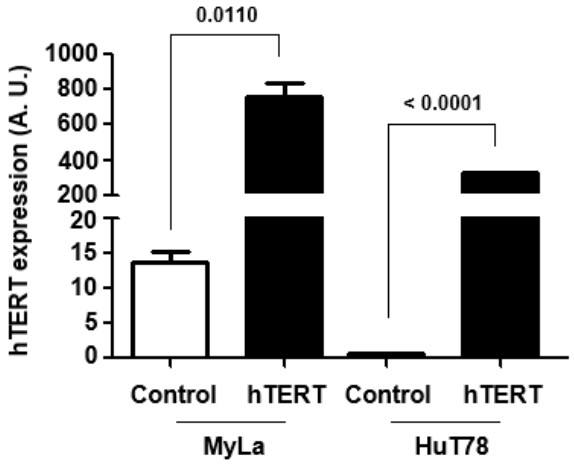

Figure 1 – hTERT overexpression in MyLa and HuT78 cell lines after lentiviral transduction

hTERT, Sp1 and AP-1 transcripts' expression

ARN isolation, reverse transcription and amplifications were described before [1]. Primer sequences used for mRNA amplification are available at **Supplemental Table 1**.

Lovastatin (LOVA)

Lovastatin was obtained from Euromedex (France) and stored at −80°C. LOVA was solubilized in dimethyl sulfoxide (DMSO). The stock solution of 10 mM was prepared and stored in small aliquots at −80°C to avoid repeated freeze thawing. To exclude adverse effects caused by DMSO, control cells were always treated with an equal amount of the solvent.

Cell viability analysis

Cell viability was assessed by flow cytometry on FACS Canto II cytometer (BD Biosciences) and quantified using FlowJo software (FlowJo®). Apoptotic/necrotic cell proportion was measured using annexin VePE antibody (BD Biosciences), according to manufacturer's recommendations, and Hoechst 33342 (H3570; Molecular Probes, Eugene, OR) was added 5 minutes before sample acquisition.

Xenografting of tumor cells in immunodeficient mice

Xerograph of CTCL tumor cells in NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice were performed accordingly to our established protocol [2] (**Figure 2**).

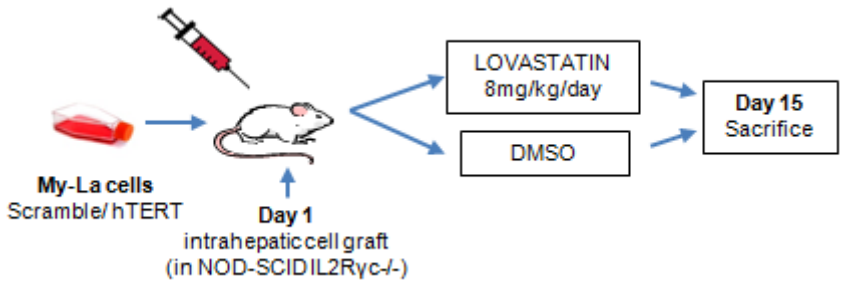

Figure 2 – Schematic representation of tumor cells xenografted in immunodeficient mice

Intrahepatic injections of hTERT MyLa cells and their controls were performed in 20 adult NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mices. Afterwards they were treated every day with 8mg/Kg of lovastatin/DMSO. After two weeks all mice were sacrificed and human cancer cells (CD30+/HLA) dissemination was estimated by flow cytometry in single-cell suspensions from mice organs.

Statistics

Statistical analysis was performed with GraphPad Prism 5.01. RT-qPCR data were collected from triplicate reactions. Data from *in vitro* functional assays were collected from experiences performed in quadruplicates. Results were presented as mean ± standard deviation. Paired Mann-Whitney test (nonparametric *T*-test) was used to compare variables. The significance level was set as $p = 0.05$.

Results

1. Lovastatin impairs dissemination capacities of CTCL cells *in vivo*.

Taking advantage of our established intrahepatic mouse model to screen therapeutic molecules [2], we were able to study the *in vivo* effect of lovastatin on tumorigenic capacities of MyLa cells (**Figure 3**).

