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L'épigénétique comme modulateur de l'expression de hTERT dans les lymphomes T cutanés

Alain Chebly

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THÈSE EN COTUTELLE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX
ET DE L'UNIVERSITÉ SAINT-JOSEPH DE BEYROUTH

ÉCOLE DOCTORALE SCIENCES DE LA VIE ET DE LA SANTE

ÉCOLE DOCTORALE SCIENCES ET SANTE

SPÉCIALITÉ : GENETIQUE

Par **Alain CHEBLY**

**L'épigénétique comme modulateur de l'expression de *hTERT*
dans les lymphomes T cutanés**

Sous la direction de : **Edith CHEVRET**
et de **Roland TOMB**

Soutenue le 15 décembre 2020

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Liste des abréviations :

- ALT : mécanisme alternatif d'allongement des télomères
- ATLL : leucémie / lymphome T adulte
- C-ALCL: Primary cutaneous anaplastic large cell lymphoma
- CD : Classe de différenciation
- CNA : Copy Number Alteration
- CST : Cdc13/Ctc1, Stn1, Ten1
- CTCF : CCCTC-binding factor zinc finger protein
- CTCL : Cutaneous T-cell lymphomas
- DDR : Réponse aux dommages à l'ADN
- DNMT : ADN méthyltransférases
- DNMTi : DNMT inhibitor
- DSS : Disease-specific survival
- EORTC: European Organization for Research and Treatment of Cancer
- ETS : E-twenty-six
- HAT : histones acétyltransférases
- HDAC : Histone désacétylases
- HMT : Histones méthyltransférases
- hMZF-2 : human Myeloid Zinc Finger protein 2
- ISCN : The International System for Human Cytogenetic Nomenclature
- LCP : Lymphomes Cutanés Primitifs
- LTC : Lymphomes T cutanés
- LTCE : Lymphomes T cutanés épidermotropes
- LDH : lactate déshydrogénase
- MF : Mycosis Fungoïde
- MFT : MF Tumor stage
- MZF : Myeloid Zinc finger protein
- ncARN : ARN non-codants
- NCCN : National comprehensive cancer network
- NGS : Next Generation Sequencing
- NHEJ : Non-homologous end joining
- OS : Overall survival
- PDC : Patient derived cells
- POT1 : Protection of telomeres protein 1
- PTCL : Lymphomes périphériques à cellules T
- RAP1 : Ras-proximate-1 / Ras-related protein 1
- RDP : Risk of disease progression
- RT : Reverse transcriptase
- SNV : Single Nucleotide Variant
- SS : Syndrome de Sézary

- TERC : TELomerase RNA Component
- TERT : TELomerase Reverse Transcriptase
- TIN2 : TRF1-interacting protein 2
- TCF : Fréquence du clone tumoral
- TCR : T cell receptor
- THOR : TERT Hypermethylated Oncogenic Region
- TPP1 : Tripeptidyl Peptidase 1
- TRF1 : Telomeric Repeat-Binding Factor 1
- TRF2 : Telomeric Repeat-Binding Factor 2
- TRFH : domaine d'homologie entre TRF1 et TRF2
- TSS : Transcription start site
- WGS : Whole genome sequencing
- WHO: World Health Organization
- WT1 : Wilms' tumor 1

Valorisation du travail de thèse :

- **Articles**

1. **Chebly A**, Prochazkova-Carlotti M, Idrissi Y, Bresson-Bepoldin L, Poglio S, Farra C, Beylot-Barry M, Merlio JP, Tomb R and Chevret. hTERT promoter behavior under HDACi influence in Sézary syndrome. *In preparation*
2. **Chebly A**, Ropio J, Peloponèse JM, Poglio S, Prochazkova-Carlotti M, Cherrier F, Ferrer J, Idrissi Y, Segal-Bendirdjian E, Chouery E, Farra C, Pham-Ledard A, Beylot-Barry M, Merlio JP, Tomb R and Chevret E. hTERT promoter methylation in cutaneous T-cell lymphomas. Promoter methylation: A player in the activation of TERT in cutaneous T-cell Lymphomas. *Under Revision*
3. **Chebly A**, Prochazkova-Carlotti M, Idrissi Y, Bresson-Bepoldin L, Poglio S, Farra C, Beylot-Barry M, Merlio JP, Tomb R and Chevret E. 5-azacytidine reduces the clonogenic capacities of SS cells. *International Journal of molecular sciences. Under Revision*
4. **Chebly A**, Chouery E, Ropio J., Kourie HR, Beylot-Barie M, Merlio JP, Tomb R and Chevret E. Diagnosis and treatment of lymphomas in the era of epigenetics. *Blood Reviews 2020, Article number 100782 in Press. Accepted*
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- **Oral Presentation**

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- **E-Poster**

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1. Introduction

1.1. *Les lymphomes cutanés primitifs*

1.1.1. *Prévalence et sous-types de la maladie*

Les lymphomes cutanés à cellules T (CTCL) regroupent un ensemble hétérogène de lymphomes non hodgkiniens issus d'un infiltrat de cellules T monoclonales au niveau de la peau et sans évidence d'extension extra-cutanée au moment du diagnostic (Kempf et al., 2019; Van-de-Velde and Zhou, 2019; Willemze et al., 2019).

Contrairement aux lymphomes ganglionnaires, les lymphomes T représentent la majorité des lymphomes cutanés primitifs (LCP). Dans les pays occidentaux, ils comptent pour environ 75% à 80% de tous les LCP (Willemze et al., 2019). Ces différents types de lymphomes arborent des caractéristiques cliniques et histologiques spécifiques. Généralement, leurs comportements cliniques et leur pronostic sont très différents de ceux des lymphomes ganglionnaires morphologiquement similaires et qui peuvent impliquer secondairement la peau. A ce titre, depuis 2005, les LCP sont inclus comme des entités à part entière dans les classifications des lymphomes : the World Health Organization–European Organization for Research and Treatment of Cancer (WHO-EORTC) 2005 et ses révisions en 2008, 2016, 2018 (Willemze et al., 2019, 2005). Les formes épidermotropes dans lesquelles les cellules migrent et colonisent l'épiderme, constituent l'entité prédominante des CTCL. Elles regroupent essentiellement le Mycosis Fungoïde (MF) et le Syndrome de Sézary (SS) (Kim et al., 2005).

Les lymphomes T cutanés primitifs peuvent s'observer chez les enfants et les adolescents, mais typiquement ils surviennent chez les personnes âgées avec un âge médian au diagnostic de 50 à 60 ans et un nombre moyen de 6,4 nouveaux cas par an et par million d'habitants (Wilcox, 2017). L'incidence de survenue d'un CTCL augmente avec l'âge, avec la plus forte incidence après 70 ans.

Parmi les CTCL, le MF est le plus fréquent, il représente environ 55% (Willemze et al., 2005). Il touche préférentiellement les hommes (sexe ratio : 1,8) de tous âges et de toutes

origines ethniques. Son incidence annuelle est estimée à 0.5/100 000, ce qui représente environ 260 nouveaux patients adultes par an en France ("Prévalence des maladies rares 2019". https://www.orpha.net/orphacom/cahiers/docs/FR/Prevalence_des_maladies_rares_par_prevalence_decroissante_ou_cas.pdf).

Le SS représente moins de 5% des CTCL (Willemze et al., 2005). Le SS se développe le plus fréquemment chez les hommes, souvent après 50 ans, et progresse rapidement. Le SS a une incidence annuelle estimée à 1/10 000 000 (Bagot, 2013). Le SS est une forme leucémique, en conséquence il est défini comme une maladie généralisée. La survie moyenne des patients atteints de SS est de 32 mois, avec une survie à 5 ans de 10 à 30%, en fonction du stade de la maladie (Willemze et al., 2005).

Le lymphome cutané primitif anaplasique à grandes cellules (Primary cutaneous anaplastic large cell lymphoma [C-ALCL]) est le deuxième sous-type le plus fréquent de CTCL après la MF (Willemze et al., 2019, 2005). L'âge moyen au diagnostic est de 60 ans avec un ratio hommes/femmes de 2-3:1 (Bekkenk et al., 2000). Le pronostic est généralement favorable, avec une survie à 10 ans chez 90% des patients (Bekkenk et al., 2000; Liu et al., 2003).

1.1.2. **Diagnostic des MF/SS**

Le diagnostic de MF/SS peut être délicat. Il nécessite la contribution des données cliniques, de l'évaluation pathologique et des études moléculaires. Les MF/SS peuvent ressembler à des dermatoses inflammatoires bénignes, et les caractéristiques histologiques spécifiques du MF peuvent être absentes au début de la maladie, même sur plusieurs biopsies. De plus, la PCR du récepteur des lymphocytes T (TCR), utilisée pour identifier la présence d'un clone de lymphocytes T dans les échantillons cliniques, présente un taux significatif de faux négatifs au stade précoce de la maladie (Kirsch et al., 2015). Des algorithmes sont proposés pour aider le clinicien dans l'établissement du diagnostic (Willemze et al., 2018). Ainsi, par exemple les recommandations pour la classification initiale des patients atteints de MF/SS sont présentées dans le tableau 1. La cytométrie en flux des cellules sanguines périphériques est recommandée pour tous les stades de MF. Cependant, elle est discutable pour les patients non-suspectés d'avoir un SS.

Tableau 1 : Les recommandations pour l'évaluation des stades des patients MF/SS (Willemze et al., 2018)

Examen physique complet : <ul style="list-style-type: none">- Types des lésions cutanées- Identification des ganglions palpables (surtout >1.5cm)- Identification des organomégalies
Biopsie cutanée : <ul style="list-style-type: none">- La zone la plus indurée- Histologie de routine et immunophénotypage- Evaluation de la clonalité T (réarrangement TCR) (optionnelle)
Examens sanguins : <ul style="list-style-type: none">- Formule numération sanguine FNS, bilan hépatique, LDH- Réarrangement TCR et relation avec le clone cutané (optionnel)- Analyse des lymphocytes anormaux par comptage des cellules de sézary avec détermination du nombre absolu et/ou cytométrie en flux (inclus CD4+/CD7- ou CD4+/CD26-) (optionnelle)
Tests radiologiques : <ul style="list-style-type: none">- CT scan thoracique et abdomino-pelvien (optionnel chez les MF de stade précoce)
Biopsie ganglionnaire : <ul style="list-style-type: none">- Biopsie excisionnelle (ganglion > 1.5cm ou irrégulier)- Histologie de routine, immunohistochimie et analyse des réarrangements TCR

Pour aider au diagnostic, des critères pathologiques ont été proposés ; mais dans la pratique clinique ils sont peu utilisés (Guitart et al., 2001). À ce jour, il n'existe pas de marqueurs moléculaires de diagnostic utilisés en clinique pour identifier de manière fiable les lymphocytes T malins des lymphocytes T bénins. Cependant, l'expression de TOX, une protéine HMG-box associée à la sélection des thymocytes, peut-être utile (Kempf, 2016; Litvinov et al., 2017, 2015; Zhang et al., 2012).

Il existe trois stades morphologiques cliniques dans le diagnostic du MF (Figure 1 et 2)
(Larocca and Kupper, 2019; Olsen et al., 2007, 2011) :

- Le patch
- La plaque
- Et la tumeur

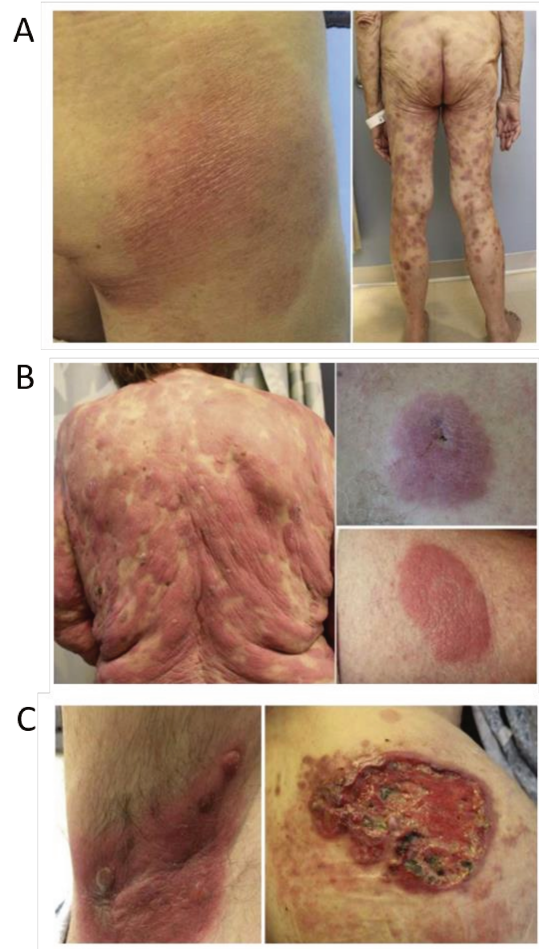


Figure 1 : (A) Patch-stage MF, (B) Plaque-stage MF et (C) Tumor-stage MF (Larocca and Kupper, 2019).

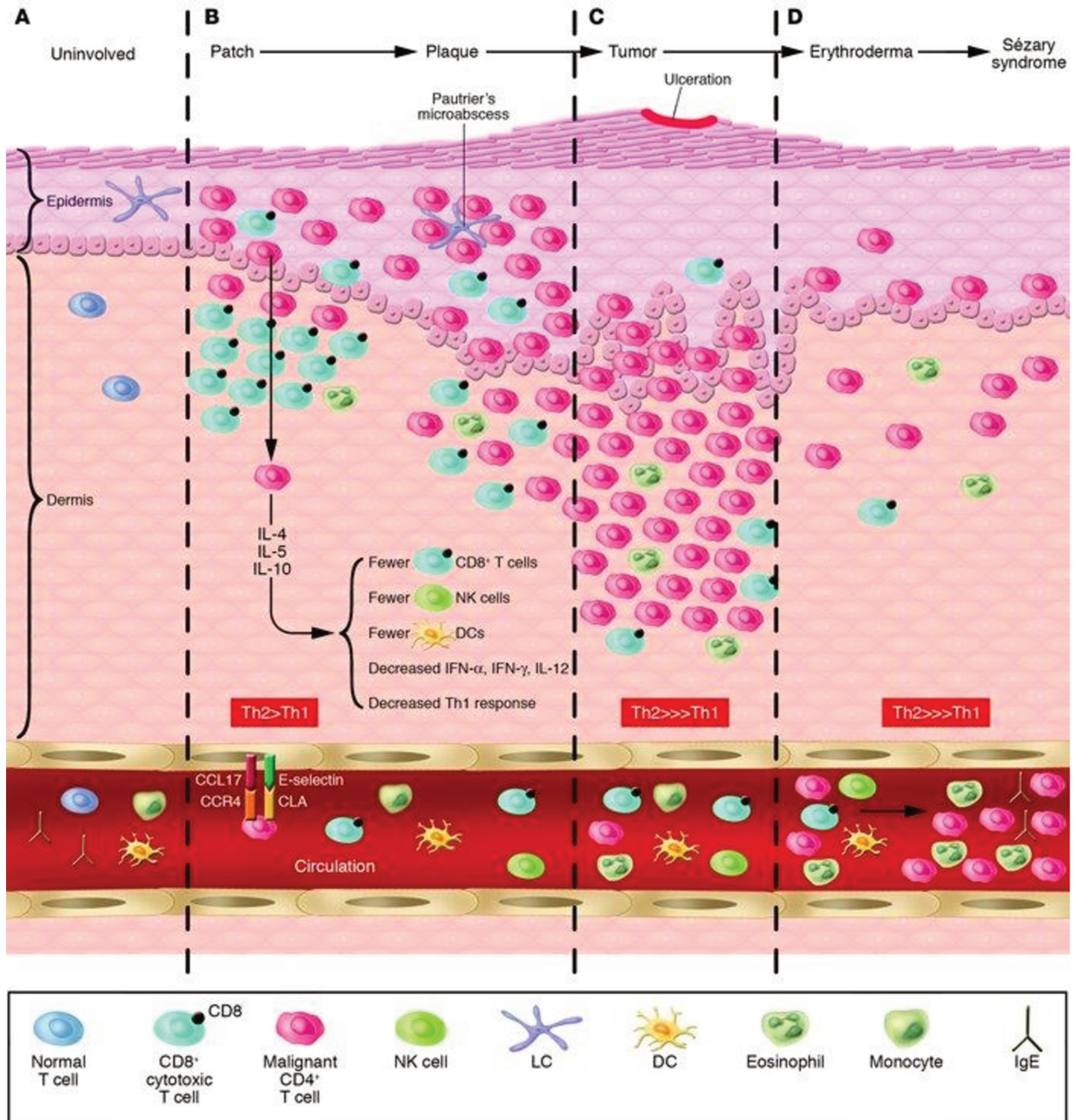


Figure 2 : La peau et le microenvironnement dans la progression du MF. (A) Une peau normale montrant les cellules de Langerhans dans l'épiderme et les cellules T dans le derme et dans la circulation. (B) Les stades MF patches et plaques, dans lesquels les cellules T malignes CD4+ se dirigent vers l'épiderme (épidermotropisme) et se rassemblent autour des cellules de Langerhans. A noter que durant ces stades, l'infiltrat épidermique et dermique contient abondamment des cellules CD8+ faisant partie de la réponse immunitaire de l'hôte. (C) Le stade MF tumorale, dans lequel la tumeur occupe le derme et le tissu subcutané et cette tumeur est composée principalement de cellules T malignes et peu de cellules T CD8+. (D) MF érythrodermique et SS (qui peut se développer à partir plusieurs années de MF) avec des cellules T malignes circulantes qui relargent des cytokines Th2 affectant le nombre et les fonctions des cellules T CD8+, cellules NK et cellules DC, et par conséquent la réponse immunitaire du hôte (Kim et al., 2005).

Chaque stade se distingue du stade précédent par une augmentation de l'épaisseur des lésions. Il existe trois sous-types de MF reconnus par l'Organisation mondiale de la santé (OMS - WHO) / Organisation européenne pour la recherche et le traitement du cancer (EORTC) avec différentes caractéristiques cliniques et histologiques (Tableau 2) (Cerroni et al., 2002; Haghighi et al., 2000; Willemze et al., 2005).

Tableau 2: Les 3 sous-types de MF (Willemze et al., 2005)

Les sous-types du MF :
Le MF folliculotropique <i>Folliculotropic MF</i>
La réticulose pagétoïde <i>Pagetoid reticulosis</i>
La chalazodermie granulomateuse <i>Granulomatous slack skin</i>

Les cliniciens doivent également être conscients de l'existence de plusieurs variantes clinicopathologiques distinctes du MF (Tableau 3) (Ahn et al., 2014).

Tableau 3 : Les différentes variantes du MF (Ahn et al., 2014)

Les variantes clinicopathologiques du MF (MF variants) :
Bullous
Hypopigmented
Ichthyosiforme
MF palmaris et plantaris (keratoderma-like)
Pigmented purpuric dermatosis-like
Papular
Poikilodermatous
Psoriasiform
Pustular
Solitary/unilesional
Syringotropic
Verrucoid

Les patients atteints de SS présentent généralement une érythrodermie, définie comme un érythème diffus touchant au moins 80% de la surface corporelle (Willemze et al., 2005). L'érythrodermie seule n'est pas un critère de diagnostic du SS. Les patients SS doivent être distingués des autres causes bénignes d'érythrodermie (Tableau 4) (Larocca and Kupper, 2019). Le Syndrome de Sézary est défini par une triade : érythrodermie, lymphadénopathie généralisée et présence de cellules T néoplasiques avec un noyau cérébriforme (cellules de Sézary, figure 3) dans la peau, les ganglions lymphatiques et dans le sang périphérique.

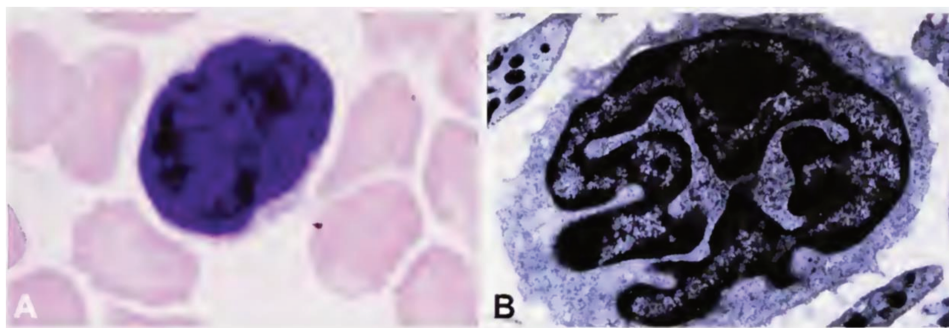


Figure 3: La morphologie d'une cellule de Sézary colorée au Giemsa (A) et ultrastructure d'une cellule de Sézary (B). (WHO, 2016)

Tableau 4: Les diagnostics différentiels devant une érythrodermie (Larocca and Kupper, 2019)

Le diagnostic différentiel des érythrodermies :
Idiopathique
Dermatite atopique
Psoriasis
Pityriasis rubra pilaris
SS
Allergie systémique
Dermatite de contact

1.1.3. *Définition du stade et pronostic*

Le stade clinique est un déterminant important du risque de progression de la maladie (RDP « risk of disease progression ») et de la survie globale (OS « overall survival ») (Agar et al., 2010).

Le recueil des données ont permis d'établir que (Agar et al., 2010) (Tableaux 5 et 6) :

- Les patients de stade IA ont une survie médiane de 35,5 ans et une survie spécifique à la maladie (DSS « disease-specific survival ») de 90% à 20 ans, ce qui est comparable aux patients sans MF. Bien que ces patients aient une évolution de la maladie indolente, à 20 ans ils ont un RDP de 18%.
- Les patients au stade IB ont une survie médiane de 21,5 ans, un DSS de 67% et un RDP de 47% à 20 ans.
- Les patients de stade IIB ont une survie médiane de 4,7 ans et un DSS de 56% à 5 ans et 29% à 20 ans. Leur RDP est de 48% à 5 ans et de 71% à 20 ans.
- Les patients de stade IIIA et IIIB ont une survie médiane de 4,7 et 3,4 ans, respectivement, et un DSS à 10 ans de 45%. Leur RDP est de 53% et 82%, respectivement.
- Les patients de stade IVA1 ont une survie médiane de 3,8 ans, un DSS de 41% à 5 ans et 20% à 10 ans. Leur RPD est de 62% à 5 ans.
- Les patients de stade IVA2 ont une survie médiane de 2,1 ans et un DSS de 23% à 5 ans et 20 % à 10 ans. Leur RPD est de 77% à 5 ans.
- Les patients au stade IVB ont une survie médiane de 1,4 an avec un DSS de 18% à 5 ans.

Tableau 5: Les stades du MF (Olsen et al., 2007)

Stade clinic	T	N	M
IA	1	0	0
IB	2	0	0
IIA	1,2	1	0
IIB	3	0,1	0
III	4	0,1	0
IVA	1-4	2,3	0
IVB	1-4	0-3	1

Tableau 6: Classification TNM originale des cas de MF (Olsen et al., 2007)

Classification	Description
T: Skin	
T ₀	Clinically and/or histopathologically suspicious lesions
T ₁	Limited plaques, papules, or eczematous patches covering < 10% of the skin surface
T ₂	Generalized plaques, papules, or erythematous patches covering ≥ 10% or more of the skin surface
T ₃	Tumors, one or more
T ₄	Generalized erythroderma
N: Lymph nodes	
N ₀	No clinically abnormal peripheral lymph nodes; pathology negative for CTCL
N ₁	Clinically abnormal peripheral lymph nodes; pathology negative for CTCL
N ₂	No clinically abnormal peripheral lymph nodes; pathology positive for CTCL
N ₃	Clinically abnormal peripheral lymph nodes; pathology positive for CTCL
B: Peripheral blood	
B ₀	Atypical circulating cells not present (< 5%)
B ₁	Atypical circulating cells present (> 5%); record total white blood count and total lymphocyte counts, and number of atypical cells/100 lymphocytes
M: Visceral organs	
M ₀	No visceral organ involvement
M ₁	Visceral involvement (must have pathology confirmation and organ involved should be specified)

L'un des plus grands défis dans la gestion du MF est l'identification des patients à risque de progression de la maladie à un stade précoce. Une avancée significative dans l'identification de ces patients provient des travaux de de Masson *et al.* (de Masson et al., 2018). Dans cette étude rétrospective monocentrique, la charge du clone malin des lymphocytes T (fréquence du clone tumoral [TCF]) dans la peau lésionnelle a permis de prédire à un stade précoce la RDP et la OS des patients. Un TCF supérieur à 25% était significativement associé à la survie sans progression et à la survie globale. Cette mesure était supérieure à la prédiction de la survie sans progression par rapport au stade (IB vs IA), à la présence de plaques, à une LDH élevée, à l'âge et à la présence d'une transformation en grandes cellules. De plus, lorsque les patients à haut risque de progression de la maladie tel que déterminé par le TCF étaient traités par radiothérapie locale, ils avaient une OS améliorée (Larocca and Kupper, 2019).

1.1.4. **Physiopathologie**

Le MF et le SS sont deux entités cliniquement distinctes, bien qu'elles présentent des similitudes phénotypiques et morphologiques (Horna et al., 2019; Larocca and Kupper, 2019).

L'évolution du MF est lente sur plusieurs années voire décennies. Dans les stades précoces (stades IA, IB et IIA), le patient présente des lésions cutanées érythémateuses en macules ou plaques parfois associées à des adénopathies périphériques palpables. Aux stades plus avancés (stades IIB, IIIA et IIIB) une ou plusieurs tumeurs cutanées sont présentes, elles peuvent, pour les formes sévères, être associées à une érythrodermie généralisée et des adénopathies périphériques palpables (Agar et al., 2010). La transformation du MF (T-MF ou MFT) en cellules larges est associée à une forme agressive de la maladie avec une réduction de la survie (Vergier et al., 2000).

Le SS est une forme agressive de CTCL. Les patients SS présentent une peau érythrodermique et un nombre de cellules T néoplasiques (cellules de Sézary) circulantes supérieur à 1000/mm³. Certains patients se présentent d'emblée avec un SS et d'autres présentent initialement des lésions cutanées classiques de MF et répondent plus tard aux critères du SS. Ces derniers sont appelés MF leucémiques, SS précédés de MF, ou SS secondaire. Selon le

NCCN («National Comprehensive Cancer Network »), tout patient qui répond aux critères d'un envahissement sanguin important (B2 dans la classification TNMB des CTCL type MF/SS, ce qui correspond aux stades IV de la classification des CTCL (IVA2 à IVB), est considéré atteint de SS (Bagot, 2013; Larocca and Kupper, 2019).

Les critères définissant le SS, en plus du nombre absolu de cellules de Sézary qui doit être supérieur ou égal à 1000 cellules/mm³, sont une augmentation des cellules CD3 ou CD4 positives conduisant à un ratio CD4/CD8 supérieur ou égal à 10, la perte d'expression de certains marqueurs lymphocytaires T comme le CD7, une augmentation relative ou absolue des lymphocytes avec la mise en évidence de lymphocytes T clonaux identiques dans le sang et dans la peau (consortium BIOMED-2) (van Dongen et al., 2003).

Les cellules SS et MF sont des cellules de Sézary au noyau cérébriforme (figure 3) qui sont dans la plupart des cas des lymphocytes T CD4+/CD8- $\alpha\beta$. Selon ce profil, les lymphocytes T présentent fréquemment une perte du CD26 et du CD7 (Olsen et al., 2007). Des études récentes de cytométrie en flux ont établi des recommandations de manière à mesurer l'implication de l'envahissement sanguin par les cellules de Sézary à tous les stades de MF/SS. Selon ces recommandations, les marqueurs CD4+CD7- ou CD4+CD26- devraient toujours être déterminés (Scarlsbrick et al., 2018).

Sur la base des analyses immunophénotypiques traditionnelles, il avait été proposé que les lymphocytes T du MF seraient plutôt de type effecteur mémoire et que les cellules de SS seraient de type central mémoire (Campbell et al., 2010; Clark et al., 2012, 2011). Cependant, de nouvelles études concluent à une hétérogénéité et une plasticité d'expression des marqueurs de maturation présents à la surface des lymphocytes T dans les SS/MF, remettant en cause l'origine de la cellule T tumorale (Horna et al., 2020, 2019; Illingworth et al., 2020).

1.1.5. *Génétique des CTCL*

1.1.5.1. *Cytogénétique*

L'utilisation des outils de cytogénétique classique et/ou moléculaire a permis, en fonction de la pathologie et du stade de la maladie, d'appréhender la complexité des caryotypes. Différentes anomalies de nombre et de structure ont été rapportées surtout pour les CTCL agressifs (MFT/SS). Les anomalies chromosomiques sont des gains au niveau des chromosomes 7, 8q, 9q, 17q and 19 (Fischer et al., 2004; Karenko et al., 1999; Mao et al., 2002; Prochazkova et al., 2007; Zettl et al., 2002), des pertes au niveau des chromosomes 2q, 9p, 10q, 17p (Batista et al., 2006; Fischer et al., 2004; Karenko et al., 1999; Mao et al., 2002; Prochazkova et al., 2003; Thangavelu et al., 1997), ainsi que des réarrangements de locus comme par exemple les réarrangements 12q21 (Karenko et al., 2005). Parmi les anomalies observées dans les C-ALCL, on trouve les gains des chromosomes 7q, 17 et 21 ainsi que les pertes des chromosomes 3p, 6q, 8p et 13q (van Kester et al., 2010). Cependant, l'ensemble de ces études conclut en une absence d'anomalies cytogénétiques spécifiques et récurrentes dans les sous-types de CTCL (Laharanne et al., 2010; Mao et al., 2011; Prochazkova et al., 2007; van Doorn et al., 2009; van Kester et al., 2010; Vermeer et al., 2008). L'absence d'anomalies cytogénétiques considérées comme potentielles cibles thérapeutiques a encouragé la recherche d'autres biomarqueurs impliqués dans la pathogenèse des CTCL et qui pourraient servir dans la recherche de nouvelles pistes thérapeutiques.

1.1.5.2. *Profils d'expression génique*

Au cours de ces cinq dernières années les études de séquençage nouvelle génération ont permis de comparer le profil d'expression génique de divers sous-types de CTCL à ceux de lymphocytes T normaux circulant ou à des cellules de peau saine ainsi qu'à celles de la peau inflammatoire. Les données issues de ces analyses ont permis de mieux appréhender la diversité et la complexité des anomalies génétiques présentes dans les CTCL et plus particulièrement pour les stades avancés (MFT et SS).

Ainsi, il a été mis en évidence que les cellules SS exprimaient des gènes normalement non-exprimés dans les cellules T saines, comme par exemple : *TWIST1*, *PLS3* (plastine 3), *NCR1* (*NKp46* et *KIR3DL1*), les deux derniers étant des marqueurs de cellules NK (Michel et al., 2013). Le gène *TWIST1* est aussi retrouvé exprimé dans de nombreux cas de MF, en particulier dans les stades avancés. L'altération de l'expression de *TWIST1* et *PLS3* pourrait être liée à une hypométhylation aberrante de l'ADN (Wong et al., 2015).

La comparaison des profils d'expression des cellules SS à celui de lymphocytes T CD4+ normaux, par la technologie RNA-seq a révélé un total de 345 transcrits significativement régulés à la hausse dans les cellules SS. Les gènes impliqués par cette augmentation d'expression se concentrent sur quelques voies de signalisation spécifiques à savoir :

- Le contrôle du cycle cellulaire,
- L'activation transcriptionnelle de *MYC*
- La régulation du système immunitaire,
- Et la signalisation des chimiokines.

Ainsi, une augmentation d'expression $\geq 5x$ des gènes importants dans la signalisation du TCR, des cytokines et des chimiokines a été observée. Cela concerne *CD3G*, *RAC2*, *PRKCQ* (*PKC θ*) et *HRAS* (de la signalisation du TCR); les récepteurs aux chimiokines *CCR4* et *CCR8*, *IL6R α* et *IL2RG*. Le gène *IL2RG* code pour la chaîne gamma commune du récepteur à l'IL-2, qui est une sous-unité à six récepteurs de cytokines comprenant les récepteurs à l'IL-15 et à l'IL-21, qui sont des facteurs autocrines importants dans les CTCL. Une expression augmentée de 19x a été observée pour le gène *TOX*. La protéine TOX est un facteur de transcription dont l'expression est tellement augmentée dans les CTCL, qu'à ce titre il a été proposé comme biomarqueur spécifique pour le MF et le SS. Des études ont révélé que *TOX* pourrait jouer un rôle dans la pathogénicité des CTCL, car il régule à la baisse l'expression de *CDKN1B* et *CDKN1C*, des protéines inhibitrices du cycle cellulaire, ainsi que celle de *RUNX3* qui peut agir comme un gène suppresseur de tumeur dans les CTCL (Dulmage et al., 2019; Haider et al., 2016; Y. Huang et al., 2015).

1.1.5.3. Altérations génétiques

Les données issues d'anciennes investigations concernent un nombre limité de patients, elles sont focalisées sur des mutations d'un gène en particulier ou sur des anomalies de nombre de copies à basse résolution. Ces investigations démontrent que les aberrations de nombres de copie (CNA) présentes dans les CTCL sont partagés avec d'autres pathologies comme les lymphomes périphériques à cellule T (PTCL), et plus particulièrement avec la leucémie / lymphome T adulte (ATLL), dont les cellules T présentent souvent un adressage à la peau (skin-homing).

Les études plus récentes de séquençage à haut débit « whole genome sequencing » (WGS) et d'analyse du nombre de copies à haute résolution ont été réalisées sur un plus grand nombre de patients (Choi et al., 2015; da Silva Almeida et al., 2015; Wang et al., 2015). Les fréquences observées des différentes anomalies varient selon les études, avec parfois des écarts importants, probablement en raison de l'hétérogénéité considérable de la maladie ; cependant, globalement, la plupart des résultats sont assez similaires (Iqbal et al., 2019).

Dans la majorité des cas de SS étudiés, environ les trois quarts des « single nucléotide variant » (SNV) sont des transitions C vers T, qui confèrent deux types de signatures mutationnelles (Iqbal et al., 2019):

- une signature de désamination spontanée au niveau de dinucléotides CpG associée à l'âge,
- et une signature associée à l'exposition à la lumière ultraviolette-B.

Cette dernière mutation dans les SS suggère que la maladie peut provenir de cellules T résident mémoire T (T_{RM}) dans la peau.

En plus des SNV, les cellules SS présentent un nombre important d'anomalies de structures. Une étude par WGS sur six cas de SS a montré la présence en moyenne de 168 jonctions anormales par cas, et ceci est dû à des anomalies de structures (Kiel et al., 2015). Bien qu'aucune anomalie récurrente n'ait été trouvée, 42 gènes de fusion potentiels ont été identifiés, dont plusieurs sont susceptibles d'avoir un impact pathogène. Dans les SS, les gènes les plus fréquemment perdus ou mutés de manière délétère sont généralement non fonctionnels dans de nombreuses autres tumeurs. La perte de 17p survient dans ~ 40% des cas, et, inclus de petites

perdes en 17p13.1, *TP53* est perdu dans au moins 50% des cas de SS (Gros et al., 2017). De plus, des mutations *TP53* ont été observées à hauteur de 20-30% (Gros et al., 2017). La perte du 9p21.3 où se situe *CDKN2A* est observée dans ~ 50% des cas et est souvent homozygote. Les pertes de *CDKN2A* et de *TP53* sont supposées conférer une résistance à l'apoptose et à la sénescence cellulaire, et augmenter la prolifération cellulaire ainsi que l'instabilité génomique (Iqbal et al., 2019).

D'autres pertes de gènes semblent être dues à la perte de régulateur du cycle cellulaire comme la perte de *RB1* (13q14.2) et de *CDKN1B* (12p13.1). Le gène *FAS* un régulateur de l'apoptose, est généralement délété ou muté. *FAS* est localisé en 10q23.3, une région qui comprend aussi le gène *PTEN*. La perte de *FAS* devrait interférer avec l'activateur de la mort cellulaire induite (AICD), ce qui représente un mécanisme homéostatique important dans les cellules T.

Les gènes impliqués dans le remodelage de la chromatine sont aussi fréquemment remaniés, tel que la perte au niveau du 1p36.1 impliquant le gène *ARID1A*. Ce gène est connu pour être délété ou muté dans de nombreuses tumeurs. Dans l'ensemble, 40 à 60% des cas de CTCL présentent une délétion ou une mutation *ARID1A*. Il s'agit d'une sous-unité ciblant les complexes de remodelage de la chromatine SWI/SNF, qui peuvent déplacer les nucléosomes le long de l'ADN. Dans un modèle de cancer du côlon, la perte d'*ARID1A* entraîne une perte ou une diminution de l'acétylation de l'histone H3K27 (Mathur et al., 2017). *ARID1A* a également un rôle dans la réparation de l'ADN, et sa perte rend les cellules sensibles aux dommages à l'ADN (Watanabe et al., 2014). *ARID5B* est aussi fréquemment (~ 40%) concerné dans les pertes au niveau du 10q, et *ARID3A* dans les pertes distales de 19p (Kiel et al., 2015). D'autres composants de SWI/SNF sont également fréquemment affectés par des pertes telles que *SMARCC4* en 3p21.31. Les délétions ou les mutations affectant *ARID1A*, *ARID5B* ou *SMARCC4* surviennent dans près de 61% des cas.

D'autres gènes mutés dans les CTCL ont également des fonctions activatrices. Par exemple, les mutations de *MLL2*, *MLL3* et *MLL4* (*KDM2D-Cet-B*) surviennent dans les SS avec des fréquences variables selon les études (entre 4% et 59%) (da Silva Almeida et al., 2015; Kiel et al., 2015). Les gènes de la famille *MLL* catalysent les mono- ; di- et triméthylation de la lysine 4

de l'histone H3 (H3K4) ; et ils semblent particulièrement importants dans la monoacétylation des « enhancers », ce qui est associé avec un accroissement de l'activité du gène (Guo et al., 2013; Kaikkonen et al., 2013). D'autres gènes régulateurs de la chromatine sont mutés dans le SS, comme *BRD9*, *CHD3* et *CREBBP*. Des modifications qui touchent la méthylation de l'ADN sont assez répandues dans le SS. Des pertes au niveau du 2p23.3, parfois homozygotes, concernant l'ADN méthyltransférase *DNMT3A*, sont décrites dans ~ 20% à 37% des cas de SS (Choi et al., 2015; da Silva Almeida et al., 2015). Des mutations et des délétions se produisent également dans la famille des gènes *TET* ce qui peut impliquer *TET2* (da Silva Almeida et al., 2015).

Un autre gène généralement perdu avec un effet important sur la transcription est *NCOR1*. Il est localisé en 17p, cette région est perdue dans près de la moitié des cas de SS. Bien que le 17p inclue également *TP53*, des mutations *NCOR1* non-sens et de décalage de cadre de lecture ont été trouvées dans 15% des cas de SS, souvent associées à la suppression de l'autre allèle, suggérant un rôle pathogénique (Kiel et al., 2015). *NCOR1* interagit avec plusieurs facteurs de transcription et recrute les histones déacétylases (HDAC) pour réprimer la transcription.

ZEB1 sur le 10p a été rapporté délété dans 36% des cas de SS et muté dans près de 10%. La protéine ZEB1 est un facteur de transcription qui généralement réprime la transcription, mais qui peut aussi avoir un rôle activateur. Parmi les gènes réprimés par ZEB1 figurent *BCL6*, *IL2* et *IL15* (Mishra et al., 2016; Papadopoulou et al., 2010; Wang et al., 2009).

Les mutations de gain de fonction qui surviennent au niveau des gènes *STAT3*, *STAT5B*, *JAK1* et *JAK3* peuvent être observées occasionnellement dans les SS. L'ensemble de ces mutations concernent ~10% des cas des SS. De plus, *STAT3 -5A* et *-5B* sont sur le chromosome 17q, bras chromosomique très fréquemment gagné dans les CTCL.

Les pertes en 10q incluent fréquemment *NFKB2* qui code pour la sous-unité inhibitrice de NFκB (p100). Dans la voie non-canonique de NFκB, l'extrémité C-terminale de p100 est éliminée par protéolyse, donnant une sous-unité p52. Cette sous-unité s'hétérodimérise avec RELB, puis active un groupe de gènes cibles de NFκB. Dans une étude, 10% des CTCL présentait des délétions 10q responsables de la formation d'une protéine NFκB2 tronquée en C-terminale (Choi et al., 2015). La protéine tronquée est capable de mimer l'activation naturelle en passant par la voie non-canonique de NFκB (Migliozza et al., 1994). Plusieurs autres mécanismes peuvent

contribuer à l'activation de NFκB dans les CTCL. Le TCR induit l'activation de NFκB, ce qui implique le recrutement et la phosphorylation d'un complexe CARD11-BCL10-MALT1 par PKCθ. Les mutations activatrices de *CARD11* ont observées dans les CTCL (6% dans une étude) (Kiel et al., 2015). Les gains ciblés de *PRKCE* qui code pour PKCθ sont fréquents. Une mutation ponctuelle récurrente dans *TNFRSF1B* codant pour *TNFR2a* a été identifiée chez 5% des cas de CTCL aboutissant à l'activation de la voie de signalisation non-canonique de NFκB (Ungewickell et al., 2015).

D'autres mutations suspectées d'avoir des répercussions plus larges sur les voies de signalisation concernent des mutations ponctuelles de *CD28* et occasionnellement des duplications en tandem partielles qui résultent en la fusion CTLA4-CD28. Cette fusion aboutit à une augmentation de la signalisation.

Des mutations de *PLCG1* qui code pour la phospholipase Cγ1, ont été rapportées à diverses fréquences (en général ~ 10%). Certaines de ces anomalies récurrentes, se traduisent par un gain de fonction. *PLCγ1* génère deux seconds messagers très importants :

- L'inosine trisphosphate (IP3), qui induit la libération de calcium du reticulum endoplasmique,
- Et le diacylglycérol (DAG), qui active plusieurs voies dans les cellules T, y compris les voies RAS-ERK et NF-κB.

Dans les CTCL, la voie RAS-ERK peut également être activé par des mutations de gain de fonction dans *NRAS*, *KRAS* et *MAPK1* (*ERK2*) (Iqbal et al., 2019; Kiessling et al., 2011).

1.1.5.4. micro ARN (miRNA, miR)

Des profils d'expression différentielles de miR peuvent être observés en fonction du stade de la pathologie CTCL (Ralfkiaer et al., 2014, 2011; Sandoval et al., 2015). Le miR-155 est régulé à la hausse dans les stades précoces de MF et le miR-21 est régulé à la hausse dans les stades plus avancés (Mishra et al., 2016). Ces deux miARN ont des fonctions oncogéniques (Onco-miR) (Esquela-Kerscher and Slack, 2006).

Chez les patients SS, à partir du même ARN primaire régulé à la hausse en raison d'une expression aberrante de *TWIST1*, les miR les plus fortement exprimés sont les miR-214, miR-199a-5p et miR-199a-3p (environ 50, 40 et 10 fois plus, respectivement) (Narducci et al., 2011).

1.1.5.5. Biomarqueurs moléculaires

Certaines des altérations génétiques mentionnées dans ce paragraphe pourraient être utilisées comme biomarqueur moléculaire. La notion de biomarqueur ou de marqueur biologique a été défini par Hulka et Wilcosky en 1988 comme des « altérations cellulaires, biochimiques ou moléculaires mesurables dans des milieux biologiques tels que les tissus, les cellules ou les fluides humains » qui peuvent aider à comprendre le diagnostic, la progression, la régression ou l'issue du traitement clinique (Hulka and Wilcosky, 1988). Les biomarqueurs diagnostiques idéaux sont systématiquement et fortement surexprimés dans les cellules malignes alors qu'ils sont absents ou presque absents dans la population cellulaire normale (Kohnken et al., 2016).

Dans les CTCL, aucun biomarqueur est actuellement utilisé pour guider la décision thérapeutique (Damsky and Choi, 2016). Il n'existe pas de biomarqueur prédictif de la transformation en grandes cellules des MF/SS, de même il n'existe pas de biomarqueurs spécifiques des cellules tumorales (Cristofolletti et al., 2019). Cette absence de biomarqueurs représente un sérieux obstacle à l'établissement d'un diagnostic précoce et à l'évaluation de la progression du lymphome. Bien qu'aucun biomarqueur ne puisse être utilisé pour diagnostiquer de manière fiable la pathologie, la combinaison de plusieurs marqueurs parmi les potentiels biomarqueurs du SS (Tableau 7) pourrait être envisagée et pourrait être étudiée de manière prospective (Cristofolletti et al., 2019).

Tableau 7 : Les biomarqueurs génomiques et moléculaires potentiellement pertinents dans le SS, incluant des variations de nombre de copies (CNV), les mutations fréquentes détectées par NGS, les micro ARN ainsi que les transcrits de fusions (Cristofolletti et al., 2019)

CNVs ¹		Somatic Mutations ²		MicroRNAs ³			
Gain	Loss	Gene	Chromosome	Up	Chromosome	Down	Chromosome
1p36.13	1p22.1	ANK3	10q21.2	miR-21	17q23.1	miR-15a	13q14.2
4q12	1p36.11 (ARID1A)	ARID1A	1p36.11	miR-143	5q32	miR-16	13q14.2
5p15.33	2p23.3 (DNMT3A)	ATM	11q22.3	miR-145	5q32	miR-17-5p	13q31.3
6p25.3	2q37.3	BRAC2	13q13.1	miR-152	17q21.32	miR-22	17p13.3
7p22.2	3q11.2-q13.2	CACNA1E	1q32.1	miR-155	21q21.3	miR-31	9p21.3
7q36.1	4p13-p12	CADPS	3p14.2	miR-199a	19p13.2	miR-107	10q23.31
8q12.1 (TOX)	4q31.3	CARD11	7p22.2	miR-200c	12p13.31	miR-146b	10q24.32
8q24.21 (MYC)	5q33.1	CREBBP	16p13.3	miR-214	1q24.3	miR-203	14q32.33
9p24.2	6p21.1	DNAH9	17p12	miR-223	Xq12	miR-205	1q32.2
10p15.1 (PRKCQ)	6q23.3-25.2 (A20/TNFAIP3)	DNMT3A	2p23.3	miR-486	8p11.21	miR-342	14q32.2
10p15.1-p12.33	7p15.3-q21.2	ENPP2	8q24.12	miR-574-5p	4p14		
10p12.33	8p23.1-p11.21	FAM47A	Xp21.1				
17q21.2 (STAT5B)	8q12.3-q22.1	FAS	10q23.3				
17q11.2-25.1	9p21.2 (CAAP)	FAT3	11q14.3				
20q13.2	9p21.3 (CDKN2A-CDKN2B)	FAT4	4q28.1	BMP2K	INPP4B	chr4	chr4
21q22.3-q11.23	10p11.22 (PTEN, FAS)	GPR158	10p12.1	CARD11	PIK3R3	chr7	chr1
	10q21.2-23.31	JAK3	19p13.11	CBLB	ZEB1	chr3	chr10
	10q23.31 (ZEB1)	KMT2C	7q36.1	CD28	CTLA4	chr2	chr2
	10q24.32-q26.11 (NFKB2)	MLL2	12q13.2	CENPE	INPP4B	chr4	chr4
	11p13-q23.2	NuRD/MTA1	14q32.33	COL25A1	NFKB2	chr4	chr10
	11q22.3 (ATM)	PDCD11	10q24.33	DHFR	ARHGAP18	chr5	chr6
	11q23.2 (USP28)	PLCG1	20q12	DHFR	RASGRF2	chr5	chr5
	12p13.2-q22	POLD1	19p13.33	FASN	SGMS1	chr17	chr10
	13q12.2-14.2 (RB1)	POT1	7q31.33	ICOS	CD28	chr2	chr2
	16q22.1-q24.3	PREX2	8q13.2	IKZF2	GLI2	chr2	chr2
	17p11.2 (NCOR1)	PRKCQ	10p15.1	IKZF2	BACH2	chr2	chr6
	17p13.1 (TP53)	PTPRK	6q22.33	IKZF2	SPAG1	chr2	chr8
	19p13.3 (E2A)	RAD51C	17q22	INPP4B	ATAD1	chr4	chr10
	22q11.23	SOCS7	17q12	IWS1	IKZF2	chr2	chr2
		STAT3	17q21.2	MAP3K7	MYB	chr6	chr6
		STAT5A	17q21.2	MBNL1	KIAA2018	chr8	chr3
		STAT5B	17q21.2	MYB	MBNL1	chr3	chr6
		TET1	10q21.3	PPFIA1	NFKB2	chr11	chr10
		TET2	4q24	RBMS1	GLI2	chr2	chr2
		TP53	17p13.1	SGMS1	ZEB1	chr10	chr10
		UNC13C	15q21.3	TOX	MYBL1	chr8	chr8
		ZEB1	10p11.22	TYK2	UPF1	chr19	chr19
				USP32	RAD51B	chr17	chr14
				USP32	TTC18	chr17	chr10

1.1.6. Traitements des CTCL

Le traitement des CTCL dépend du stade de la maladie et de l'état général du patient. Aux stades précoces, les MF (IA-IIA) peuvent être contrôlés avec des thérapies appliquées localement sur la peau. Les traitements systémiques sont recommandés en deuxième ligne pour des stades de MF IA, IB et IIA. La survie à 5 ans de ces patients est d'environ 90%, comparés aux 30% à 50% pour les stades avancés (IIB-IVB). La chimiothérapie, la polychimiothérapie, ainsi que les nouveaux agents thérapeutiques sont recommandés pour les stades avancés de la maladie (Scarisbrick et al., 2015; Trautinger et al., 2017).

Le traitement d'un patient SS prend en compte l'étendue de la maladie, l'impact du traitement sur la qualité de vie, les facteurs pronostiques, l'âge du patient et les comorbidités. Le traitement d'un patient SS privilégie toujours les traitements qui préservent le système immunitaire du patient. En effet, comme la pathologie SS est associée à un dysfonctionnement de l'immunité cellulaire qui entraîne une réponse altérée aux infections et aux cellules tumorales, l'utilisation de thérapies immunosuppressives pourrait conduire à une infection massive. L'infection est une cause fréquente de décès chez les patients SS. Un autre principe du traitement de la pathologie SS est la surveillance vigilante de la colonisation microbienne et de l'infection (Cristofolletti et al., 2019; Talpur et al., 2008).

Bien que de nombreux traitements soient disponibles (Tableau 8), la plupart des réponses au traitement sont partielles et non durables. Par conséquent, le protocole thérapeutique comprend des traitements successifs, seuls ou en combinaison. De plus, l'effet du traitement sur la survie globale est inconnu (Spicknall, 2018), à l'exception de la greffe allogénique de moelle osseuse (BMT). Elle permet d'obtenir des rémissions complètes et durables, cependant, elle est associée à une morbidité et une mortalité élevées et n'est actuellement envisagée que chez les patients jeunes, relativement en bonne santé et qui présentent un stade avancé de la maladie (Molina et al., 2005).

Tableau 8 : Les traitements disponibles pour les CTCL (Cristofolletti et al., 2019)

Les traitements disponibles pour le SS :	
Photophérèse extracorporelle	Thérapie photoimmune
Interferon- α (IFN- α)	Modificateur de la réponse biologique
Bexarotene	Chimiothérapie
Methotrexate	Chimiothérapie
Psoralen plus ultraviolet A (PUVA)	Photochimiothérapie
Vorinostat	Inhibiteur HDAC
Romidepsin	Inhibiteur HDAC
Brentuximab	anti-CD30
Alemtuzumab	anti-CD52
Mogalumizumab	anti-CCR4
Polychimiothérapie	Chimiothérapie

Compte tenu de l'efficacité limitée des thérapies existantes, les patients à un stade avancé de la maladie sont encouragés à participer à des essais cliniques. Plusieurs agents sont en cours de développement et d'essais cliniques pour le traitement des MF/SS (Tableau 9).

Tableau 9 : Les agents thérapeutiques en essais cliniques pour les CTCL (Larocca and Kupper, 2019)

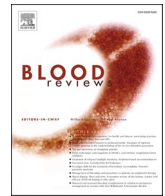
Agent thérapeutique	Phase d'essai clinique
Mogamulizumab (anti-CCR4 antibody)	Phase III (NCT01728805)
E7777 (cytotoxic IL-2 fusion protein)	Phase II (NCT01871727)
MRG-106 (miR-155 antagonist)	Phase II (NCT02580552)
Duvelisib (PI3K inhibitor)	Phase II (NCT02783625)
Ruxolitinib (JAK 1/2 inhibitor)	Phase II (NCT02974647)
TTI-621 (SIRPaFc IgG4, anti-CD47)	Phase I (NCT02663518)

1.1.7. Article *“Diagnosis and treatment of lymphomas in the era of epigenetics”*

Dans la revue de la littérature intitulée "Diagnosis and treatment of lymphomas in the era of epigenetics" nous avons fait l'inventaire des modifications épigénétiques connues dans tous les lymphomes T et B avec un focus particulier pour les CTCL. Nous avons défini leurs physiologies et détaillé leurs implications pathologiques.

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Review

Diagnosis and treatment of lymphomas in the era of epigenetics

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ABSTRACT

Lymphomas represent a heterogeneous group of cancers characterized by clonal lymphoproliferation. Over the past decades, frequent epigenetic dysregulations have been identified in hematologic malignancies including lymphomas. Many of these impairments occur in genes with established roles and well-known functions in the regulation and maintenance of the epigenome. In hematopoietic cells, these dysfunctions can result in abnormal DNA methylation, erroneous chromatin state and/or altered miRNA expression, affecting many different cellular functions. Nowadays, it is evident that epigenetic dysregulations in lymphoid neoplasms are mainly caused by genetic alterations in genes encoding for enzymes responsible for histone or chromatin modifications. We summarize herein the recent epigenetic modifiers findings in lymphomas. We focus also on the most commonly mutated epigenetic regulators and emphasize on actual epigenetic therapies.

1. Introduction

Lymphomas or lymphoid neoplasms are a heterogeneous group of cancers affecting the lymphatic system. They arise from a proliferation of B-cells, T-cells or cells of the innate lymphoid system (Natural killer (NK) cells, NK-like T-cells and $\gamma\delta$ T-cells). The term lymphoma is most commonly used for mature or peripheral lymphoid cells neoplasms while precursor lymphoid neoplasms correspond to leukemia/lymphoma [1]. These lymphoproliferative disorders represent the fifth most common cancer worldwide. More than 100 different types of lymphomas exist, presenting different clinical characteristics and treatment

procedures [1]. Lymphomas are broadly classified as Hodgkin's lymphoma (HL) and non-Hodgkin's lymphomas (NHLs). HL is a rare B-cell malignant neoplasm considered as one of the most curable cancers worldwide [2]. In adults, the most common NHLs are: diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), Burkitt lymphoma (BL), small lymphocytic lymphoma (SLL), peripheral T-cell lymphoma (PTCL) and anaplastic large-cell lymphoma (ALCL). For optimal management, it is important to correctly classify lymphomas according to the cell of origin and the tumor aggressiveness. Indeed, lymphomas' nature and classification are used to guide therapeutic decisions. Hence, cytotoxic therapies are applied to treat aggressive high-grade

Abbreviations: ABC-DLBCL, Activated B-cell like diffuse large B-cell lymphoma; AITL, Angioimmunoblastic T-cell lymphoma; ALCL, Anaplastic large-cell lymphoma; ATLL, Adult T-cell leukemia/lymphoma; C-ALCL, Cutaneous anaplastic large-cell lymphoma; BL, Burkitt lymphoma; CBCL, Cutaneous B-cell lymphoma; CLL, Chronic lymphocytic leukemia; CTCL, Cutaneous T-cell lymphoma; DLBCL, Diffuse large B-cell lymphoma; DNMT, DNA methyltransferase; DNMTi, DNA methyltransferase inhibitors; FL, Follicular lymphoma; GC-DLBCL, Germinal center B-cell like diffuse large B-cell lymphoma; HAT, Histone acetyltransferases; HDAC, Histone deacetylases; HDACi, HDAC inhibitors; HL, Hodgkin lymphoma; HMT, Histone methyltransferases; HMTi, Histone methyltransferases inhibitors; IDH, Isocitrate dehydrogenase; KAT, Lysine acetyltransferases; KDM, Lysine demethylases; MCL, Mantle cell Lymphoma; KMT, Lysine methyltransferases; MF, Mycosis fungoides; MfT, Mycosis fungoides tumor stage; NGS, Next-generation sequencing; NHLs, Non hodgkin lymphomas; PCLBCL-LT, Primary cutaneous diffuse large B-cell lymphoma- Leg type; PCFCL, Primary cutaneous follicle center lymphoma; PMBCL, Primary mediastinal B-cell lymphoma; PRC2, Polycomb repressive complex 2; PTCL, Peripheral T-cell lymphoma; SLL, Small lymphocytic lymphoma; SS, Sézary syndrome; TET, Ten-eleven translocation; tFL, Transformed follicular lymphoma.

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lymphomas, while immunochemotherapies such as the anti-CD20 combined to chemotherapy improved the treatment of B-cell lymphomas [3–5]. Targeted therapies against several cellular markers such as anti-CD30, CD52, CD158, CCR4 and BCL2 were developed and are currently used to treat lymphomas [6–11]. Recent achievements in the generation of CAR T-cells led to promising results in curing aggressive or refractory B/T-cell lymphomas [12]. However, there is a need for new combination therapies using cytotoxic agents, targeted therapies, immune modulators or epigenetic modulators to define novel personalized therapies either as first line or second line treatments.

In addition to the primary site, the cell of origin subtype and the differentiation stage, lymphomas' classification also considers the presence of genetic abnormalities. Some types of NHLs are characterized by specific genetic abnormalities such as chromosomal translocation (for example t(8;14)(q24;q32) in BL and t(14;18)(q32;q21) in FL) or gene mutations (for example *TP53* mutations or deletions in SLL). In parallel to genetic abnormalities, common epigenetic dysregulations are observed. They contribute either to tumor transformation; or appear initially in neoplasms lacking highly recurrent initiating genetic alterations, like in most systemic peripheral T-cell lymphomas (PTCL) or cutaneous T-cell lymphoma (CTCL). This led to the expansion of epigenetic studies exploring the implication of new biomarkers [13].

The term "epigenetics" was coined by Conrad Hal Waddington in 1942, in order to understand and describe cell differentiation [14]. Nowadays, epigenetics refers to gene expression changes that are not related to DNA sequence alterations but to the chemical compounds modifications as DNA methylation, histone modifications, and to non-coding RNAs expression such as microRNAs (miRNAs, miRs) [15]. Epigenetic modifications regulate broad DNA-based processes, as transcription, DNA repair and replication. During gametogenesis, an epigenetic reprogramming of the genome occurs. After zygote formation, paternal and maternal genomes undergo epigenetic changes that erase most of the parental epigenetics marks [16,17]. During early embryogenesis specific epigenetic marks happen and are maintained during the organism's lifespan, thus mediating tissues' functions by ensuring genetic programming and cellular differentiation [18]. Changes in gene activities mediated by epigenetics were characterized over the years, and some epigenetic marks were reported as hallmarks of solid and hematologic malignancies including lymphomas. These hallmarks can be inherited, but interestingly many of them are reversible [19].

Over the years, the reversible status of epigenetic aberrations has led to the development of the promising field of epigenetic therapy [20]. Distinct patterns of DNA methylation, histone marks, miRNAs expression and recently long non-coding RNAs (lncRNAs) have been shown to be importantly involved in the maintenance of T-cells identity and the development of B-cells, suggesting that epigenetic modifications are a

key mechanism in several types of lymphomas [21–24]. In this review, we highlight and focus on recent advances in our understanding of the most common epigenetic modifications and treatments in lymphomas.

2. DNA methylation

Cytosine methylation of mammalian genomic DNA, in the context of CpG islands, was the first epigenetic mark described in 1975. It is still considered as the most studied epigenetic modification of all time because it plays a major role in the control of gene expression in mammalian cells [25]. DNA methylation is associated with heterochromatin formation and transcriptional repression such as genomic imprinting and chromosome X inactivation [26,27].

2.1. DNA methylation writers

DNA Methyltransferases (DNMT), the enzymes that catalyze DNA methylation, are mainly classified into: DNMT1, DNMT3A and DNMT3B (Fig. 1) [28]. Each DNMT play a key role in the physiological hematopoiesis where DNA methylation is dynamically regulated (discussed in the following paragraphs) [29–32]. DNMT1 is called "maintenance DNMT" due to its role in methylating the unmethylated strand of newly generated hemimethylated DNA during cell division. DNMT3A and DNMT3B are called genome-wide "de novo DNMT" because they can methylate the unmethylated cytosines during embryogenesis. DNMT3A and DNMT3B are down-regulated in adult somatic cells, but are over-expressed in diverse tumors [28,33–35]. In a wide variety of hematologic malignancies including lymphomas, somatic mutations in the DNMT genes were identified [36].

2.1.1. DNMT1

DNMT1 maintains 5mC status during cell proliferation. Indeed, when DNMT1 is non-functional, the hematopoiesis is suppressed and the lymphomagenesis is delayed. *DNMT1* was reported to be implicated in the *de novo* methylation during tumorigenesis, playing a major role in the prevention and maintenance of the tumor phenotype in *MYC*-induced T-cell lymphomas [37]. In BL patients, DNMT1 is overexpressed emphasizing its pivotal role in BL pathogenesis beyond its role in normal B-cell differentiation [38–40], however some critiques could be addressed regarding the methods used in these articles especially in the choice of controls.

2.1.2. DNMT3A

Mutations in *DNMT3A* have been recurrently found in different hematopoietic diseases including T-cell lymphomas [25,41]. In T-cell neoplasms, a high frequency of bi-allelic mutations are observed,

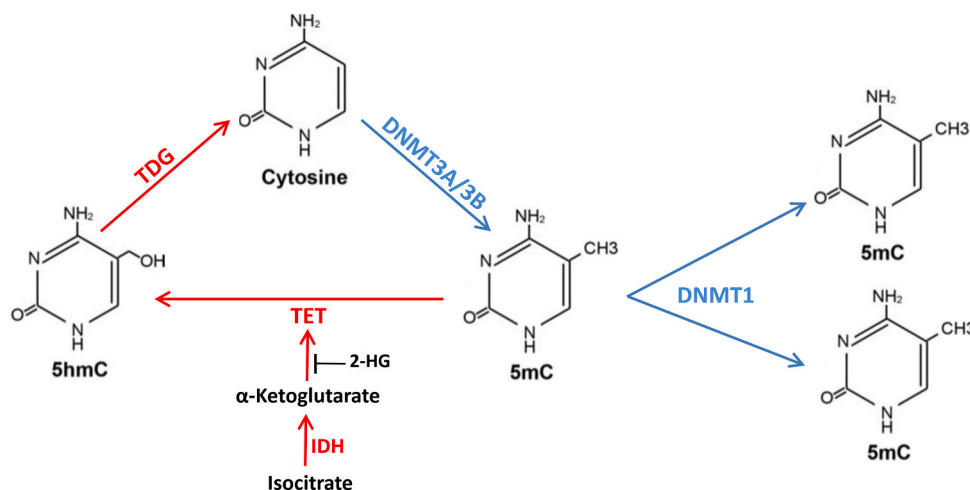


Fig. 1. Methylation (in blue) and demethylation (in red) at the fifth position of cytosine of CpG dinucleotides. DNMT3A and DNMT3B enzymes methylate the cytosine (5mC), and DNMT1 maintain the methylation during cell division. TET proteins promote demethylation by catalyzing the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC). IDH proteins promote Isocitrate transformation into α -ketoglutarate, which is essential for TET catalytic function. The final stage of the demethylation is mediated by the Thymine DNA Glycosylase (TDG), allowing excision and replacement with unmethylated cytosine. DNMT: DNA methyltransferases; IDH: Isocitrate dehydrogenase; TET: Ten-eleven translocation; 2-HG: 2-hydroxy-glutarate.

suggesting that a complete loss of DNMT3A is a critical event during the development of these neoplasms [25]. Hence, between 11 and 33% of angioimmunoblastic T-cell lymphoma (AITL) patients carry a *DNMT3A* loss-of-function mutation [42,43]. *DNMT3A* mutations co-occur with *TET2* (Ten-Eleven Translocation 2) mutations at a high frequency (73%) especially in AITL and PTCL, suggesting an oncogenic cooperation between *DNMT3A* and *TET2* mutations, involving cytosine methylation and demethylation processes [43]. In PTCL subtypes, such as AITL and follicular T-cell lymphoma, *TET2* and *DNMT3A* mutations were observed first, in clonal hematopoiesis of indeterminate potential (CHIP) stage where a hematopoietic stem and progenitor cell (HSPC) acquire somatic mutations that confer a selective advantage, followed afterwards by either *RhoA* and/or *IDH2* mutations (Fig. 2) [43–48]. *DNMT3A* mutations have also been observed in CTCL with a reduced or complete loss of expression in Sézary syndrome (SS), an aggressive subtype of CTCL [49,50].

2.1.3. DNMT3B

DNMT3B is predominantly reported in BL. In nearly 86% of BL patients, *DNMT3B* is overexpressed. It contributes, with DNMT1, to the BL's DNA methylation pattern [40]. While in BL, the overexpression of DNMT3B was proven to be a direct transcriptional target of the MYC oncoprotein and therefore considered as MYC-dependent, it was reported in DLBCL as a prognostic factor associated with shortened survival and treatment resistance [51,52]. Additionally, an overexpression of all the DNMTs (DNMT3B, DNMT3A and DNMT1) was reported in BL.

2.2. DNA methylation profiles

In addition to somatic mutations in genes regulating the DNA methylation in lymphomas, different methylation profiles were described. Some of these latter were linked to disease severity [53]. Among tumor cells of the same tumor, "methylation heterogeneity" refers to the differences in the methylation profiles and in the methylation levels. It increases with disease severity and it was shown to be associated with a poor outcome in FL, SLL and DLBCL [53–56]. Remarkably, at diagnosis the methylation heterogeneity can be used to distinguish good responders to treatment. Relapsed DLBCL patients showed therefore a higher degree of methylation heterogeneity at diagnosis compared to non-relapsed patients, suggesting a relapse-associated methylation signature in this lymphoma [54]. Interestingly, at relapse, the reduction of intra-tumor methylation heterogeneity evokes a clonal tumor cells selection, and sustains the idea that methylation heterogeneity is dynamic and can be used as a prognostic pre and post-treatment biomarker. Moreover, hypermethylation of *SMAD1* (Small mothers

against decapentaplegic homolog 1), a regulator of TGF- β , was found in chemoresistant DLBCL patients. The hypermethylation of *SMAD1* was responsible for its silencing. *SMAD1* reactivation and chemosensitization were reestablished after exposure to a low dose of DNMT inhibitors, supporting the idea that in some lymphomas, more specifically in DLBCL, DNA methylation can predict the response to treatment [57]. On the other hand, *MGMT* (O-6-Methylguanine-DNA Methyltransferase) promoter hypermethylation was reported to be associated with a significant increase in overall survival and progression-free survival in multi-treated DLBCL patients [58].

Global genomic methylation and gene specific methylation (or focalized methylation) were widely studied in lymphomas. For instance, in SLL, a disease similar to chronic lymphocytic leukemia (CLL) affecting lymph nodes, a global hypomethylation was described almost 30 years ago and was confirmed through the years [59,60]. In FL and DLBCL patients, a global genome hypomethylation with focalized hypermethylation of tumor suppressor genes (Fig. 3), such as *KLF4* (Kruppel-like factor 4) or *p16(INK4a)* (Cyclin-dependent kinase inhibitor 2A) was described [61–66]. Similarly, in mantle cell lymphoma (MCL) several tumor suppressor genes including *p15(INK4b)* (Cyclin-dependent kinase inhibitor 4B), *HOXD8* (Homeobox protein Hox-D8), *MLF1* (Myeloid leukemia factor 1) and *PCDH8* (Protocadherin-8), were reported to be hypermethylated and silenced [65]. In CTCL, malignant T cells present widespread promoter hypermethylation of tumor suppressor genes involved in cell cycle, DNA repair and apoptosis pathways, including *BCL7a*, *PTPRG*, and *p73* [67]. Genome of SS cells is characterized by chromosomal instability with many numerical and structural chromosomal alterations due to extensive DNA hypomethylation [68,69]. In SS, 126 hypermethylated gene promoters were identified including multiple established and potential tumor suppressors genes, such as the tumor suppressor *GNMT* (Glycine N-methyltransferase) and the regulator of hematopoietic stem cells proliferation *GOS2* (G0/G1 switch gene 2) [69].

2.3. DNA methylation erasers

The reversible status of DNA methylation or demethylation was identified after the discovery of the family of dioxygenases proteins TET (ten-eleven translocation). DNA demethylation is a dynamic process involving TET and IDH (isocitrate dehydrogenase) proteins. DNA demethylation plays important roles in the transcriptional activation of silenced genes (Fig. 1) [70–73]. Several studies showed that *TET* or *IDH* genes may be implicated in the tumorigenesis, since the inactivation of these latter can result in abnormal histone/DNA methylation patterns [74,75].

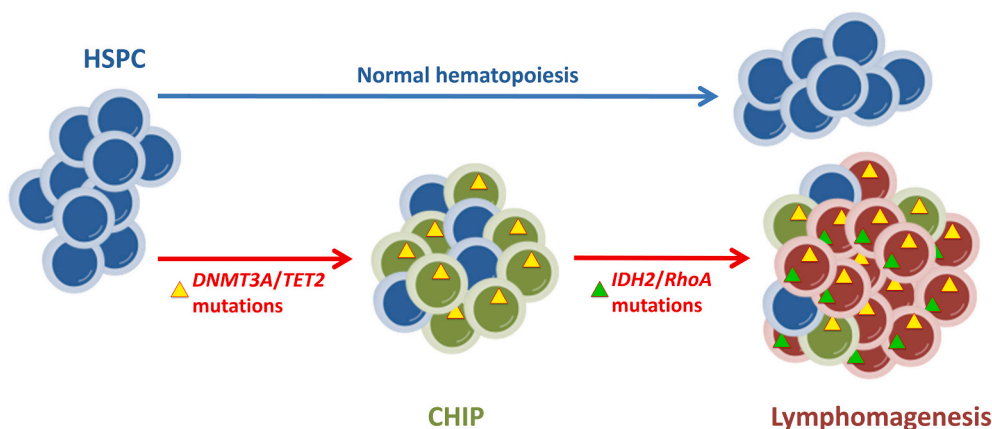


Fig. 2. Mutations in epigenetic regulators such as *TET2/DNMT3A* may cause aberrant differentiation and self-renewal in HSPC, leading to CHIP. At this oligoclonal hematopoiesis stage, additional mutations occur, such as mutations in *IDH2* and/or *RhoA*, leading to lymphomagenesis. HSPC: Hematopoietic Stem and Progenitor Cell; CHIP: Clonal Hematopoiesis of Indeterminate Potential.

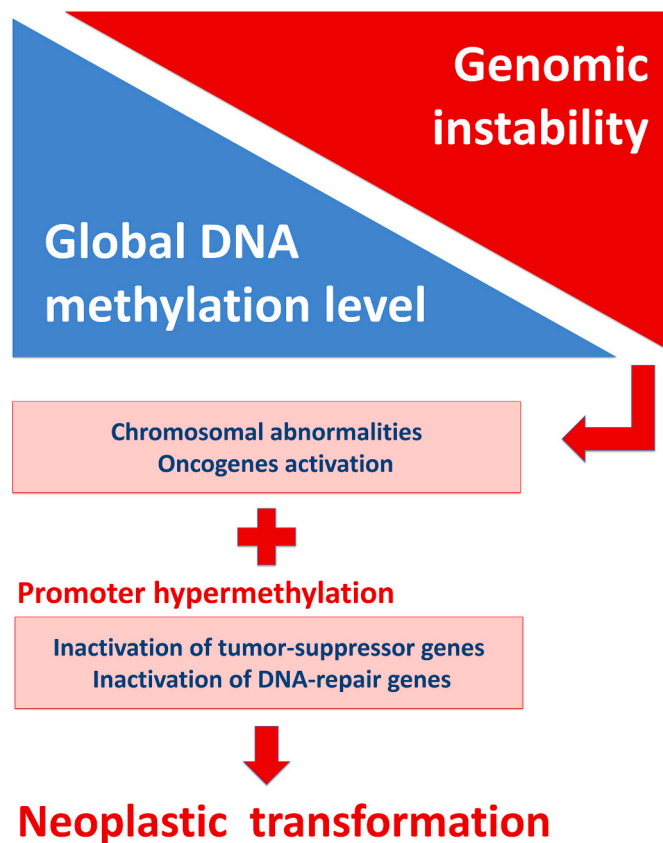


Fig. 3. The dual role of DNA methylation in neoplastic transformation. Global DNA hypomethylation can lead to genomic instability, while focalized DNA hypermethylation can inactivate tumor-suppressor and DNA-repair genes.

2.3.1. TET family members

TET1, *TET2* and *TET3* have distinct expression patterns among different cell types [76]. Somatic mutations in these genes were reported in solid tumors, such as colorectal cancer, clear-cell renal cell carcinoma and metastatic castration-resistant prostate cancer [77–79]. While, *TET1* and *TET3* mutations are very rare in hematological malignancies [80], *TET2* mutations were observed in a wide range of hematopoietic neoplasms including both myeloid and lymphoid malignancies [81–83]. Mutations in *TET2* are mostly heterozygous and are either missense present in the C-terminal catalytic domain or nonsense/frameshift located in the N-terminal region leading thus to a premature truncation of the corresponding protein [84]. However, regardless their C-terminal or N-terminal position, these mutations have been associated with an advanced stage disease and a shorter progression-free survival in T-cell lymphoma [85]. The *TET2* gene is among the most frequently mutated genes in the clonal hematopoiesis, but some studies showed that *TET2* mutations alone are not sufficient to induce a lymphoid transformation [86]. Additional supportive oncogenic events are essential to drive lymphomagenesis. Hence, mutations in *JAK2*, *KRAS*, *NRAS*, *SRSF2*, and in the epigenetic regulators *EZH2* and *DNMT3A* have been found to coexist with *TET2* mutations [87–89].

2.3.2. IDH2

IDH2 mutations in AITL can indirectly inactivate the enzymatic activity of TET, due to the conversion of α -ketoglutarate (α -KG) to the “oncometabolite” D-2-hydroxyglutarate (D2HG) [42,90]. Twenty to 45% of AITL patients had a mutated *IDH2*. The mutation is almost exclusively present at the position p.R172 of the protein [42,91,92]. *TET2* and *IDH2* mutations are not mutually exclusive in PTCL including AITL [91,93]. It is suggested that *IDH2*^{R172} mutations participate to AITL pathogenesis displaying a unique gene expression profile in comparison

to other PTCL-NOS (Not otherwise specified) subtypes [94]. Additionally, AITL samples with *IDH2* mutations displayed an important increase in H3K27me3 levels and DNA hypermethylation in gene promoters involved in T-cell receptor signaling and T-cell differentiation [94]. *IDH2* mutations as a sole abnormality did not show any prognostic value in lymphomas [90].

2.4. DNA methylation conclusion

Globally, mutations in genes responsible for DNA methylation and demethylation were reported in lymphomas leading to erroneous methylation patterns. These mutations can be commonly shared among different subtypes of lymphomas or specific to a unique subtype. Mutations leading to an aberrant focalized DNA hypermethylation can silence tumor suppressor genes, and mutations leading to wide genomic DNA hypomethylation can cause genomic instability. A comprehensive evaluation of the therapeutic response to either chemotherapy or demethylating agents may help to predict which patients may respond to personalized therapies.

3. Histone modifications

The N-termini of the four core histones, H2A, H2B, H3 and H4, known as histone tails, extend out of the nucleosome and are susceptible to a range of post-translational modifications. The main modifications are methylation, acetylation, phosphorylation, glycosylation, ubiquitination and sumoylation (sumo: small ubiquitin-related modifier) [95–99]. These modifications do not only alter the overall compaction of chromatin but, also regulate genes expression in a highly coordinated manner. The histone tail modifications are controlled by specific enzymes that are reported to be implicated in hematological malignancies including lymphomas (discussed in the following paragraphs) [100].

3.1. Histone methylation

Methylation on lysine (K) is associated with transcriptional activation or repression, depending on which residue is methylated and the degree of its methylation [101]. This mechanism is mediated by lysine methyltransferases (KMT or HMT) and lysine demethylases (KDM or HDM). Monomethylation of the histone H3 on lysine 4 (H3K4), on lysine 36 (H3K36) and on lysine 79 (H3K79) is associated with transcriptional activation, while dimethylation and trimethylation of histone H3 on lysine 9 (H3K9me2, H3K9me3) and histone H3 on lysine 27 (H3K27me2, H3K27me3) is associated with transcriptional repression (Fig. 4).

3.1.1. Lysine methyltransferases

KMT2 is a family of methyltransferases allowing H3K4 methylation and promoting transcription by inducing an open chromatin conformation. Mutations in *KMT2A* (*MLL1*), *KMT2B* (*MLL2*), *KMT2C* (*MLL3*) and *KMT2D* (*MLL2* or *MLL4*) were reported in hematopoietic neoplasms. Mutations in *KMT2D* were reported in 27% of Germinal Center (GC)-DLBCL and in 20% of Activated B-cell like (ABC)-DLBCL biopsies. *KMT2D* mutations generate a truncated protein lacking the entire C-terminal of conserved domains, and participate in malignant transformation [102]. *KMT2D* is one of the most frequently mutated genes in FL (70% to 90%) and in DLBCL (30%) [102,103]. Mutations in *KMT2D* result in the loss of its enzymatic function leading to an impaired histone methyltransferase (HMT) activity. This facilitates lymphomagenesis by remodeling the epigenetic landscape of cancer precursor cells [104]. In FL, *KMT2D* mutation occurrence is an early oncogenic event underlying its role as a key player in the development of the “common progenitor cell” population [105–107]. Mutations involving the other KMT2 family members *KMT2A*, *KMT2B* and *KMT2C* are rare in lymphomas, with some missense mutations of unknown significance located outside their enzymatic domains [108].

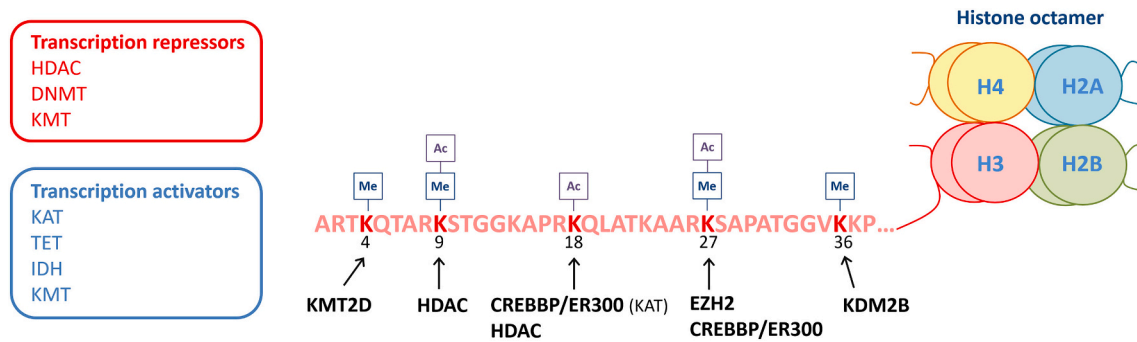


Fig. 4. This Fig. shows the histone octamer (H2A, H2B, H3 and H4) with histone H3 acetylation (Ac) and methylation (Me) in lymphomas as well as the enzymes responsible for active (in blue) or inactive (in red) transcription. Histone acetylation is associated with active transcription, and is controlled by histone lysine acetyltransferases (KAT, or HAT) and histone deacetylases (HDAC). Histone methylation is associated with transcriptional activation or repression, depending on which residue is methylated and the degree of methylation. Histone methylation is controlled by histone lysine acetyltransferases (KMT, or HMT) and histone demethylase (KDM).

DNMT: DNA methyltransferases; IDH: Isocitrate dehydrogenase; TET: Ten-eleven translocation.

3.1.2. *EZH2*

EZH2 is the catalytic unit of PRC2 (Polycomb repressive complex 2), containing the SET (Su(var)3-9, Enhancer-of-zeste and Thritorax) domain which catalyzes the methylation of the H3K27 residue. *EZH2* is the most frequently mutated PcG (Polycomb Group) member in hematological malignancies [109]. It plays a dual role in hematological tumorigenesis. Indeed, *EZH2* acts as an oncogene in Natural killer/T-cell (NKT) and B-cell lymphomas, while it acts as a tumor suppressor in T-cell acute lymphoblastic leukemia (T-ALL), myelodysplastic syndromes (MDS) and MDS/Myeloproliferative neoplasms (MPN) [110–113]. Morin et al. were the first to report mutations in this KMT leading to an increase of H3K27me3 levels in GC lymphomas [103,114]. *EZH2* mutations in FL cluster to 3 amino acid positions (Y641, A682 and A692) and are located within its catalytic domain SET [103,114–116]. *EZH2* mutations at tyrosine residue 641 (Y641) were found in ~20% of DLBCL patients, ~10% of BL patients and ~4% of primary mediastinal B-cell lymphoma (PMBCL) patients [117]. These gain-of-function mutations in *EZH2* increase gene repression via H3K27 hypermethylation and result in repression of plasma cell differentiation signatures. Overexpression of *EZH2* was also reported in NKT lymphoma and CLL/SLL [118,119]. Mutant *EZH2* as a sole abnormality is not sufficient to induce lymphomagenesis, but was shown to accelerate tumorigenesis driven by BCL2 and MYC [120,121].

3.1.3. Lysine demethylases

The roles of the KDMs in the course of some diseases remain largely unknown. However, mutations and deletions of KDMs were reported in lymphoid neoplasms. Approximately, 5% of DLBCL patients carry a mutated or deleted *KDM2B*, a gene with a H3K36 histone demethylase activity [102]. However, more information are needed regarding the function of *KDM2B* in cancers and whether its regulation is tumor and stage dependent [122]

3.2. Histone acetylation

Acetylation occurs on a lysine at different positions in the histone tail, leading to open chromatin structure and active transcription by allowing the binding of transcription factors. Acetylation of histone H3 on lysine 9 (H3K9), lysine 14 (H3K14) and lysine 27 (H3K27) are among the most studied lysine acetylations. Lysine acetylation and deacetylation are controlled by histone lysine acetyltransferases (KAT or HAT) and histone deacetylases (HDAC), respectively. KAT includes CREBBP (CBP), EP300 (p300), KAT2B (PCAF), KAT5 (Tip60) and KAT6A (MOZ), while HDACs include HDAC1-11 and SIRT1-7 [123]. The 18 discovered HDACs are divided into four subclasses: I, II, III and IV. Class I (HDACs 1, 2, 3 and 8) has an exclusive nuclear localization, class II (HDACs 4, 5, 6,

7, 9 and 10) has a nuclear and cytoplasmic localization, class III includes SIRT family of proteins, and class IV (HDAC11) has an exclusive cytoplasmic localization [124,125]. The precise mechanism of HDACs in lymphomagenesis and especially in T-cell malignancies was investigated under the intervention of HDAC inhibitors, but it is still not fully elucidated. The oncogenic participation of some KATs and HDACs is discussed next.

3.2.1. Histone acetyltransferases

CREBBP (CBP) and *EP300* (p300) are frequently mutated in B-cell lymphomas, mainly in DLBCL and FL. *CREBBP* mutations are found in 40% of FL and 15~30% of DLBCL while *EP300* mutations are found in around 5 to 10% of FL and DLBCL [108,115]. These alterations are usually monoallelic and lead to the inactivation of KAT coding domain, suggesting that KAT are haploinsufficient tumor suppressors in B-cell lymphomas. Also, the reduced KAT activity contributes to B-cell tumorigenesis [126,127]. *CREBBP* inactivating mutations exert pleiotropic effects on gene expression, probably mediated by defective BCL6 and p53 activities through a reduced acetylation of these proteins (Fig. 5) and an impairment of plasma cell differentiation [126–129]. Deleterious *CREBBP* mutations (p.Q839* and p.S1207fs) were identified in CTCL and more specifically in SS [49]. *CREBBP* mutations were also found in 26% of patients with primary cutaneous diffuse large B-cell lymphoma-leg type (PCLBCL-LT) [130]. Germline mutations of *CREBBP/EP300* are found in patients with Rubinstein-Taybi syndrome, that can contribute to the occurrence of some rare tumors including lymphomas in these patients [131]. Remarkably, *CREBBP/EP300* deficiency seems to exert a role predominantly in B-cell malignancies, while it is less frequent in solid tumors [132,133]. Recently, the *CREBBP/EP300* synthetic lethal interaction mechanism has been unveiled, paving the way to a potential targeting of *CREBBP*-mutated DLBCL/FL cells, using selective small inhibitors of *CREBBP* and *EP300* [134].

3.2.2. Histone deacetylases

Different HDACs were reported to play a role in the normal and aberrant differentiation of B and T-cells. HDAC-BCL6 complexes contribute to lymphomagenesis through deregulation of pathways involved in cell proliferation and survival [135]. In GC lymphomas, *CREBBP* mutations disable the acetylation process and result in an unopposed deacetylation by BCL6-SMRT-HDAC3 complexes of the “B-cell signaling” and “immune response” genes enhancers promoting lymphomagenesis [126]. HDAC7 was reported to have an anti-oncogenic effect and to be frequently under-expressed in B-cell malignancies including BL [136]. HDAC6 was differentially expressed in lymphomas, with a weak expression in B-cell lymphomas and an overexpression in T-cell lymphomas [137,138]. Additionally, HDAC6 inhibition showed

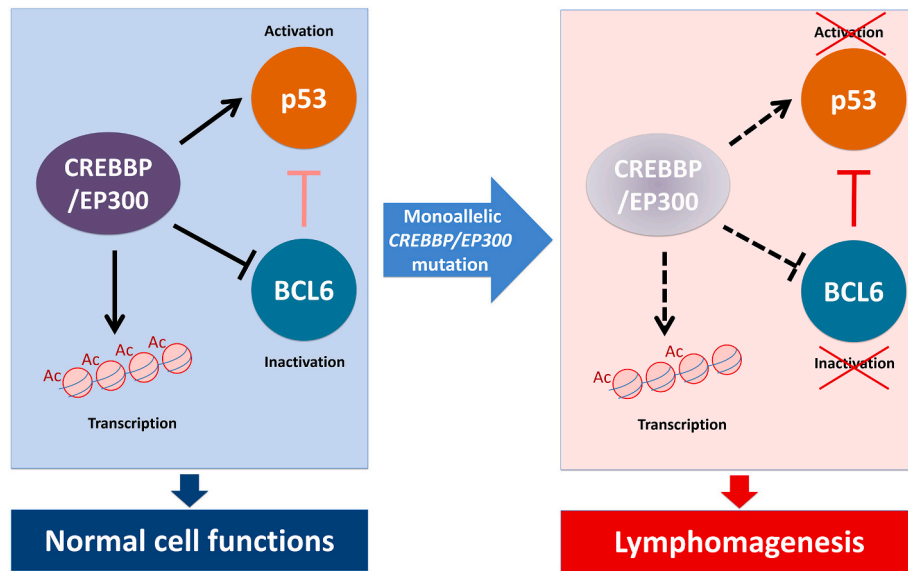


Fig. 5. Monoallelic *CREBBP* (*CBP*) or *EP300* (*p300*) mutations lead to the inactivation of KAT coding domain. Mutated *CREBBP* or *EP300* result in reduced acetylation of histone (H3) and non-histone proteins. And will participate in the lymphomagenesis by the activation of BCL6 and the repression of the tumor suppressor p53.

evident antitumoral effect in MCL [139]. In DLBCL, the overexpressed HDAC1 was associated with a poor survival. To date, the available information showed that HDACs mutations and copy number alterations (CNAs) are not frequent in lymphomas. So far, *HDAC1*, *HDAC4* and *HDAC7* mutations or CNAs were reported in DLBCL patients [103,108,115,140].

3.3. Histone modifications conclusion

To sum up, histone methylation and histone acetylation represent the most studied histone modifications in lymphomas. Mutations in genes regulating histone modifications were observed as well as an abnormal specific methylation or acetylation profiles. While the oncogenic role of some mutations was well demonstrated, only correlative data exist for other mutations, awaiting for functional research studies. Histone phosphorylation, glycosylation, ubiquitination and sumoylation remain not well explored in the field of tumorigenesis and precisely in lymphomagenesis. So far, most of the studies were focused on the genes controlling the global histone modifications. Therefore, targeted studies focusing on specific genes expression that might be altered by histone modifications are needed to explain the dysregulations that are not caused by DNA mutations or methylation.

4. MicroRNAs (miRNAs)

The first discovery of miRNAs came in 1993 from two published papers describing a locus in *Caenorhabditis elegans* (*C. elegans*) involved in the developmental timing of this species [141,142]. In 2001, Lee and Ambros identified further 15 *C. elegans* miRNAs and used the miRNA term for the first time [143]. Since that time, more than 25,000 miRNAs have been identified in over 200 different species, including more than 2,500 human miRNAs [144,145]. However, it is still debatable whether miRNAs regulation should be clearly classified among epigenetic regulators or not.