We observed that at the liver, the injection site, lovastatin produced no effect on MyLa controls, while hTERT MyLa cells population was significantly decreased (**Figure 3A**). Few MyLa cells disseminated to the spleen, even when overexpressing *hTERT* (**Figure 3B**). We observed that MyLa cells subtly migrated to the lungs. The impact of lovastatin on MyLa cell dissemination to these two organs was not obvious. (**Figure 3C**). MyLa cells strongly disseminated to the kidneys after *hTERT* overexpression. Lovastatin statistically decreased this ability (**Figure 3D**).

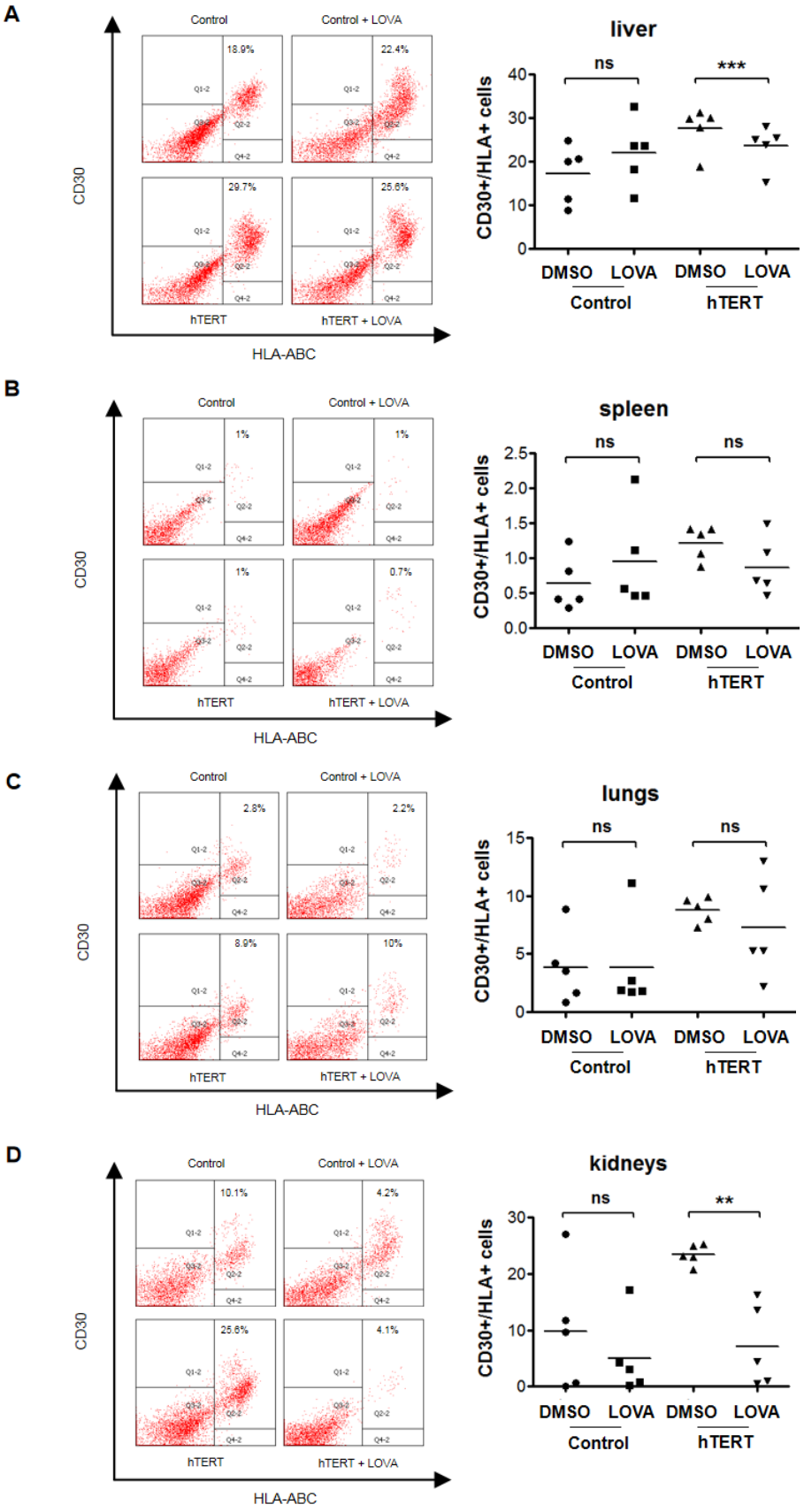

Figure 3 – *in vivo* lovastatin impact on tumorigenic properties

The impact of lovastatin (LOVA) on MyLa cells was analyzed through xenograph of immunodeficient mice.