MiRNAs are small (21 to 25 nucleotides) non-coding RNAs, encoded by genes that are distributed throughout the entire genome. Many of them are located in non-coding regions; others are located within an intron or UTR (Untranslated region) of a coding gene [146]. MiRNAs regulate the post-transcriptional gene expression that is ensured by mRNA cleavage and degradation or translational repression. It is

estimated that around two-third of human genes are directly regulated by miRNAs, suggesting that the latter are involved in most cellular processes [147]. Several recent studies showed that miRNAs are involved in normal and pathogenic hematopoiesis, including lymphomagenesis [148–151]. They act as strong negative regulators of hematopoiesis by blocking important genes implicated in the final stages of cells differentiation [152]. In cancers, miRNAs can function as oncogenes or tumor suppressors and they are named respectively onco-miRs and suppressor-miRs [153]. MiRNAs are considered as potential tumor biomarkers, as their expression levels can be used to classify tumors according to their diagnosis, subtypes and stages. But, so far no single miRNA identified can be used solely as a biomarker [154]. Deregulated miRNAs expression can be due to aberrant genomic gains or losses but alternatively to other epigenetic modifications such as DNA methylation (Fig. 6) [155–157]. MiR-124a was the first miRNA reported to be inactivated by DNA methylation [158].

4.1. Commonly deregulated MicroRNAs in lymphomas

Over the years, many miRNAs expression profiles were reported in cancers and lymphomas but sometimes with a lack of reproducibility among studies. The most commonly dysregulated miR in lymphomas is the upregulated miR-155, also reported in all cancers [159–167]. The cluster miR-17~92 was upregulated in different lymphomas' subtypes [161,163,168–171] while miR-150 was found downregulated [161,163,172–175]. Table 1 summarizes the upregulated and down-regulated miRNAs in lymphomas, as well as their profiles and signatures.

4.2. MicroRNAs in the subtypes of the same pathology

MiRNAs can be differentially expressed among the different classifications of the same pathology. For example, it has been reported that different subtypes of DLBCL, GC-DLBCL and ABC-DLBCL may be distinguishable by their miRNAs profiles, but others were not able to confirm these results. This might be explained by the different techniques used and/or the interlaboratory low reproducibility in miRNAs quantification [168,176]. While, ABC-DLBCL is associated with high expression of miR-21, miR-146a, miR-155, miR-221 and miR-363, GC-DLBCL express high levels of miR-17~92 cluster and miR-421

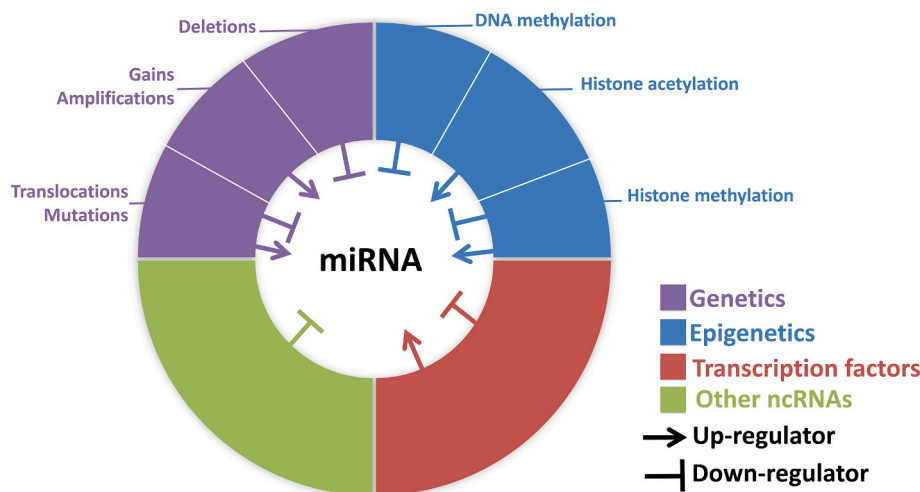


Fig. 6. This Fig. shows the different mechanisms that can regulate the maturation and the expression of miRNAs. Other than their own regulation and maturation, upregulated or downregulated miRNAs can result from genetic alterations (copy number gains or losses, mutations or translocations), epigenetic modifications (DNA/histone methylation or histone acetylation), transcription factors expression and even some other non-coding RNAs (ncRNAs) such as competing endogenous RNA (ceRNA).

[168,169,177]. Additionally in the t(14;18) negative subset of FL, the downregulation of miR-16, miR-26a, miR-101, miR-29c and miR-138 was associated with changes in the expression of genes related to cell cycle, apoptosis and B-cell differentiation [178]. In the t(8;14) negative BL cases, miR-34b could be responsible for the overexpression of the MYC oncogene [179]. Furthermore, the differences between miRNAs profiles in ALK(+) and ALK(-) ALCL included an upregulation of miR-135b in ALK(+) ALCL cell lines and human samples, while the oncogenic miR-155 showed a higher expression in ALK(-) ALCL [165,166,180].

4.3. MicroRNAs affecting the prognosis

Correlative studies identified a great number of miRNAs that were found deregulated and implicated in the prognosis or the therapeutic response. Among them, miR-135a was the first miRNA reported to be associated with the survival outcome in HL disease through direct targeting of JAK2. HL patients with low levels of miR-135a had a shorter disease-free survival compared to those with high levels of this miRNA [181]. In DLBCL, low expressions of the miR-27b and miR-34a were correlated to a poor prognosis, and miR-21 expression was proposed to be an independent prognostic factor in ABC and GC-DLBCL [182–185]. In FL, the downregulated miR-451 and the upregulated miR-338-5p may be used as biomarkers to predict the invasion of FL cells into the bone marrow [186]. After transformation of FL into a high-grade lymphoma, it is named transformed FL (tFL) [187,188]. In this transformation, two miRNAs were reported to be differentially expressed: miR-17-5p was found to be upregulated while miR-31 was downregulated [189]. In MCL, the downregulation of miR-223 and the upregulation of miR-20b and miR-18b correlated with more severe clinical features, while miR-15b was reported to be involved in the transformation to an aggressive MCL [174,190–192]. In CTCL, the high levels of miR-155 and low levels of miR-200b may predict decreased overall survival [167]. Whereas, the deregulation of miR-106b-5p, miR-148a-3p and miR-338-3p predicted the disease progression in early stage Mycosis Fongoides (MF), a subtype of CTCL [193].

4.4. MicroRNAs conclusion

Taken together, miRNAs profiling revealed numerous upregulated onco-miRNAs and downregulated suppressor-miRNAs. A low reproducibility exists between single center studies presumably due to the use of different methodologies (Microarrays, PCR, RNA-Seq...), and the analysis of different tissues for the same subtype of lymphoma (cell lines, blood cells, serum, lymph nodes, fresh tumor biopsies or FFPE tissues).

Therefore, multi-center studies focusing on miRNAs as biomarkers in the treatment of lymphomas might unveil promising information, as some miRNAs showed important correlations with drug resistance or sensitivity.

5. Epigenetic therapies

The first approved epigenetic drugs for clinical use were the inhibitors for DNMT in MDS and for HDAC in CTCL. Conversely, these drugs did not show any significant activity in solid tumors. Numerous new drugs targeting epigenetic modifications are presently under clinical trials investigations for patients with hematological neoplasms including lymphomas. Here, we report previously approved epigenetic drugs and new promising drugs being tested (Table 2).

5.1. DNA-methyltransferase inhibitors

The first use of a DNA-methyltransferase inhibitors (DNMTi) goes back to the 1970s, when 5-azacytidine was used as a cytidine-analog-derived chemotherapeutic agent that was revealed to have pronounced toxicity [194]. Years later, a revolutionary discovery was made when a low dose of 5-azacytidine was capable to reduce DNA methylation in cell culture and to induce severe phenotypic changes with less cytotoxic effects [195,196]. It was later shown that 5-azacytidine can be classified as an epigenetic drug that reduces global DNA methylation levels [195]. Since then, 5-azacytidine proved to be efficient particularly in patients with MDS. As a consequence, 5-azacytidine was the first epigenetic drug approved by the Food and Drug Administration (FDA) and used in cancer therapy [197,198].

On the molecular level, DNMTi incorporate into the DNA of proliferative cells and target the DNMT1 leading to its proteasomal degradation [199]. Therefore, the original methylation pattern is continuously lost during cell divisions. Increased evidences can explain the DNMTi anti-tumor effects: DNMTi can demethylate the promoters of aberrantly silenced tumor suppressor genes, initiating their reactivation [200]. DNMTi can also demethylate gene bodies, resulting in the downregulation of oncogenes [201], and DNMTi can activate the immune system, either by the re-expression of dormant antigens or by the upregulation of endogenous retroviral transcripts [202,203].

On the clinical level, the efficacy of DNMTi (i.e. 5-azacytidine) in lymphoid neoplasms is less prominent than in MDS or acute myeloid leukemia (AML). While 5-azacytidine is still not approved for the treatment of AITL patients, a clinical trial reported prominent responses to 5-azacytidine not only in AITL patients with an associated myeloid neoplasm but also in 4 out of 7 AITL patients without a myeloid

Table 1 (continued)

SLL/ CLL	in patients who failed to achieve a complete response	miR-92a miR-155	[256] [164,256]
Downregulated		miR-15a /miR-16-1 miR-129-2 miR-9 family, mainly miR-9-3 miR-181b miR-150	[255,257] [258] [259] [260] [172,256]

Table 2
Epigenetic therapies in lymphomas.

Drug/Agent	Epigenetic effect	Lymphoma	Status
Vorinostat	HDACi	CTCL	Approved
Romidepsin	HDACi	CTCL	Approved
Belinostat	HDACi	Relapsed/refractory PTCL	Approved
Decitabine	DNMTi	Relapsed/refractory DLBCL Relapsed/refractory T-lymphoblastic lymphoma	Clinical trials
Tazemetostat	EZH2i (HMTi)	DLBCL, FL, tFL and PMBCL	Clinical trials
CPI-1205	EZH2i (HMTi)	B-cell lymphomas	Clinical trials
SHR2554	EZH2i (HMTi)	Relapsed/refractory mature lymphoid neoplasms	Clinical trials
PF-06821497	EZH2i (HMTi)	FL DLBCL	Clinical trials
MAK683	EZH2i (HMTi)	DLBCL	Clinical trials
Entinostat	HDACi	ALCL AITL	Clinical trials
Panobinostat	HDACi	DLBCL CTCL MCL AITL PTCL	Clinical trials
Resminostat	HDACi	CTCL, MF, SS	Clinical trials
Abexinostat	HDACi	DLBCL FL MCL	Clinical trials
Mocetionostat	HDACi	DLBCL FL	Clinical trials
Cobomarsen	miR-155 inhibitor	MF DLBCL ATLL	Clinical trials

association. This indicated that the effect of 5-azacytidine on AITL is not restricted to patients with associated myeloid neoplasm [204]. Currently, decitabine (5-aza-2'-deoxycytidine) is in phase 4 trial used as monotherapy in relapsed and refractory DLBCL (NCT03579082), and in relapsed or refractory T-lymphoblastic Lymphoma (NCT03558412). Decitabine is being investigated in few other trials whether alone or in combination with other drugs, as in a phase 2 trial in combination with SHR-1210 (PD-1 antibody) in relapsed or refractory HL (NCT03250962). The use of DNMTi as a monotherapy in lymphomas is still debatable, meanwhile several ongoing studies show that DNMTi in combination with standard chemotherapy could improve clinical response [205,206]. A new regimen called D-COP combining low dose decitabine with COP (Cyclophosphamide+Vindesine+Bonisone) is being tested as a treatment for relapsed and refractory DLBCL (NCT03494296). Another protocol is being tested in a phase 1/2 trial for relapsed or refractory PMBCL patients combining SHR-1210, GVD chemotherapy (Gemcitabine, Vinorelbine and Doxorubicine) and low dose decitabine (NCT03346642). Recently, new DNMTi with significantly improved pharmacological properties were identified such as dicyanopyridine (DCP). These DNMTi are non-DNA-incorporating molecules generating extensive antitumor activity and more robust results in term of DNA methylation [207].

5.2. Histone methyltransferases inhibitors

Among histone methyltransferases (HMT), EZH2 was considered as a promising therapeutic target. The interest in EZH2 as a target came from the identification of its activating mutations in FL and GC-DLBCL leading to an increased enzymatic activity. EZH2-PRC2 inhibitors were thus developed and showed encouraging results in clinical trials [117,208,209].

Tazemetostat (EPZ-6438, E7438), an orally administrated EZH2 inhibitor is showing very promising results in phase 1/2 clinical trial in advanced solid tumors, DLBCL, FL, tFL and PMBCL (NCT01897571). Data from this trial showed good clinical activity in FL and in DLBCL patients with both wild-type and mutated *EZH2* [210]. A phase 2 trial is studying tazemetostat's activity exclusively in mutated *EZH2* patients with relapse or refractory B-cell lymphomas (NCT03456726). The "Epi-RCHOP" refers to the combination of tazemetostat and R-CHOP chemotherapy (Rituximab + Cyclophosphamide + Doxorubicin + Vincristine + Bonisone) in phase 1/2 clinical trial in newly diagnosed DLBCL patients (NCT02889523). The "Epi-R-CHOP" trial was suspended following the development of a secondary T-cell lymphoma in one pediatric case receiving tazemetostat in another study. Hence, the FDA put in 2018 a partial hold on the enrolment of trials investigating tazemetostat. However, the hold was lifted allowing new enrollments to reopen, and a phase 2 "Epi-R-CHOP" trial is expected [211].

Recently, several novel EZH2 inhibitors molecules were developed and some of them are being tested in clinical trials. In phase 1 trials, two small molecules are being tested; CPI-1205 in patients with B-cell lymphomas (NCT02395601) and SHR2554 in patients with relapsed or refractory mature lymphoid neoplasms (NCT03603951). Recently, a study was terminated in phase 1 clinical trial of GSK2816126 molecule showing modest anticancer activity at tolerable doses of this molecule in DLBCL, tFL, other NHLs and solid tumors (NCT02082977) [212]. More molecules are being tested clinically, such as PF-06821497 and MAK683 in patients with FL and DLBCL (NCT03460977 and NCT02900651).

5.3. Histone deacetylase inhibitors

HDAC proved to interact, in the cytoplasm, with some proteins involved in carcinogenesis (such as p53) and showed to be involved in several cell functions such as cell cycle regulation, stress response, protein degradation, cytokine signaling and apoptosis [213–216]. Due to these findings, it was rational to consider HDAC as potential targets in the context of epigenetic therapies using HDAC inhibitors (HDACi).

The failure of multi-agent chemotherapies in treating MfT and PTCL urged the search for novel therapeutical tools and strategies. This coincided with the discovery of first HDACi molecules: Vorinostat (Suberoylanilide hydroxamic acid, SAHA) and romidepsin. They rapidly emerged into clinical trials due to their anti-tumor activity and their efficacy in preclinical studies on T-cell lymphomas [217,218]. Vorinostat inhibits mainly HDAC class I and II, while romidepsin is specific to HDAC class I. Two clinical trials confirmed the safety and the clinical activity of vorinostat in the treatment of CTCL, leading to its approval in 2006 for CTCL treatment [219–221]. This approval made vorinostat the second epigenetic drug to be approved for the treatment of a hematological malignancy [221]. Romidepsin's efficacy was confirmed by two other clinical trials and resulted in FDA approval for the treatment of

relapsed or refractory CTCL in 2011 [222]. In 2014, the HDACi belinostat (PXD-101) was approved by the FDA for the treatment of patients with relapsed or refractory PTCL [223]. To date, the exact chromatin changes in CTCL patients treated with HDACi remain unknown. It has been shown that HDACi manipulate DNA accessibility in both CTCL and host CD4+ T cells, suggesting an impact on host immunity in patients [224].

Moreover, romidepsin and vorinostat are being further tested as monotherapy or in combination with other drugs in more than 50 and 70 clinical trials, respectively. Other HDACi are being studied in several phase 1/2 trials for the treatment of B-cell or T-cell lymphomas, such as entinostat (MS-275), panobinostat (LBH589), resminostat (4SC-201), abexinostat (PCI24781), mocetinostat (MGCD0103) and others. Most of the HDACi failed to be efficient in DLBCL. In the light of these results, a genomic-based stratification approach in patients with B-cell lymphomas might be necessary prior to treatment.

5.4. Targeting miRNAs

As previously discussed, miR-155 is the most investigated miRNA in cancer. Several groups have hypothesized that miR-155 might be a therapeutic target in MF [225,226]. Consequently, Cobomarsen (MRG-106), a synthetic locked nucleic acid-modified oligonucleotide inhibitor of miR-155, was developed [227]. Recent studies demonstrated that Cobomarsen inhibits cell proliferation and induces apoptosis in MF and in human lymphotropic virus type 1 (HTLV-1+)-related CTCL [227]. Currently, it is being tested in three clinical trials in patients with MF, DLBCL, CLL or in adult T-cell leukemia/lymphoma (ATLL) (NCT02580552, NCT02580552, NCT03837457).

6. Conclusion

Among the epigenetic regulators identified so far, the vast majority have well-defined roles in normal lymphomagenesis, and their fine regulation is intimately involved in normal immune cells functions [228]. In lymphomas, epigenetic dysregulation correlated with disordered lymphocyte functions [229], and different lymphomas' specific epigenetic signatures were highlighted [148,167,168,230,231]. The epigenetic signature can define lymphomas' molecular subtypes, and help to confirm the diagnosis or to predict the prognosis.

Amid the broad types of epigenetic regulators, DNA methylation, remains the most studied epigenetic mark ever. However, during the last decade, researchers deployed efforts to focus on others epigenetic alterations such as histones modifications and miRNAs expressions. Notwithstanding the prompt growth of published data proposing miRNAs as biomarkers in lymphomas, we are still far from their clinical validation and implementation. Indeed, contradictory miRNAs biomarkers signatures for the same pathology were reported, probably related to the lack of standard normalization approaches and to the abundance of single-center investigations. In order to establish accurate miRNAs signatures, it would be thus necessary to adopt multi-center studies together with standardized approaches. By following these methodological recommendations, pertinent epigenetic biomarkers with clinical relevance and potential therapeutic targets could be identified.

7. Future considerations

While targeting miRNAs remains more challenging at the clinical level, epigenetic therapy has already proven effective in the world of cancer including lymphomas. Practically, DNMTi, HMTi and HDACi are being used. Several drugs targeting epigenetic modifiers have demonstrated evident activity with ongoing trials exploring new combinations among epigenetic drugs and chemotherapies or even immunotherapies. Regardless of the remarkable progress achieved in the field of cancer epigenetic therapies, further insights are needed into the biological

consequences of epigenetic therapies as well as the identification of response mechanisms. This will improve our understandings in restoring the aberrant epigenome, in order to define personalized therapies. In conclusion, despite the new approved epigenetic treatments available nowadays, we are still in need of newer drugs and/or to define patients who will benefit from combination therapies, especially for those with aggressive lymphomas. Epigenetic modifications can alter the accessibility of the transcription factors to their binding sites on the DNA sequence leading to an erroneous activation or silencing. The links between the transcription factors and the epigenetic modifications represent an exciting field to be investigated with attractive perspectives in the development of novel therapeutic approaches.

8. Practice points

- Frequent epigenetic dysregulations including DNA methylation, histone modifications and non-coding RNAs are reported in lymphomas.
- Genetic alterations in the genes encoding for histone/chromatin modifiers are frequently responsible for the epigenetic alterations in lymphomas.
- Epigenetic dysregulations can be specific to a certain subtype or commonly shared among different subtypes of lymphomas.
- Epigenetic therapies such as DNMTi, HMTi and HDACi are used for the treatment of lymphomas.
- New epigenetic therapies are currently being tested as monotherapies or in combination.

9. Research agenda

- Development of new epigenetic drugs especially for aggressive lymphomas.
- Randomized clinical trials to evaluate new epigenetic drugs or new combinations with chemotherapies and/or immunotherapies.
- Focalized studies on the interactions between the transcription factors and the epigenetic modifications.

Author Contributions

All authors made substantial contributions to this study and they approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

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1.2. Les télomères

L'organisation de la molécule d'ADN, support de l'information génétique, est organisée dans les chromosomes de manière à protéger l'intégrité de celui-ci et par conséquent celle de l'information génétique. Ainsi chaque bras chromosomique se termine par une séquence d'ADN particulière hautement conservée. Chez les vertébrés, cette séquence est formée de la répétition en tandem de l'hexamère 5' (-TTAAGGG-)n 3' formant environ 10 à 15 kb chez les humains. Cette extrémité particulière du chromosome a été dénommée télomère indépendamment par Hermann Müller and Barbara McClintock à la fin des années 1930 (Pfeiffer and Lingner, 2013; Shay and Wright, 2019; Webb et al., 2013).

1.2.1. Structure et organisation des télomères

Les télomères comprennent une région double brin proximale et une région simple brin distale (Figure 4A) avec des régions interstitielles et sub-télomériques qui séparent les répétitions d'ADN du reste du chromosome (de Lange, 2018).

L'extrémité simple brin, riche en G, comprend entre 50 et 300 nucléotides. De manière à protéger l'extrémité chromosomique, l'ensemble des répétitions télomériques se replie sur elle-même en formant une large boucle, dénommée boucle T pour « télomérique » (T-loop) (Nandakumar and Cech, 2013). La partie terminale simple brin se retrouve séquestrée au niveau d'une structure tricaténaire dénommée boucle D pour « déplacement » (D-loop) (Lei et al., 2004; Loayza and De Lange, 2003) (Figure 4B).

L'extrémité monocataire riche en G peut également s'organiser en une structure hélicoïdale à quatre brins connue sous le nom de G-quadruplex (Figure 4D). Une telle organisation implique l'empilement dans un même plan de quartets de guanines. Chaque quartet est formé par l'association de quatre guanines dans un arrangement de liaison hydrogène de type Hoogsten cyclique (Frees et al., 2014; Martínez and Blasco, 2018). La formation de tétrades de guanines assure la stabilisation de l'extrémité chromosomique non plus dans une structure à deux brins (comme dans le duplexe) mais dans une structure à quatre brins. Les G-quadruplexes

se caractérisent par une très grande stabilité thermodynamique et une cinétique de formation très favorable (l'assemblage se faisant de façon intramoléculaire) (Monchaud, 2017). Ces structures compactes et stables, en plus de former une coiffe télomérique, inhibent l'accès à la télomérase (Lin and Yang, 2017). Bien que ces structures aient été observée *in vivo* par résonance magnétique nucléaire, leur fonction biologique reste inconnue (Lin and Yang, 2017; Rhodes and Lipps, 2015; Zhang et al., 2010).

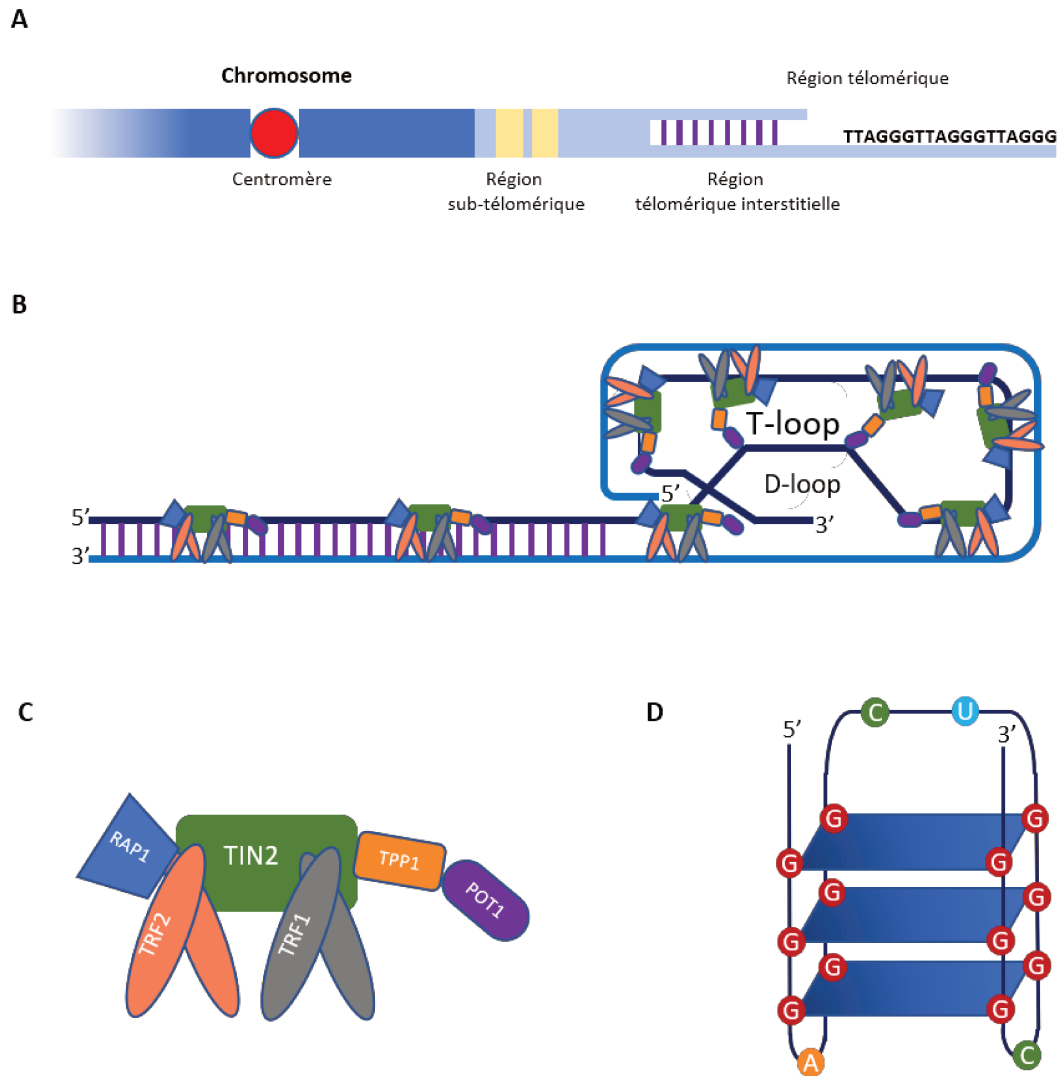


Figure 4 : Représentations schématiques : (A) des régions sub-téломérique et téломérique, montrant les répétitions des nucléotides incluant une région double-brin et une région simple-brin. (B) La boucle T (T-loop) et la boucle D (D-loop) formées par les complexes Shelterin et la queue d'ADN. (C) Le complexe Shelterin formé par les protéines « Telomeric-repeat-binding factor 1 and 2 » (TRF1 et TRF2), « protection of telomeres 1 » (POT1), « TRF1-interacting nuclear protein 2 » (TIN2), « TIN2-interacting protein » (TPP1), et « repressor-activator protein 1 » (RAP1). (D) La structure G-quadruplexe. D'après Frees et al., 2014 et Martínez and Blasco, 2018.

1.2.2. Protéines téломériques

En plus de leur stabilisation par l'adoption d'une conformation tri-dimensionnelle particulière, les téломères sont stabilisés par une association à plusieurs protéines.

Comme toute chromatine, l'ADN télomérique est empaqueté autour des histones. Les télomères des eucaryotes supérieurs sont principalement composés d'hétérochromatine riche en histone 3 triméthylée sur la lysine 9 (H3K9me3), en histone 4 triméthylée sur la lysine 20 (H4K20me3) et une isoforme de la protéine hétérochromatine (HP) (Chow et al., 2018; Galati et al., 2013; Vaquero-Sedas and Vega-Palas, 2011). Les histones méthyltransférases (HMT), SUV39H1 et SUV39H2, favorisent la méthylation des résidus H3K9. Une fois tri-méthylée, la H3K9me3 recrute les protéines HP1. Par la formation de dimères, les protéines HP1 jouent un rôle crucial dans la compaction de la chromatine en établissant des ponts moléculaires entre les nucléosomes voisins (Decottignies, 2014). Une fois liées aux H3K9me3, les protéines HP1 recrutent à leur tour, les HMT capables de triméthyliser H3K9, créant ainsi de nouveaux sites de liaison pour HP1 (Chaib et al., 2011). Afin d'éviter la propagation de la forme hétérochromatine au-delà de la région télomérique des protéines insultrices comme les CTCF « *CCCTC-binding factor zinc finger protein* » se lient à la chromatine. Ces protéines recrutent des histones acétyltransférases (HAT) capables de s'opposer à l'action des HMT (Decottignies, 2014). En conclusion, l'hétérochromatine télomérique joue un rôle dans le maintien de l'intégrité structurale du télomère. La perte de l'hétérochromatine se traduit par une conformation plus ouverte de la chromatine, une fonction télomère défectueuse, et ceci peut conduire à une instabilité chromosomique (Schoeftner and Blasco, 2009).

Pour structurer de manière aussi particulière les séquences télomériques, des protéines spécifiques interagissent avec ces séquences en modulant leur organisation structurale et fonctionnelle. Ces protéines au nombre de six constituent le complexe shelterin (de Lange, 2018) (Figure 4C). L'abondance de ces protéines shelterin fait qu'elles sont en mesure d'interagir avec l'ensemble de l'ADN télomérique qu'il soit en double ou simple brin (Takai et al., 2010). Chez les humains les shelterin sont les « Telomeric-repeat-binding factor 1 and 2 » (TRF1 et TRF2), le « protection of telomeres 1 » (POT1), le « TRF1-interacting nuclear protein 2 » (TIN2), la « TIN2-interacting protein » (TPP1), et la « repressor-activator protein 1 » (RAP1) (de Lange, 2005). La protéine POT1 se lie à l'ADN simple brin, TRF1 et TRF2 se lient à l'ADN double brin, les autres protéines agissent comme des adaptateurs et modulent les interactions entre les composantes des shelterin (Martínez and Blasco, 2011; Shay and Wright, 2019). Le complexe shelterin

fonctionne comme une unité dynamique qui régule la longueur des télomères, empêche les extrémités chromosomiques d'être reconnues comme des dommages à l'ADN et réprime les signaux de réponse aux dommages à l'ADN (DDR). Les protéines TRF1 et TRF2 régulent négativement la longueur des télomères et favorisent la réplication des télomères (Lin et al., 2014). Le domaine TRFH (domaine d'homologie entre TRF1 et TRF2) de TRF2 régule la formation de la boucle T dont l'assemblage et le désassemblage est coordonné au cours du cycle cellulaire. TRF1 et TRF2 inhibent aussi le mécanisme de réparation de l'ADN par la jonction d'extrémités non homologues (NHEJ) (Schmutz and de Lange, 2016). TIN2 relie TRF1 et TRF2 en se liant aux deux protéines simultanément. TIN2 recrute TPP1 en formant un triple complexe TIN2-TPP1-TRF2 (Hu et al., 2017). Cette interaction fournit une base structurale pour l'assemblage du complexe shelterin (Kim et al., 2017). Dans cette triple association, TPP1 est essentiel au recrutement de POT1 au niveau des télomères. POT1 interagit avec une forte spécificité à l'ADN simple brin et par son association à TPP1 il interagit à l'ADN double brin (Hu et al., 2017). La protéine RAP1 qui ne se lie pas directement à l'ADN, forme un complexe avec TRF2 réprimant ainsi les recombinaisons télomériques homologues (Martinez et al., 2010; Rai et al., 2016). Le couple RAP1-TRF2 empêche la localisation, au niveau des télomères, de protéines comme la polyADD-ribose polymérase 1 et la SLX4 « SLX4 structure-specific endonuclease subunit » (Rai et al., 2016).

Des complexes protéiques autres que celui des shelterins contribuent à la régulation et au maintien des télomères (Pinto et al., 2011; Rai et al., 2016). Ces complexes sont soit directement recrutés au niveau des télomères soit indirectement via des interactions avec les composants shelterin (Pinto et al., 2011). Ainsi, le CST, un complexe protéique hétérotrimérique composé du « conserved telomere protection component 1 » (CTC1), du « suppressor of cdc13a » (STN1) et du « telomeric pathway with STN1 » (TEN1), se localise sur l'ADN simple brin et intervient dans la protection et la longueur des télomères. Au moment de la réplication le complexe CST interagit avec l'ADN primase Pol alpha (Rice and Skordalakes, 2016). Les protéines RTEL1 « helicase regulator of telomere length 1 » et RAD51 sont impliquées dans la réplication et la recombinaison de l'ADN télomérique (Diotti and Loayza, 2011; Vannier et al., 2013).

1.2.3. La réplication des télomères et ses conséquences

Léonard Hayflick a démontré dès 1960 que les cellules normales en culture arrêtent leur division après 40 à 60 passages (Shay and Wright, 2000). Cet arrêt de division s'explique par la réplication incomplète du « brin retardé » responsable du raccourcissement progressif des télomères. La perte des longueurs télomériques est estimée entre 50 et 250 pb par mitose (Heidenreich and Kumar, 2017; Ohki et al., 2001; Tomita, 2018). Ce raccourcissement agit comme un "comptage moléculaire" qui conduit finalement à la sénescence répllicative appelée aussi « limite de Hayflick » ou à la mort des cellules par apoptose (Shay and Wright, 2011). Dans les cellules proliférantes des tissus en auto-renouvellement, comme les cellules hématopoïétiques, les cellules de la peau ou les cellules de l'épithélium gastro-intestinal, le raccourcissement des télomères est atténué par l'activité de la télomérase (Heidenreich and Kumar, 2017; Shay and Wright, 2019). Cependant, la plupart des cellules souches adultes et des cellules somatiques ne présentent pas une activité télomérase suffisante pour maintenir indéfiniment la longueur de leurs télomères. Par conséquent, les télomères de ces cellules se raccourcissent jusqu'à atteindre une longueur critique provoquant ainsi la sénescence des cellules (Blasco, 2005). Ce processus, qui limite la capacité répllicative des cellules, est considéré comme un mécanisme suppresseur des tumeurs (O'Sullivan and Karlseder, 2010), car afin d'acquies un état de prolifération illimité il est nécessaire que la cellule tumorale arrive à maintenir une certaine longueur télomérique.

1.3. La télomérase

La télomérase est en mesure de neutraliser le raccourcissement des télomères lié à la réplication incomplète de l'ADN. Le nombre de répétitions ajoutées par la télomérase est un mécanisme finement contrôlé dont la perturbation peut engendrer différentes pathologies liées aux télomères (Heidenreich and Kumar, 2017). Dans la majorité des cancers humains (80 à 90%), la télomérase est activée de manière pathologique permettant l'immortalisation cellulaire (Bodnar et al., 1998; Nakamura et al., 1997; Umbricht et al., 1999). Dans les autres tumeurs, la longueur des télomères est régulée par le mécanisme d'allongement alternatif des télomères (ALT) qui est basé sur la recombinaison homologue des télomères (Gaspar et al., 2018).

Bien que la maintenance des télomères soit nécessaire pour assurer l'immortalité des cellules tumorales, *hTERT* exerce aussi d'autres fonctions cellulaires (Li and Tergaonkar, 2014; Low and Tergaonkar, 2013). Ces fonctions, non reliées aux télomères, sont impliquées dans l'oncogenèse incluant par exemple la régulation de l'activité mitochondriale, la prolifération cellulaire et l'apoptose, les voies de signalisation (WNT/B-catenin, NF- κ B), la réparation des dommages à l'ADN, l'inflammation et la métastase (Koh et al., 2015; Nguyen and Wong, 2020; Ségal-Bendirdjian and Geli, 2019). Le mécanisme moléculaire par lequel la réactivation de la télomérase confère aux cellules tumorales de nombreuses propriétés « non-canoniques » caractéristiques du cancer est toujours mal connu (Koh et al., 2015; Ségal-Bendirdjian and Geli, 2019).

1.3.1. ***Structure de la télomérase***

La télomérase humaine est composée d'une sous-unité catalytique à activité transcriptase inverse (TERT « TElomerase Reverse Transcriptase ») et d'un composant ARN (TERC « TElomerase RNA Component »), qui sert de matrice pour l'élongation des séquences télomériques. A ces deux éléments s'ajoutent des molécules accessoires dont le rôle est de réguler la biogenèse de la télomérase ainsi que sa localisation et ses fonctions subcellulaires (Figure 5) (Arndt and MacKenzie, 2016; Collins, 2006; Podlevsky and Chen, 2012).

L'activité de la télomérase est régulée d'une manière fine et précise depuis la transcription et la biosynthèse de ses composants jusqu'à son recrutement au niveau des télomères pour sa fonction canonique ou sur ses autres cibles pour ses fonctions non-canoniques. Dans la majorité des cellules, il a été démontré que l'expression de la sous-unité catalytique de la télomérase *hTERT* est le facteur limitant pour assurer une activité télomérase efficace (Liu et al., 2000). Cela a été démontré par le fait qu'une simple expression ectopique de *hTERT* était suffisante pour induire une immortalisation de certaines cellules humaines. Depuis, l'expression de l'ARNm de *hTERT* est considérée suffisante pour reconstituer une activité télomérase (Bodnar et al., 1998; Meyerson et al., 1997; Vaziri and Benchimol, 1998). La sous-unité TERC est quant à elle présente dans les cellules de manière constitutive. Dans le paragraphe suivant nous allons nous intéresser au gène *hTERT* et aux mécanismes moléculaires qui influencent sa régulation.

1.3.2. **Le gène *hTERT***

Le gène *hTERT* est localisé sur le bras court du chromosome 5 en 5p.15.3.3, environ à un mégabase du télomère 5p occupant à peu près 35kb (Bryce et al., 2000; Cong et al., 1999). Il est composé de 16 exons et de 15 introns. Le promoteur de *hTERT* est localisé dans une région d'ilots de CpG de 4Kb localisé aux alentours de -1800 à +2300 par rapport à l'ATG (Choi et al., 2007). Le cœur du promoteur (core promoter) quant à lui est positionné entre 330 bp en amont et 37bp en aval par rapport à l'ATG (Lee et al., 2019; Liu et al., 2017).

La région promotrice de *hTERT* est dépourvue de la TATA box et de la CAAT box, mais elle est riche en sites de liaisons pour de nombreux facteurs de transcription (STAT3, ER, NF- κ B, PAX5, PAX8, c-Myc, HIF-1/USF, ETS, p52, KLF4, VDR, AP-1, EGFR, MAD1, E2F...) (Cong et al., 1999; Lewis and Tollefsbol, 2016; Ramlee et al., 2016; Takakura et al., 1999) (Figure 6).

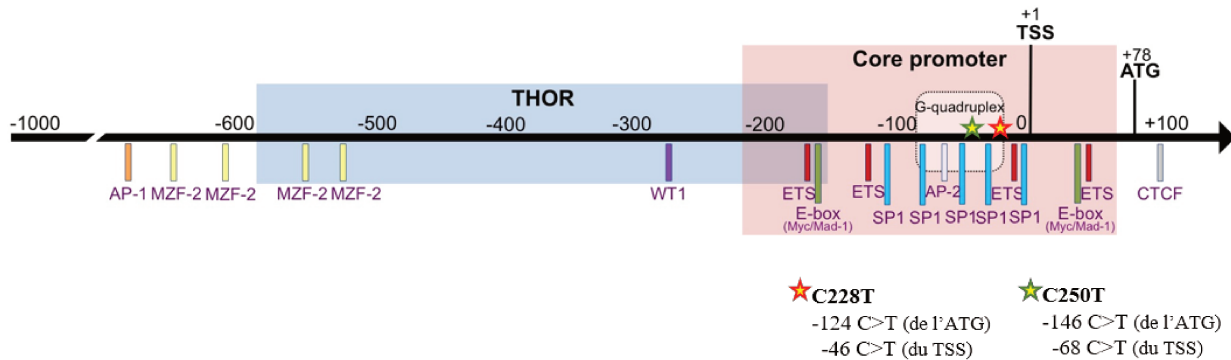


Figure 6 : La région distale du promoteur de *hTERT* montrant le "core promoteur" et THOR (TERT Hypermethylated Oncogenic Region). Le site d'initiation de la transcription (TSS, +1) et le site d'initiation de la traduction (codon start ATG, +78). Les sites de liaisons des facteurs de transcriptions ainsi que la structure G-quadruplexe qui peut être adoptée par le promoteur *hTERT* sont aussi schématisés. D'après Chebly *et al.*, 2020.

Pour appréhender la complexité de la fixation des facteurs de transcription au niveau du promoteur de *hTERT*, nous allons nous intéresser à l'oncogène c-Myc. Cet oncogène se dimérise avec son partenaire Max puis il se lie sur le promoteur de *hTERT* dans la région du cœur-promoteur au niveau de la séquence E-box (Figure 6). Cette liaison active la transcription du gène *hTERT* (Cong *et al.*, 1999; Wu *et al.*, 1999). c-Myc peut aussi coopérer avec SP1 « specificity protein 1 » et bien que chacun d'eux se fixe sur un site de liaison spécifique sur le promoteur de *hTERT*, ensemble ils augmentent la transcription de *hTERT* (Kyo *et al.*, 2000; Oh *et al.*, 2001). D'autres facteurs sont capables de réguler la transcription de *hTERT* en agissant sur c-Myc. Ceci est le cas de p300 qui active l'expression du gène *hTERT* en interagissant et en stabilisant c-Myc (Faiola *et al.*, 2005). Dans les fibroblastes du cordon ombilical, il a été montré que SIRT1 augmente l'expression de *hTERT* via FOXO3a qui active l'expression de c-Myc (Yamashita *et al.*, 2012). De façon similaire, la kinase Aurora-A active l'expression de c-Myc ce qui induit une augmentation de l'expression de *hTERT* (Yang *et al.*, 2004).

Le facteur de transcription NF-κB régule la transcription du gène *hTERT* soit de manière directe par une association au niveau de la région proximale du promoteur (Aravindan *et al.*, 2011; Natarajan *et al.*, 2008), soit de manière indirecte en modulant des régulateurs situés en amont du gène *hTERT* (Sheng *et al.*, 2006; Sinha-Datta *et al.*, 2004). Dans de nombreuses lignées cellulaires cancéreuses il a été rapporté que STAT3 joue un rôle important dans la régulation de

l'expression de *hTERT* (Chung et al., 2013; Konnikova et al., 2005). En plus des activateurs de *hTERT*, des répresseurs tels que E2F1 et MAD1 agissent sur le promoteur de *hTERT* (Oh et al., 2000; Zhang et al., 2014; Zhu et al., 2008).

Il est intéressant de noter que certains facteurs de transcription sont capables d'exercer un double rôle sur la transcription de *hTERT*. Ainsi, SP1, que nous avons déjà mentionné comme activateur de l'expression de *hTERT* lorsqu'il se fixe sur les motifs GC-box de la région promotrice de *hTERT* (Kyo et al., 2000; Liu et al., 2009, p. 1), joue un rôle opposé dans les cellules somatiques à télomérase négative. L'inhibition de l'expression de *hTERT* dans ces cellules est due à la fixation de SP1 dans la région proximale du promoteur et au recrutement de protéines histones déacétylases (HDAC) (Hou et al., 2002; Won et al., 2002). Les facteurs AP1 « activator protein 1 » et HIF2a « hypoxia-inducible factor 2-alpha » sont d'autres exemples de facteurs capables d'interagir avec des sites spécifiques de la région promotrice de *hTERT* est d'avoir un double effet sur l'expression du gène (Lou et al., 2007; Takakura et al., 2005).

La présence des facteurs de transcription est essentielle à la régulation de la transcription de *hTERT*. Cependant la transcription d'un gène implique non seulement la liaison de facteurs de transcription au niveau du promoteur ou des régions « enhancer », mais aussi la régulation de l'accessibilité à l'ADN, ce dernier processus est contrôlé par des mécanismes épigénétiques (Liu et al., 2007, p. 3, 2016; Takakura et al., 2001; Xu et al., 2001) .

En résumé, tous les régulateurs que nous venons de mentionner coopèrent de manière rigoureuse et coordonnée afin de s'assurer que le gène *hTERT* soit silencieux dans la plupart des cellules somatiques normales, tandis qu'il doit être exprimé au bon moment au bon endroit et en quantité correcte uniquement dans peu de cellules, telles que les lymphocytes activés et les cellules souches/progénitrices. Cependant, cet équilibre est perturbé dans les cellules tumorales, probablement en raison de l'expression de régulateurs positifs ou l'extinction/séquestration de régulateurs négatifs (Liu et al., 2016).

Dans les cellules cancéreuses la réactivation de *hTERT* peut être due à des mécanismes génétiques ou épigénétiques. L'un de ces mécanismes peut survenir seul et être suffisant pour ré-activer la télomérase (F. W. Huang et al., 2015; Rowland et al., 2020). Cependant il n'est pas à

exclure que la génétique et l'épigénétique coopèrent pour aboutir à la réactivation fine et précise de *hTERT* dans la cellule tumorale.

En plus des régulateurs endogènes de *hTERT* que nous venons de discuter, certains virus tumoraux codent pour des protéines qui stimulent la transcription de *hTERT*. Ces régulateurs exogènes les plus connus comprennent le Virus d'Epstein-Barr (EBV), le cytomégalovirus (CMV), le virus Kaposi de l'herpès associé au sarcome (KSHV), le virus du papillome humain (HPV), le virus de l'hépatite B (VHB), le virus de l'hépatite C (VHC) et le virus de la leucémie à cellules T humaines-1 (HTLV-1) (Bellon and Nicot, 2008; Strååt et al., 2009).

Dans le paragraphe suivant nous nous focaliserons sur les mécanismes génétiques qui peuvent survenir au niveau du promoteur de *hTERT* tel que les mutations somatiques, les amplifications et les réarrangements génomiques du locus de *hTERT*.

Les mutations

Initialement découvertes dans les mélanomes (Horn et al., 2013; Huang et al., 2013), les mutations du promoteur de *hTERT* sont des événements fréquents et récurrents dans de nombreux types de cancer (carcinome hépatocellulaire, carcinome de la thyroïde, glioblastome multiforme, cancer urothélial, mélanome) (Heidenreich and Kumar, 2017; Huang et al., 2013; Killela et al., 2013; T. Liu et al., 2014; Liu et al., 2013; Nault et al., 2013; Rachakonda et al., 2013; Vinagre et al., 2013; Wang et al., 2014). Mais elles sont plus rarement observées dans les cancers du poumon, du sein, du foie, de la prostate, les tumeurs gastro-intestinales, et les cancers hématologiques (Killela et al., 2013; Tiantian Liu et al., 2014; Stoehr et al., 2015; Vinagre et al., 2013). Les mutations au niveau du promoteur de *hTERT* surviennent, de manière prépondérante et mutuellement exclusive, au niveau de deux sites sur le chromosome 5 : à 1,295,228 et à 1,295,250 ou à -124 et -146bp depuis l'ATG (-46 et -68 depuis le TSS) (Figure 6). Ces mutations aboutissent à un changement cytosine vers thymidine (C>T). Ces transitions vers un dimère de pyrimidine sont notées soit :

- C228T (chr5, 1 295 228 C > T) ou -124 C>T (de l'ATG) ou -46 C>T (du TSS).
- C250T (chr5, 1 295 250 C > T) ou -146 C>T (de l'ATG) ou -68 C>T (du TSS).

Ces mutations somatiques génèrent des séquences consensus *de novo* de liaison pour les facteurs de transcription de la famille ETS « E-twenty-six » et augmentent l'expression de *hTERT* (Horn et al., 2013; Huang et al., 2013). Parmi les membres de la famille des facteurs de transcription ETS, les protéines de liaison GA (GABPA ou GABPB1) sont spécifiquement recrutées aux sites de mutations et activent la transcription de *hTERT* dans les cellules tumorales (Bell et al., 2015; Mancini et al., 2018). Il a ainsi été proposé que les mutations du promoteur contribuent à la tumorigenèse selon un mécanisme constitué de deux phases (Chiba et al., 2017) :

- Au cours de la phase initiale, au lieu d'empêcher le raccourcissement de tous les télomères, les mutations stabilisent les télomères les plus courts ce qui prolonge la durée de vie de la cellule.
- Au cours de la deuxième phase, les télomères extrêmement courts conduisent à une instabilité génomique, la télomérase est alors régulée de manière à être encore plus active et ceci soutient la prolifération cellulaire

Cette proposition du rôle des mutations du promoteur de *hTERT* se réfère aux fonctions canonique et non canonique de la télomérase.

Les amplifications

La région contenant le gène *hTERT* en 5p15.33 est souvent amplifiée dans les cellules cancéreuses (Cao et al., 2008). La recherche systématique de l'amplification de *hTERT*, effectuée sur une cohorte de 6835 patients représentant 31 tumeurs différentes, a révélé que 4% de ces tumeurs présentent une amplification de *hTERT* (Barthel et al., 2017). Les amplifications de *hTERT* sont observées fréquemment dans les cancers de l'ovaire, les adénocarcinomes du poumon, les carcinomes squameux du poumon, les carcinomes de l'œsophage, et les carcinomes corticosurrénaux. L'augmentation du nombre de copies de *hTERT* s'accompagne d'une augmentation de l'expression du gène et corrèle avec un mauvais pronostic dans les tumeurs du sein, l'adénocarcinome pulmonaire, le carcinome de la thyroïde, et le carcinome à cellules de Merkel (cancer rare de la peau) (Gay-Bellile et al., 2017; Leão et al., 2018; Wang et al., 2016; Zhu et al., 2006).

Les réarrangements

Un autre mécanisme de régulation positive de *hTERT*, observé initialement dans le neuroblastome, correspond à des réarrangements génomiques dans le locus de *hTERT* en 5p15.33 (Peifer et al., 2015). Les réarrangements sont regroupés dans une région à 50 kb en amont du site de transcription de *hTERT*. Ils conduisent à la juxtaposition de super-enhancers actifs à proximité immédiate du locus de *hTERT* ce qui provoque un remodelage de la chromatine et une expression accrue du gène. Ce type de réarrangement survient principalement dans le neuroblastome à haut risque et il est mutuellement exclusif avec les amplifications du gène *N-MYC* et les mutations du gène *ATRX* (Peifer et al., 2015).

Les variants

Le gène *hTERT* est un gène à copie unique. Il présente un seul site d'initiation de la transcription soumis à une régulation par le mécanisme d'épissage alternatif. Jusqu'à présent, chez l'homme, plus de 20 variants d'épissage sont identifiés. Le variant du transcrit complet ou « full-length » contient les 16 exons. Ce transcrit est formé de quatre domaines spécifiques nécessaires à la production de l'enzyme télomérase catalytiquement active :

- Le domaine N-terminal de la télomérase (TEN) situé dans l'exon 1
- Le domaine de liaison à l'ARN de la télomérase (TRBD) qui couvre les exons 2 et 3
- Un domaine de transcriptase inverse (RT) qui couvre les exons 4 à 13
- Et un domaine d'extension c-terminale (CTE) qui couvre les exons 14 à 16

Le domaine RT fournit spécifiquement les sites actifs nécessaires pour catalyser l'activité enzymatique de la télomérase, il participe à l'assemblage et à la stabilisation appropriés du complexe hTERT/hTERC et il facilite la processivité de l'enzyme télomérase (Cech, 2004; Mitchell and Collins, 2000; Rouda and Skordalakes, 2007). Uniquement, le transcrit complet « full-length » est traduit en une protéine fonctionnelle présentant une activité télomérase (Wong et al., 2014). Tous les autres variants (α , β , γ et leurs combinaisons), qui sont formés par des pertes dans un ou plusieurs exons ou par l'insertion d'un exon (Hrdlicková et al., 2012), ne présentent pas de séquences codantes pour le domaine de la transcriptase inverse, ils sont supposés agir comme des dominants négatif (Wong et al., 2014).

1.3.3. Régulation épigénétiques de l'expression de *hTERT*

Les mécanismes épigénétiques modulent l'expression d'un gène sans altérer sa séquence nucléotidique, mais en modifiant par exemple l'accessibilité des facteurs de transcription à leurs sites de liaisons. Les facteurs épigénétiques constituent un groupe d'éléments qui modulent la transcription de *hTERT*. La méthylation de l'ADN d'une part ; les modifications des protéines histones d'autre part, ainsi que l'effet des ARN non codants (ncRNA) sont impliquées dans la régulation de la transcription de *hTERT* (Ge et al., 2006; Liu et al., 2016; Takakura et al., 2001; Xu et al., 2001) (Figure7).

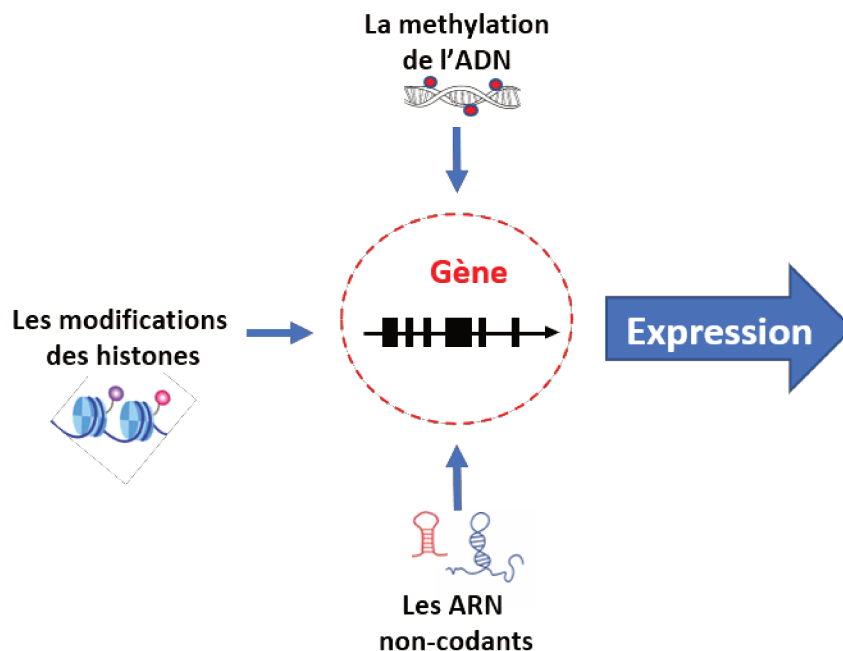


Figure 7: Les modifications épigénétiques qui régulent l'expression des gènes. D'après Chebly et al.

1.3.3.1. La méthylation de l'ADN

La méthylation de l'ADN survient dans le contexte de séquences de dinucléotide 5'CG-3' appelés site de méthylation CpG. Sur ces cytosines suivies par une base guanine, les ADN méthyltransférases (DNMT) peuvent ajouter un groupe methyl (-CH₃) sur le carbone 5 de la guanine. Les séquences CpG sont réparties sur l'ensemble du génome, normalement dans les régions non-codantes. Lorsque la fréquence en dinucléotides CpG est élevée, ces régions sont

appelées îlots CpG. Près de 80% des sites CpG sont méthylés dans les régions inter-géniques alors que la plupart des sites dans le promoteur et dans l'exon 1 sont non-méthylés (Deaton and Bird, 2011). Les îlots CpG sont généralement localisés près des promoteurs des gènes là où l'initiation de la transcription survient. A peu près 70% des promoteurs des gènes humains contiennent des îlots CpG et par conséquent la méthylation de l'ADN a été proposée comme un régulateur de l'expression des gènes (Deaton and Bird, 2011; Saxonov et al., 2006).

La méthylation de l'ADN est catalysée par plusieurs DNMT : DNMT3A, DNMT3B et DNMT1. Les DNMT3 (A et B) sont impliquées dans la méthylation *de novo* de l'ADN et la DNMT1 est responsable de la méthylation du brin nouvellement synthétisé lors de la réplication de l'ADN (Irvine et al., 2002). La méthylation de l'ADN au niveau des promoteurs a été reconnue comme l'un des moyens les plus fréquents et les plus stables parmi les mécanismes qui contrôlent l'expression des gènes. Canoniquement, la méthylation de l'ADN est associée à la mise sous-silence du gène (Bird, 1992; Keshet et al., 1986). Ainsi, au niveau de gènes activement transcrits, le promoteur a tendance à être non-méthylé, car la méthylation de l'ADN empêche la liaison de facteurs de transcription activateur ou modifie l'architecture de la chromatine pour lui donner une conformation moins adaptée à la transcription (Larsen et al., 1992; Straussman et al., 2009).

Dans les cellules cancéreuses, les études révèlent des variations dans le statut de méthylation de l'ADN. Il a été observé, une hypométhylation globale des sites CpG tout le long des corps des gènes et une hyperméthylation des îlots CpG au niveau des régions promotrices (Herman and Baylin, 2003). Cette hyperméthylation spécifique est surtout associée à l'inhibition de l'expression de gènes suppresseurs de tumeurs (Hatada et al., 2006), cependant d'autres gènes peuvent échapper à cette règle générale et c'est le cas du gène *hTERT*. En effet, dans la plupart des cellules cancéreuses la majorité des îlots CpG du promoteur de *hTERT* sont hyperméthylés alors que *hTERT* est exprimé (Azouz et al., 2010; Castelo-Branco et al., 2016; Leão et al., 2019; Lee et al., 2019; Lewis and Tollefsbol, 2016; Liu et al., 2017; Svahn et al., 2018). Le mécanisme précis par lequel le statut de méthylation du promoteur de *hTERT* se traduit par l'activation du gène est à déterminer. Toutefois, pour expliquer cette observation il a été proposé que l'hyper-méthylation pourrait diminuer l'affinité d'activateurs de la transcription et permettre l'association de répresseur de la transcription. Selon une autre hypothèse, la méthylation du

promoteur de *hTERT* pourrait empêcher la liaison des facteurs répresseurs et permettre l'activation spécifique du promoteur par des facteurs transcriptionnels particuliers. Cette hypothèse est à mettre en relation avec le statut de méthylation de la région promotrice de *hTERT*. En effet, la portion proximale du cœur du promoteur, celle qui permet la fixation d'activateurs essentiels de l'expression du gène est pratiquement toujours hypométhylées alors que la région en amont du cœur du promoteur est souvent hyperméthylée (Lee et al., 2019; Rowland et al., 2020).

La modulation du niveau de méthylation de la région promotrice de *hTERT* peut influencer l'interaction des facteurs dont la liaison est méthylation-sensible. Par exemple :

- Sur le promoteur de *hTERT*, le CTCF se fixe près du TSS et il réprime la transcription de *hTERT*. La méthylation de l'ADN empêche la liaison de CTCF et par conséquent active *hTERT* (Choi et al., 2010; Renaud et al., 2005).
- WT1, un autre répresseur de *hTERT*, présente aussi une liaison sensible au statut de méthylation. Dans les cellules tumorales, les sites de liaison pour WT1 présentent un taux accru de sites CpG méthylés, ce qui empêche la fixation de WT1 et par conséquent l'expression de *hTERT* est activée (Guilleret et al., 2002; Shin et al., 2003).
- L'oncogène c-Myc présente lui aussi une liaison méthylation-sensible. Sa liaison est absente ou réduite quand ses sites de liaison sont méthylés, ce qui se traduit par une diminution de l'expression de *hTERT* (Prendergast and Ziff, 1991).

Une autre explication impliquerait un mécanisme plus complexe dans lequel la méthylation de l'ADN ainsi que l'architecture de la chromatine interagiraient ensemble (Ng and Bird, 1999). En effet, la méthylation de l'ADN, en modulant l'accessibilité des facteurs de transcription, peut contribuer aux changements de conformation de la chromatine et par conséquent modifier l'expression des gènes (Bert et al., 2013). Les changements de conformations spécifiques engendrés par la méthylation du promoteur de *hTERT* peuvent induire un recrutement différent de facteurs de transcription, aboutissant à l'expression de *hTERT* dans les cellules tumorales (Lewis and Tollefsbol, 2016).

1.3.3.2. Les modifications des histones

Il existe plusieurs modifications post-traductionnelles des histones, comme leur acétylation, leur méthylation ou leur phosphorylation, qui peuvent influencer la compaction de la chromatine, l'accessibilité de l'ADN et par conséquent l'expression des gènes (Bannister and Kouzarides, 2011). Généralement, l'histone 3 triméthylé sur la lysine 4 (H3K4me3) et l'histone 3 acétylé sur la lysine 27 (H3K27ac) constituent des marques associées à une chromatine active, alors qu'une augmentation en H3K9me et/ou H3K27Me3 est associée à une répression de la transcription d'un gène (Portela and Esteller, 2010).

La méthylation, l'acétylation et la phosphorylation des histones sont impliquées dans la régulation de la transcription d'*hTERT* (Ge et al., 2006; Liu et al., 2007, p. 3, 2016; Takakura et al., 2001; Xu et al., 2001). Dans les cellules humaines, l'acétylation/désacétylation des histones s'est avérée être une caractéristique commune de l'activation/répression de *hTERT* (Liu et al., 2007, p. 3, 2016; Takakura et al., 2001; Xu et al., 2001). Les cellules tumorales qui expriment fortement *hTERT* présentent un taux global élevé de certaines marques d'histones tel que H3ac, H4ac, H3K9ac et H3K4me2/3 (Atkinson et al., 2005).

Il a été montré que les facteurs de transcription Myc/Max/Mad et Sp1 interagissaient avec le recrutement des histones acetyltransférase (HATs) ou histone désacétylases (HDACs) au niveau du promoteur de *hTERT*, en fonction du statut du promoteur et du contexte cellulaire (Liu et al., 2016; Takakura et al., 2001; Xu et al., 2001). Le facteur SMYD3 joue un rôle important dans l'oncogenèse. À l'instar d'une histone méthyltransférase, il est capable de se lier directement à une séquence CCCTCC sur le promoteur *hTERT* et de catalyser spécifiquement la triméthylation de H3K4, grâce à laquelle la transcription du gène *hTERT* est activée. La triméthylation de H3K4 médiée par SMYD3 est nécessaire pour une occupation optimale de c-Myc et Sp1 sur le promoteur de *hTERT* (Hamamoto et al., 2004; Liu et al., 2007). Tout ceci montre que les facteurs épigénétiques échangent et coopèrent intimement avec les facteurs de transcription, de manière à exercer leurs effets régulateurs sur la transcription de *hTERT*.

1.3.3.3. Les ARN non-codants

A ce jour, le classement des ARN non-codants (ncARN) parmi les modifications épigénétiques n'est pas encore clairement établi. Les ncRNA incluent les microARN (miRNA/miR), les petits ARN de transfert dérivés (tsRNA), les PIWI-interacting ARN (piRNA), les ARN circulaires (circRNA) et les ARN longs non-codants (lncRNA). Durant les dernières années, les études portant sur les ncRNA en oncologie sont croissantes surtout pour les miR. Ceci est lié à l'implication de ces miR dans certaines voies de signalisation qui constituent des pistes thérapeutiques prometteuses (Slack and Chinnaiyan, 2019). Dans cette introduction, nous allons aborder seulement les miR impliqués dans la régulation de *hTERT*.

Dans les cellules tumorales, les miR influencent la production de la protéine hTERT principalement par une liaison directe au niveau de la région 3' non traduite (3'-UTR), modifiant ainsi les capacités prolifératives, d'apoptose, de migration, d'invasion et de chimiosensibilité de la cellule.

Le miR-138, par exemple, est significativement diminué dans les lignées cellulaires de carcinomes anaplasiques de la thyroïde (ATC). Sa surexpression, puis sa liaison au niveau de la séquence 3'-UTR de *hTERT*, diminue dans les cellules la quantité de la protéine hTERT (Hrdličková et al., 2014; Mitomo et al., 2008). De plus, le miR-615-3p, transcrit à partir d'un intron du gène *HOXC5*, régule négativement *hTERT* en ciblant son 3'-UTR. Il a été démontré que le miR-615-3p ensemble avec *HOXC5* sont capables de coopérer et de faire une boucle de régulation de type « feedforward » qui orchestre transcriptionnellement et post-transcriptionnellement l'inhibition de *hTERT* au cours de la différenciation cellulaire (Yan et al., 2018). Le miR-1182 joue un rôle variable dans l'inhibition de *hTERT*, en fonction du type de cellule tumorale. Ainsi quand il cible le cadre ouvert de lecture (« open reading frame » ORF) de *hTERT*, cela se traduit par la réduction de la prolifération et des métastases dans les cellules du cancer gastrique. Et, quand il cible le 3'-UTR de *hTERT*, il augmente la chimiosensibilité des cellules cancéreuses de la vessie (D. Zhang et al., 2015; Zhou et al., 2016). A côté d'un ciblage direct sur *hTERT*, les miR peuvent réguler indirectement le gène *hTERT* en influençant des régulateurs situés en amont. Par exemple, le

miR-34a cible FOXM1 et c-Myc, tous les deux capables d'activer la transcription de *hTERT* et en conséquence moduler l'activité de la télomérase et la senescence cellulaire (Xu et al., 2015).

En plus des miR qui régulent négativement l'expression de *hTERT*, certains miR sont capables de réguler positivement l'expression de *hTERT*. Par exemple, dans le cancer cervical, le miR-346 et le miR-138 sont en compétition pour la même région 3'-UTR de *hTERT*. Le miR-138 diminue l'expression de *hTERT*, alors que le miR-346 augmente l'expression de *hTERT* (Song et al., 2015).

PITX1 exerce un rôle suppresseur sur *hTERT*, et PITX1 est une cible directe du miR-19b. Par conséquent, de manière indirecte miR19b augmente l'expression de *hTERT* (Ohira et al., 2015). Le miR-202, capable de cibler MXD1, inhibe la liaison de MXD1 sur le promoteur de *hTERT* ce qui favorise la liaison de c-MYC sur le promoteur, aboutissant à une augmentation de l'expression de *hTERT* (Farhana et al., 2015).

Dans les lignées cellulaires de cancer colorectal, le miR-21 agit sur la voie de signalisation PTEN/ERK1/2 en ciblant le gène *PTEN*, ce qui régule positivement l'expression de *hTERT* (Feng et al., 2011; Yang et al., 2015).

Les miRNAs qui régulent négativement et positivement *hTERT* sont répertoriés dans le Tableau 10 (Jie et al., 2019; Lewis and Tollefsbol, 2016).

Tableau 10: Les miR répresseurs et activateurs de *hTERT* (Jie et al., 2019; Lewis and Tollefsbol, 2016).

Fonctions	miR	Mode d'action	Pathologie
Répresseurs	miR-138	Ciblant le 3'UTR de <i>hTERT</i>	Carcinome anaplasique de la thyroïde
	miR-138-5p	Potentiellement inhibe <i>hTERT</i>	Cancer colorectal
	miR-422a		
	miR-299-3p	Ciblant le 3'UTR de <i>hTERT</i>	Cancer du larynx
	miR-512-5p	Ciblant le 3'UTR de <i>hTERT</i>	Carcinome épidermoïde de la tête et du cou
	miR-661	Ciblant le 3'UTR de <i>hTERT</i>	Gliome
	miR-1182	Ciblant le cadre ouvert de lecture (ORF) de <i>hTERT</i>	Cancer gastrique
	miR-1182	Ciblant le 3'UTR de <i>hTERT</i>	Cancer de la vessie
	miR-1207-5p	Ciblant le 3'UTR de <i>hTERT</i>	Cancer gastrique
	miR-1266		
	miR-491-5p	Ciblant le 3'UTR de <i>hTERT</i>	Cancer cervical
	miR-133a		
	miR-138		
	miR-491		
	miR-532	Ciblant le 3'UTR de <i>hTERT</i>	Cancer ovarien
	miR-3064		
	miR-498		
miR-34a	Ciblant la voie FOXM1/c-MYC	Carcinome hépatocellulaire	
miR-615-3p	Ciblant le 3'UTR de <i>hTERT</i>	Divers cancers	
Activateurs	miR-346	Compétition avec le miR-138	Cancer cervical
	miR-19b	Inhibition de PITX1	Mélanome
	miR-202	Via la voie MXD1-MYC/MAX	Cancer pancréatique
	miR-21	Via la voie PTEN/ERK1/2	Cancer colorectal

1.4. Problématique

Aux stades précoces les lymphomes T cutanés épidermotropes (LCTE) sont souvent indolents avec une survie proche de celle de la population générale, la majorité ne progressent pas vers un stade plus avancé. Cependant, chez une minorité de patients une progression rapide peut survenir et d'autres patients peuvent se présenter d'emblée à un stade avancé, au-delà du stade IIB, avec une médiane de survie de 1 à 5 ans (Duvic et al., 2018). Jusqu'à ce jour, aucun marqueur spécifique annonciateur de l'évolution agressive des LCTE est connu (Olsen et al., 2015). A l'heure actuelle, les traitements visent à obtenir une régression des lésions ou à ralentir la progression mais n'ont pas d'action curative dans les stades avancés (DeSimone et al., 2015). Les stratégies thérapeutiques existantes aboutissent souvent à une résistance progressive aux traitements (Froehlich et al., 2019). Seule l'allogreffe de cellules souches hématopoïétiques pourrait constituer une approche curative (Cristofolletti et al., 2019).

Par conséquent et notamment pour les LCTE de stades avancés, une meilleure connaissance des événements moléculaires initiateurs ou promoteurs de la progression de la maladie vers une forme avancée tumorale ou leucémique permettrait le développement d'approches thérapeutiques innovantes, plus efficaces et bien tolérées.

Récemment, les études de séquençage à haut débit ont décrit un large spectre d'altérations génétiques, comprenant des mutations dans des gènes impliqués dans la méthylation de l'ADN, la modification des histones et le remodelage de la chromatine (Chevret et al., 2016; van Doorn et al., 2016; Poglio et al., 2018).

L'efficacité thérapeutique des inhibiteurs d'histone deacetylase (HDACi) dans cette pathologie conforte l'implication que pourrait avoir les altérations épigénétiques dans la lymphomagenèse LCTE (van Doorn et al., 2016). Cette mise en évidence de gènes impliqués dans les modifications épigénétiques apporte un début de rationalité à l'utilisation d'HDACi dans le traitement des LCTE. En effet, dès 2006, aux Etats-Unis, la Food and Drug Administration (FDA) a autorisé l'utilisation du vorinostat, puis en 2009 celle de la romidepsine (Ott et al., 2017), alors que les mécanismes moléculaires mis en jeu par ces molécules étaient mal définis. En France, ces molécules sont des solutions thérapeutiques de seconde intention dans le traitement des LCTE

agressifs. Elles sont obtenues via une procédure d'autorisation temporaire d'utilisation (ATU) (Mishra et al., 2016). Aujourd'hui, le vorinostat et la romidepsine ont montré leur efficacité dans le traitement des LTC (McClure et al., 2018; Mervis et al., 2018). Cependant, les mécanismes moléculaires à l'origine de l'efficacité thérapeutique de ces inhibiteurs sont à déterminer (McClure et al., 2018). En effet, peu d'études *in vitro* et *in vivo* ont été réalisées avant l'utilisation de ces molécules en essais cliniques (Deschamps et al., 2019).

De manière générale, l'immortalité répliquative des cellules tumorales peut être acquise par activation de la télomérase. Ainsi, ~ 90% des cellules tumorales expriment la télomérase alors que les cellules saines environnantes ne l'expriment pas faisant de l'expression de la télomérase un marqueur de transformation tumorale (Shay et al., 2012). L'activité de la télomérase est due à la présence de hTERT. L'expression de *hTERT* est régulée essentiellement au niveau transcriptionnel. Au niveau du promoteur de *hTERT* se trouve une région régulatrice très riche en îlots CpG et contenant de nombreux sites de liaison pour des facteurs de transcription activateurs et répresseurs (Akincilar et al., 2016). De plus, *hTERT* est localisé dans une région où la chromatine est condensée dans les cellules normales (Azouz et al., 2010), ce qui corrèle avec sa non-expression. Cet état de condensation de la chromatine peut être modulé par des modificateurs épigénétiques agissant soit sur l'ADN (par exemple sur la méthylation des îlots CpG) soit sur les histones en modulant leur niveau d'acétylation ou de méthylation ou de phosphorylation par exemple. Les variations de méthylation de l'ADN et les modifications portant sur les histones peuvent agir en synergie ou de façon indépendantes afin de moduler l'état de condensation de la chromatine et donc l'accessibilité des séquences régulatrices de l'ADN aux facteurs de transcription. L'utilisation d'agents épigénétiques tels que les inhibiteurs spécifiques des ADN méthyltransférases (DNMT) et d'histones désacétylases (HDAC), offrent de nouvelles approches thérapeutiques (Gnyszka et al., 2013; Chun et al., 2015; Duvic et al., 2015).

Des travaux antérieurs effectués par notre équipe ont montré que la télomérase est activée dans les LTCE en absence d'amplifications ou de réarrangements du gène *hTERT* (Chevret et al., 2014). L'inhibition de la télomérase dans ces cellules se traduit par l'arrêt de la prolifération

et par la mort des cellules, faisant de ces tumeurs des candidates potentielles à un traitement ciblé anti-téломérase (Chevret et al., 2014). Cependant, les mécanismes contrôlant l'activation de la téломérase dans les LCTE restent inconnus. Récemment nous avons établi que dans ces lymphoproliférations, les mutations hotspot du promoteur de *hTERT* ne peuvent pas être responsables de l'expression de la téломérase (Ropio *et al.* submitted).

1.5. Objectifs

Au vu de l'ensemble de ces données, l'objectif principal de ma thèse a été d'explorer des mécanismes épigénétiques qui pourraient être responsables de l'activation de la téломérase dans les LTC.

Tout d'abord nous avons établi le statut de méthylation de l'ADN au niveau de la région régulatrice du promoteur de *hTERT* dans des cellules de lymphomes cutanés. Puis nous avons comparé les profils de méthylation des cellules tumorales entre elles et aux cellules hématopoïétiques saines et souches-progénitrices (CD34+). Ce travail est présenté dans le chapitre 3.

Par la suite, nous avons identifié une région d'intérêt et identifier les facteurs de transcription potentiellement présents dans cette région. Ce travail est présenté dans le chapitre 4.

Et pour finir nous avons utilisé des modulateurs épigénétiques et analysé leurs impacts sur les marques épigénétiques identifiées, ainsi que sur les capacités tumorigènes des cellules de lymphomes cutanés *in vitro*. Ce travail est présenté dans les chapitres 5 et 6.

2. Le matériel biologique utilisé et la cytogénétique des CTCL

Pour réaliser l'ensemble de ces travaux, j'ai utilisé cinq lignées cellulaires de lymphomes T cutanés primitifs épidermotropes (HuT78 et MyLa) et non-épidermotropes (les Mac) disponibles dans notre équipe de recherche au laboratoire U1053 :

- La lignée HuT78, lignée commerciale de syndrome de Sézary ATCC, Molesheim, France. (Bunn and Foss, 1996).
- La lignée MyLa, lignée de mycosis fongoïde de stade supérieur à IIIB transmise par le Dr K. Kaltoft, Institute of Human Genetics, Aarhus University. (Kaltoft et al., 1992).
- Et, les lignées Mac1, Mac2A et Mac2B, sont trois lignées de lymphomes anaplasiques à grandes cellules établies à partir du même patient à des stades différents de la pathologie : indolent pour Mac1 et agressif pour Mac2A et Mac2B. Ces lignées nous ont été gracieusement données par le Pr M. Kadin, Boston University and Roger Williams Medical Center, Providence, Rhode Island. (Kadin et al., 1994)

Ainsi que des lignées récemment développées dans notre équipe. Ces lignées dérivent de patients atteints par le syndrome de Sézary, SS (SS PDC = Patients-derived cells). Elles sont dénommées : L1, L2, L4 et L5 (Poglio et al., 2020).

Nous avons aussi utilisé des cellules tumorales isolées à partir de sang total prélevé sur six patients SS de stades supérieurs à IIIB (T4NxMOB1 ou T4NxMOB2), hospitalisés dans l'unité d'onco-dermatologie du service de Dermatologie du CHU de Bordeaux (Pr Beylot-Barry) : Il s'agit dans mon manuscrit des patients 5, 6, 7, 8, 9 et 10 (Formulaire de consentement en **annexe 1**).

Pour avoir des données en provenance de sujets sains, nous avons utilisé des lymphocytes T CD4+ et les cellules CD34+ isolés à partir du sang total de donateurs sains provenant de l'Établissement français du sang, EFS (CODECOH n°DC-2015-2412, convention N°16PLER023).

Avant de débiter l'investigation épigénétique du promoteur de *hTERT* nous avons fait la caractérisation cytogénétique des lignées commerciales. Pour cela, le caryotype conventionnel et le caryotype par hybridation *in situ* fluorescente multicolore (mFISH) ont été réalisés sur HuT78, MyLa, Mac1, Mac2A et Mac2B. Cette caractérisation se justifie par le fait qu'une lignée cellulaire, même si les conditions de cultures sont très surveillées, peut dériver au cours du temps et acquérir des changements dans ses caractéristiques (Geraghty et al., 2014; Wenger et al., 2004).

Le caryotype de ces lignées est presque diploïde « near-diploid » avec un nombre de chromosomes entre 44 et 49, sauf pour la lignée Hut78 qui est presque triploïde « near triploid ». Elle présente entre 77 et 81 chromosomes. L'analyse cytogénétique met en évidence la complexité des caryotypes notamment pour les stades avancés de la pathologie : stade MF transformé (MFT) pour la lignée MyLa (Figure 8) et stade SS pour la lignée HuT78. La lignée Mac1 (C-ALCL) est la lignée qui présente le moins d'anomalies chromosomiques, alors que les deux lignées Mac2A et Mac2B (C-ALCL) dérivées du même patient que Mac1 mais à un stade tumoral, présentent un caryotype plus de complexe que celui de Mac1. Les caryotypes observés ont montré des similitudes avec les caryotypes décrits dans la littérature avec peu d'anomalies additionnelles (Kaltoft et al., 1992; Netchiporouk et al., 2017).

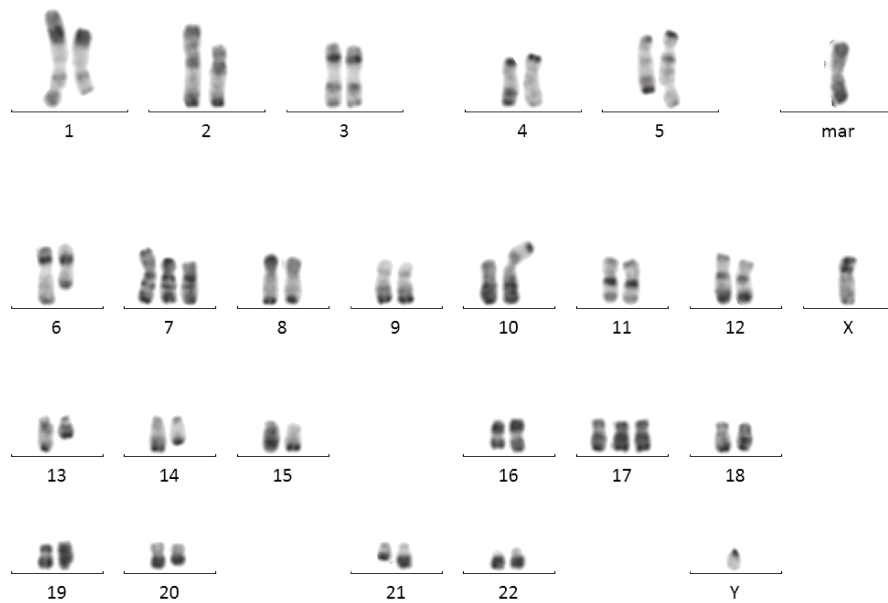


Figure 8 : Le caryotype conventionnel de la lignée cellulaire MyLa présentant 49 chromosomes avec plusieurs remaniements complexes.

Les formules chromosomiques sont rapportées selon les recommandations du ISCN (The International System for Human Cytogenetic Nomenclature) 2016 et sont regroupées dans le Tableau 11.

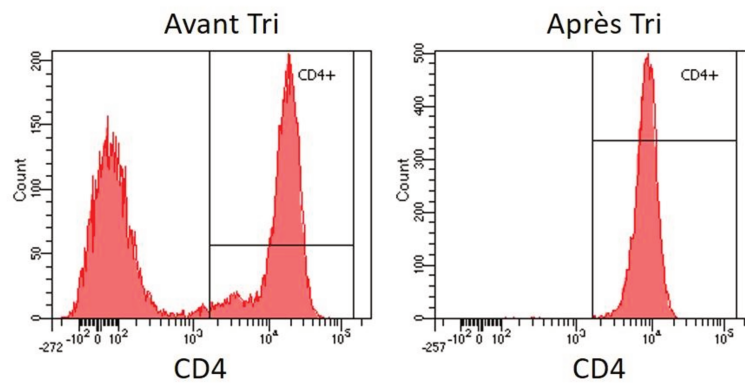
Tableau 11: Les formules chromosomiques des 5 lignées cellulaires

Cell line	Chromosomal formula according to ISCN 2016
MyLa	<p>47~49,XY,del(1)(q32)[10],der(1)t(1;15)(p36;q15)[10], del(2)(p?16)[8],der(2)t(2;14)(q32;q12)[10],t(4;5)(q13;q15)[10], der(6)?add(6)(p22)del(6)(q15;q23)[10],del(7)(p15)[10],+del(7)(p1?5)[10], del(9)(p21)[9],der(10)t(10;13)(p14;q13)[10],der(13)t(13;16)(q14;?q23)[10], der(14)(2pter→2p13::14p12→14q12::2q32→2qter)[10], der(15)t(1;15)(q32;q15)[10],+17[10],der(18)t(18;?19)(q23;?p13)[10], der(19)t(?11;19)(?;p13)[10],der(20)t(20;?)(q13;?)[10], der(21)t(?16;21)(?;q22)[10],+mar[10][cp10]</p>

HuT78	<p>77~81<3n>,Y,t(X;13)(p11;q14)x2[5],+?der(2;20)(p10;p10)[5],+3[5], der(3)t(3;10)(q29;q24)x2[5],+?4[5],+?4[5],der(4) (4pter→4p?14::4?q21→4?p14::4?q25→4?q21::16p?11→16p?13::13q14→13qter) x2[5],+?5[3],t(5;6)?(p10;p10)x2[5],+der(6)t(4;6)(q2?1;p?21)x2[5], der(7)t(7;10)(p14;q11)x2[5],-9[5],der(10)t(7;10)(q11;q22)[4],del(10)(q24)[5], ?del(11)(q?) [5],?del(12)(p13)[4],-13[4],+14[5],del(14)(q?11q?24)x2[5], +15[4],16[5],+17[5],der(18)t(2;18)(p22;p11)x2[5],+der(19)t(19;20)(q13;q11)[5], der(19)t(19;20)(q13;q11)[5],der(19)t(19;22)(q10;q10)x2[5], +der(20)t(19;20)(?;p11)[4],del(20)(?q11q13)[3], der(20)(:9p11→9p24::20p13→20qter)x2[5],der(21)t(11;21)(q?13;q10)[5][cp5]</p>
Mac1	<p>45~47,XY,der(4)?t(4;5)(p16;?) [7],t(8;9)(p22;p2?4)[7],-13[5], der(14)?(:9::13q?32→13p10::14p?10→14qter)[7],-18[7],+21[7][cp7]</p>
Mac2A	<p>45~46,X,-Y[10],?dup(3)(?q25q28)[10],del(5)(?q12q25),del(6)(q15)[10], t(8;9)(p22;p2?4)[10],der(10)t(6;10)(q15;p11)[9],+12[5],del(12)(q11q13)[10], ?i(14)(q10)[9],der(15)t(2;15)(p11;p12)[10],del(16)(q11q22)[10], inv(20)(?p13q13)[10],-22[10],+mar[10][cp10]</p>
Mac2B	<p>44~45,X,-Y[10],del(6)(q15)[10],der(8)t(8;9)(p22;p2?4)[10], der(9)t(8;9)(p22;p2?4)?inv(9)[10],der(10)t(6;10)(q15;p11)[10], del(12)(q11q13)[10],dup(12)(q12q13)[10],der(15)t(2;15)(p11;p12)[10], der(21)t(21;?)(p11;?) [9],der(22)t(22;14)(p11;?) [7],+mar[4][cp10]</p>

Les résultats de cytogénétiques des cinq lignées ont été publiés dans un article qui évalue les différentes techniques utilisées pour mesurer la longueur des télomères dans les cancers hématologiques, en considérant les CTCL comme modèle (Ropio *et al.*, 2020) : Cet article se trouve en **annexe 2** (Ropio, J., Chebly, A. et al Reliable blood cancer cells' telomere length evaluation by qPCR. Cancer medicine 2020). Le caryotype des lignées L1, L2, L4 et L5 est publié dans Poglio *et al.* 2020.

Les caryotypes des cellules de patients 5 à 10, n'a pas été réalisé. En revanche pour ces cellules l'immunophénotype a été réalisé selon les modalités établies pour les lignées L1, L2, L4 et L5 (Poglio *et al.* 2020) ce qui a permis récupérer les cellules tumorales (Figure 9).



Patient	% des cellules CD4+ ou TCRVb+ avant tri	% des cellules CD4+ ou TCRVb+ après tri
5	49.6% (CD4)	98.2%
6	41.8% (TCRV8)	97.0%
7	21.5% (TCRVB17)	97.1%
8	25.9% (CD4)	98.7%
9	50% (CD4)	99.2%
10	30.3% (CD4)	99.3%

Figure 9 : Figure montrant le taux de cellules tumorales chez les 6 patients SS, avant et après tri selon le CD4 ou le TCRvB.

3. La méthylation du promoteur de *hTERT*

Les lymphomes T-cutanés (CTCL) sont des tumeurs télomérase-positives exprimant le gène *hTERT* dans lesquelles ni l'amplification ni les mutations hotspot du promoteur ne peuvent expliquer la réexpression de *hTERT*. Comme le promoteur de *hTERT* est riche en îlots CpG, nous avons étudié la contribution de mécanismes épigénétiques dans sa réexpression. Nous avons analysé le statut de méthylation du promoteur de *hTERT* dans des lignées cellulaires CTCL, des cellules de patients et des cellules de donneurs sains. Les analyses de méthylation ont montré un profil de méthylation commun exclusivement dans les cellules tumorales, englobant une région distale hyperméthylée entre -650 pb et -150 pb du TSS et une région proximale hypométhylée de -150 pb à +150 pb. Il est intéressant de noter que la région hyperméthylée correspond à la région oncogénique hyperméthylée (THOR) récemment décrite comme étant associée à la réactivation de la télomérase dans de nombreux cancers, mais jusqu'à présent non étudiée dans les lymphomes. De plus, nous avons évalué l'effet sur THOR de deux inhibiteurs d'histones désacétylases (HDACi), la romidepsine et le vorinostat, tous deux approuvés pour le traitement des CTCL; ainsi que d'un inhibiteur de l'ADN méthyltransférase (DNMTi) 5-azacytidine, non approuvé pour les CTCL. Les résultats obtenus révèlent que les cellules tumorales présentent un degré de méthylation de THOR spécifique comparé aux cellules non tumorales et que la méthylation de THOR est relativement stable même sous la pression des médicaments épigénétiques « épidroques ».

3.1. Article "Promoter methylation: A player in the activation of TERT in cutaneous T-cell Lymphomas"

Promoter methylation: A player in the activation of hTERT in cutaneous T-cell lymphomas.

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Search Terms:	DNA methylation, hTERT, Cutaneous T-cell lymphomas, Rare tumors

Abstract:

Cutaneous T-cell lymphomas (CTCL) are telomerase positive tumors expressing hTERT in which neither amplification nor promoter hotspot mutations could explain the hTERT re-expression. Since hTERT promoter is rich in CpG, we investigated the contribution of epigenetic mechanisms in its re-expression. We analyzed hTERT promoter methylation status in CTCL cell lines, patient cells and healthy-donor's cells. Gene-specific methylation analyses revealed a common methylation pattern exclusively in tumor cells, encompassing a hypermethylated distal region from -650bp to -150bp and a hypomethylated proximal region from -150bp to +150bp. Interestingly, the hypermethylated region matches with the recently described TERT Hypermethylated Oncogenic Region (THOR) reported to be associated with telomerase reactivation in many cancers, but so far not reported in lymphomas. Additionally, we assessed the effect on THOR of two histone deacetylase inhibitors (HDACi), romidepsin and vorinostat, both approved for CTCL treatment as well as a DNA methyltransferase inhibitor (DNMTi) 5-azacytidine, unapproved for CTCL. Overall, the findings reported here reveal a distinct methylation pattern of THOR in CTCL tumor cells and they suggest that THOR methylation is relatively stable even under epigenetic drugs' pressure.