2. Lovastatin impairs cell viability, and induces cell death of CTCL cells

The *in vitro* impact of lovastatin on MyLa and HuT78 cells' viability was assessed by flow cytometry. This analysis allowed us not only to assess cell viability, but also to check the induction of cell death (**Figure 4**). HuT78 cells were more sensitive than MyLa cells to lovastatin treatment. After 48h HuT78 were all almost dead, while this was observed for MyLa cells only after 72 hours (data not shown).

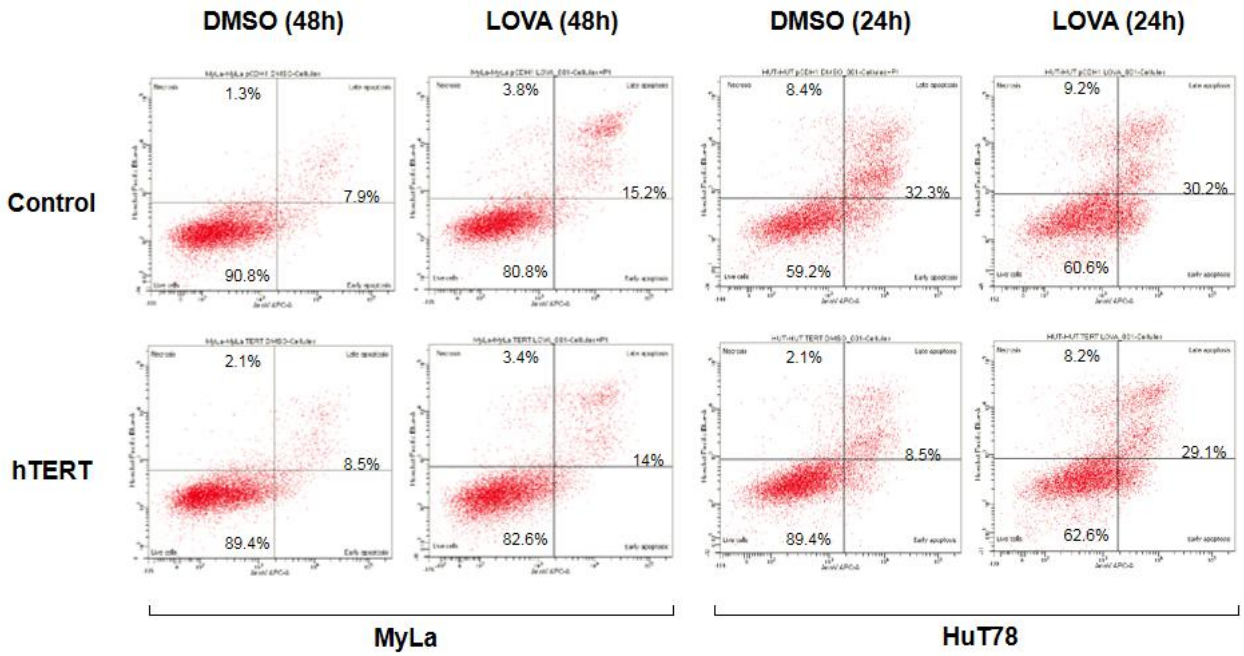

Figure 4- *in vitro* lovastatin impact on cell viability

The impact of lovastatin (LOVA) treatment on MyLa and HuT78 cells was analyzed by flow cytometry.

On MyLa controls, lovastatin induced a 10% decrease of cell viability as it induced cell death both by apoptosis and necrosis. On hTERT MyLa cells lovastatin produced a 7% decrease of cell viability through the induction of cell death, mostly by apoptosis. On HuT78 controls, lovastatin produced no effect on cell viability. On the other hand, lovastatin induced a 30% decreased of hTERT HuT78 cells' viability, as cells entered in cell death both by apoptosis and by necrosis (**Figure 4**).