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1 **Promoter methylation: A player in the activation of**
2 ***hTERT* in cutaneous T-cell lymphomas.**

3
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31
32 **KEYWORDS:**

33 Cutaneous T-cell lymphomas, CTCL, DNA methylation, hTERT, Telomerase, HDACi.

34
35 **ABBREVIATIONS:**

36 CTCL: Cutaneous T-cell lymphomas

37 DNMTi: DNA methyltransferase inhibitors

38 HDACi: Histone deacetylase inhibitors

39 hTERT: human Telomerase Reverse Transcriptase gene

40 NTC: Non-treated cells

41 SS: Sézary Syndrome

42 SS PDC: Sézary Syndrome Patient-Derived cells

43 TF: Transcription Factor

44 THOR: TERT Hypermethylated Oncogenic Region

45 T-MF: Transformed tumor stage Mycosis Fungoides

46 TSS: Transcription Start Site

47 WT1: Wilms' Tumor 1

48 **ABSTRACT**

49 Cutaneous T-cell lymphomas (CTCL) are telomerase positive tumors expressing *hTERT* in which
50 neither amplification nor promoter hotspot mutations could explain the *hTERT* re-expression. Since
51 *hTERT* promoter is rich in CpG, we investigated the contribution of epigenetic mechanisms in its re-
52 expression. We analyzed *hTERT* promoter methylation status in CTCL cell lines, patient cells and
53 healthy-donor's cells. Gene-specific methylation analyses revealed a common methylation pattern
54 exclusively in tumor cells, encompassing a hypermethylated distal region from -650bp to -150bp and
55 a hypomethylated proximal region from -150bp to +150bp. Interestingly, the hypermethylated region
56 matches with the recently described *TERT* Hypermethylated Oncogenic Region (THOR) reported to
57 be associated with telomerase reactivation in many cancers, but so far not reported in lymphomas.
58 Additionally, we assessed the effect on THOR of two histone deacetylase inhibitors (HDACi),
59 romidepsin and vorinostat, both approved for CTCL treatment as well as a DNA methyltransferase
60 inhibitor (DNMTi) 5-azacytidine, unapproved for CTCL. Overall, the findings reported here reveal a
61 distinct methylation pattern of THOR in CTCL tumor cells and they suggest that THOR methylation
62 is relatively stable even under epigenetic drugs' pressure.

63 INTRODUCTION

64 Cutaneous T-cell lymphomas (CTCL) encompass a heterogeneous group of rare T
65 lymphoproliferative disorders, characterized by clonal proliferation of malignant T-cells involving
66 the skin as a primary site. They include cutaneous anaplastic large cell lymphoma (C-ALCL), mycosis
67 fungoides (MF) and Sézary syndrome (SS) (1). While most C-ALCL and MF have an indolent course,
68 some MF may progress to a transformed tumor stage (T-MF) of poor prognosis. SS can be developed
69 in a patient affected many years with MF, but it arises more frequently as erythroderma associated
70 with a frank leukemic variant (2). Treatment of MF/SS can be very challenging especially in the
71 aggressive forms of the disease. Several therapies are being used and the choice of the therapeutic
72 agent is stage-dependent. It includes different drugs, such as: bexarotene, methotrexate, interferon-
73 alpha, histone deacetylases inhibitors (HDACi) or the recently introduced monoclonal antibodies such
74 as mogamulizumab, brentuximab vedotin or IPH4102. While chemotherapies only allow short-lived
75 responses, allogeneic stem cell transplantation is the only curative option (3,4).

76 In cancer cells, replicative immortality can be acquired due to telomerase reactivation driven
77 by the acquisition of expression of the catalytic subunit of the human telomerase reverse transcriptase
78 gene (*hTERT*) (5). The *hTERT* gene can be upregulated through genetic mechanisms including
79 promoter's mutations and less frequently gene amplifications or rearrangements (6) and through
80 epigenetic mechanisms involving DNA methylation, histones modifications and non-coding RNAs
81 effects (7–12). Recently, a 433-base pair sequence in *hTERT* promoter containing 52 CpG was
82 reported hypermethylated in many cancers, but was not investigated in lymphomas (13). This region
83 was named TERT Hypermethylated Oncogenic Region (THOR) and is known today to participate in
84 telomerase activation when hypermethylated (13,14). Binding sites of three transcription factors are
85 located in the THOR region: two transcriptional silencers WT1 (Wilms' tumor 1) and MZF-2
86 (myeloid zinc finger 2) and one transcriptional enhancer c-MYC that binds to an Enhancer box (Ebox)
87 (15).

88 In a previous work, our team reported that the telomerase is activated in CTCL with the
89 absence of amplifications or rearrangements in the *hTERT* locus (16). In a complementary study we
90 stated the absence of *hTERT* hot spot promoter mutations in these types of tumors (Ropio *et al.* In

91 preparation). Since little is known about the mechanisms underlying changes during tumorigenesis
92 (17) and since *hTERT* promoter epigenetic investigation was never reported in CTCL, we present
93 herein a pioneer exploration of the epigenetic mechanisms that could contribute to *hTERT* re-
94 activation in CTCL. We evaluate THOR methylation status in CTCL cell lines and in SS patient-cells
95 in comparison to healthy cells (CD34+ and CD4+lymphocytes). We explore THOR methylation
96 under the pressure of a demethylating agent, unapproved for CTCL; and we describe the effect of
97 clinically-approved HDAC inhibitors on THOR methylation status.

98

99 **MATERIALS AND METHODS**

100 **Cell lines, SS patient-derived cells and cell culture**

101 Five CTCL cell lines were studied: Myla (T-MF) (kindly provided by Dr K. Kaltoft,
102 Denmark), HuT78 (SS) (ATCC, France), Mac1, Mac2A and Mac2B (C-ALCL) (DSMZ, Germany).
103 Cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% of fetal bovine
104 serum (Eurobio, France) and 100U/mL of penicillin and streptomycin (Gibco). Four SS patient-
105 derived cells (PDC) obtained from four SS patients (patients 1 to 4) were also investigated. They were
106 cultured as previously described (18). All cell cultures were incubated at 37°C in a humidified
107 incubator with 5% CO₂.

108

109 **SS patients and tumor cells isolation**

110 Ten SS patients, 8 females and 2 males, with a median age of 69.5 years (range 52-93) were
111 recruited to this study. The diagnosis was established in accordance with the criteria of the WHO-
112 EORTC (World Health Organization and the European Organization for Research and Treatment of
113 Cancer) (1). All of them presented a B2 stage, eight a T4 stage, one a T3 stage and one a T2b stage.
114 PDC were obtained from Patients 1 to 4 as mentioned above and fresh SS cells from patients 5 to 10.
115 SS patients were blindly investigated by researchers who were not aware of the clinical management
116 or the prescribed treatment for these patients. Peripheral blood mononuclear cells (PBMC) were
117 isolated using Pancoll (Pan Biotech, Germany). Clonal TCR $\nu\beta$ was determined using IOTest® Beta
118 Mark TCR $\nu\beta$ Repertoire Kit (Beckman Coulter, France). Cells were sorted manually according to

119 the TCRv β result or by using a BD FACSAria™ II cell sorter (BD Biosciences, France). This study
120 was carried out in accordance with the standards set by the Declaration of Helsinki.

121

122 **Controls and healthy donors**

123 Seven healthy age-matched donors were recruited from the *Etablissement Français du Sang*
124 (EFS) in Bordeaux (DC 2015 2412-18PLER012). PBMC were isolated from peripheral blood
125 samples, using Pancoll (Pan Biotech, Germany). CD4⁺ cells were manually sorted using CD4
126 Microbeads human kit (Miltenyi Biotec, Germany), and separated into two pools: A (3 donors) and
127 B (4 donors). Progenitor/stem cells CD34⁺ were collected from 20 healthy donors at the EFS and
128 pooled together.

129

130 **Chemicals**

131 Drugs included in this study were two HDACi used to treat CTCL: romidepsin and vorinostat
132 (Euromedex, France). Based on previous reports (19,20), 1x10⁶ SS PDC (1, 2, 3 and 4) were exposed
133 to romidepsin (10nM) or vorinostat (3 μ M) for 48h. For the demethylating agent, Hut78 cell line and
134 SS PDC (2 and 3) were exposed to 3nM, 1.7nM and 2.3nM of 5-azacytidine, respectively, for 72h. 5-
135 azacytidine is a DNA methyltransferase inhibitor (DNMTi), not approved for CTCL treatment.

136

137 **DNA/RNA isolation and cDNA synthesis**

138 Genomic DNA was extracted using Quick-DNA Microprep kit (ZYMO Research, USA).
139 Total RNA was isolated using the Direct-zol™ RNA MiniPrep kit (ZYMO Research). DNA and RNA
140 concentrations were measured using the Nanodrop ND-1000 spectrophotometer. cDNA was
141 synthesized from 100ng of RNA using the SuperScript II reverse transcriptase kit (Invitrogen, France).

142

143 **Locus-specific bisulfite sequencing**

144 Genomic DNA was bisulfite converted using the EZ-DNA Methylation kit (ZYMO Research). The
145 region from -650bp to +150bp relative to the transcription start site (TSS) of *hTERT* was amplified
146 by PCR using GO-Taq-G2-Hotstart (Promega, USA). Primers were bisulfite-specific and completely

147 devoid of CpG sites as previously described (7,11). Forward and reverse primers sequences as well
148 as PCR conditions are listed in supplementary table 1. Amplicon lengths were verified and PCR
149 products were purified using Macherey-Nagel extraction kit. Purified amplicons were cloned into the
150 p-GEMT easy vector system I (Promega) and then Competent *E. coli* (Promega) were transformed
151 using the ligation product. Bacterial suspensions were enriched in SOC medium (New England
152 Biolabs, USA). Colonies were grown overnight on LB (Luria-Bertani)-Agar containing 32µg/ml
153 Xgal, 120µg/ml IPTG and 100µg/ml Ampicillin. After white colonies selection and checking the
154 DNA insertion by PCR, colonies were incubated overnight for enrichment in LB medium with
155 100µg/ml Ampicillin at 37°C under agitation. Plasmid DNA was isolated using Nucleospin plasmid
156 kit (Macherey Nagel, Germany). Each sample was performed in duplicate. Ten to 30 clones were
157 extracted and sequenced. DNA sequences were analyzed using ChromasPro software (Technelysium,
158 Australia) and bisulfite images were obtained using QUMA (Riken, Japan, <http://quma.cdb.riken.jp>)
159 (21).

160

161 ***hTERT* and *WT1* expression analysis by quantitative real-time PCR (qRT-PCR)**

162 cDNAs were amplified by qRT-PCR using Takyon™ No Rox SYBR® MasterMix dttP Blue
163 (Eurogentec, France) and the following primer sets: *hTERT* gene, forward primer: 5'-
164 GCATTGGAATCAGACAGCAC-3', and reverse primer: 5'-CCACGACGTAGTCCATGTTC-3',
165 housekeeping gene *TBP*: forward primer: 5'-CACGAACCACGGCACTGATT-3', and reverse
166 primer: 5'-TTTTCTTGCTGCCAGTCTGGA-3'. For Wilms' Tumor 1 (*WT1*) gene, qRT-PCR was
167 performed using WT1 PrimePCR™ SYBR® Green Assay (Biorad, USA) according to the
168 manufacturer's instructions. qRT-PCR analyses were run on a Stratagene Mx3005P system (Agilent
169 Technologies, USA). Each sample was performed in triplicate and the mean value was calculated.
170 Values are expressed in arbitrary units (A.U.).

171

172 **Luciferase assay**

173 Luciferase assays were performed as previously described by Gazon *et al.* (22). Briefly, 293T
174 cell line was used to set up the protocol, then HuT78 and MyLa cells were transfected with a plasmid

175 DNA mixture containing 100 ng/μl of pGL3-hTERT-378-Luc reporter plasmid (23), 100 ng/μl of
176 pActin-βgal and the indicated amount of pAD/WT1-IRES-nAMcyan (Gift from Edward McCabe,
177 Addgene #29756). HuT78 and MyLa were electroporated using Gene Pulser Xcell Electroporation
178 Systems (Biorad). Forty-eight hours post-transfection, cells were washed with cold PBS and then
179 lysed in 1x passive lysis buffer (Promega). Luciferase and β-galactosidase assays were both
180 performed in a Spark 10M multiplate reader (Tecan, Switzerland) with Genofax A, Genofax B kit
181 (Yelen, France) and Galacto-Star kit (Life Technologies, USA), respectively, as described by the
182 manufacturer. Each experiment was performed in triplicate and Luciferase activities were normalized
183 for transfection efficiency based on β-galactosidase.

184

185 **WT1 ChIP-qPCR assay**

186 *WT1* ChIP-qPCR assays were performed by Active Motif (USA). Briefly, *WT1* ChIP-qPCR
187 assay was performed using 30 μg of chromatin obtained from cultured cells (HuT78, SS PDC 1, 2
188 and 3) or primary human T lymphocytes (Healthy CD4⁺ cells) and 8 μg of WT1 antibody sc-192
189 (Santa Cruz Biotechnology, USA). qPCRs were performed using primer pairs (Supplementary table
190 2) designed for the region of interest (*hTERT* -323) and for 2 positive controls (TAL1-2k and *hTERT*-
191 709). A negative control was also used, consisting of a primer pair that amplifies a region in a gene
192 desert on chromosome 12 (Untr12). Data were normalized to the genomic DNA for the particular cell
193 type.

194

195 **Telomerase activity by TRAP assay**

196 Telomerase activity was assessed in CTCL cell lines and SS PDC (1, 2, 3 and 4) using the
197 TRAP assay (TRAPeze telomerase detection kit; S7700, Millipore). Protein extracts were used to
198 extend a synthetic telomeric DNA by PCR amplification (1 cycle of 30°C for 30 min, followed by a
199 telomeric PCR amplification: 95°C for 3 min, 2 cycles of 95°C for 20 sec and 49°C for 20 sec,
200 followed by 30 cycles of 95°C for 20 sec and 60°C for 20 sec with signal acquisition) on a Stratagene
201 Mx3005P system (Agilent Technologies). Each sample was run in duplicate with a control DNA.

202

203 **Statistical analysis**

204 General statistical analyses were performed using the Mann Whitney test (GraphPad
205 Prism, version 5). *P* values of less than 0.05 were considered statistically significant.

206

207 **RESULTS**

208 **CTCL cell lines and SS patients' tumor cells express *hTERT***

209 Healthy controls CD4+ and CD34+ showed *hTERT* expression of 0.47 and 0.95 A.U.
210 respectively (figure 1). Compared to healthy controls, CTCL cell lines expressed the highest *hTERT*
211 levels (ranging from 2.7 to 8.2 A.U.) (figure 1). In SS PDC *hTERT* was expressed. While PDC 3
212 showed *hTERT* expression level similar to cell lines (6 A.U), PDC 1, 2 and 4 showed *hTERT*
213 expression levels in the same ranges of those of healthy controls 0.60, 0.88 and 0.50 A.U., respectively
214 (figure 1). In SS patients' fresh cells, *hTERT* was expressed at lower levels (0.07 to 0.12) than healthy
215 controls (0.47 and 0.95), except for patient 10 with 0.60 A.U. (figure 1). Also, we found that *hTERT*
216 expression correlated with telomerase activity in CTCL cell lines and in SS PDC with an R^2 equal to
217 0.7502 (Supplementary figure 1).

218

219 **THOR is methylated in CTCL cell lines and SS PDC**

220 A common *hTERT* promoter methylation pattern in CTCL cell lines and in SS PDC was
221 revealed by locus-specific bisulfite sequencing (figure 2). This pattern comprises a hypermethylated
222 distal region between -650bp and -150bp from the TSS; as well as a hypomethylated proximal region
223 between -150bp and +150bp including the TSS and the ATG start codon. The hypermethylated region
224 in CTCL cell lines corresponds to the recently identified region known as THOR (figure 2A). Among
225 CTCL cell lines (figures 2B-F), HuT78 presented the highest levels of THOR methylation with an
226 average of 87% (figure 2C), followed by MyLa with 83% (figure 2B) and Mac1 with 64% (figure
227 2D). Mac2A (figure 2E) and Mac2B (figure 2F), compared to Mac1, showed lower THOR
228 hypermethylation levels with an average of 49% and 45%, respectively. As in cell lines, SS PDC 1,
229 2, 3 and 4, presented hypermethylated THOR levels: 73%, 67%, 50% and 53%, respectively (figures
230 2G-J). On the contrary, THOR was hypomethylated in both healthy pools CD4+ (figures 2K and 2L)

231 and CD34+ (figures 2M and 2N), 11% and 7.5% respectively. Moreover, compared to healthy cells,
232 the THOR methylation level was significantly increased in all CTCL cell lines and in the studied SS
233 PDC ($P < 0.0001$).

234

235 **THOR hypermethylation is a specificity of tumor cells**

236 In order to strengthen our findings regarding THOR methylation profiles in cultured CTCL
237 cells, we studied the methylation status of *hTERT* promoter in fresh patient cells. For each patient,
238 tumor cells (clonal TCR $\nu\beta$ positive cells) and normal cells used as individual controls (TCR $\nu\beta$
239 negative cells) were sorted and analyzed. Strikingly, THOR methylation levels were prevalently
240 observed higher in tumor cells than in normal cells. A significant difference ($P < 0.0001$) was observed
241 in patients 5 (figure 3A), 6 (figure 3B), 7 (figure 3C), and 9 (figure 3E), with an average methylation
242 level of 46%, 35%, 42% and 56% respectively in tumor cells, and 4%, 10%, 5% and 13% respectively
243 in normal cells. A significant difference was also found in patients 8 (figure 3D) and 10 (figure 3F)
244 ($P = 0.0455$ and $P = 0.0079$, respectively) with lower THOR methylation levels in tumor cells (15% and
245 22.9%, respectively for tumor cells; and 6.5% and 11.5% respectively for normal cell). Figure 3G
246 summarizes THOR methylation levels in the aforementioned six SS patients. In all healthy cells
247 explored (CD4+, CD34+ and SS patients' normal cells), THOR was hypomethylated with a
248 methylation level ranging from 4 to 13% (figure 4). In our study, a cut-off value of 15% was used for
249 SS patients which is quite similar to that of 16.1% used by Lee *et al.* (13).

250

251 **WT1 overexpression reduces *hTERT* activation**

252 Regarding the transcription factors' binding sites on THOR, while the MZF-2 binding sites
253 were hypermethylated in all tumor samples and the Ebox site was hypomethylated in almost all tumor
254 samples (87%, 13/15 cell lines and patients); WT1 binding site presented different methylation levels
255 between tumor samples (cell lines and patients). For this reason, we focused on WT1 and we assessed
256 by qRT-PCR, the expression levels of WT1 in SS cells (Hut78 cell line and SS PDC). Interestingly,
257 HuT78 and SS PDC 1, 2 and 3 expressed *WT1* mRNA (supplementary figure 3A). WT1 protein
258 expression was verified by Western blot analysis (supplementary figure 3B). Next, we evaluated the

259 effect of WT1 overexpression on *hTERT* promoter in two aggressive MF/SS cell lines: MyLa and
260 HuT78. We noticed in these latter that WT1 overexpression reduced significantly the *hTERT*
261 activation in a dose-dependent manner (figure 5A): in MyLa: $P < 0.0001$ with 10 and 20 μg of WT1
262 while in HuT78: $P = 0.0051$ and 0.0026 with 10 and 20 μg of WT1, respectively.

263

264 **WT1 binding on *hTERT* promoter**

265 The obtained results pertaining to the WT1 overexpression and its impact on *hTERT*
266 expression, urge us to evaluate the physical interaction between WT1 and *hTERT* promoter using a
267 ChIP-qPCR approach. Since this assay requires important amounts of cells to be analyzed, SS PDC
268 1, 2 and 3 were selected because these patients were epigenetic therapy free; and HuT78 was selected
269 since it was the only SS cell line in this study. WT1 ChIP-qPCR results showed faint WT1 binding
270 signals at *hTERT* -323 (a region of *hTERT* promoter located around -323bp from TSS) in HuT78 cell
271 line and in all SS PDC studied (figure 5B). The pool of normal CD4+ cells, used as a normal control,
272 also showed a faint binding of WT1 to the *hTERT* promoter region targeted in our study. In all cells
273 investigated, no binding events were detected with the negative control primers (Untr12).
274 Contrastingly, significant signals for WT1 binding were observed with the positive control primer
275 pair TAL1 (-2k) in all SS PDC analyzed, showing an enrichment over background between 11 and
276 19-fold whereas no signals were detected in the HuT78 cell line. In addition, the normal CD4+
277 control, HuT78 cell line and PDC (1, 2 and 3), did not present WT1 binding signals with the additional
278 positive control *hTERT* -709.

279

280 **THOR hypermethylation is insensitive to HDACi**

281 Since two HDACi (romidepsin and vorinostat) are approved for CTCL treatment, without
282 clear molecular investigations, we studied the effect of these two drugs focusing on *hTERT* expression
283 in SS cells. *hTERT* expression decreased significantly ($P < 0.001$) in SS PDC 1, 2 and 3 after
284 romidepsin and vorinostat treatments (figure 6A). Surprisingly, in patient 4, *hTERT* expression was
285 not altered the same way as in the other patients. In fact, *hTERT* expression increased slightly with
286 romidepsin treatment and remained unchanged with vorinostat (figure 6A). Methylation levels of

287 *hTERT* promoter in SS PDC 1, 2 and 3 showed weak changes between non-treated cells (NTC) and
288 cells treated with either vorinostat or romidepsin (figure 6B). Indeed, after HDACi treatments
289 methylation levels and profiles remained quite the same throughout the entire promoter. In patient 4,
290 a slight decrease in THOR methylation status was observed only after romidepsin treatment in
291 comparison with NTC, with a statistical significance of $P= 0.00063$. No particular methylation or
292 demethylation of any CpG site was observed after romidepsin or vorinostat treatments
293 (supplementary figure 4).

294

295 **THOR hypermethylation is insensitive to 5-azacytidine**

296 Since HDACi did not exert any effect on THOR methylation in SS PDC, we analyzed the
297 effect of the demethylating agent 5-azacytidine on *hTERT* expression and promoter methylation in
298 HuT78 cell line and SS PDC 2 and 3. While *hTERT* expression decreased significantly after 5-
299 azacytidine treatment in SS PDC 2 and 3 ($P<0.01$) and in HuT78 ($P<0.001$) (figure 7A), the
300 methylation status of *hTERT* promoter remained unchanged throughout the entire promoter, showing
301 the same methylation levels and profiles: a highly methylated distal region and a poorly methylated
302 proximal region (figure 7B).

303

304 **DISCUSSION**

305 Since *hTERT* gene promoter comprises dense CpG islands (figure 2A), several studies
306 reported that its methylation is essential for its transcription (17,24,25). In Sézary Syndrome, a
307 subtype of rare aggressive primary cutaneous T cell lymphomas, an epigenomic analysis revealed
308 more than one hundred hypermethylated gene promoters, but *hTERT* was not mentioned among these
309 genes (26). To examine whether *hTERT* promoter methylation is likely to be involved or not in *hTERT*
310 expression, we carried out a pioneer epigenetic study using bisulfite Sanger sequencing in five CTCL
311 cell lines, four SS PDC, six fresh SS patients' cells and pools of healthy cells. Standard bisulfite
312 sequencing is considered to be time-consuming, however the consistency of the results generated by
313 this technique in gene-specific methylation studies overcome this inconvenience. Moreover, when

314 compared to other global genomic bisulfite techniques, standard bisulfite sequencing shows the ability
315 to detect, in the region of interest, the methylation status cell-by-cell of all consecutive CpGs (27).

316 Our analyses identified a common methylation pattern in all analyzed CTCL tumor cells.
317 This pattern encompasses a hypermethylated distal region from -650bp to -150bp and a
318 hypomethylated proximal region from -150bp to +150bp. This dual methylation pattern was
319 previously described in different pathologies (7,11,28). The hypermethylated region matches with the
320 433-base pair region containing 52 CpGs and recently named *TERT* Hypermethylated Oncologic
321 Region (THOR) (13,14). THOR is reported to be associated with telomerase reactivation in many
322 cancers but it was not studied till now in cutaneous lymphomas. Our results, suggest that in CTCL
323 the association between THOR methylation and *hTERT* expression is a prevalent phenomenon.
324 Focusing on aggressive CTCL, freshly collected SS patients' cells showed the same methylation
325 profile seen in cell lines and PDC, allowing the exclusion of any possible artifact of cell culture.
326 Nevertheless, THOR methylation is involved in *hTERT* reactivation in CTCL, without a direct
327 correlation, in concordance with previous reports (13), suggesting the implication of additional
328 "players" in this complex regulation, as for instance the hypomethylated proximal promoter region of
329 *hTERT* (28). The observed *hTERT* expression levels in CTCL cells were earlier described as sufficient
330 to promote the oncogenesis (16). In addition to THOR hypermethylation, other epigenetic
331 mechanisms and/or cis/trans regulatory elements participating in *hTERT* re-expression in CTCL
332 remain to be elucidated. Such studies would pave the way for understanding the exact biological role
333 of THOR in *hTERT* re-expression.

334 Furthermore, we observed that patients' normal cells do not harbor a methylated THOR,
335 supporting previously published results and confirming that THOR hypermethylation is an exclusive
336 epigenetic mark of tumor cells that can be used as a hallmark of cancer cells (29). Our data emphasize
337 also the complexity of *hTERT* gene expression and regulation. Indeed, THOR was observed to be
338 hypomethylated in normal CD4+ and CD34+ cells while *hTERT* was expressed, suggesting that this
339 expression in stem/progenitor and normal cells do not involve THOR methylation.

340 Several TERT-activator transcription factors (TFs), as ETS, c-MYC, SP1 and NFkB, and
341 TERT-repressive TFs, as WT1 and MZF-2, are known to bind to *hTERT* promoter (30,31). In acute

342 promyelocytic leukemia, Azouz *et al.* showed that the methylation of the distal domain of *hTERT*
343 promoter (including THOR) is associated with a decrease of WT1 binding to *hTERT* promoter and
344 sustained *hTERT* expression (11). We, therefore, investigated the role of WT1 in the pathogenesis of
345 CTCL. We verified that WT1 mRNA and protein are expressed in CTCL. WT1 expression levels did
346 not seem to be affected by THOR methylation levels in HuT78 and SS PDC. Then we observed that
347 WT1 overexpression reduced *hTERT* expression. Strengthened by this observation, we looked for the
348 physical interaction between WT1 and THOR in SS cells. Our data suggest that *hTERT* modulation
349 expression in CTCL may occur independently of WT1 binding to the THOR region. However, it is
350 known that low expressed or low binding levels of some TFs constitute a challenge to be identified.
351 Hence, further investigations are required in order to confirm whether, in CTCL, the binding of the
352 downregulating TFs (WT1 and MZF-2) to THOR is methylation sensitive and whether other binding
353 TFs might be present in this region.

354 A link exists between DNA methylation and histone modifications. This link is mediated by
355 a group of proteins with methyl DNA binding activity that localize to methylated DNA and recruit
356 other protein complexes such as histone deacetylases (HDAC) and histone methyltransferases
357 (32,33). Since the exact mechanism behind the effectiveness of HDACi treatments in SS patients
358 remains unknown (34), we investigated first the methylation status of *hTERT* promoter using two
359 HDACi treatments approved in MF/SS patients. Interestingly after *in vitro* HDACi treatments, *hTERT*
360 expression levels decreased in all SS PDC, while methylation patterns of *hTERT* promoter including
361 THOR remained unchanged, except for one PDC: patient 4, the only patient who had previously
362 received romidepsin. This observation may suggest a possible drug resistance mechanism. In the other
363 patients, THOR remained hypermethylated and *hTERT* proximal promoter encompassing TSS and
364 ATG remained hypomethylated. In a previous study using vorinostat in non-small cell lung cancer,
365 Li *et al.* observed a repression of the telomerase expression and a reduction of *hTERT* methylation
366 levels near the TSS (around -200bp to +160bp), but THOR was not investigated (35). In our study,
367 the TSS region was already hypomethylated and rationally cannot be more demethylated. Altogether,
368 these data suggest that HDACi reduced *hTERT* expression only in patients who did not receive
369 previous epigenetic therapies. Besides, we proved that other epigenetic drugs such as 5-azacytidine,

370 a demethylating agent, did not exert a demethylation on *hTERT* promoter including THOR in SS,
371 while it reduced *hTERT* expression. In other pathologies, it has been reported that *hTERT* expression
372 decreased after treatments with demethylating agents and it was accompanied sometimes with a
373 promoter demethylation, suggesting that the demethylation of *hTERT* promoter could be pathology-
374 dependent (36,37). Altogether, our results suggest that *hTERT* promoter in CTCL is resistant to
375 epigenetic drugs, indicating that these drugs can alter *hTERT* expression in an indirect way. In the
376 light of these observations, we report that the rapid and toxic effect of epigenetic drugs (HDACi and
377 DNMTi) in CTCL does not affect the THOR methylation status.

378

379 **CONCLUSIONS**

380 Taken together, our findings strongly suggest that THOR hypermethylation is a hallmark of
381 neoplastic CTCL cells associated with *hTERT* activation. Additionally, we propose that THOR
382 hypermethylation might be used as a biomarker of cancer cells in SS patients. By adding CTCL to
383 the list of tumors analyzed for THOR methylation, our findings represent a significant step forward
384 towards a better understanding of the mechanisms involved in telomerase activation and its regulation
385 by epigenetic drugs in this pathology. Our data provide a starting point for further investigations to
386 assess relationships between THOR methylation status, *hTERT* expression and TFs binding to THOR
387 in order to fully understand the sophisticated molecular mechanism of *hTERT* activation in CTCL.
388 The advent of new gene-specific targeting tools (17) will help to establish causality between *hTERT*
389 promoter DNA methylation and *hTERT* expression, paving the way to a better understanding of the
390 clinical response to epigenetic drugs in CTCL patients with advanced-stage.

391 **SUPPLEMENTARY MATERIALS**

392 Supplementary Methods.

393 Supplementary Table 1 and 2.

394 Supplementary Figure 1, 2, 3 and 4.

395

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408

409 **AUTHORS CONTRIBUTIONS**

410 AC performed DNA/RNA/proteins extractions, bisulfite sequencing, tumor cells isolation, western
411 blot, statistical analyses, analyzed and interpreted the data, wrote the manuscript and prepared the
412 figures; AC, JR, JF and YI performed qPCR analyses; JF performed TRAP assay; AC, SP, MPC, and
413 FC performed flow cytometry analyses, cell cultures and HDACi/DNMTi treatments; JMP performed
414 luciferase assay; MBB and APL recruited SS patients and followed them up; ESB, JMP, JPM, MBB,
415 EIC, CF, and RT provided helpful advices and assisted in editing the manuscript; EdC conceived and
416 designed the study, revised and edited the final version of this manuscript. All authors read and
417 approved the final version of the manuscript.

418

419 **CONFLICTS OF INTERESTS**

420 The authors declare no conflict of interest.

421

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522 **FIGURE LEGENDS**

523

524 **Figure 1: *hTERT* expression in cell lines and patients' cells.**

525 *hTERT* mRNA levels quantified by fluorescence real-time reverse transcriptase PCR in CTCL cell
526 lines, in SS patient-derived cells (SS PDC), in SS patient cells (SS Patients) and in healthy CD4+ and
527 CD34+ cells. *hTERT* mRNA levels were normalized to the expression of the *TBP* gene and expressed
528 in arbitrary unit (A.U). TBP: TATA-box Binding Protein located on 6q27.

529

530 **Figure 2: *hTERT* gene promoter methylation including THOR in CTCL cells and healthy 531 controls.**

532 (A) *hTERT* gene promoter including THOR (Chr5:1,295,321–1,295,753;GRCh37/hg19) containing
533 52 CpG represented each by a vertical dash. (B to N) Methylation profiles of CTCL cells (red) and
534 controls (blue): full black dots represent methylated CpGs whereas empty dots represent
535 unmethylated CpGs. For CTCL cell lines: MyLa is represented in (B), HuT78 in (C), Mac1 in (D),
536 Mac2A in (E) and Mac2B in (F). For SS PDC: PDC 1 is represented in (G), PDC 2 in (H), PDC 3 in
537 (I) and PDC4 in (J). Healthy CD4+ controls: two pools are represented in (K) and (L). Normal
538 stem/progenitor cells: two pools of normal CD34+ cells are represented in (M) and (N).

539

540 **Figure 3: *hTERT* gene promoter methylation including THOR in Sézary syndrome patient cells.**

541 Graph (A) to (F) showing the difference between methylation profiles of tumor cells (red) and normal
542 cells (blue), in patient 5 (A), patient 6 (B), patient 7 (C), patient 8 (D), patient 9 (E) and in patient 10
543 (F). Chart (G) showing THOR methylation levels in tumor (red) and normal (blue) cells in each of
544 the 6 SS patients' cells. THOR: TERT hypermethylated oncogenic region.

545

546 **Figure 4: THOR methylation status.**

547 The difference in THOR methylation averages between normal cells in blue and tumor cells in red in
548 all cells studied.

549

550 **Figure 5: Effect of the transcription factor WT1 on *hTERT* promoter in CTCL.**

551 Graph (A) presents the results of luciferase assay showing the effect of empty vector (Mock), 10µg
552 and 20µg of WT1 on *hTERT* promoter activation in HuT78 and MyLa cell lines. Graph (B) shows
553 the results of CHIP-qPCR using a WT1 antibody targeting the TERT-323 region in SS PDC 1, 2, 3,
554 HuT78 a SS cell line and healthy CD4+ (Control). SS PDC: Sézary Syndrome Patient-Derived Cells.

555

556 **Figure 6: HDACi treatments in Sézary syndrome patient-derived cells.**

557 Graph (A) shows *hTERT* expression in NTC, romidepsin and vorinostat -treated cells. Graph (B)
558 shows THOR methylation % in NTC, romidepsin and vorinostat -treated cells. HDACi: Histone
559 DeAcetylases inhibitors; NTC: Non-Treated Cells.

560

561 **Figure 7: 5-azacytidine treatment in Sézary cells.**

562 Graph (A) shows hTERT expression in NTC and 5-azacytidine -treated cells. Graph (B) shows THOR
563 methylation % in NTC and 5-azacytidine -treated cells. ATG: Start codon; NTC: Non-Treated Cells;
564 SS PDC: Sézary Syndrome Patient Derived Cells; TSS: Transcription Start Site.
565

566 **SUPPLEMENTARY FIGURE LEGENDS**

567
568 **Supplementary figure 1: Correlation between *hTERT* expression level and telomerase activity.**
569 *hTERT* mRNA levels and telomerase activity evaluated by TRAP assay are correlated in CTCL cell
570 lines and SS PDC with $R^2 = 0.7502$. CTCL: Cutaneous T-Cell Lymphomas; SS PDC: Sézary
571 Syndrome Patients-Derived Cells.
572

573 **Supplementary figure 2: Correlation between THOR methylation status and hTERT**
574 **expression level.**

575 Correlations and R^2 coefficients between THOR methylation status and hTERT expression level in
576 CTCL cell lines (A), SS PDC (B) and SS patients (C). CTCL: Cutaneous T-Cell Lymphomas; SS
577 PDC: Sézary Syndrome Patients-Derived Cells; SS: Sézary Syndrome.
578

579 **Supplementary figure 3: WT1 mRNA and protein expression.**

580 (A) WT1 mRNA levels normalized to the expression of the *TBP* gene and expressed in arbitrary unit
581 (A.U.) and (B) WT1 protein levels by western blot in HuT78 cell line, in SS PDC 1, 2 and 3 and in
582 MCF7 as positive control using the stain-free technology. SS PDC: SS PDC: Sézary Syndrome
583 Patients-Derived Cells.
584

585 **Supplementary figure 4: hTERT promoter methylation profiles after HDACi treatments.**

586 *hTERT* promoter methylation profiles of SS PDC 1, 2, 3 and 4 in NTC and in romidepsin or vorinostat
587 -treated cells. HDACi: Histone DeAcetylases inhibitors; SS PDC: SS PDC: Sézary Syndrome
588 Patients-Derived Cells, NTC: Non-Treated Cells.
589