3. Lovastatin impacts *hTERT* expression in CTCL

We investigated the effect of lovastatin on MyLa and HuT78 cells overexpressing hTERT as well as, in their respective controls (**Figure 5**).

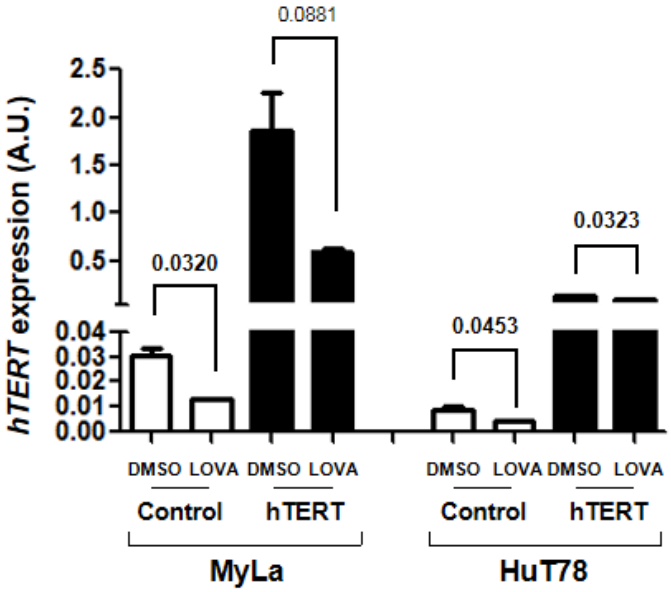

Figure 5. Lovastatin impact on *hTERT* expression

The impact of lovastatin (LOVA) on MyLa and HuT78 cells was analyzed by RT-qPCR.

Lovastatin decreased *hTERT* expression in control MyLa cells (p = 0.0320) as well as in hTERT MyLa cells (p = 0.0881). The same effect was observed on HuT78 cells, as we observed a decrease of *hTERT* expression in control HuT78 cells (p = 0.0453), as well as in hTERT HuT78 cells (p = 0.0323).

4. Indication that *hTERT* transcription regulation is sub-type specific

The expression of Sp1, as well as some c-Jun (c-Jun and JunD) and c-Fos family (c-Fos and Fra2) members were analyzed by RT-qPCR in wild type MyLa and HuT78 cells (**Figure 6**).

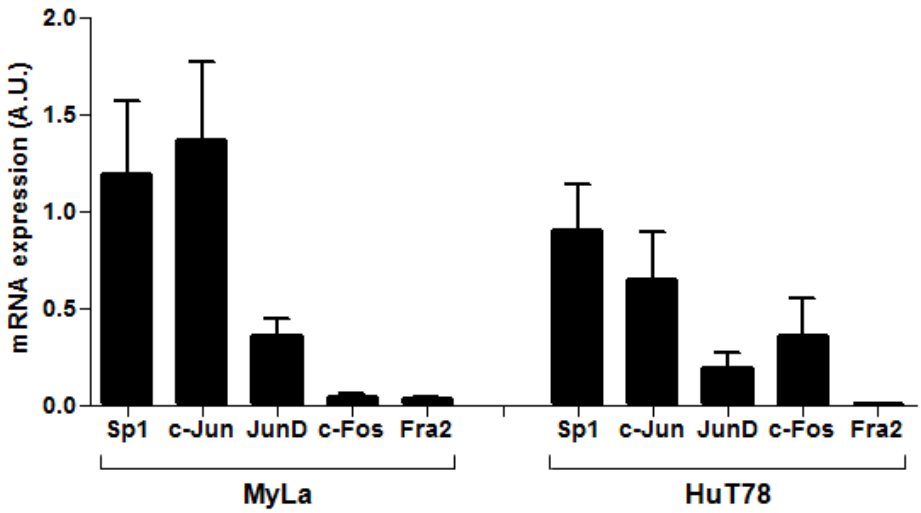

Figure 6. Expression of transcription factors that regulate *hTERT* transcription

The expression of Sp1, as well as some c-Jun and c-Fos family members was analyzed by RT-qPCR in MyLa and HuT78 cells.