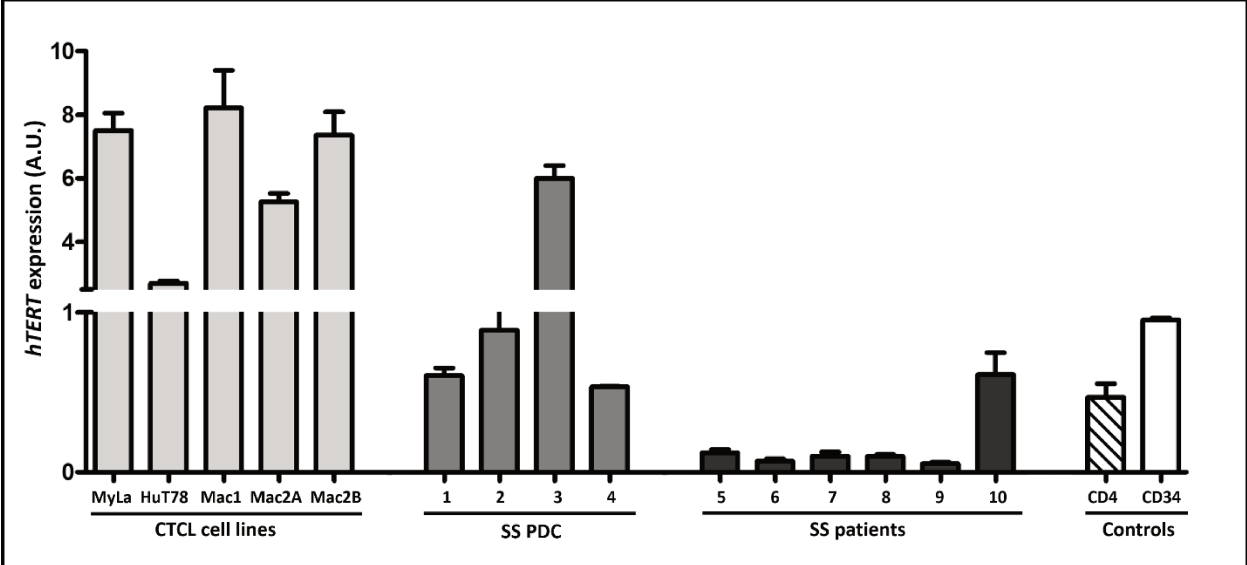


Figure 1

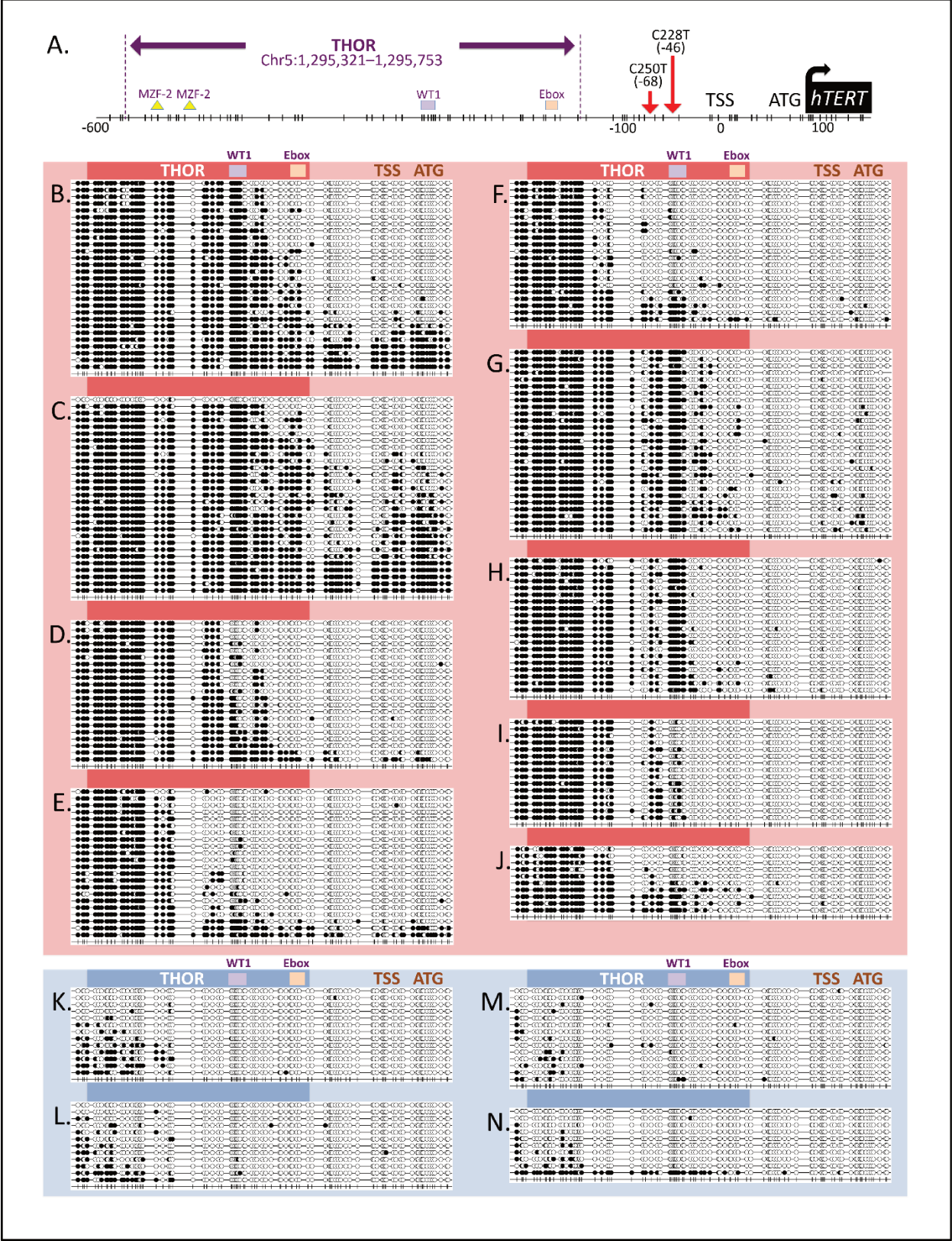


Figure 2

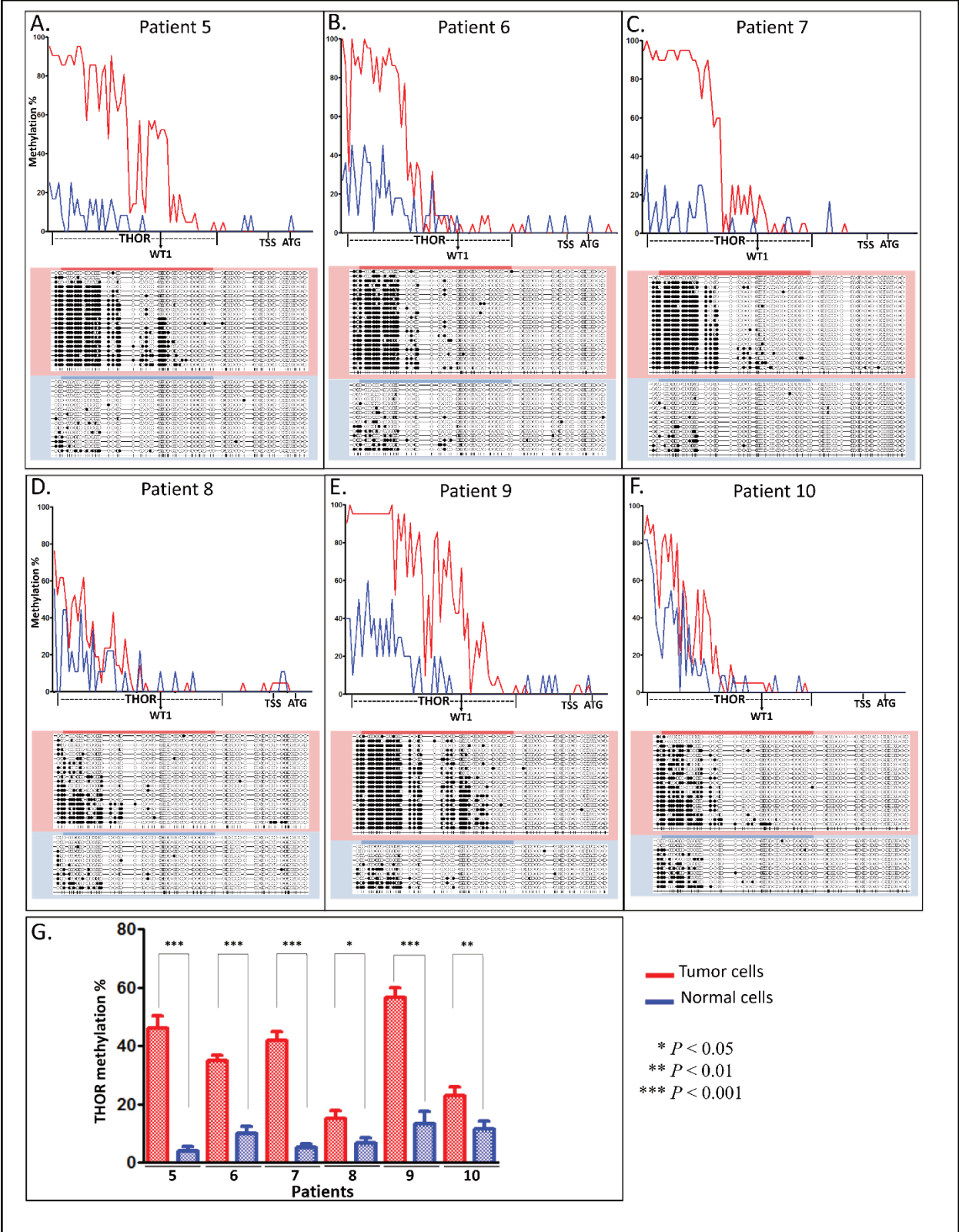


Figure 3

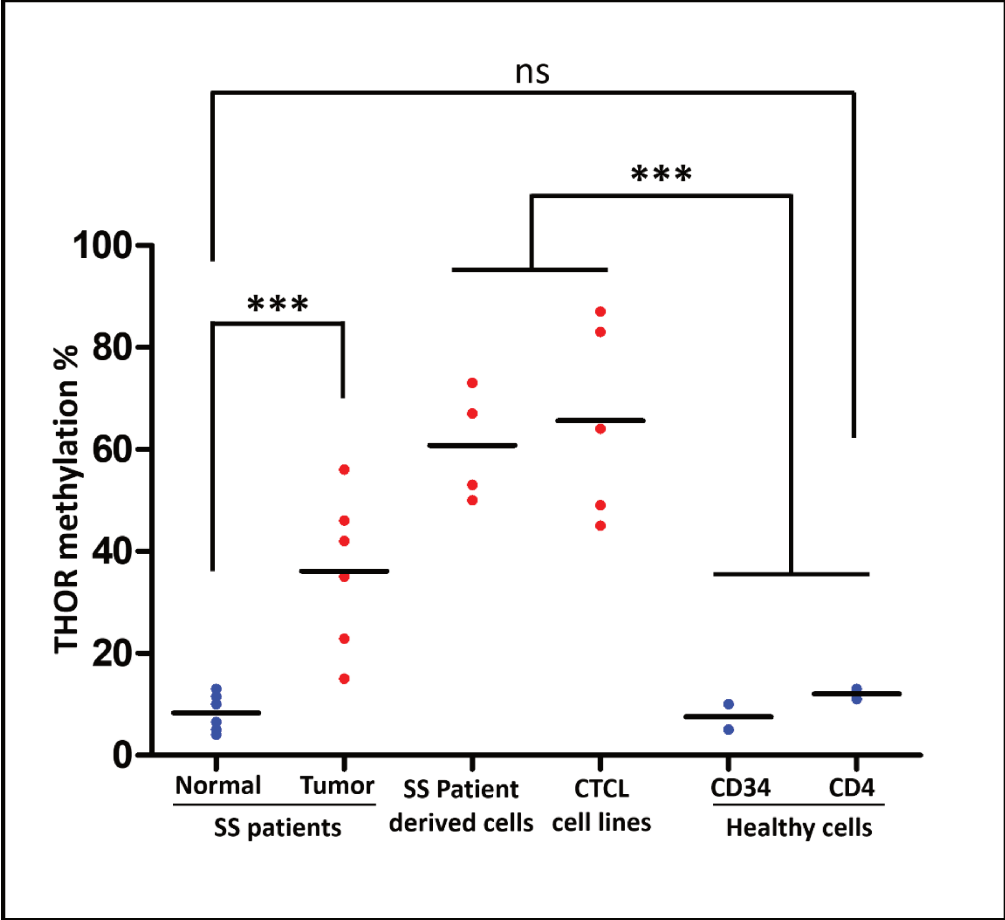


Figure 4

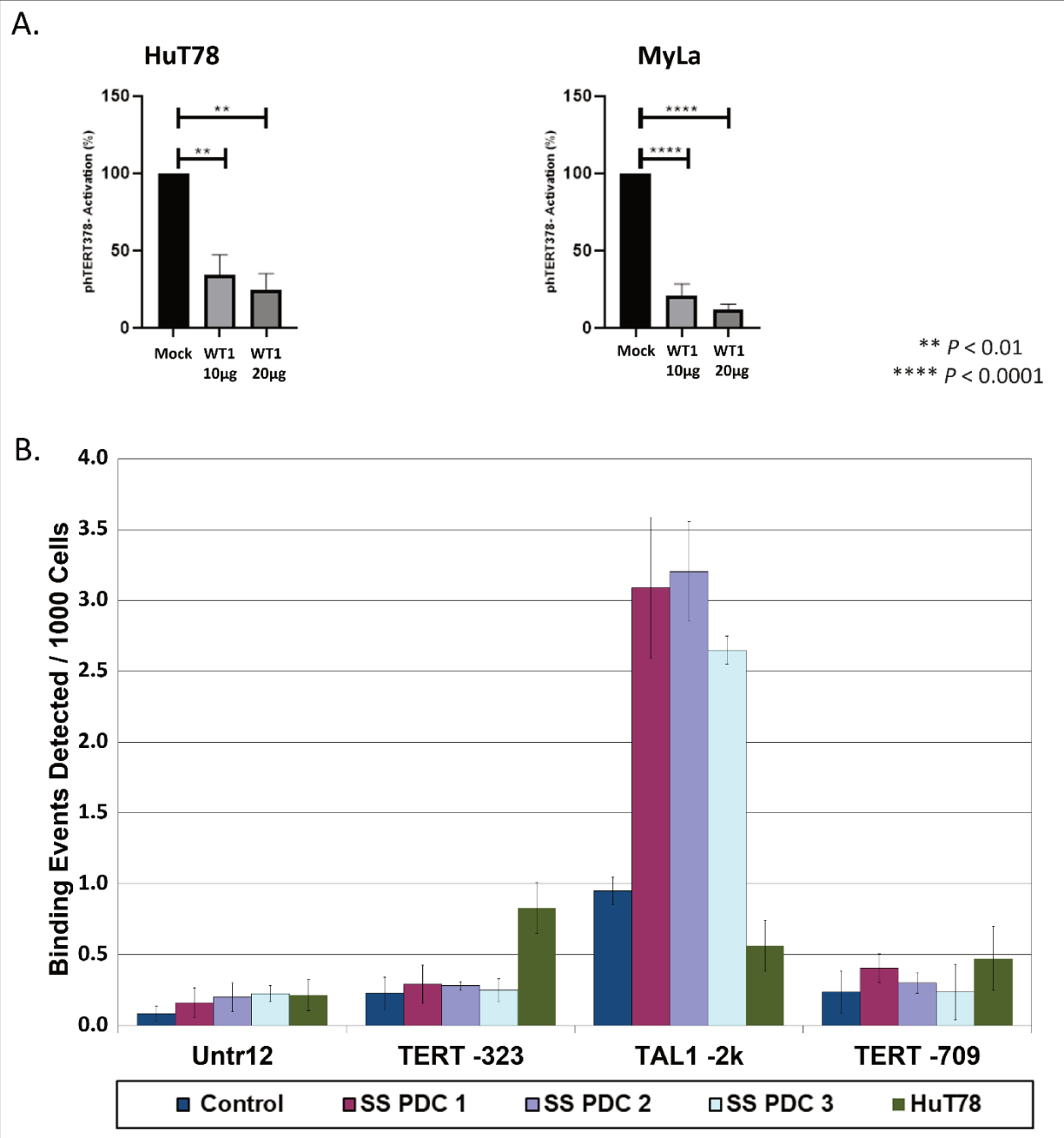


Figure 5

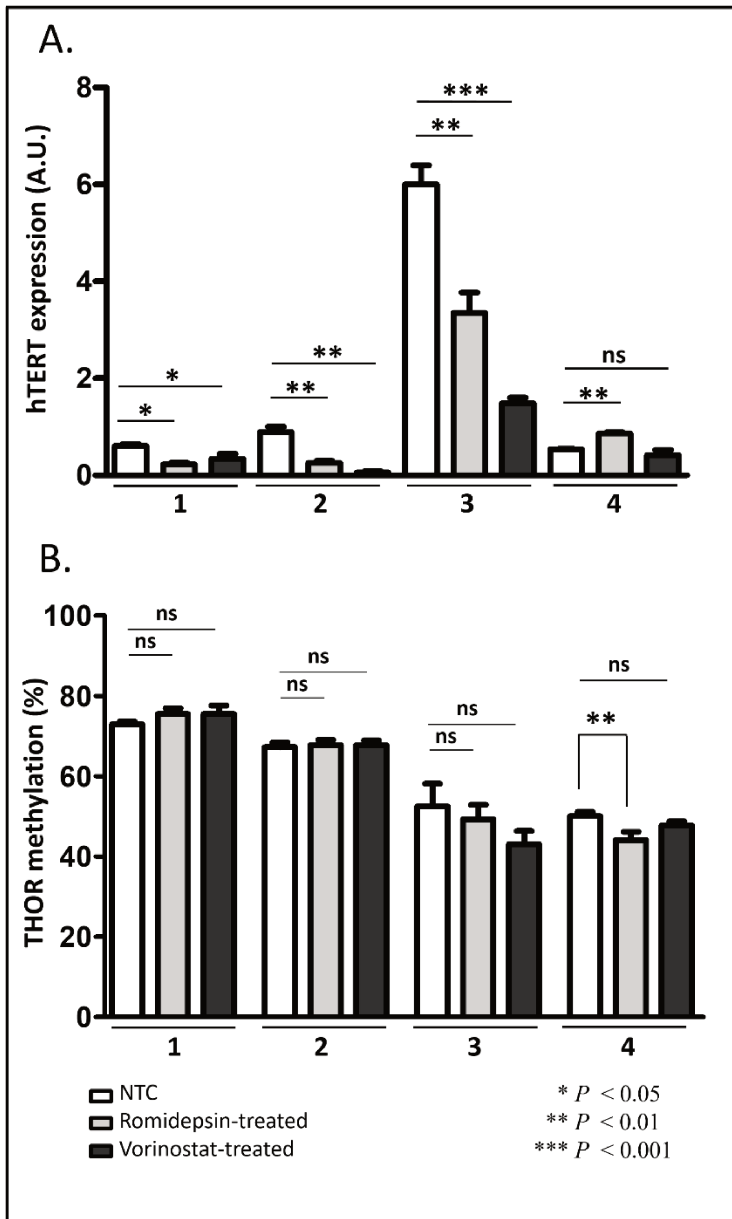


Figure 6

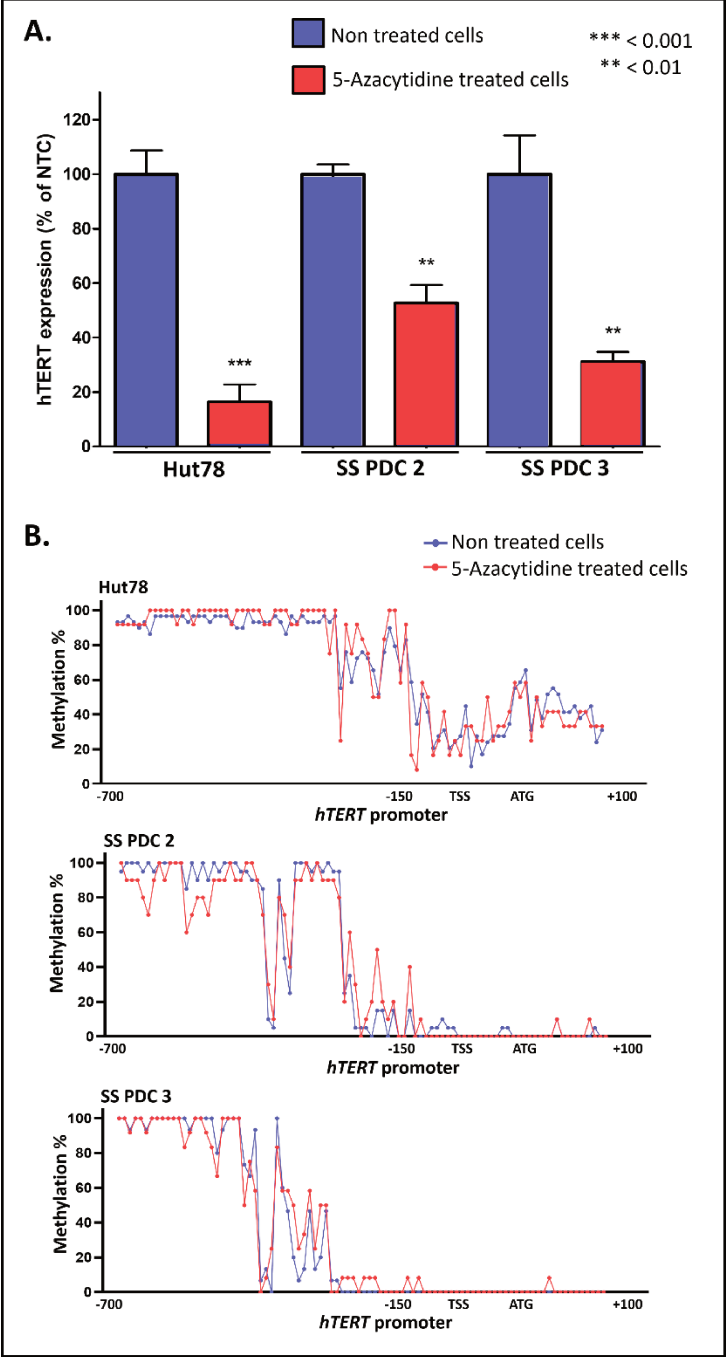


Figure 7

Supplementary table 1: *hTERT* Bisulfite PCR conditions and primers sequences

Forward Primer	5' GGTTTGTGTTAAGGAGTTTAAGT 3'	
Reverse Primer	5' CCAACCCTAAAACCCCAAAC 3'	
PCR program	2 min: 94°C	
	30s: 94°C	35 cycles
	30s: 58°C	
	1min: 72°C	
5min: 72°C		

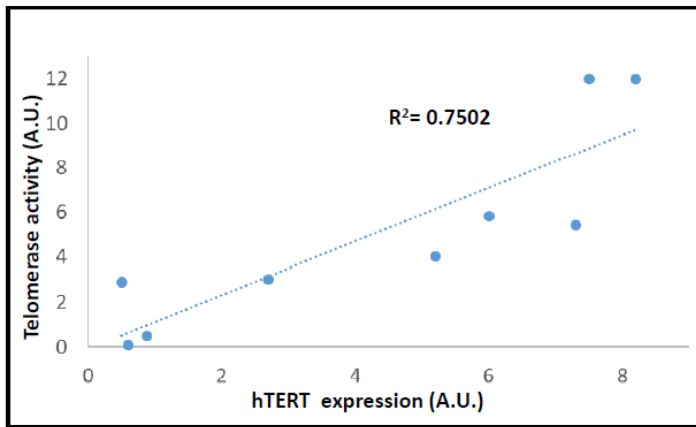
Supplementary table 2: Primer sequences used for *WT1* ChIP-qPCR

Region	Primer sequence	Purpose
<i>hTERT</i> -323 -F	AGCGGAGAGAGGTCGAATC	Region of interest
<i>hTERT</i> -323 -R	AGGGCCTCCACATCATGG	
<i>hTALI</i> -2k -F	CAGAAGGGCAGCAAACAAAC	Positive control
<i>hTALI</i> -2k -R	GTGTCCTGTTGGGCAGTGTG	
<i>hTERT</i> -709 -F	GAGCAAACCACCCCAAATC	Positive control
<i>hTERT</i> -709 -R	TCCATTTCCACCCCTTCTC	
Untr12	Active Motif Human negative control primer set 1, #71001	Negative control

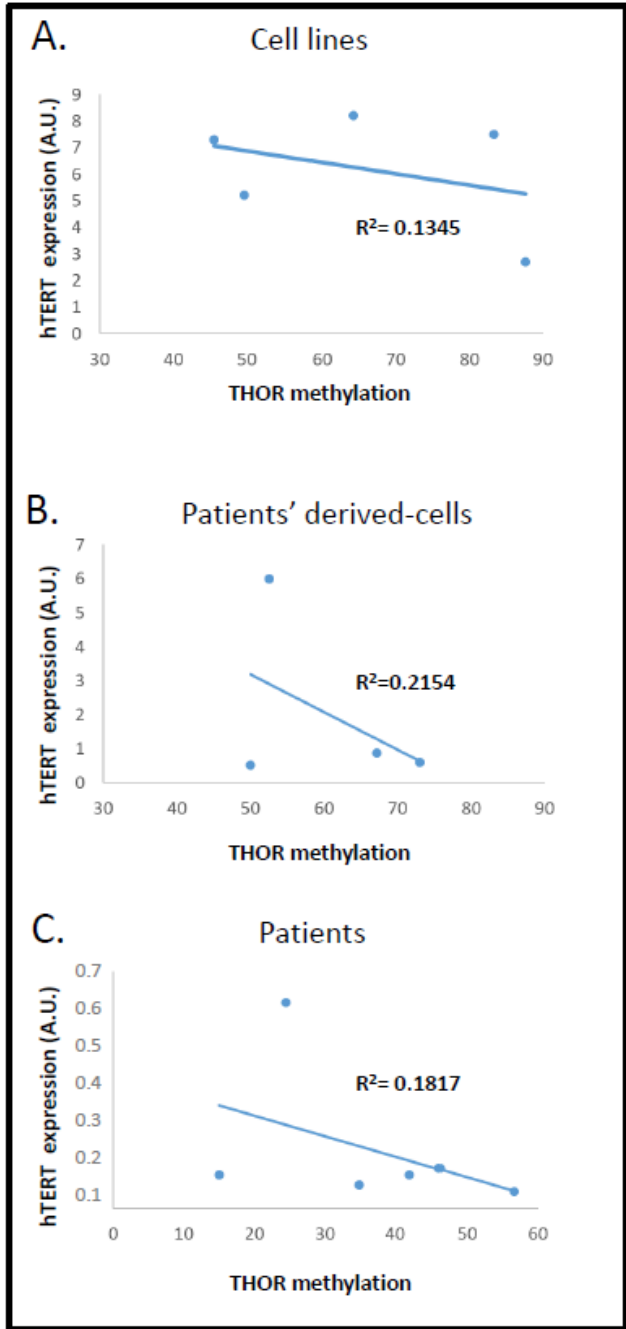
SUPPLEMENTARY METHODS

Western Blot

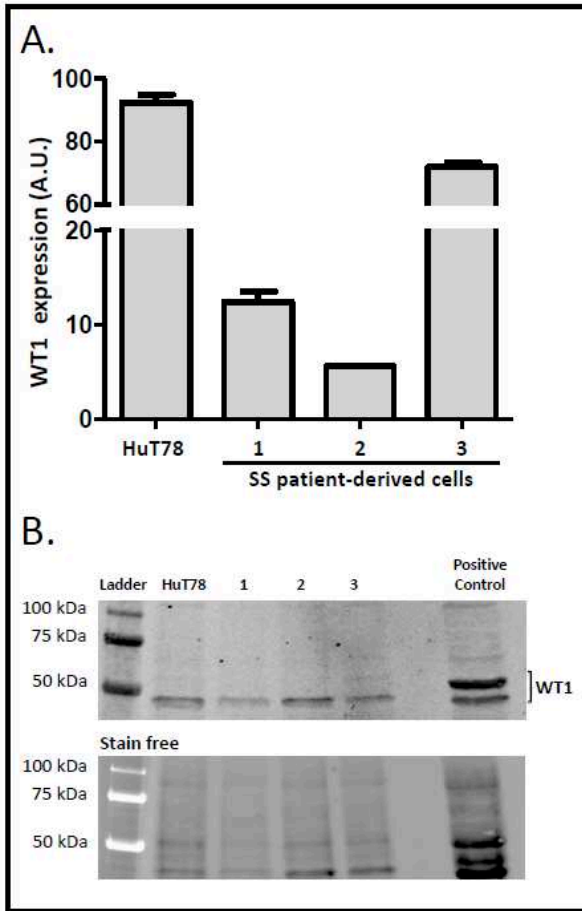
Western Blot assay was performed according to the manufacturer's recommendations (Bio-Rad, USA). Briefly, protein extracts from HuT78 cell line, SS PDC 1, 2 and 3 and MCF7 cell line (positive control expressing WT1, recommended by the manufacturer) in addition to All Blue Prestained Protein ladder were separated by SDS-PAGE on 8–16% TGX Stain-Free™ Protein Gels (Bio-Rad) for approximately 45 min at 150V in TGX buffer (Bio-Rad). Stain-free gels were activated by exposure to UV for 1 min. Proteins were transferred to nitrocellulose membranes (Bio-Rad) using the Bio-Rad Trans-Blot Turbo Transfer System for 7 min. Total proteins on membranes were detected using the Stain-free method. Membranes were blocked with TBST with 5% BSA for 1 hour. Membranes were then incubated with primary antibody (WT1 monoclonal antibody, Santa Cruz Biotechnology) diluted 1:500 in TBST with 5% BSA at 4°C overnight. Excess of primary antibody was removed by washing the membranes three times in TBST for 10 min each. The secondary antibody (peroxidase-conjugated anti-mouse DyLight800) diluted 1:5000 was incubated with the membrane in TBST with 5% BSA for 1 hour. Excess of secondary antibody was removed by washing the membranes three times in TBST for 5 min each. Membranes were visualized using Bio-Rad ChemiDoc™ Imager. Detection and quantification of bands' intensities were done using Image Lab software (Bio-Rad).



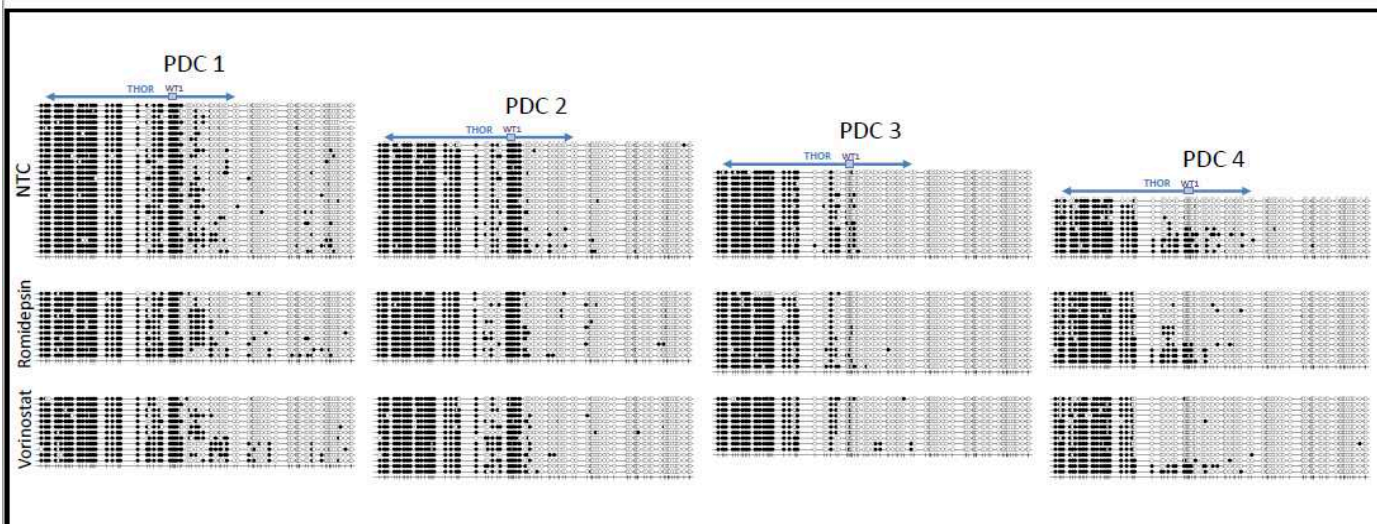
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4

4. Le facteur de transcription hMZF-2

Notre travail sur le niveau de méthylation de la région distale du promoteur de *hTERT*, montre que le statut de méthylation de *hTERT* est stable même sous la pression de médicaments épigénétiques. Ceci nous conduit à nous intéresser à d'autres acteurs susceptibles d'agir sur le promoteur de *hTERT* pour moduler son expression. Nous nous sommes intéressés aux facteurs de transcription présents dans la région de THOR.

Deux facteurs de transcription ayant un rôle répresseur sur l'expression de *hTERT* sont localisés dans cette région : le facteur Wilm's Tumor 1 (WT1) et le facteur human Myeloid Zinc Finger protein 2 (hMZF-2). Notre étude sur l'implication de hMZF-2 dans la réactivation de *hTERT* n'a pas été réalisée en raison d'une absence d'outils spécifiques à son investigation. Nos recherches bibliographiques ont donné lieu à un commentaire au sujet de ce facteur.

Dans le manuscrit "hMZF-2 the mysterious transcription factor", nous avons donné un aperçu des connaissances actuelles sur le facteur de transcription hMZF-2, nous avons souligné les limites de certaines investigations, le manque d'études concernant la liaison de hMZF-2 au promoteur *hTERT*, l'absence d'études fonctionnelles et les informations ambiguës concernant hMZF-2.

Nous avons écrit ce commentaire, afin de soulever plusieurs questions concernant l'existence et le statut de hMZF-2, facteur de transcription très souvent positionné sur le promoteur *hTERT* sans preuves biologiques et moléculaires de son existence.

4.1. Article "hMZF-2, the elusive transcription factor"



*h*MZF-2, the Elusive Transcription Factor

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Keywords: telomerase, transcription factor, *hTERT*, Mzf, myeloid zinc finger 1, myeloid zinc finger, MZF-2, myeloid zinc finger 2

INTRODUCTION

The myeloid zinc finger (MZF) protein family encompasses different transcription factors (TFs) including the myeloid zinc finger protein 1 (MZF-1), also known as zinc finger protein 42 (ZNF42) (Hromas et al., 1991). Assessing the role of MZF-1 in the granulocyte colony-stimulating factor (G-CSF)-induced differentiation of neutrophil in mice, Murai et al. (1997) unexpectedly isolated a novel MZF cDNA form that they named MZF-2. They suggested that MZF-1 and MZF-2 are produced from a single gene by using two alternative transcription initiation sites (Murai et al., 1997). The newly MZF-2 isolated was predicted to be longer than MZF-1. In this initial report by Murai et al. (1997) the human and the murine MZF-2 (*h*MZF-2 and *m*MZF-2, respectively), were predicted to have a 75.3% identity between their amino acids (aa) sequences. The *h*MZF-2 and *m*MZF-2 proteins contain 13 zinc finger motifs each, which are identical to those reported in the MZF-1 protein (Morris et al., 1994; Murai et al., 1997). It was also proposed that both *h*MZF-1 and *h*MZF-2 most likely recognize and bind to the same consensus sequences (5'-AGTGGGA-3' and 5'-CGGGGAGGGGAA-3') (Murai et al., 1997). In a complementary study, the same authors investigated only the *m*MZF-2 form and evaluated its transcriptional regulatory ability in myeloid cells (Murai et al., 1998). In this review, we question the actual existence of *h*MZF-2 as a transcription factor involved in *hTERT* expression and regulation.

*h*MZF-2 and *hTERT* Gene

According to the above reports, the *h*MZF-2 protein was supposed to bind to the distal region of the recently identified telomerase reverse transcriptase (TERT) hypermethylated oncogenic region (THOR) (Figure 1). THOR epigenetic modifications were shown to be a crucial regulator of the *hTERT* gene re-expression in solid tumors and leukemia (Lee et al., 2019) (Figure 1). Indeed, *hTERT* expression, a limiting factor of the telomerase activity (TA), is elevated in 85 to 90% of human cancers, thus promoting survival, proliferation, and invasion capacities of tumor cells (Ramlee et al., 2016). *hTERT* can be regulated through the binding of TFs (either repressors or activators) to its promoter region. MZF-2 was classified among the suppressors of the *hTERT* gene in human and canine (Long et al., 2005; Kyo et al., 2008). Due to the lack of appropriate and validated *h*MZF-2 antibodies, no chromatin immunoprecipitation experiments were done, and therefore, the binding of MZF-2 to the *hTERT* promoter was reported only as a result of indirect *in vitro* experiments. So far, Fujimoto et al. (2000) predicted that *h*MZF-2 can bind to 4 sites, all of them being located on the *hTERT* promoter at positions -514, -543, -619, and -687 (Figure 1). Since

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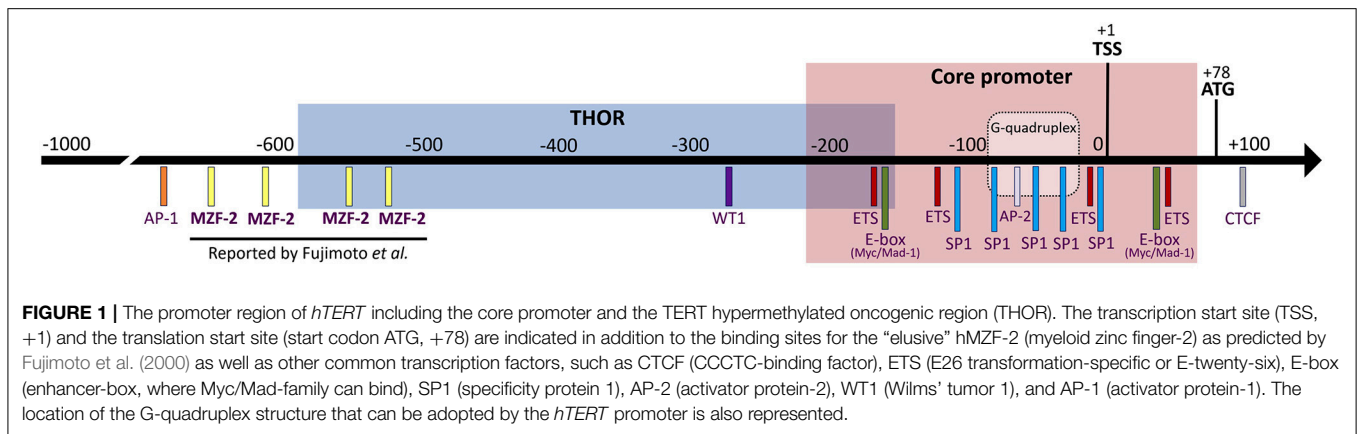
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Ségal-Bendirdjian E, Merlio J-P,
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*h*MZF-2, the Elusive Transcription
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this initial report, these four binding sites were presented in several figures of book chapters or review articles on telomerase regulation, including recently published ones (Ducrest et al., 2002; Pericuesta et al., 2006; Jafri et al., 2016; Lewis and Tollefsbol, 2016; ElHajj et al., 2017; Heidenreich and Kumar, 2017; Eitsuka et al., 2018; Srinivas et al., 2020), without any additional report that stated unambiguously the existence of hMZF-2 while the presence of other regulators of the *hTERT* gene, located further upstream of the transcription start site (TSS), were clearly reported to influence the *hTERT* expression, such as the activator protein 1 (AP-1), vitamin D (3) receptor (VDR), signal transducer and activator of transcription 3 (STAT3), and nuclear factor κ B (NF- κ B) (Ramlee et al., 2016).

hMZF-2 in the Databases

Blasting the forward and reverse primers (CCGGAGATGG GTCACAGTCC and TTGCTGAACACCTTGCCAC) used by Fujimoto et al. to amplify *MZF-2* transcripts (Fujimoto et al., 2000), we obtained very significant alignments with *MZF-1* and its mRNA variants. Such findings can be explained by the hypothesis that *MZF-2* is transcribed from the same gene as *MZF-1* (Murai et al., 1997). Moreover, the human form hMZF-2 sequence is still absent in the genomic and proteomic databases, while the murine form remains to be validated. In the UCSC Genome Browser on Human (genome.ucsc.edu), the OMIM (omim.org), the NCBI (ncbi.nlm.nih.gov/gene), and the *Ensembl* (ensembl.org) databases, only *MZF-1* exists. In the GeneCards database (genecards.org), a search for “*MZF-2*” directs to the *MZF-1* gene and to the biological region LOC110806263 which refers to the TERT 5’ regulatory region on the *hTERT* promoter and citing the paper by Fujimoto et al. (2000). In the proteomic database UniProt (uniprot.org), information concerning *MZF-2* in mouse (*Mus musculus*) is available under the label “experimental evidence at transcript level,” but no information is indicated for the human *MZF-2* form.

DISCUSSION

In a recent review article published in 2020, Brix et al. (2020) regrouped information on *MZF-1* and its role in

regulating cancer invasion. They also discussed *MZF-1* transcript variants. They stated that the first *MZF-1* isoform isolated and characterized was believed to be the full-length *MZF-1* (485 aa) until the identification of the long isoforms (734 aa), named *MZF-2a* in mouse and *MZF1B/C* in human (Brix et al., 2020). Brix et al. defined hMZF-2 as the largest form of hMZF-1, or “full-length hMZF-1” (Brix et al., 2020). However, the 734 aa full-length hMZF1 (*MZF1B/1C*) differs in length from the 775 aa hMZF-2 predicted initially by (Murai et al., 1997; Peterson and Morris, 2000) (Supplementary Figure 1). As for the structural domains in *MZF*, the SCAN domain that mediates interactions between members of a mammalian subfamily of zinc-finger transcription factors is shared between *MZF-1* and *mMZF-2* (uniport.org), while this information is not available for hMZF-2.

Herein, we summarize the available information regarding *MZF-2* published as original research articles (Murai et al., 1997, 1998; Fujimoto et al., 2000) and those published in review articles (Ducrest et al., 2002; Pericuesta et al., 2006; Jafri et al., 2016; Lewis and Tollefsbol, 2016; ElHajj et al., 2017; Heidenreich and Kumar, 2017; Eitsuka et al., 2018; Srinivas et al., 2020). All the published reports, as well as the search in genomic databases, lead us to be doubtful about the real existence of the human form hMZF-2. From these reports, it is not clearly demonstrated whether hMZF-2 is another isoform of hMZF-1. Twenty-three years after its discovery, data concerning hMZF-2 genomic or proteomic sequences are still unpublished. No antibody against the hMZF-2 protein is available. If it is true that hMZF-2 refers to the full-length hMZF-1 as mentioned by Brix DM et al. in 2020, why is this information lacking in the genomic databases? Most of the hMZF-2 original research articles were published before the availability of a reference genome. However, we aimed to highlight the lack of biological evidence that confirm the existence of hMZF-2, functionally differentiate hMZF-2 from hMZF-1, and unequivocally state its ability to regulate the *hTERT* gene. Therefore, we urgently suggest that the four theoretical hMZF-2-binding sites on the *hTERT* promoter should be no longer assigned to this “elusive” transcription factor until further clear experimental evidence is reported (Figure 1). Indeed, the precise identification of the TFs’ binding sites on the promoter of the oncogene *hTERT* would refine insights into the epigenetic regulation of *hTERT* activity in cancer.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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grants from INSERM, the National Center for Scientific Research (CNRS), and the Ligue Nationale Contre le Cancer (Comité Ile-de France).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2020.581115/full#supplementary-material>

Supplementary Figure 1 | Pairwise alignment comparison between the human MZF-1 and the mouse MZF-2 proteins showing that the N-terminal region is missing in the full length MZF-1.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. L'effet de la 5-azacytidine sur *hTERT* et sur la clonogénicité des cellules de Sézary

Le syndrome de Sézary (SS) est une variante leucémique agressive des lymphomes T-cutanés (CTCL) dans laquelle le gène human Telomerase Reverse Transcriptase (*hTERT*) est réexprimé. Plusieurs thérapies existent pour le traitement des SS mais elles ne permettent pas de réponses durables. Nous avons montré que la 5-azacytidine, une épidroque qui appartient aux inhibiteurs de l'ADN méthyltransférase (DNMTi), peut réduire l'expression de *hTERT* sans modifier le niveau de méthylation de son promoteur. La 5-azacytidine est approuvée pour le traitement de plusieurs hémopathies malignes, mais pas pour les CTCL. Ici, nous avons utilisé le test de Soft Agar pour analyser l'effet fonctionnel de la 5-azacytidine sur les capacités clonogéniques des cellules SS. Nos données ont révélé que la 5-azacytidine exerce un effet antitumoral dans les cellules de SS, et que la diminution de l'expression de *hTERT* s'accompagne d'une diminution des capacités clonogéniques des cellules tumorales SS.

5.1. Article “5-azacytidine reduces the clonogenic capacities of SS cells”



1 *Brief Report*

2 **5-azacytidine reduces the clonogenic capacities of SS**
3 **cells**

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21

22 **ABSTRACT**

23 Sézary syndrome (SS) is an aggressive leukemic variant of cutaneous T-cell lymphomas (CTCL) in

24 which the human Telomerase Reverse Transcriptase (*hTERT*) gene is re-expressed. Current available

25 treatments do not provide long-term response. We previously reported that 5-azacytidine, a DNA

26 methyltransferase inhibitors (DNMTi) epigenetic drug, can reduce the *hTERT* expression without altering the

27 methylation level of *hTERT* promoter. 5-azacytidine is approved for the treatment of several

28 hematological disorders, but not for CTCL. Here, we describe the use of the soft agar assay to analyze

29 the functional effect of the 5-azacytidine on the clonogenic capacities of SS cells. Our data revealed

30 that 5-azacytidine exerts an anti-tumor effect in SS cells, and that the downregulation of *hTERT*
31 expression is associated with a decrease in the clonogenic capacity of SS tumor cells.

32 **KEYWORDS:** Sézary Syndrome, Cutaneous T-cell lymphomas, 5-azacytidine, Epidrugs,
33 Epigenetics, DNA methylation, hTERT, Telomerase.

34 BACKGROUND

35 Telomerase activation through re-expression of the human telomerase reverse transcriptase
36 (*hTERT*) is found in 85-90% of human cancers and is believed to play a crucial role in tumor cells
37 replicative behavior and immortality [1,2]. While *hTERT* re-expression in cancer cells can be
38 attributed to genetic alterations such as *hTERT* gene locus rearrangements, amplifications or promoter
39 mutations; in cutaneous T-cell lymphomas (CTCL) these mechanisms fail to explain *hTERT*
40 upregulation [3,4] (Ropio *et al.* Submitted). Recent studies, reported an association of *hTERT*
41 promoter hypermethylation at a specific region named THOR (TERT Hypermethylated Oncogenic
42 Region) with telomerase reactivation in various human cancers. This led to the assumption that THOR
43 may be a molecular biomarker [5]. Since THOR was not yet investigated in CTCL, we decided to
44 study its role and implication in this pathology and we came to a conclusion that THOR's status could
45 be a hallmark of cancer cells in CTCL (Chebly *et al.* Submitted).

46 Within the primary CTCL spectrum, Sézary syndrome (SS) is a rare and aggressive leukemic
47 variant in which telomerase re-expression holds a crucial role in telomere maintenance and
48 tumorigenic properties [4,6]. The lack of specific biomarkers for neoplastic cells in SS [7], makes
49 telomerase expression a potentially promising therapeutically targetable biomarker. The treatment of
50 SS is extremely challenging, with therapeutic options for first-line therapies including extracorporeal
51 photopheresis or immunomodulating agents such as interferon- α . Second-line therapies involve
52 targeted immunotherapies (anti-CD30, anti-CD52, anti-CCR4, anti-CD158k) and single/multiagent
53 chemotherapies [7,8]. Although various treatments are available, most responses are partial and not
54 sustainable except for allogeneic bone marrow transplantation [7,9]. Within this context, other
55 therapeutic approaches have emerged. Hence, histone deacetylase inhibitors (HDACi) have been

56 shown to be of benefit for patients with hematological malignancies and particularly in CTCL [10].
57 Two HDACi, romidepsin and vorinostat, were approved by the Food Drug Administration (FDA) for
58 the treatment of relapsed/refractory CTCL. Through histone deacetylation, HDACi can modulate
59 DNA packaging, leading to an alteration in gene accessibility and therefore gene transcription.
60 Besides HDACi, other DNA packaging modifiers are available such as DNA methyltransferase
61 (DNMT) inhibitors, 5-azacytidine and 5-aza-2'-deoxycytidine (Decitabine, DAC). These DNMTi are
62 FDA-approved for the treatment of myelodysplastic syndrome (MDS) and acute myeloid leukemia
63 (AML), but not for CTCL.

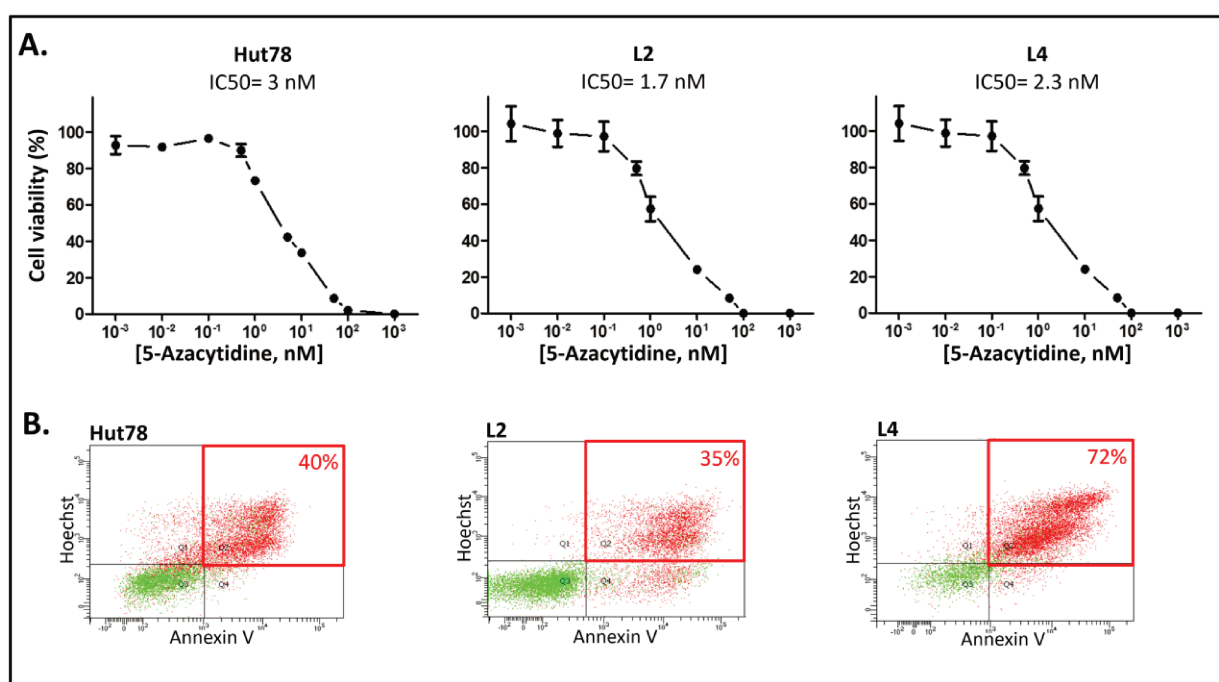
64 In a previous study, we reported that 5-azacytidine reduced *hTERT* expression levels in SS cells
65 *in vitro*, while maintaining THOR's methylation status (Chebly *et al.* Submitted). Herein, we analyze
66 the effect of 5-azacytidine on the clonogenic capacities of SS cells.

67

68 RESULTS AND DISCUSSION

69 While focusing on SS, an aggressive leukemic form of CTCL, we investigated the effect of the
70 hypomethylating agent 5-azacytidine on *hTERT* promoter methylation status and *hTERT* expression.
71 Telomerase expression was detected in all SS samples analyzed, which is consistent with previous
72 findings showing that SS are telomerase positive tumors [4] (Chebly *et al.* Submitted). The IC50
73 values for 5-azacytidine in SS cells determined at 72h, were 3nM, 1.7nM and 2.3nM, in HuT78, L2
74 and L4, respectively (Figure 1A). Consequently, cells were treated for 72h with 5-azacytidine using
75 the calculated IC50 values. Compared to non-treated cells (NTC), *hTERT* expression levels dropped
76 to 17%, 52% and 31% in 5-azacytidine treated HuT78, L2 and L4, respectively. This decline occurred
77 with no change in the methylation status of *hTERT* promoter, in line with our previously reported

78 findings (Chebly *et al.* submitted). Apoptosis/necrosis analyses by flow cytometry, using
 79 AnnexinV/Hoescht assay, revealed a majority of AnnexinV+Hoescht+ treated cells, thus indicating
 80 that these were in late apoptosis (Figure 1B), and confirming that 5-azacytidine alone could trigger
 81 T-cells apoptosis [11]. Moreover, it has been reported that 5-azacytidine in combination with
 82 romidepsin synergistically induced apoptosis in CTCL cells *in vitro* [12].