MyLa cells expressed high levels of Sp1 and c-Jun transcription factors and to, while very low levels of c-Fos and Fra2 were detected. HuT78 cells expressed high levels of Sp1, c-Jun, JunD and c-Fos transcription factors, while expression of Fra2 transcription factor was not detected.

Discussion and perspectives

The main canonical function of telomerase concerns its mechanisms of action related with telomere length maintenance. However, additional non-telomeric roles related with tumorigenesis and cancer cell properties emerged from its catalytic subunit, *hTERT* [3-5]. CTCL are tumors in which, besides maintenance of telomere length, telomerase exerts additional functions [1]. Therefore, telomerase provides an attractive therapeutic target to CTCL treatment. Here, we investigated the potential of *hTERT* as a therapeutic target for CTCL treatment. This is of particular interest since *hTERT* expression is a transversal characteristic of CTCL cells [1]. We tested lovastatin, a commonly used agent in clinic, for CTCL treatment and assess its effects on *hTERT* expression as well as on its telomere-independent functions in two cell lines representative of aggressive CTCL sub-types (T-MF and Sz). We demonstrated that lovastatin induced a decrease of *hTERT* expression in CTCL cells. We also showed that lovastatin decreased *in vitro* cell viability and dissemination cell

capacities *in vivo*. Furthermore, given the known contribution of *hTERT* overexpression to disease aggressiveness [1], we can assume that their decrease after lovastatin treatment is in part related to hTERT. However, the possibility of other lovastatin targets contributing to reduction in the aggressive phenotype still prevails.

hTERT transcription activation is a major key event regulating hTERT expression. It is a multi-factorial process thus the contribution of transcription factors cannot be neglect, even if none of them clearly account for cancer-specific *hTERT* expression [6, 7]. Sp1 and AP-1 transcription factors are important for *hTERT* regulation [8]. Sp1 is a transcription factor that binds to GC-box motifs, while AP-1 transcription factor consists of either Jun/Jun homodimers or Fos/Jun heterodimeric complexes [9, 10]. Homo- and hetero-dimers bind to numerous promoters, including *hTERT* [10]. Our preliminary investigation on these transcription factors may be indicative that *hTERT* transcription regulation is mediated by different homo- and hetero-dimers of AP-1 transcription factors in different CTCL subtypes. Nevertheless, we did not investigate all c-Jun and c-Fos family members neither their physical binding to *hTERT* promoter. Lovastatin were found to act in part, by impairing the binding of c-Jun and Sp1 transcription factors to a gene promoter [11]. Thus lovastatin *hTERT* transcription impairment in CTCL cells may be due to this mechanism. This has to be confirmed on CTCL cells. We planned to acess this problematic by ChIP-qPCR, in collaboration with Jean-Marie Peloponnese.

These results are a first clue, to our knowledge, pointing for a statin as a therapeutic agent for CTCL treatment.

Supplemental data

Table 1 – Primer sequences used to RT-qPCR amplification

Primers	Primer sequence
cFOS	
Fw	5'- CCAACCTGCTGAAGGAGAAG -3'
Rv	5'- AGATCAAGGGAAGCCACAGA -3'
FOSB	
Fw	5'- TTGCACCTTACTTCCCCAAC -3'
Rv	5'- AGGAGTCCACCGAAGACAGA -3'
FRA1	
Fw	5'- GCCCACTGTTTCTCTTGAGC -3'
Rv	5'- GATGGAGAGTGTGGCAGTGA -3'
FRA2	
Fw	5'- CCTCCATGTCCAACCCATAC -3'
Rv	5'- GACGCTTCTCCTCCTCTTCA -3'
cJUN	
Fw	5'- GCAGCCCAAACTAACCTCAC -3'
Rv	5'- TAGCCATAAGGTCCGCTCTC -3'
JUNB	
Fw	5'- TGGAACAGCCCTTCTACCAC -3'
Rv	5'- AGGCTCGGTTTCAGCAGTTT -3'
JUND	
Fw	5'- CGCCTGGAAGAGAAAGTGAA -3'
Rv	5'- GTTGACGTGGCTGAGGACTT -3'
hTERT	
Fw	5'- GCATTGGAATCAGACAGCAC -3'
Rv	5'- CCACGACGTAGTCCATGTTC -3'
TBP	
Fw	5'- CACGAACCACGGCACTGATT -3'
Rv	5'- TTTTCTTGCTGCCAGTCTGGA -3'

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Conclusion-Perspectives

Cutaneous T-cell lymphomas (CTCL), although a rare disease, have been increasing over the years. Cutaneous lymphomas are the second most common group of extra-nodal non-Hodgkin lymphomas, with as estimated incidence of 1/100000 in Western countries (Willemze, Hodak et al.). CTCL diagnosis may be difficult during the early stages, when the disease presents with an indolent clinical behavior, because they share features with common dermatologic conditions (Bagherani and Smoller). Even if CTCL in vast majority of cases presents as an indolent disease, they can progress to tumor stages with the involvement of internal organs, presenting though an aggressive clinical behavior (Willemze, Jaffe et al. 2005). Some patients however, may present at diagnosis a CTCL with aggressive behavior, which is characterized by high heterogeneity of tumor cells.

hTERT is a central player in development and progression of many cancers, and there are evidences to such an involvement in CTCL. In this work we aimed to deepen knowledge in *hTERT* regulating mechanisms to try to bring to light the contribution of this gene to CTCL lymphomagenesis. Hence, it may help to identify biomarkers which can help the diagnosis and to identify patients at risk for progression as well to find targets for effective therapy solutions against CTCL.

The *TERT-CLPTM1L* region of chromosome 5p15.33 is a multi-cancer susceptibility locus that encodes the reverse transcriptase subunit, *hTERT*, of the telomerase enzyme. Numerous cancer-associated single-nucleotide polymorphisms (SNPs) have been identified within the *hTERT* gene. Here we showed that SNPs in the promoter and gene coding region associates with risk for CTCL. Our results must be confirmed in larger population before adding CTCL to the list of cancers affected by *hTERT* SNPs.

Activation of *hTERT* transcription is a major step controlling *hTERT* expression (Akincilar, Unal et al. 2016). Based on our results, we can exclude *hTERT* promoter mutations as a mechanism involved in *hTERT* transcription activation in CTCL. In hematological malignancies, the epigenetic plasticity of *hTERT* promoter is a determinant mechanism controlling telomerase expression (Ropio, Merlio et al. 2016). Epigenetic regulation seems to play an important role in CTCL. Indeed, romidepsin, a

potent histone deacetylase (HDAC) inhibitor was approved by the Food and Drug Administration (FDA) for the treatment of relapsed/refractory CTCL patients showing an overall response rate of 34% to 35% (Bates, Robey et al.). Furthermore, the combination of romidepsin and azacitidine (a DNA methyltransferase inhibitor) exerts synergistic antiproliferative effects and induction of apoptosis in CTCL cell lines and tumor cells derived from Sézary syndrome patients (Rozati, Cheng et al.). However, the epigenetic context of *hTERT* is yet to be explored, as well as its contribution to responses of CTCL cells to epigenetic regulators.

While the mechanism underlying telomerase expression is still unknown, we highlighted the major role of post-transcriptional mechanisms regulating telomerase non canonical functions in CTCL. Indeed, by modulating *hTERT* splicing pattern we induced specific biological effects on *in vitro* CTCL cells' proliferation, viability and tumorigenic capacities. Hence, we showed that *hTERT* splicing mechanisms are related with disease aggressiveness, which provides a rational to try to target this mechanism. Indeed, modulating the mode of hTERT pre-mRNA splicing is providing a new precept of therapy for cancer and aging-related diseases (Liu, Wang et al. 2017). Ligand 12459 (triazine derivative) was reported to inhibit telomerase activity through the decrease of α+β+ transcript while increasing α+β-. In addition, ligand 12459 was also reported to stabilize the formation of G-quadruplex structures. Thus, 12459 could inhibit telomerase activity through the stabilization of G-quadruplex structures on the telomere ends (Gomez, Lemarteleur et al. 2004). Chemically modified antisense oligonucleotides were also reported to potentially change the splicing patterns of hTERT pre-mRNA. 2′-O-methyl-Phosphorothioates (2′-O-methyl-PTOs) complementarily combine on the splicing site located at the junction of intron 5 and exon 6 in the hTERT pre-mRNA, inducing a decrease of α+β+ transcript while the α-β+ and α-β-transcripts increased. The change in the hTERT pre-mRNA splicing pattern consequently led to a remarkable suppression of telomerase activity, cell growth, and apoptosis (Brambilla, Folini et al. 2004). To our knowledge this kind of therapeutic approaches were not tested in CTCL.