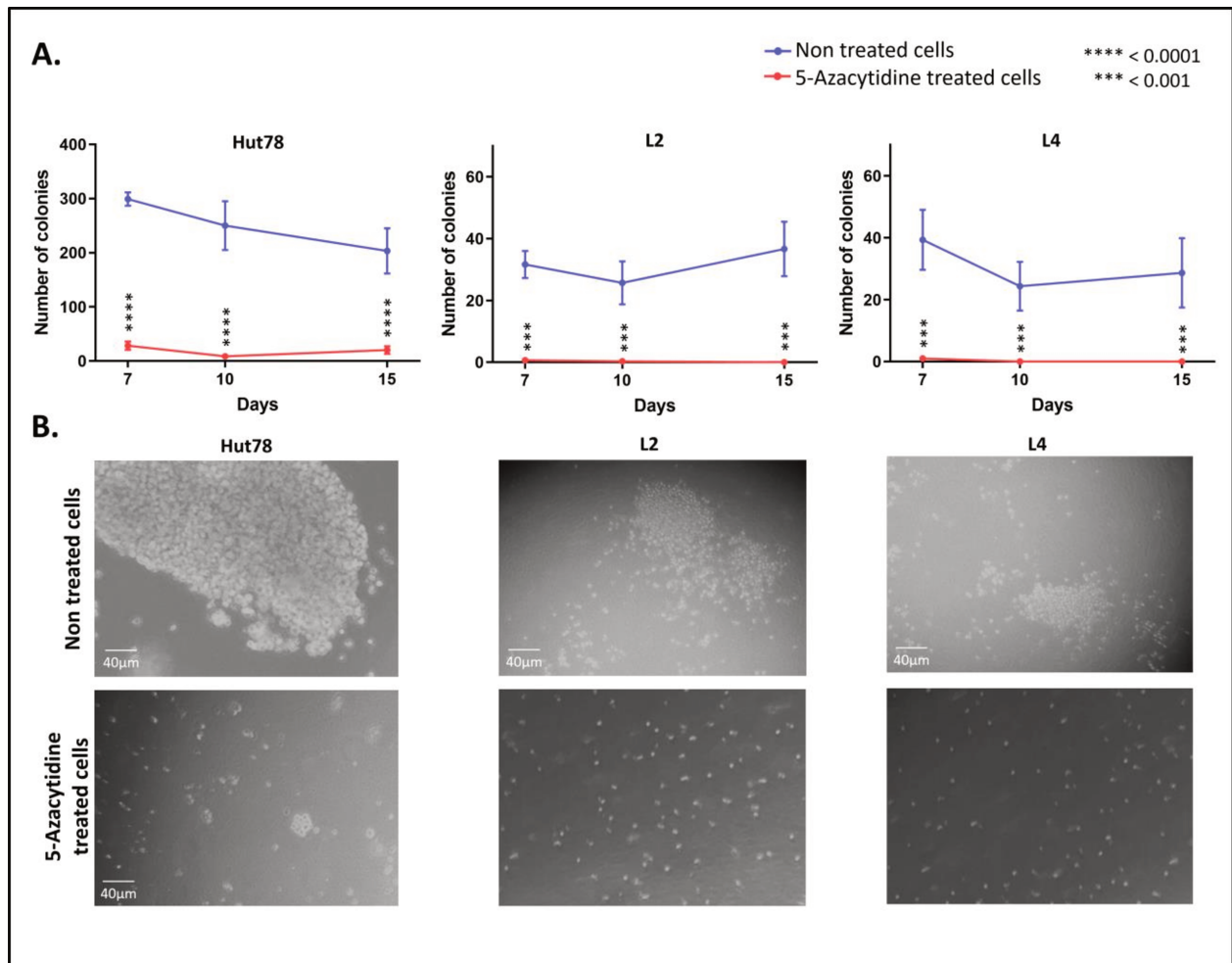


83
 84 **Figure 1: 5-azacytidine treatment in HuT78 cell line, L2 and L4.**

85 (A) Graphs showing cell viability % after 72h using different concentrations of 5-azacytidine to
 86 determine the IC50. (B) Flow cytometry plots showing the apoptosis/necrosis analyses of 5-
 87 azacytidine treated cells and displaying the % of late apoptotic cells.

88

89 Furthermore, soft agar assay allowed comparison of SS cells' *in vitro* tumorigenic capacities
 90 between non-treated cells (NTC) and after 72h of 5-azacytidine treatment. During soft agar assay, the
 91 drug's pressure was released. While in Hut78 NTC, the number of colonies (>8 cells) per well was
 92 299 at day 7 and 203 at day 15, this number was significantly lower in 5-azacytidine treated cells
 93 ($P < 0.0001$) with 28 colonies per well at day 7 and 20 at day 15 (Figure 2A).



94

95 **Figure 2: The effects of 5-azacytidine on the clonogenicity of SS cells.**

96 (A) Graphs showing the number of colonies per well in NTC (blue) and 5-azacytidine treated cells
 97 (red). (B) Images of the observed colonies in NTC and after 5-azacytidine treatment, in HuT78 cell
 98 line, L2 and L4.

99

100 Microscopic observations by two independent individuals revealed that Hut78 NTC colonies were

101 countless and huge in NTC, while they looked smaller with unhealthy appearance in treated cells

102 (Figure 2B). In L2, the number of colonies per well was 32 at day 7 and 37 at day 15. This number

103 significantly decreased ($P < 0.001$) in 5-azacytidine treated cells to 1 per well at day 7 and 0 at day 15

104 (Figure 2A). In L4, the number of colonies per well was 39 at day 7 and 29 at day 15 then it dropped

105 down ($P < 0.001$) in treated cells to 1 per well at day 7 and 0 at day 15 (Figure 2A). After 72 hours of

106 5-azacytidine treatment, L2 and L4 cells lost their capacity to form colonies *in vitro* (Figure 2B). It

107 has been reported that 5-azacytidine and/or DAC have the potential to reduce the clonogenicity of

108 various cancer cells, including leukemia, colorectal cancer, uveal and skin melanoma [13–15], our
109 data demonstrate a comparable effect on SS cells. Furthermore, SS treated cells showed a limited
110 proliferative capacity concomitant with the decrease in *hTERT* expression while compared to NTC,
111 suggesting a correlation between cell proliferation and *hTERT* expression. In a previously published
112 work, we stated that the inhibition of *hTERT* expression in SS cells leads to dramatic cell death *in*
113 *vitro* [4]. The correlation observed here, might be explained by an indirect effect of 5-azacytidine on
114 *hTERT* expression, most probably through targeting of upstream genes or *hTERT* gene regulators or
115 even histones modifications [16].

116

117 MATERIALS AND METHODS

118 One SS cell line HuT78 (ATCC, France) and two SS patient-derived cells (L2 and L4) [17]
119 were investigated in this study. The diagnosis of both SS patients was established in accordance with
120 the criteria of the WHO-EORTC (World Health Organization and the European Organization for
121 Research and Treatment of Cancer) [18]. HuT78 was cultured in RPMI 1640 medium (Gibco, USA)
122 supplemented with 10% of fetal bovine serum (Eurobio, France) and 100U/mL of penicillin and
123 streptomycin (Gibco). Cells were cultured without antibiotics for 48h before 5-azacytidine treatment.
124 L2 and L4 cells were cultured as previously described [17]. All cell cultures were incubated at 37°C
125 in a humidified 5% CO₂ incubator.

126 The half maximal inhibitory concentration (IC₅₀) was determined at 72h using the luminescent
127 cell viability assay CellTiter-Glo® (Promega, USA). Thirty million cells of HuT78, L2 and L4 were
128 treated for 72h with the calculated 5-azacytidine IC₅₀ value. *hTERT* expression level was verified by
129 qPCR and the methylation status of *hTERT* promoter was tested by locus specific bisulfite

130 sequencing, as previously reported (Chebly *et al.* Submitted). SS cells colony-formation capacity
131 (clonogenicity) was determined using soft agar assay, after releasing the 5-azacytidine pressure.
132 Statistical analyses were performed by Mann Whitney test on GraphPad Prism software (version 8.4).
133 *P* values of less than 0.05 were considered statistically significant. All analyses were performed in
134 biological and technical replicates.

135

136 CONCLUSION

137 Altogether, our data show that *in vitro* anti-tumor effect of 5-azacytidine is associated with
138 massive cell death, *deceleration* of *cell proliferation* and decrease in tumor cells' clonogenic
139 capacities. At *hTERT* gene level, 5-azacytidine treatment altered *hTERT* expression leading to a
140 reduced expression along with a drop in tumorigenic capacities of SS cells *in vitro*. Our findings raise
141 questions about the mechanism of action responsible for *hTERT* downregulation induced by 5-
142 azacytidine. This epidrug could have induced global demethylation resulting in an upregulation of
143 genes involved in further repression of *hTERT* promoter; or could have altered histone conformation
144 adopted on *hTERT* promoter, thus restricting accessibility of transcription factors to their binding
145 sites.

146

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149

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154

155 **Author Contributions**

156 Conceptualization: AC and EC; Methodology: AC, MPC, LBB, YI and EC; Formal Analysis: AC,
157 MPC and EC; Investigation: AC, MPC, YI and EC; Resources: AC, MPC and EC; Data Curation:
158 AC, MPC, CF and EC; Writing – Original Draft Preparation: AC; Visualization: AC; Writing –
159 Review & Editing: EC, SP, LBB, CF, JPM, MBB and RT; Funding Acquisition: EC; Supervision:
160 JPM and EC.

161

162 **Conflicts of Interest**

163 The authors declare no conflict of interest

164

165 **Ethics approval and consent to participate**

166 This study was approved by the institutional review board of Bordeaux University, in accordance
167 with the Declaration of Helsinki on ethical principles for medical research involving human subjects.

168

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6. Les modifications des histones sur *hTERT*

La romidepsine et le vorinostat sont des inhibiteurs de l'histone désacétylase (HDACi) approuvés pour le traitement des lymphomes T-cutanés (CTCL), y compris le syndrome de Sézary (SS). Les mécanismes d'action exacts des HDACi ainsi que les réponses moléculaires à ces médicaments restent mal compris. La pathologie SS est une tumeur télomérase positive, dans laquelle les mécanismes moléculaires responsables de l'expression du gène *hTERT* n'ont pas encore été complètement découverts. Comme la réactivation de ce gène pourrait être induite par des modifications d'histones, nous avons analysé l'effet de la romidepsine et du vorinostat sur l'expression de *hTERT* ainsi que sur la dynamique des marques d'histones dans les cellules SS. Notre étude confirme que dans les cellules SS les HDACi peuvent réduire l'expression de *hTERT*. D'un point de vue fonctionnel les HDACi diminuent les capacités clonogènes de ces cellules. Les résultats obtenus au niveau du promoteur *hTERT* suggèrent que la marque d'histone H3K27ac, associée à une transcription active, et la marque d'histone H3K27me3, associée à la répression transcriptionnelle, sont subtilement modifiées en réponse aux traitements à la romidepsine ou au vorinostat. Ceci confirme la plasticité des marques histone présentes dans la région promotrice de *hTERT*. Et, suggère au niveau du promoteur, un équilibre précaire mais finement régulé entre la quantité de marques activatrice et répressive semble nécessaire pour induire une réponse appropriée du gène *hTERT*.

6.1. **Article “*hTERT* promoter behavior under HDACi influence in Sézary syndrome”**

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***hTERT* promoter behavior under HDACi influence in Sézary syndrome.**

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22 **ABSTRACT**

23 Romidepsin and vorinostat are histone deacetylase inhibitors (HDACi) approved for the treatment of
24 Cutaneous T-cell lymphomas (CTCL) including Sézary syndrome (SS). The exact mechanisms of
25 action of HDACi as well as the molecular responses to these drugs remain not well understood.
26 Moreover, SS pathology is a telomerase positive tumor, in which the molecular mechanisms
27 responsible for *hTERT* gene expression have not yet been fully uncovered. As gene reactivation could
28 be induced by histones modifications, we analyzed the effect of romidepsin and vorinostat on *hTERT*
29 expression along with histone marks dynamics in SS cell line and SS Patient derived cells. Our study
30 confirms that HDACi can reduce *hTERT* expression as well as the clonogenic capacities of SS cells.
31 Additionally, it suggests that H3K27ac histone mark, associated with active transcription, and
32 H3K27me3 histone mark, associated with transcriptional repression, at *hTERT* promoter are deftly
33 altered in response to HDACi treatments. Our data sustained previous reports on *hTERT* gene
34 regulation showing that it is unique relatively to its peculiar roles in cells. The *hTERT* promoter region
35 is remarkable; it does not behave or even respond to drugs in a simplistic way, highlighting the
36 importance of identifying new targetable biomarkers that are implicated in *hTERT* regulation.

37

38 **KEYWORDS:** Cutaneous T-cell lymphomas, Sezary syndrome, Histone modifications, HDACi,
39 Telomerase, hTERT, THOR.

40 INTRODUCTION

41 Histone deacetylase inhibitors (HDACi) are a group of drugs that can inhibit the functional
42 activities of histone deacetylases (HDAC) leading to an increased histone acetylation (1). Besides their
43 direct roles in epigenetic regulations, HDACi have important roles in cancer cells, inducing cell death,
44 apoptosis and cell cycle arrest (2,3). The first HDACi approved by the food and drug administration
45 (FDA) were vorinostat (suberoylanilide hydroxamic acid, SAHA) in 2006 and then romidepsin
46 (depsipeptide, FK-228) in 2009 both for Cutaneous T-cell lymphomas (CTCL) (4,5). To date, four
47 HDACi were approved by the FDA (Romidepsin, vorinostat, panobinostat and belinostat) and they are
48 mainly used for the treatment of selected hematological malignancies including CTCL, Peripheral T-
49 cell lymphomas (PTCL) and Multiple myeloma (4–7). These drugs are currently tested in additional
50 hematological disorders and solid tumors (lung, breast and prostate cancers) as monotherapies or in
51 combination with different drugs (demethylating agents, proteasome inhibitors or chemotherapies) (8).
52 Since the exact mechanism of action and efficacy of HDACi is still unclear, the identification of novel
53 biomarkers to predict the response to treatment is needed.

54 Sézary syndrome (SS) is a rare leukemic subtype of CTCL, characterized by the presence of
55 clonal neoplastic T cells with cerebriform nuclei (known as Sézary cells) in the skin, lymph nodes, and
56 peripheral blood (9). Although SS patients present an aggressive clinical course, available treatments
57 provide partial and not durable responses, except for the allogenic stem cell transplantation (10).
58 Chevret *et al.* reported that CTCL are telomerase positive tumors expressing *hTERT*, the catalytic
59 subunit of the telomerase (11). In this pathology, emerging evidences suggested that epigenetic
60 modifications could be the mechanism responsible for *hTERT* reactivation. Hence, TERT
61 Hypermethylated Oncogenic Region (THOR) a 433bp region on *hTERT* promoter, and histone
62 modifications on *hTERT* were reportedly associated with *hTERT* activation in a large number of
63 cancers (12–14). Our team reported a pioneer investigation on THOR methylation status in CTCL
64 cells, sustaining the implication of THOR in telomerase activity in cutaneous lymphomas (Chebly et
65 al. submitted).

66 In the current work, we explore the influence of two HDACi, romidepsin and vorinostat, on
67 *hTERT* expression. Also, we analyze the dynamics of histone marks at the genomic level and on
68 *hTERT* promoter as a consequence of HDACi treatments. Furthermore, we report the functional
69 impact of romidepsin and vorinostat on SS cells' tumorigenic capacities *in vitro*.

70

71 **MATERIALS AND METHODS**

72 **Cell lines, SS patient-derived cells and cell culture**

73 HuT78 a SS cells line (American Type Culture Collection, ATCC, France) were maintained in
74 RPMI 1640 medium (Gibco, USA) supplemented with 15% of fetal bovine serum (Eurobio, France).
75 SS patient-derived cells (Patient 1 and 2) were cultured as previously described (15). All cell cultures
76 were incubated in a humidified incubator at 37°C with 5% CO₂.

77

78 **Chemicals and IC50 determination**

79 Two HDACi were used: romidepsin and vorinostat (Euromedex, France). In order to treat SS
80 cells with these drugs at the half maximal inhibitory concentration (IC50), 40,000 cells per well were
81 plated in 96-well plates with romidepsin (ranging between 0.01 and 100 nM) or vorinostat (ranging
82 between 0.001 and 50 μM), or without any drug. Plates were incubated for 48h at 37°C in a
83 humidified incubator with 5% CO₂. Cell viability was determined using the luminescent cell viability
84 assay CellTiter-Glo® (Promega, USA). Luminescence levels were quantified using a FlexStation® 3
85 (Molecular Devices, USA).

86

87 **RNA isolation, cDNA synthesis and *hTERT* expression by quantitative real-time PCR (qRT- 88 PCR)**

89 Total RNA was isolated using Direct-zol™ RNA MiniPrep kit (ZYMO Research). RNA
90 concentrations were measured using Nanodrop ND-1000 spectrophotometer. cDNA was synthesized
91 from 200ng of isolated RNA using SuperScript II reverse transcriptase kit (Invitrogen, France).

92 cDNAs were amplified by qRT-PCR using Takyon™ No Rox SYBR® MasterMix dttP Blue
93 (Eurogentec, France) using the following primer sets for *hTERT* gene: forward primer: 5'-
94 GCATTGGAATCAGACAGCAC-3', and reverse primer: 5'-CCACGACGTAGTCCATGTTC-3'. The
95 housekeeping gene *TBP* was used for normalization: forward primer: 5'-
96 CACGAACCACGGCACTGATT-3', and reverse primer: 5'-TTTTCTTGCTGCCAGTCTGGA-3'.
97 qRT-PCR analyses were run on a Stratagene Mx3005P system (Agilent Technologies, USA). Each
98 sample was performed in triplicate and the mean value was calculated. Values are expressed in
99 percentage relatively to Non-treated cells (NTC) (100%).

100

101 **Proteins extraction and western blot analyses**

102 Proteins were extracted from HuT78, SS Patient 1 and 2 (not treated cells (NTC) and romidesosin or
103 vorinostat –treated cells) using RIPA buffer. Western Blot assay was performed according to the
104 manufacturer's recommendations (Bio-Rad, USA). Briefly, protein extracts from HuT78 cell line, SS
105 Patient 1 and 2 in addition to All Blue Prestained Protein ladder were separated by SDS-PAGE on 8–
106 16% TGX Stain-Free™ Protein Gels (Bio-Rad) for 45min at 150V in TGX buffer (Bio-Rad). Stain-
107 free gels were activated by exposure to UV for 1min. Then, proteins were transferred to nitrocellulose
108 membranes for 7min using the Bio-Rad Trans-Blot Turbo Transfer System. Membranes were blocked
109 with TBST and 5% BSA for 1 hour. Membranes were then incubated with primary antibodies (Histone
110 H3K27me3 antibody, Histone H3K9me3 antibody, Histone H3K4me3 antibody, Histone H3K27ac
111 antibody and Histone H3K9ac antibody, Active Motif, USA) diluted to 1:1000 in TBST with 5% BSA
112 at 4°C overnight. Excess of primary antibody was removed by washing the membranes three times in
113 TBST. Then, the membrane was incubated with the secondary antibody (peroxidase- conjugated anti-
114 rabbit DyLight800) diluted to 1:5000 in TBST with 5% BSA for 1 hour. Excess of secondary antibody
115 was removed by washing the membranes three times in TBST. Membranes were visualized using Bio-
116 Rad ChemiDoc™ Imager. Total proteins on membranes were detected using the Stain-free method.
117 Detection and quantification of bands' intensities were done using Image Lab software (Bio-Rad).

118

119 **H3K27ac and H3K27me3 ChIP-qPCR assay**

120 H3K27ac and H3K27me3 ChIP-qPCR assays were performed by Active Motif. Briefly, 30 µg
121 of HuT78, SS Patient 1 or SS Patient 2 chromatin as well as 4 µg of H3K27me3 antibody (Active
122 Motif #39155) or 4 µg of H3K27ac antibody (Active Motif #39133) were used for the ChIP. In total,
123 five primer sets were selected for the qPCR analyses targeting four regions upstream and one region
124 downstream *hTERT* TSS (17). Two positive control primer pairs were used (GAPDH for H3K27ac
125 and MYT1 for H3K27me3) as well as two negative control primer pairs (amplifying a region in a gene
126 desert on chromosome 12 Untr12 for H3K27ac, and targeting the promoter region of the active gene
127 ACTB for H3K27me3). The primer sequences are indicated in the supplementary table 1.

128

129 **Soft agar assay**

130 Soft agar assay was used to determine the colony-forming capacity of SS cells. This assay was
131 carried out as described previously (11) with few modifications. Briefly, cells were IC50 treated
132 during 48 hours. Their viability were check and an equal amounts of NTC or HDACi-treated cells
133 (50,000 cells) were seeded in 6-well plates, in the soft agar “upper layer” containing the appropriate
134 cell culture media free of HDACi treatment. Plates were incubated in a humidified incubator at 37°C
135 with 5% CO₂. All wells were screened daily by microscopy and the colonies were counted for each
136 condition. Fresh culture media were added every 3 days. Each condition was performed in triplicate
137 and the mean values of the number of colonies were calculated.

138

139 **Statistical analysis**

140 Data analyses were carried out using GraphPad Prism (version 8) (San Diego, USA). All of the
141 experiments were performed at least three independent times. Comparisons between the different
142 groups were analyzed by Mann Whitney test with $P < 0.05$ considered statistically significant.

143

144 **RESULTS**

145 **IC50 values using romidepsin and vorinostat in SS cells**

146 After 48h of treatment with either romidepsin or vorinostat, cell viability was determined. Drug
147 concentrations causing 50% of cell death (IC50 values) were: for romidepsin 1.56 nM in HuT78, 1.85
148 nM in SS Patient 1 and 21.40 nM in SS Patient 2; and for vorinostat 0.254 μ M in HuT78, 0.830 μ M in
149 HuT78 in Patient 1 and 2.44 μ M in Patient 2 (figure 1B). Following these results, 65 million cells of
150 HuT78, Patient 1 and Patient 2 were treated either with romidepsin or vorinostat using the adequate
151 calculated IC50 value, in parallel with NTC controls.

152

153 ***hTERT* expression level decreased after romidepsin and vorinostat treatments**

154 Forty-eight hours after romidepsin or vorinostat treatments the *hTERT* expression levels were
155 examined. Values were normalized to the NTC *hTERT* expression level. Compared to NTC, the
156 *hTERT* expression levels in HuT78 decreased of 37% with romidepsin and 36% with vorinostat (figure
157 2). In SS Patient 1, *hTERT* expression levels diminished by 56% with romidepsin and 83% with
158 vorinostat (figure 2). In Patient 2, *hTERT* expression levels were reduced by 84% with romidepsin and
159 91% with vorinostat (figure 2).

160

161 **Global histone modifications after romidepsin and vorinostat**

162 In order to identify differential effects at the histone level, we examined selected histone marks based
163 on their implication in *hTERT* functions. Five histone marks were explored: H3K27me3, H3K9me3,
164 H3K27ac, H3K9ac and H3K4me3 using western blot analyses in SS cells after 48h of HDACi
165 treatments (figure 3A). The common global effect was a significant fold-change increase for all the
166 histone marks studied except for 3 conditions (Figure 3B). This increase was more pronounced for
167 H3K9ac and H3K27ac histone marks (fold change varied from 2.4 to 744.7) and more significantly in
168 SS Patient cells than in cell line. In order to give a straightforward message, all histone changes
169 observed are presented in a table (figure 3B).

170

171 **HDACi mediated H3K27ac and H3K27me3 patterns at *hTERT* promoter**

172 The active histone mark H3K27ac and the repressive mark H3K27me3 were studied by ChIP followed
173 by qPCR targeting 4 regions on *hTERT* promoter (TERT-1102, TERT-773, TERT-323 and TERT-
174 233) as well as one region in *hTERT* gene (TERT+1310) (figure 4A). Among these regions, TERT-
175 323 and TERT-233 are located within THOR. Epidrugs' effects appeared more pronounced in Patients
176 cells than in HuT78 cell line. As shown in Figure 4, globally at all position investigated, the binding of
177 the active histone mark H3K27ac was more sensitive to romidepsin than to vorinostat in all samples
178 studied, with fold changes up to 3.9 with romidepsin compared to 2.7 with vorinostat. The same effect
179 was observed for the repressive mark H3K27me3 in HuT78 and SS patient 1. Interestingly, the -233
180 region located in THOR was more sensitive to epidrugs than all the other regions investigated on
181 *hTERT* promoter, with an increase in both active H3K27ac and repressive H327me3 marks.

182

183 **Alteration of SS cells' clonogenic capacities by HDACi**

184 Soft agar assay was used to evaluate the modifications in the clonogenic capacities of SS cells
185 previously treated with romidepsin or vorinostat. In soft-agar wells, cells were cultured without any
186 drug's pressure. HuT78 NTC were highly clonogenic with roughly 140 colonies per well between day
187 7 and day 21; with a continuous increase in the size of the colonies (figure 5A and 5B), while the
188 colonies observed in romidepsin and vorinostat -treated HuT78 cells were smaller in size and less
189 numerous with almost zero colonies at day 21 (figure 5A). Among NTC conditions, SS Patients (1 and
190 2) were less clonogenic than HuT78. NTC colonies of Patient 1 and 2 were around 42 colonies and 31
191 colonies per well, respectively (figure 5A). Romidepsin and vorinostat -treated SS patient cells were
192 not able to form colonies even at day 21 (0 to 1 colony per well were counted) (figure 5B).

193

194 **DISCUSSION**

195 Our team previously demonstrated that DNA methylation at THOR on *hTERT* promoter is
196 associated with telomerase reactivation in CTCL (Chebly et al. submitted). While, it is reported that
197 DNA methylation can engage in crosstalk with other epigenetic pathways including histone
198 modifications (18,19), the interplay between DNA methylation and histone modifications has not yet
199 been fully explored (20). To unveil epidrugs' molecular mechanisms on *hTERT* regulation, we
200 previously investigated the effect of two HDACi (romidepsin and vorinostat) both approved for CTCL
201 and we observed that these drugs can reduce telomerase expression in SS tumor cells without altering
202 the methylation status of *hTERT* promoter (Chebly et al. submitted). Therefore, we carried an
203 investigation of the dynamics of five histone marks at the global level and two histone marks at
204 *hTERT* promoter in SS cells treated with HDACi. Also, we evaluated the functional impact of these
205 two epidrugs on SS cells' clonogenic capacities *in vitro*.

206 We confirmed herein that *hTERT* expression falls in romidepsin or vorinostat -treated SS cells
207 (Chebly et al. submitted). This decrease was accompanied with a drop in the clonogenic capacities of
208 SS cells. The colonies formed by treated cells were less numerous and smaller in size. HDACi are
209 known to impact the clonogenicity of cancer cells, such as the romidepsin in bladder cancer (21) and
210 the vorinostat in polycythemia vera (PV) hematopoietic progenitors expressing JAK2^{V617F} (22). Our
211 results obtained in SS cells are in accordance with these observations. Additionally, HDACi were
212 reported to induce global acetylation, which is recognized as DNA damages leading to cell death in
213 apoptosis-susceptible CTCL cells (23,24). This effect of HDACi can explain our observations
214 regarding the lasting toxic effect of HDACi even after releasing the drug's pressure, causing cell death
215 in SS cells previously treated by HDACi. Besides, it was reported that *hTERT* exerts non-canonical
216 functions in CTCL impacting the clonogenicity of tumor cells (11). Our present data support this
217 observation and suggest a role for HDACi in reducing the clonogenic capacities of SS cells by
218 reducing *hTERT* expression.

219 Five histone marks (active and repressive marks) were studied by western blot in romidepsin
220 and vorinostat treated cells compared to untreated cells in order to evaluate the global changes of these

221 marks in SS: H3K27me3, H3K9me3, H3K27ac and H3K9ac and H3K4me3. An obvious increase in
222 the acetylated histone marks H3K9ac and H3K27ac was observed in patient's cells (up to 345 and 744
223 fold changes). It is already known that H3K9ac and H3K27ac denote active regions at enhancers and
224 promoters reflecting active gene transcription (25,26). The increase of these two acetylated marks
225 observed in our study could be explained by the inhibition of the HDACs that are the main targets of
226 the epidrugs HDACi (1). Interestingly, also methylated histone marks showed an increase after
227 HDACi treatments, more pronounced in Patient 2. Our results illustrate the coordination between
228 histone methylation and histone acetylation in response to HDACi (27), confirming that epigenetic
229 regulation can affect various molecular elements to generate a specific response.

230 To unambiguously explore *hTERT* promoter histone marks, we looked at the dynamics of
231 H3K27ac and H3K27me3 histone marks under the pressure of HDACi treatments. These two histone
232 marks were previously reported to play a role in *hTERT* activation (13,14). High levels of H3K27me3
233 at the *hTERT* promoter were found in telomerase-negative human primary cells (13), while H3K27ac
234 is known to mark active enhancers, showing peaks that juxtaposed *hTERT* to strong enhancers
235 elements in neuroblastoma (14). The H3K4me3 mark of active chromatin also was exhibited at *hTERT*
236 promoter but in cancer cell lines carrying heterozygous promoter mutations (13). In SS, *hTERT*
237 promoter is reported to be lacking of hotspot mutations (Ropio et al. Submitted). The *hTERT* promoter
238 ChIP-qPCR investigation revealed changes in the active and repressive histone marks with romidepsin
239 and vorinostat treatments. Overall after vorinostat treatment, the enrichment observed in the repressive
240 histone mark H3K27me3 was more prominent than the changes observed in the H3K27ac active mark.
241 Surprisingly, the active and the repressive histone marks were increased with both treatments, while
242 *hTERT* expression was reduced suggesting a relation between epigenetic regulation and the global
243 crosstalk. Among the *hTERT* promoter regions analyzed, only the -233 region seems to be recurrently
244 sensitive to HDACi, indicating a possible role of this region in *hTERT* epigenetic regulation. The
245 increase in H3K27ac and H3K27me3 levels might probably result in an alteration of the histones'
246 marks balance on *hTERT* promoter leading to a gene repression; or leaving the chromatin accessible to

247 a competition between activator and repressive transcription factors. These subtle changes seem to
248 surpass a critical and important threshold for *hTERT* expression. Such physiopathological thresholds
249 remain to be defined and explored. These features may represent the plasticity of the epigenetic
250 regulation of *hTERT* in SS cells, confirming that epigenetic regulations under HDACi could be the
251 result of a cascade and a coordination between several elements and/or pathways that can be drug-
252 dependent and cancer type-dependent (28). Furthermore, according to the outcomes of single agents
253 HDACi or during guided combination therapies, there are still many aspects and effects to elucidate
254 (29). In this context, the study of additional *hTERT* promoter histone marks might help to draw the full
255 image of the epigenetic response to vorinostat and romidepsin in SS cells and would also help to
256 understand the regulation of *hTERT* gene in cancer treatment.

257 In conclusion, our findings suggest that HDACi treatments can reduce *hTERT* expression and
258 consequently the clonogenic capacities of SS cells. While, HDACi can modify the protein expression
259 of several histone marks at the genomic level, the H3K27ac and H3K27me3 at *hTERT* promoter seem
260 to be subtly altered. These results confirm that *hTERT* promoter does not behave in a simplistic way
261 (20) and suggest the implication of other “players” in the complex dynamic regulation of *hTERT* gene.
262 Altogether, our study provides new insights regarding the regulation of *hTERT* expression by HDACi
263 in SS cells. Also, it constitutes a basis towards more targeted epigenetic studies in order to unveil the
264 mechanism of action of epidrugs in CTCL lymphomagenesis and to predict the response to epigenetic
265 treatments. This prediction may facilitate the selection of patients that could benefit from epigenetic
266 therapy.

267

268 **ABBREVIATIONS**

269 CTCL: Cutaneous T-cell lymphomas; HDACi: Histone deacetylases inhibitors; NTC: Non-treated
270 cells; SS: Sézary syndrome; THOR: TERT hypermethylated oncogenic region; TSS: Transcription
271 start site; WHO-EORTC: World Health Organization-European Organization for Research and
272 Treatment of Cancer.

273

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278

279 **AUTHORS' CONTRIBUTIONS**

280 AC wrote the manuscript and prepared the figures. AC, MPC, LBB, SP and YI performed the
281 experiments and interpreted the results. CF, MBB, JPM and RT provided helpful advices and assisted
282 in editing the manuscript. EC conceived the study, revised and edited the final version of this
283 manuscript. All authors read and approved the final version of the manuscript.

284

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289

290 **COMPETING INTERESTS**

291 The authors declare that they have no competing interests.

292

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368

369 **FIGURE LEGENDS**

370

371 **Figure 1: IC50 values of romidepsin and vorinostat.**

372 Graphs showing cell viability results after 48h of different concentrations of romidepsin (A) or
373 vorinostat (B) in HuT78 cell line, SS Patient 1 and SS Patient 2 cells. IC50 values are expressed in nM
374 for romidepsin and μ M for vorinostat.

375

376 **Figure 2: *hTERT* expression after romidepsin and vorinostat treatments.**

377 *hTERT* gene expression after 48h of romidepsin or vorinostat pressure in HuT78, SS Patient 1 and SS
378 Patient 2 cells using quantitative PCR (qPCR). Results are represented as percentages of the
379 expressions in Non-treated cells (NTC).

380

381 **Figure 3: Global analysis of histone modifications in SS cells.**

382 Western blot analyses with antibodies against repressive (H3K27me3 and H3K9me3) and active
383 histone marks (H3K27ac, H3K9ac and H3K4me3) in HuT78 cell line, SS Patient 1 and SS Patient 2
384 cells (A). Western blot analyses were done using the Stain-Free technology for normalization and
385 quantification. Table (B) shows the increase in histone marks expressed in fold-change. L: Protein
386 Ladder, N: non-treated cells, R: romidepsin treated cells and V: vorinostat treated cells.

387

388 **Figure 4: Chromatin analysis at *hTERT* gene.**

389 Chromatin immunoprecipitation (ChIP) was followed by quantitative PCR (qPCR) using five pairs of
390 primers to amplify regions on *hTERT* gene (A) (TERT-1102; -773, -323, -233 and +1310) as well as a
391 negative control (Untr12 for H3K27ac and ACTB-145 for H3K27me3) and a positive control
392 (GAPDHpro for H3K27ac and MYT-1772 for H3K27me3) before (NTC) and after romidepsin and
393 vorinostat treatments in HuT78 cell line, SS Patient 1 and SS patient 2. ChIP results of H3K27ac and
394 H3K27me3 were normalized to the input values (B).

395

396 **Figure 5: Soft Agar assay in SS cells before and after romidpsin and vorinostat treatments.**

397 (A) Colonies formation in HuT78, SS Patient 1 and SS Patient 2 without treatment (NTC, black line)
398 or after a previous 48h treatment with romidepsin (blue line) or vorinostat (red line). (B) Size and
399 shape of the colonies in NTC, romidepsin- and vorinostat- treated cells after 3 weeks in Soft Agar.