We tested a commonly clinical agent on CTCL cells and we obtained very promising results. Indeed, we demonstrated that lovastatin impairs *hTERT* expression as well as it induces a decrease of cell viability *in vitro* and cell dissemination *in vivo*. Given the known contribution of *hTERT* overexpression to disease aggressiveness

(Chevret, Andrique et al.), we can assume that the decrease of aggressive cancer cell abilities after lovastatin treatment is in part related to hTERT. The molecular mechanism behind lovastatin effect on CTCL cells is yet to be investigated. Our first clue is based on the effect of lovastatin on colorectal cancer. Indeed, lovastatin was shown to restrict metastatic capacities of colorectal cancer in part by impair the binding of c-Jun and Sp1 transcription factors to MACC1 promoter, thereby inhibiting its expression (Juneja, Kobelt et al.).

Despite their heterochromatic state, telomeres are transcribed giving rise to long noncoding RNAs called TERRA (telomeric repeat-containing RNA). TERRA molecules play critical roles in telomere biology and here we showed that they are involved in CTCL lymphomagenesis. Based on our results, TERRA seems to regulate telomere length-dependent telomerase activity. Functional studies to confirm the role of individual TERRA molecules are required to validate our hypothesis. Shelterin association contributes to telomere stability and prevents unwanted DNA-damage repair. Alteration in the structure and function of any of shelterin components may contribute to initiation and progression of cancer, and so it will be of great interest to understand their impact on CTCL lymphomagenesis. Furthermore, POT1 was found mutated, in 10% of CTCL patients, stimulating telomere elongation and telomere dysfunction (Pinzaru, Hom et al.). In addition to shelterin, an increasingly large number of proteins are associated with telomeres. These proteins also perform crucial functions, highlighted by the fact that they are altered or mutated in several fatal degenerative syndromes as well as cancers (Kalan and Loayza ; Patel, Vasan et al. ; Sarek, Marzec et al. ; Stewart, Chaiken et al.). The complete protein composition present at CTCL telomeres is not known, nor are defined the changes that occur during tumorigenesis in telomeric chromatin composition. Arguably, one of the most important challenges of the telomere field is to define these changes and characterize their functions.

Altogether the present work provides valuable insight into telomere biology of CTCL. We contributed to the identification of crucial regulating mechanisms of telomerase as well as we identified new players in CTCL lymphomagenesis, providing new fields of research.

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Title: Telomere biology of cutaneous T-cell lymphomas

Abstract : Telomere biology plays critical roles in cellular homeostasis. They are at the borderline between tumor suppression and tumor initiation. Although telomere dysfunction resulting from replicative attrition constrains tumor growth by engaging DNA-damage signaling pathways, it can also promote tumorigenesis. Expression of telomerase enables telomere-length homeostasis and allows tumor cells to escape the antiproliferative barrier posed by short telomeres. Telomere biology is involved in lymphomagenesis of Cutaneos T-cell lymphomas (CTCL), thus we interested in the contributions of hTERT, the catalytical subunit of telomerase, and telomeric repeat-containing RNA (TERRA) to disease initiation and/or progression.

We investigated transcriptional and post-transcriptional regulating mechanism of hTERT and we highlighted the major role of alternative mRNA splicing in regulating telomerase non canonical functions in CTCL. We unveiled the participation of TERRA in CTCL lymphomagenesis, which seems to regulate telomere length-dependent telomerase activity. We finally tested the value of lovastatin, a cholesterol-lowering and potential anti-neoplastic agent commonly used in clinic, as an anti-telomerase drug, on CTCL cells. Lovastatin impaired hTERT transcription, decreasing cell viability *in vitro* and cell dissemination *in vivo*. Altogether these data provide important insights into telomere biology of CTCL. We contributed to the identification of crucial regulating mechanisms of telomerase as well as we identified new players in CTCL lymphomagenesis, providing new fields of research.

Keywords : CTCL, telomeres, telomerase, hTERT, TERRA

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