400

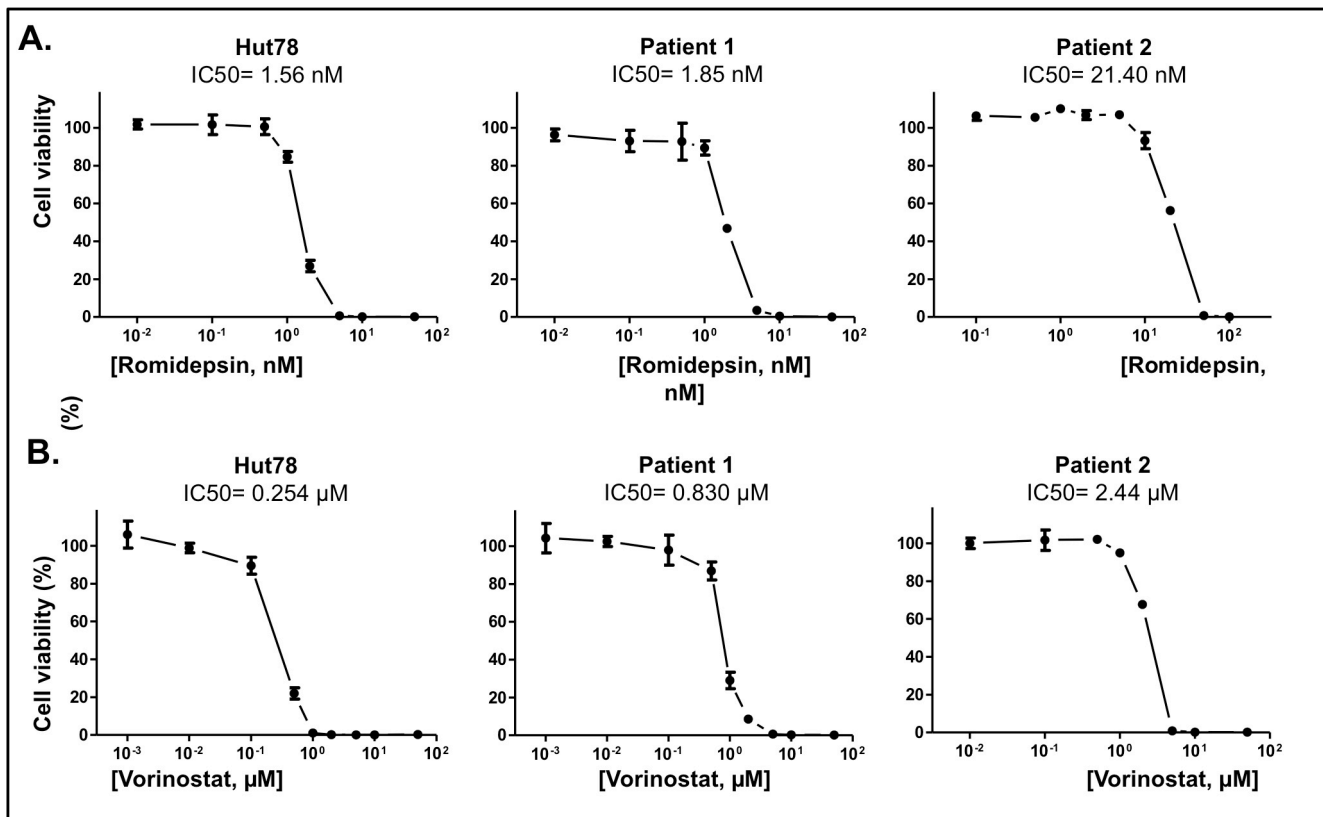


Figure 1

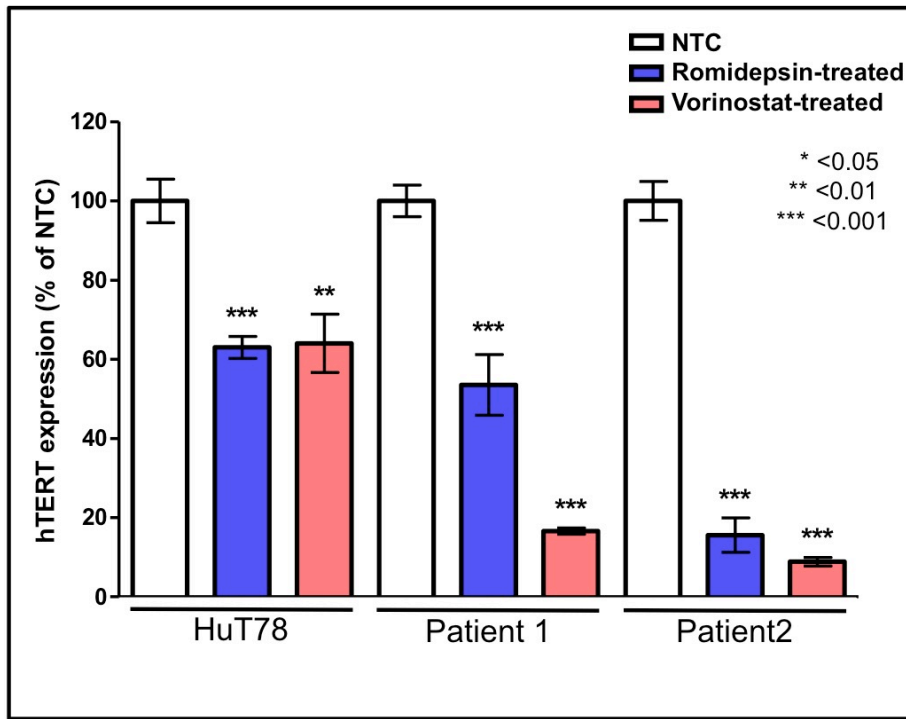


Figure 2

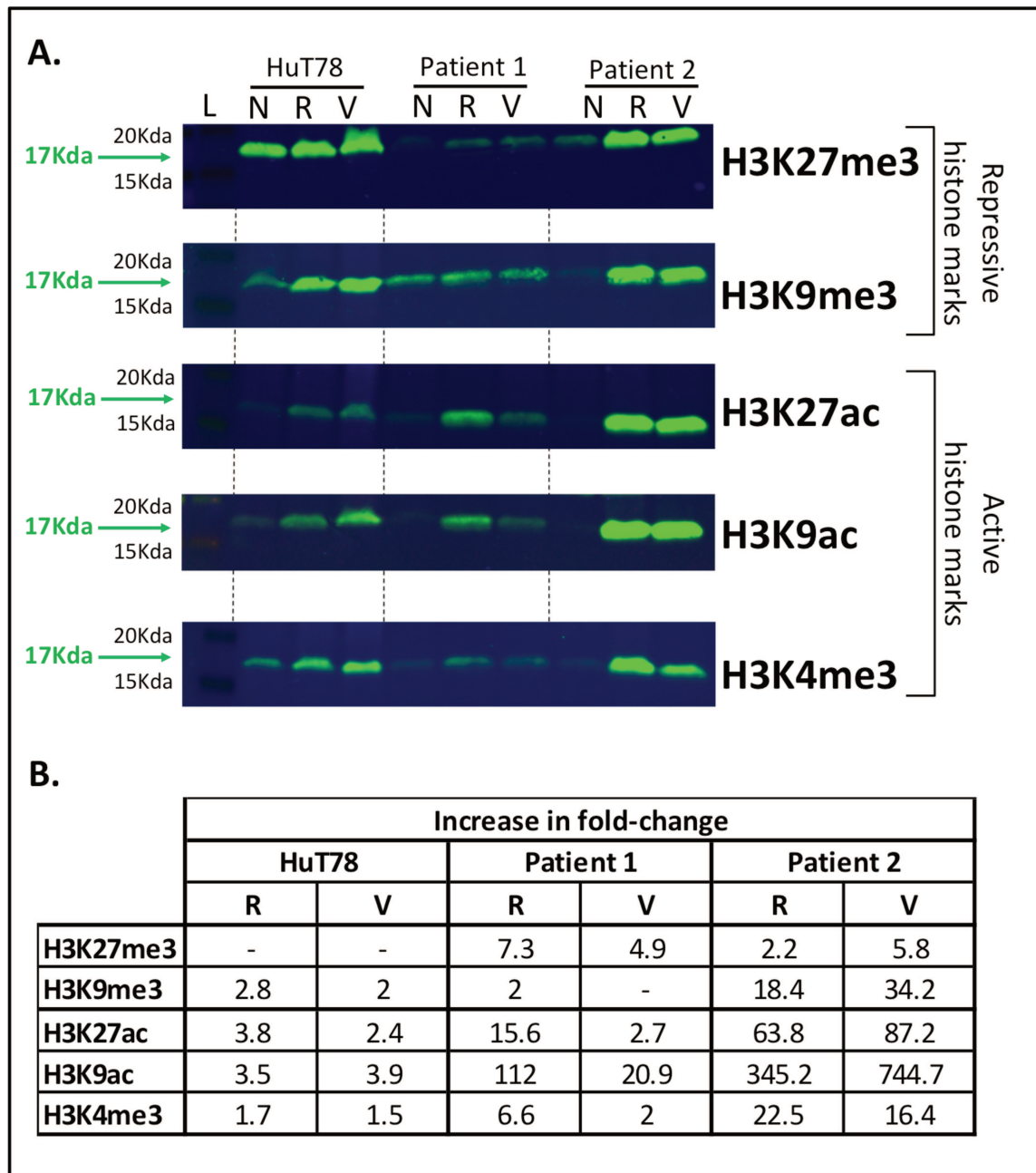


Figure 3

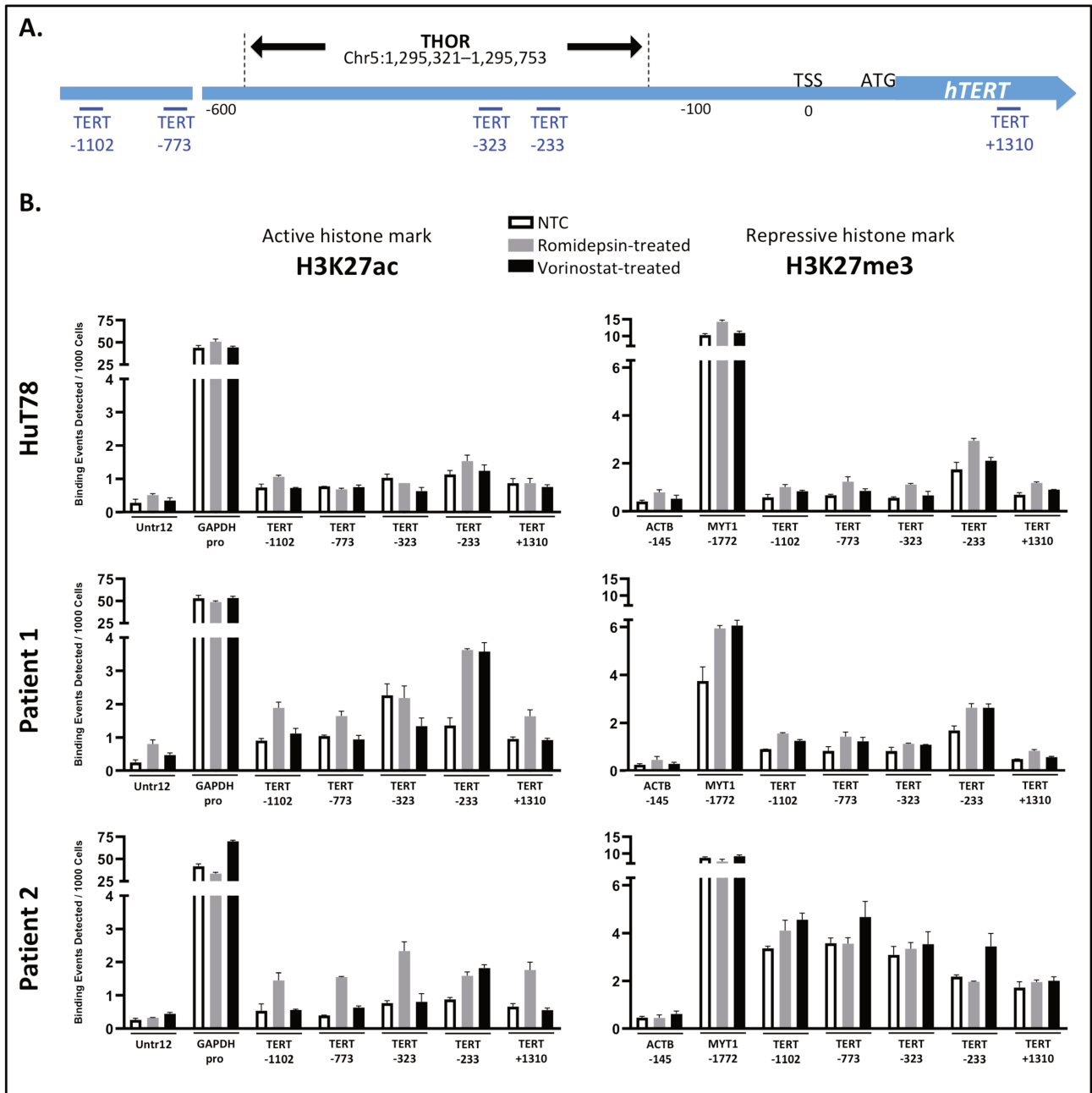


Figure 4

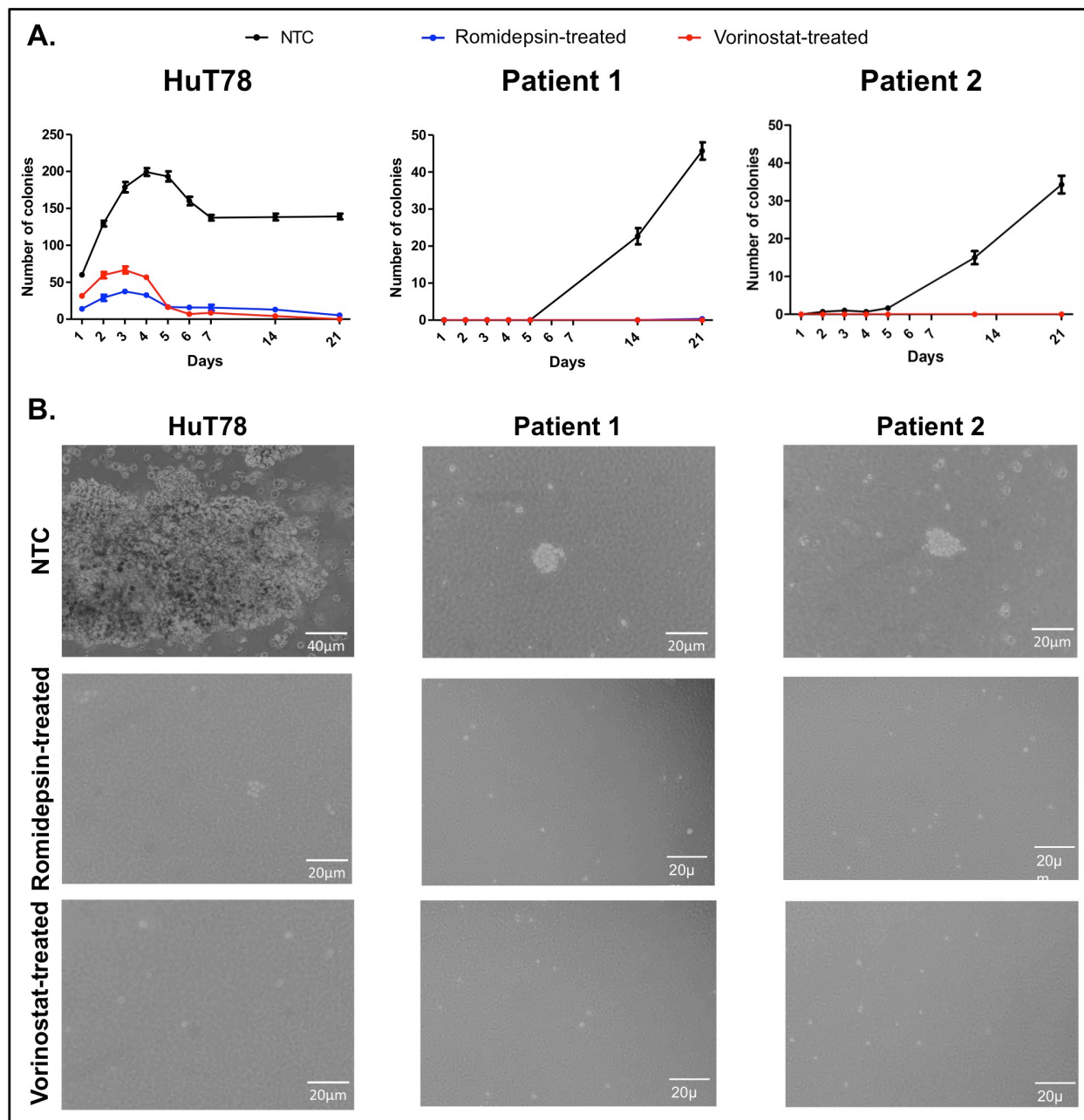


Figure 5

7. Discussion générale

Durant ma thèse, mon objectif a été d'étudier des mécanismes épigénétiques impliqués dans l'expression de la télomérase dans les CTCL et plus spécifiquement dans le syndrome de Sézary, une forme avancée et leucémique de CTCL (Willemze et al., 2005).

Les CTCL représentent un groupe hétérogène de lymphoproliférations T caractérisé par une expansion clonale de lymphocytes T tumoraux impliquant la peau comme site primaire. Aux stades précoces ils sont souvent indolents (Agar et al., 2010). Cependant, chez une minorité de patients une progression rapide peut survenir et d'autres patients se présentent d'emblée à un stade avancé, au-delà du stade IIB (médiane de survie : 1 à 5 ans). Aucun marqueur spécifique annonciateur de l'évolution agressive n'est connu. A l'heure actuelle, les traitements visent à obtenir une régression des lésions ou à ralentir la progression mais n'ont pas d'action curative dans les stades avancés (Scarisbrick et al., 2015; Trautinger et al., 2017).

Récemment, les études de séquençage à haut débit ont décrit un large spectre d'altérations génétiques impliquant des gènes régulateurs de l'épigénétique (gènes impliqués dans la méthylation de l'ADN, la modification des histones et le remodelage de la chromatine). Cette mise en évidence apporte un début de rationalité à l'utilisation de modulateurs épigénétiques dans le traitement des CTCL. Cependant, les mécanismes moléculaires à l'origine de l'efficacité thérapeutique de ces modulateurs sont à déterminer, car peu d'études *in vitro* et *in vivo* ont été réalisées avant l'utilisation de ces molécules en essais cliniques (Mervis and McGee, 2019).

Des travaux antérieurs de notre équipe ont montré que la télomérase, un marqueur de transformation tumorale, est impliquée dans la lymphomagenèse cutanée et qu'elle pourrait être sujette à une régulation épigénétique (Chevret et al., 2014) (Ropio et al., Submitted). Cependant, rien n'est connu sur la régulation épigénétique de *hTERT* dans les CTCL.

Afin d'étudier ces mécanismes, notre stratégie d'étude a reposé sur l'utilisation des quelques lignées cellulaires de CTCL disponibles à ce jour (Bunn and Foss, 1996; Kadin et al., 1994;

Kaltoft et al., 1992), sur des lignées de SS toutes récemment développées dans notre équipe (Poglio et al., 2020), ainsi que sur des cellules fraîchement isolées de patients SS.

Dans ce contexte, nous avons tout d'abord étudié la méthylation de l'ADN au niveau du promoteur de *hTERT* car il s'agit d'un mécanisme fréquent et stable de contrôle l'expression des gènes. Dans de nombreuses tumeurs, aussi bien solides qu'hématologiques, il a été démontré durant les 10 dernières années que le promoteur de *hTERT* pouvait présenter deux régions diamétralement opposées dans leur statut de méthylation, une région hypométhylée proche du TSS et une région distale hyperméthylée (Azouz et al., 2010; Lee et al., 2019; Rowland et al., 2020). Cette région hyperméthylée correspond à 433 paires de bases du promoteur et contenant 52 sites CpG, et elle n'a jamais été investiguée dans les lymphomes cutanés (Lee et al., 2019).

Dans la première partie de cette étude, nous avons analysé la méthylation du promoteur de *hTERT* par la méthode de séquençage bisulfite. Cette méthode bien que fastidieuse, a l'avantage de d'analyser les profils de méthylation "cellule par cellule". Nous avons ainsi identifié dans les CTCL, le double profil de méthylation du promoteur de *hTERT* avec une région distale hyperméthylée et une région proximale hypométhylée. La région distale hyperméthylée a été récemment dénommée THOR (TERT Hpermethylated Oncologic Region) (Lee et al., 2020, 2019). Nous avons observé que la méthylation de THOR est une spécificité des cellules tumorales des patients SS, car dans les cellules saines des patients SS, le promoteur de *hTERT* est hypométhylé au niveau de cette région. Ce travail sur les CTCL permet d'intégrer les CTCL à la liste des tumeurs qui présentent la région -650 à -150 approximativement hyperméthylée tout en exprimant le gène *hTERT*. Ce profil de méthylation pourrait être utilisé comme un biomarqueur des cellules tumorales dans les CTCL.

Il est connu que les changements de conformation de la chromatine engendrés par la méthylation du promoteur de *hTERT* peuvent induire un recrutement différentiel des facteurs de transcription. La région promotrice de *hTERT* est capable de s'associer à de nombreux facteurs de transcription activateurs et inhibiteurs. Pour poursuivre notre travail, nous nous sommes intéressés aux facteurs de transcription qui pouvaient interagir au niveau de la région hyperméthylée identifiée dans les cellules CTCL. Parmi les facteurs de transcription qui se lient

dans cette région, un seul site présentait un profil de méthylation différenciellement méthylé entre les cellules tumorales et les cellules saines. Il s'agissait du site de fixation du facteur de transcription répresseur WT1 (Wilms' Tumor 1). Pour cette raison, nous avons investigué l'implication de WT1 dans l'expression de *hTERT* dans les CTCL. Le test de Luciférase a montré que la surexpression de WT1 dans les lignées CTCL est capable de réduire l'expression de *hTERT*, confirmant que WT1 est un répresseur de *hTERT* dans les cellules CTCL (Kyo et al., 2008). Ensuite, nous avons étudié l'interaction physique entre WT1 et la chromatine du promoteur de *hTERT* par une approche ChIP-qPCR. Les résultats de cette investigation dans les CTCL sont en faveur d'une indépendance d'expression de *hTERT* par rapport à WT1. Afin de confirmer ce constat, il serait nécessaire d'effectuer l'étude de c-MyC, facteur de transcription activateur qui se lie sur la boîte enhancer (Ebox), en parallèle avec l'étude de WT1.

Dans la seconde partie de ce travail nous avons utilisé des agents thérapeutiques pour moduler l'épigénétique de la région promotrice de *hTERT* en ciblant l'ADN ou les histones. Plusieurs modifications post-traductionnelles des histones peuvent influencer la compaction de la chromatine, et par conséquent l'accessibilité de l'ADN et l'expression du gène. Deux HDACi approuvés pour le traitement des patients SS (romidespsine et vorinostat) ainsi qu'une drogue déméthylante (5-azacytidine) non approuvé pour les patients SS, ont été utilisés dans ce travail. De façon intéressante, les trois épidrogués utilisées bien qu'appartenant à différentes familles de thérapies épigénétiques ont montré des résultats moléculaires identiques sur *hTERT* ainsi qu'une similitude d'efficacité *in vitro* sur les cellules SS. Ces trois thérapies permettent de diminuer l'expression du gène *hTERT*, ainsi que de diminuer les capacités clonogènes des cellules et ceci sans altération ni du niveau ni du profil de méthylation de la région hyperméthylée ou du reste du promoteur de *hTERT*. La persistance du niveau de méthylation en présence de 5-azacytidine a été observée pour le gène *COX-2* dans des fibroblastes pulmonaires fibreux. Dans cette étude la 5-azacytidine provoquait la déméthylation d'un régulateur de *COX-2* (Evans et al., 2016). Des résultats similaires ont été observés pour d'autres gènes dans d'autres types cellulaires suggérant que la 5-azacytidine pouvait réguler de manière indirecte l'expression d'un gène. Nos résultats dans les CTCL sont aussi en faveur d'une régulation indirecte de la 5-azacytidine sur le promoteur de *hTERT*. Soit ce traitement agit sur un ou plusieurs facteurs

inhibiteurs de *hTERT* soit il agit sur les modifications post-traductionnelles histones (Allis and Jenuwein, 2016; Martin and Zhang, 2005; T. Zhang et al., 2015; Zhou et al., 2011). Pour tester cette hypothèse il sera nécessaire d'étudier les marques d'histones de la région promotrice de *hTERT* dans des cellules non traitées et dans des cellules traitées à la 5-azacytidine.

Jusqu'à présent, il n'a pas été possible d'établir de manière concluante la causalité entre la méthylation du promoteur et le changement d'expression provoqué par les médicaments actuellement disponibles pour moduler la méthylation de l'ADN. Les inhibiteurs d'ADN methyltransferase DNMTi, comme la 5-azacytidine agissent de manière globale, et ne peuvent donc pas démontrer la relation causale directe entre le statut de méthylation à un locus spécifique et la régulation transcriptionnelle correspondante. La question demeure de savoir dans quelle mesure la méthylation du promoteur *hTERT* est impliquée dans le changement d'expression. Investiguer la relation de causalité à un locus donné est possible grâce à l'avènement de nouveaux outils de ciblage spécifique utilisant la technologie CRISPR-Cas9 (CRISPR-dCas9-Based Targeted Demethylation) (Urbano et al., 2019).

Nous avons ensuite, analysé à l'échelle globale 5 marques d'histones : H3K27me3, H3K9me3, H3K4me3, H3K27ac et H3K9ac dans des cellules traitées ou non avec la romidespsine ou le vorinostat. Après le traitement une augmentation globale et significative de ces 5 marques a été observée. Localement sur le promoteur de *hTERT*, les deux marques d'histones étudiées l'une activatrice, H3K27ac et l'autre répressive H3K27me3 ont montré un enrichissement au niveau de la région -223 localisée dans la région distale hyperméthylée. Les changements des marques histones sur *hTERT* suggèrent la présence d'un certain équilibre entre marque activatrice et marque répressive qui a été modifié suite aux thérapies HDACi *in vitro* et qui est associé à une diminution d'expression du gène. Nous pouvons nous poser la question si la combinaison de ces deux HDACi aurait eu un effet synergique et entraîné des changements plus drastiques sur la présence d'une marque par rapport à l'autre.

D'un point de vue fonctionnel, des études *in vitro* ont montré que la combinaison romidepsine/5-azacytisine exerce des effets antiprolifératifs et induit l'apoptose dans des lignées cellulaires CTCL et dans des cellules issues de patients (Rozati et al., 2016). Une étude clinique a

évalué la combinaison vorinostat/5-azacytosine/chimiothérapie chez des patients atteints de lymphomes à un stade réfractaire ou en rechute (Nieto et al., 2016). Les auteurs concluent que cette association est efficace, qu'elle présente peu de risque et qu'une évaluation plus approfondie est nécessaire. En effet, des études approfondies des mécanismes moléculaires n'ont pas été établies dans ces études. En conséquence, l'impact moléculaire de la combinaison de ces épiprodrogues (HDACi + agent déméthylant) sur les marques épigénétiques tel que les histones et la méthylation du promoteur des gènes en général et plus spécifiquement sur celui de *hTERT* dans les cellules CTCL/SS restent à explorer. Ainsi que l'effet de la combinaison de ces épiprodrogues sur la modulation de l'expression de *hTERT*.

Dans la littérature, il est rapporté que pour un même DNMTi, les gènes peuvent réagir différemment. En conséquence, le traitement aux DNMTi en déméthylant des régions promotrices peut modifier l'expression de ces gènes (Seelan et al., 2018). En revanche, pour d'autres gènes la méthylation du promoteur, tout comme l'expression du gène, restent inchangées après un traitement par ces mêmes DNMTi. Les gènes qui présentent un tel comportement sont qualifiés de « réfractaires » (Fukagawa et al., 2015; Sayar et al., 2015). D'autres auteurs décrivent que des DNMTi peuvent provoquer un changement d'expression de certains gènes sans modifier leur profil de méthylation. Cet effet des drogues DNMTi est qualifié d'effet secondaire ou d'effet indirect (Evans et al., 2016; Lee et al., 2009; Seelan et al., 2018; Wozniak et al., 2007). Cet effet indirect peut agir de différentes manières, soit par :

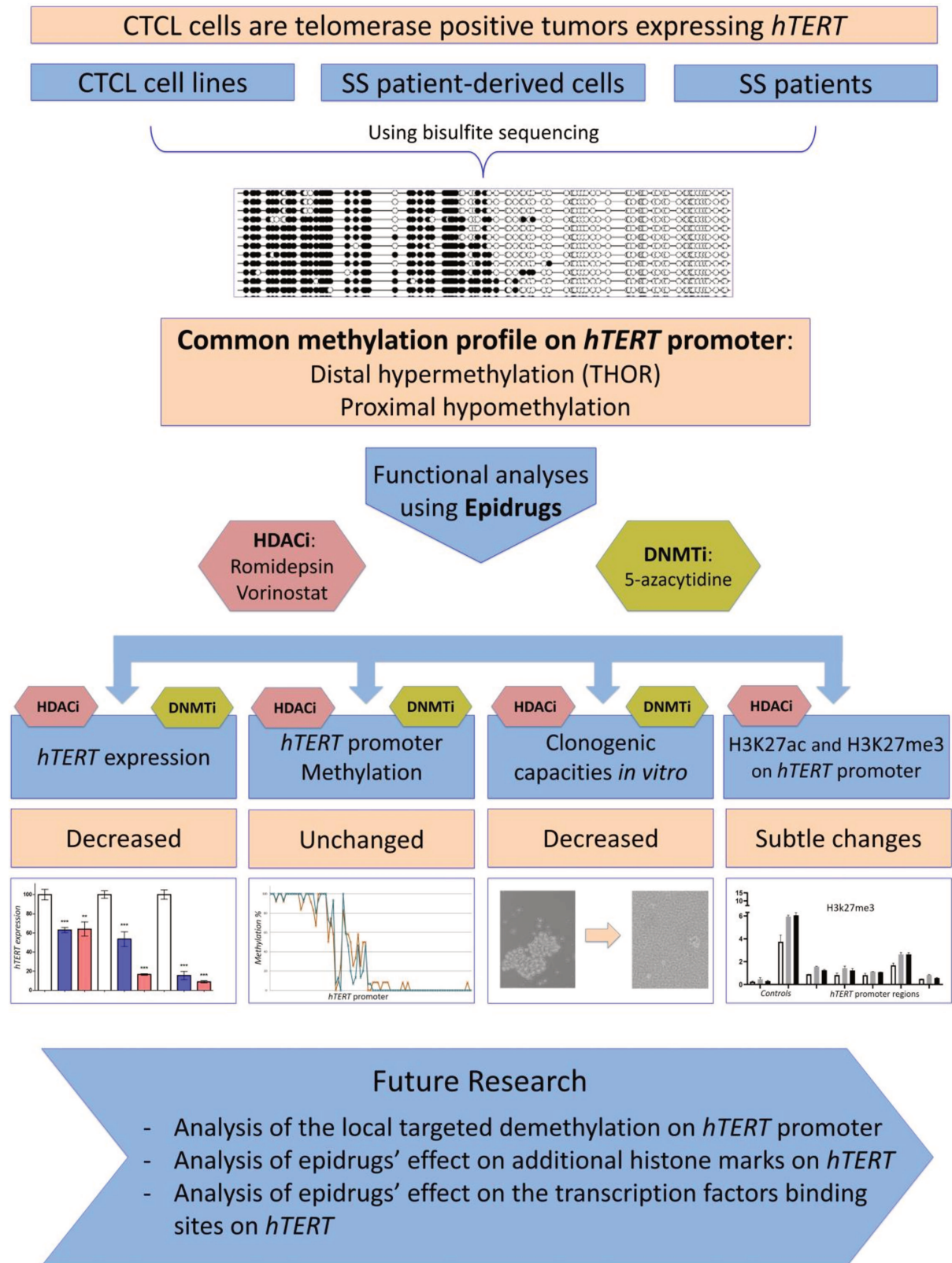
- la déméthylation d'un facteur de transcription qui contrôle l'expression d'un gène,
- ou par la déméthylation d'une région régulatrice (ex. par un « Enhancers »),
- ou par une réponse secondaire aux dommages à l'ADN
- ou bien par les mécanismes de réparation,
- ou encore par les modifications des histones

L'ensemble de ces données nous permettent de proposer une hypothèse pour interpréter nos observations : nous suggérons un effet indirect/secondaire de la 5-azacytosine sur le gène *hTERT* dans les CTCL. Cet effet abouti à une diminution de l'expression de *hTERT* sans changement du statut de méthylation du promoteur. La modification d'expression de *hTERT* pourrait être liée à

la réactivation d'un facteur de transcription suppresseur. Selon cette hypothèse, le facteur E2F1 représente un bon candidat. Car, en plus de son rôle comme régulateur négatif de la transcription de *hTERT*, ce facteur a été décrit comme pouvant impacter la croissance cellulaire et l'apoptose (Crowe et al., 2001; Fueyo et al., 1998). Une étude fonctionnelle de l'implication de ce facteur de transcription dans la régulation de l'expression de *hTERT* dans les CTCL en réponse aux épidroques semble être pertinente et, pourrait être envisagée en perspective ce travail doctoral.

En conclusion, les travaux réalisés lors de cette thèse permettent de compléter les connaissances sur la régulation de *hTERT* dans les CTCL. Une régulation épigénétique de ce gène semble probable. Et, cette régulation s'opère vraisemblablement de manière indirecte. Les futures études devront rechercher l'effet de la déméthylation locale de la région hyperméthylée du promoteur de *hTERT* en mettant à profit les nouvelles technologies telles que la technique CRISPR-dCas9-Based Targeted Demethylation, et même les techniques de NGS. En parallèle, l'étude de l'effet des épidroques sur des marques d'histones, différentes de celles que nous avons analysées, ainsi que l'étude des interactions avec le promoteur de *hTERT* de facteurs de transcriptions activateurs ou répresseurs, permettront de spécifier la place de l'épigénétique, dans la régulation de l'expression de *hTERT* dans les CTCL.

8. « Graphical Abstract »



9. Références bibliographiques

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10. Annexe 1

Modèle du formulaire de consentement éclairé signé par les patients.

**CONSENTEMENT POUR LE DON D'ELEMENTS DU CORPS HUMAIN ET
D'INFORMATIONS BIOMEDICALES A DES FINS DE RECHERCHE**

Je confirme que j'ai été informé(e) des modalités de recueil des échantillons et des informations associées ainsi que des conditions de gestion des collections dans le cadre du *Centre de Ressources Biologiques-Tumorothèque (CRB-Tumorothèque) du CHU de Bordeaux*.

J'ai pu poser toutes les questions à l'équipe médicale.

J'ai bien compris que ma participation était volontaire et que j'étais libre de modifier ou révoquer mon consentement à tout moment sans aucune justification, sans que cela n'ait de conséquence sur mon suivi médical.

Mon consentement ne décharge pas le CRB-Tumorothèque de sa responsabilité et je conserve tous mes droits garantis par la loi.

Je donne mon accord pour que les échantillons et produits dérivés, ainsi que les données biocliniques associées puissent être gérés par le CRB-Tumorothèque afin qu'ils puissent être :

- utilisés à des fins de recherche fondamentale et appliquée dans le domaine de ma pathologie
- utilisés à des fins de toute autre recherche scientifique sous contrôle de la pertinence scientifique et éthique de l'utilisation par le CRB-Tumorothèque
- inclus dans des recherches de **marqueurs génétiques** constitutionnels de manière anonyme sans que le résultat ne permette d'identifier ma personne ou ma famille
- utilisés pour le développement de produits qui pourraient être commercialisés après l'aboutissement de la recherche, sans qu'aucun bénéfice financier ne puisse me revenir.

J'ai bien conscience que :

- je peux demander à tout moment la destruction de mes échantillons conservés en le demandant au **CRB-Tumorothèque du CHU de Bordeaux - Hôpital Haut-Lévêque Avenue de Magellan – 33604 PESSAC cedex.**
- je n'aurai aucun retour financier
- aucun bénéfice direct ne peut être attendu des recherches scientifiques qui pourront être faites grâce à mes échantillons
- je pourrai obtenir des informations globales sur les activités du CRB-Tumorothèque et les avancées de la recherche en m'adressant au Pr. MERLIO, Médecin Responsable du CRB-Tumorothèque (adresse ci-dessus).
- J'accepte que le CRB-Tumorothèque puisse recontacter mon médecin si des informations importantes pour mon suivi médical sont obtenues lors des recherches.

Nom et prénom du patient :

.....

Date de naissance:

.....

Etiquette Patient

- J'accepte** que les « surplus biologiques » de mes prélèvements soient utilisés à des fins de recherche
- J'accepte** que l'examen de mes caractéristiques génétiques constitutionnelles soit réalisé pour les recherches le nécessitant
- Je m'oppose à l'utilisation de mes prélèvements à des fins de recherche ou à l'examen de mes caractéristiques génétiques

Date :

Signature du patient :

Nom et signature de la personne ayant recueilli le consentement

.....

Signature :




11. Annexe 2

Article : Ropio, J., **Chebly, A.** et al. Reliable blood cancer cells' telomere length evaluation by qPCR.

Cancer medicine. 2020.

ORIGINAL RESEARCH

Reliable blood cancer cells' telomere length evaluation by qPCR

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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 into 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, taking advantage of our expertise in telomere homeostasis investigation in primary cutaneous T-cell lymphomas. Furthermore, our data emphasize the requirement of samples with both, high DNA quality and high tumor cells representation.

KEYWORDS

cancer, qPCR, southern blot, telomere length, tumor

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1 | INTRODUCTION

Telomeres are highly conserved repetitive (TTAGGG)_n DNA-protein structures located at the ends of eukaryotic chromosomes.^{1,2} They have important functions in 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 telomerase, an enzyme specialized in the elongation of telomeric ends.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 reexpression 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 arm chromosome (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 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).^{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}

One of the main hurdles when studying cancer cells is the scarce biological material recovered which constraints molecular biology analysis. Thus, qPCR approaches present a substantial advantageous tool for cancer cells' telomere length evaluation. 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 in telomere homeostasis investigation in primary cutaneous T-cell lymphomas (CTCL). CTCL are a heterogeneous group of lymphoproliferations including entities with indolent, intermediate, and aggressive clinical behavior, in which we previously reported that telomere shortening was associated with disease aggressiveness.³⁰

2 | MATERIAL AND METHODS

2.1 | Cell lines

Five CTCL cell lines were analyzed in this study. Three cutaneous anaplastic large cell lymphoma (c-ALCL): Mac1, Mac2A, and Mac2B³¹ (DSMZ), one transformed mycosis fungoides (T-MF): MyLa 2973,³² kindly provided by Dr K. Kaltoft (Aarhus, Denmark) and one Sézary syndrome (Sz): HuT78³³ (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% fetal bovine serum (Eurobio), except for HuT78 cells, which were supplemented with 20% fetal bovine serum. All cell lines were maintained at 37°C with 5% CO₂ and regularly tested for mycoplasma contamination.

2.2 | 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).³⁴ Healthy donors ($n = 21$, $52 \leq \text{age} \leq 97$, 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 a written consent.

2.3 | 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, 5 to 10 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.

2.4 | 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 metaphase cells spreads. Cytogenetic preparations were performed as previously described.³⁵ 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.

2.5 | DNA Extraction

Genomic DNA was extracted, by a salt precipitation method adapted from Roylance *et al.*³⁶ Briefly, about 3 to 5×10^6 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 proteinase K buffer solution (2mg/ml proteinase K, 2mM EDTA, 1% SDS), prepared freshly prior to use. Suspensions were incubated overnight at 43°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 4°C during quality assessment and qPCR analysis, otherwise it was stored at -20°C.

2.6 | 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 = $\sum OD_i / \sum (OD_i / L_i)$, where OD_i is the chemiluminescent signal and L_i is the length of the TRF fragment at position i .

2.7 | 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. Fifty nanograms of target DNA was added to a reaction containing the pair of primers (telomere or KLK3) and Takyon™ No Rox SYBR® MasterMix dTTP Blue (Eurogentec), in a total reaction volume of 25µl, 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 as follows:

Telc 5'-TGTTAGGTATCCCTATCCCTATCCCTATCCC TATCCCTATCCCTAACA-3'.

Telg 5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTT GGGTTAGTGT-3'.²⁴

KLK3-forward 5'-AGGCTGGGGCAGCATTGAAC-3'.

KLK3-reverse 5'-CACCTTCTGAGGGTGAACCTTG-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). Triplicate values were accepted when the standard deviation of Ct was

below 0.5 among replicates. Results were calculated by the standard curve method.

2.8 | qPCR absolute telomere length measurement

Telomere length was calculated by means of Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell). 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). Twenty nanograms of target DNA was added to a reaction containing the pair of primers (telomere or SCR) and FastStart Essential DNA Green Master (Roche), in a total reaction volume of 20 μ l, 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 95°C for 10 minutes, followed by 32 cycles of 95°C for 20 seconds, 52°C for 20 seconds and 72°C for 42 seconds, with signal acquisition).

Data were collected from duplicate reactions for each sample (cell lines, patients, and healthy donors). 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 ($2^{-\Delta\Delta C_t}$). The final result represents the average telomere length per chromosome.

2.9 | 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 Pearson's Correlation and R^2 coefficient of correlation and *P* values were reported. Four independent biological samples were analyzed for each cell line. Data obtained with cells from one sample were considered as one experiment (*n*). The significance level was set as *P* = .05.

3 | RESULTS

3.1 | CTCL cells cytogenetic analysis

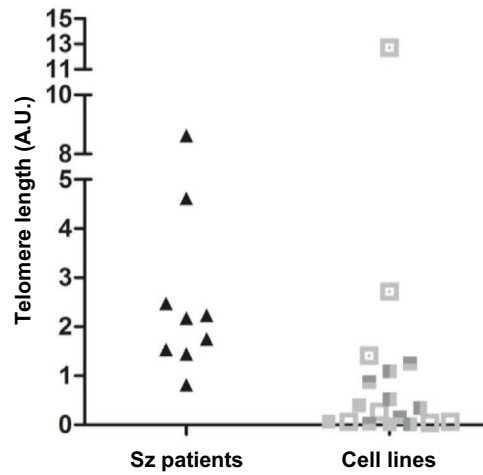
Cytogenetic investigation consisted of analyzing the karyotype for all cell lines (MyLa, HuT78, Mac1, Mac2A, and Mac2B). Thus, chromosomal rearrangements (Table S1) and ploidy (Table 1) were determined. HuT78 cell line was

	Diploid telomere length (kb)	Ploidy	Corrected telomere length (kb)
Cell lines			
Mac1	1.075 ± 0.035	Near-diploid	1.075 ± 0.035
Mac2A	4.452 ± 0.147	Near-diploid	4.452 ± 0.147
Mac2B	2.811 ± 0.093	Near-diploid	2.873 ± 0.095
MyLa	12.592 ± 0.416	Near-diploid	12.471 ± 0.412
HuT78	1.858 ± 0.061	Hypertriploid	1.279 ± 0.042
Mean			4.320 ± 0.143
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

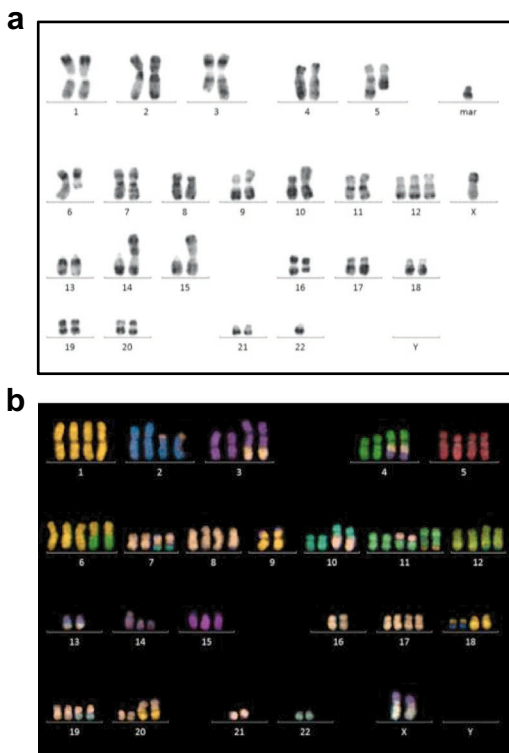
TABLE 1 CTCL cells' absolute telomere length estimated by absolute qPCR

CTCL cells' telomere length

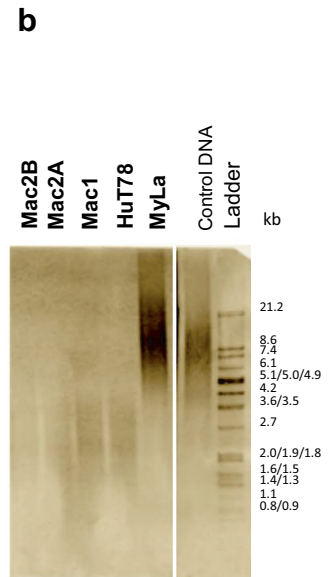
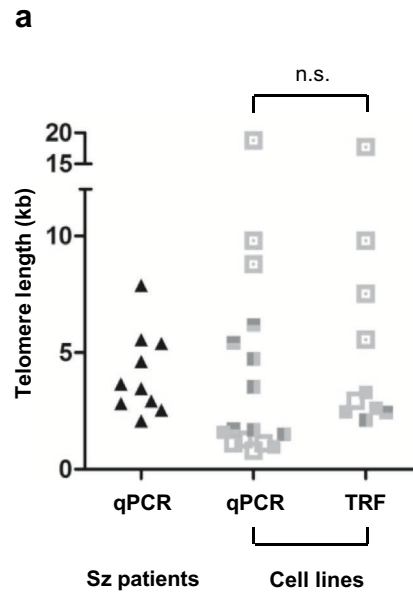
A Relative measurement



B Cytogenetic analysis



C Absolute measurement



Legend: ▲ Sz patients □ Mac1 ■ Mac2A ■ Mac2B □ MyLa ■ HuT78
 Cell lines

FIGURE 1 CTCL cells' telomere length assessment. (A) Relative telomere length measurement by a standard relative qPCR assay. (B) Cytogenetic analysis of CTCL cells (a) Conventional karyotype of a near-diploid cell and (b) mFISH of a hypertriploid karyotype (C) Absolute telomere length measurement (a) by qPCR and by TRF. The mean cell lines' telomere length estimated by qPCR (4.320 ± 0.143 kb) was similar to that estimated by TRF (5.652 kb), $P = .5040$. (b) TRF blot. Arbitrary units (AU); Cutaneous T-cell lymphoma (CTCL); Deoxyribonucleic acid (DNA); Kilobases (kb); Nonstatistically significant (n.s.) Quantitative real-time polymerase chain reaction (qPCR); Sézary (Sz); Terminal restriction fragment (TRF)

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 Table S1. For Sz patients, the complex karyotype was determined by mFISH. All Sz patients (1 to 9) were near-diploid, except patient 10 who was triploid.

4 | CTCL CELLS TELOMERE LENGTH

4.1 | Relative and absolute telomere length measurements

We measured the relative and the absolute telomere length of Sz patients at one point and four independent biological samples for CTCL cell lines (Figure 1).

The relative telomere length was assessed by means of a standard qPCR method (Figure 1A), with a mean variation between measurements (inter-CV) of 13.6% and an individual sample variation (intra-CV) of 8.4%. Using this method, we were able to measure the telomere length of 9 (out of 10) Sz patients, since we never succeeded to amplify neither the reference gene nor the telomeres for one patient (Figure 1A). In cell lines the absolute telomere length was assessed by qPCR (inter-CV of 6.7% and intra-CV of 2.5%) and by measuring the TRF length means (inter-CV of 6.3%) (Figure 1C). These two methodologies were applied only on cell lines due to the huge amounts of DNA required for TRF analysis, which was a limiting factor for analyzing Sz patients.

qPCR absolute telomere lengths were calculated considering cell ploidy: the average telomere length per chromosome was calculated by dividing the cell average telomere length over the number of chromosomes per cell (Table 1).

With this absolute qPCR method we succeeded to calculate the telomere length for all Sz patients (Figure 1Ca). Obtained results, using different telomere length measurement methods, were concordant and allowed us to conclude that Mac1 and HuT78 presented the shortest telomeres, with stable telomere length variation between independent biological samples (Figure 1A and Figure 1Ca). Mac2A and Mac2B presented longer telomeres than HuT78 and Mac1, as well as more variability in their telomere length (Figure 1A and Figure 1Ca). MyLa was the cell line with the longest telomeres among all the cell lines we studied, as well as the one with the highest variability in their telomere length measurement (Figure 1A and Figure 1Ca). The mean cell lines' telomere length estimated by qPCR (4.320 ± 0.143 kb) was similar to that estimated by TRF (5.652 kb), $P = .5040$ (Figure 1Ca).

Telomere length results estimated by TRF correlated with results from relative (Figure 2A) and absolute (Figure 2B) qPCR approaches ($R^2 = 0.6254$, $P = .0194$ and $R^2 = 0.8319$, $P = .0016$, respectively). Telomere length estimation by qPCR-based assays (Figure 2C), strongly correlated with each other ($R^2 = 0.8738$, $P < .0001$).

4.2 | DNA sample quality

When analyzing Sz patients' telomere length, we observed the occurrence of an "outlier" far from patients' average telomere length (Figure 3Aa). We verified samples' quality by agarose gel electrophoresis and we found that it was due to DNA degradation (Figure 3Ab). Thus, this patient was excluded from this study. This was further investigated in two cell lines, one with short telomeres and another one with long telomeres (Figure 3B). When DNA was degraded by heating (Figure 3Ba), the telomere lengths significantly increased (Figure 3Bb). We compared the *KLK3* (reference

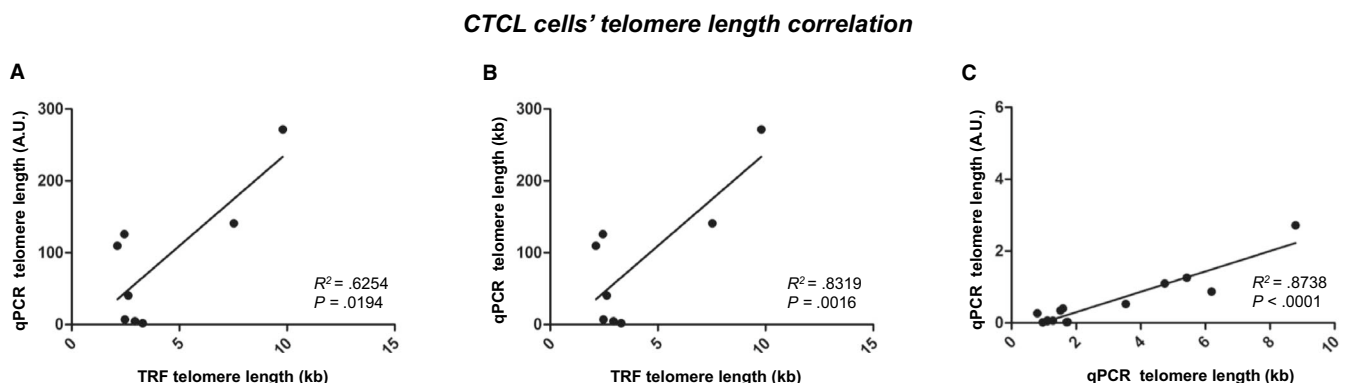
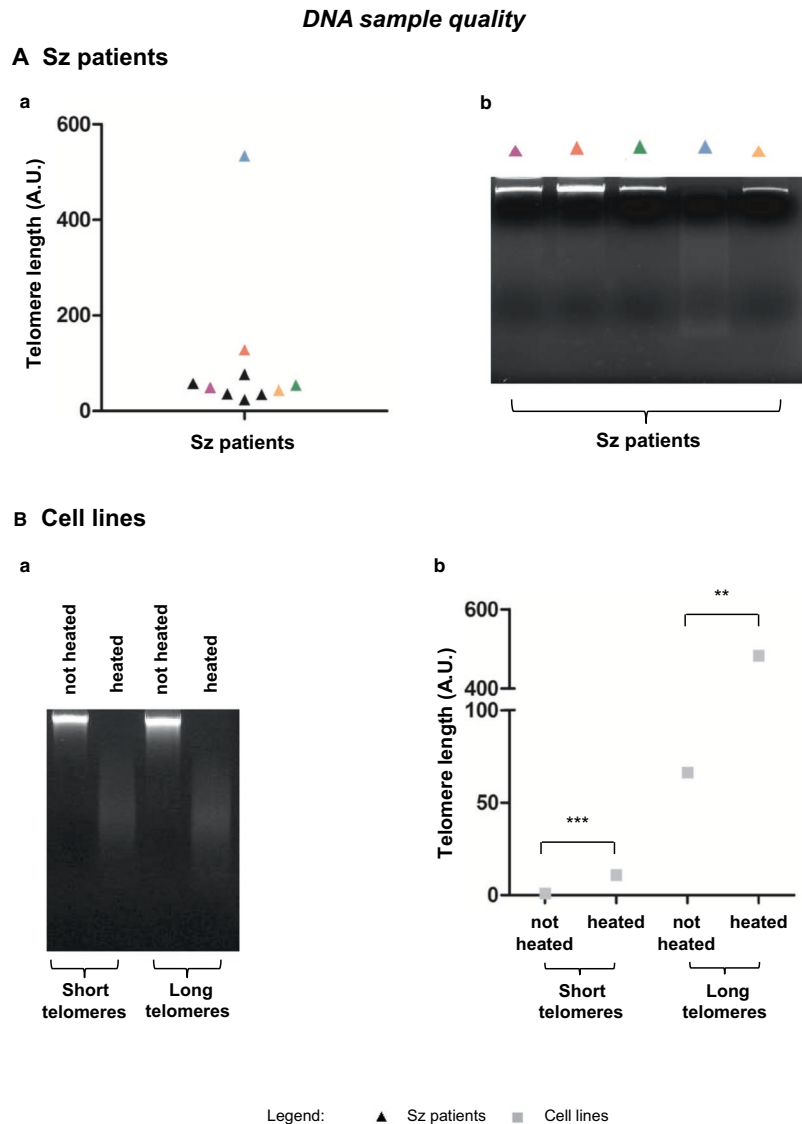


FIGURE 2 CTCL cells' telomere length assays correlation. Telomere length results estimated by TRF correlated with results from relative qPCR (A) and with results from absolute qPCR (B). Telomere length estimation by qPCR-based assays correlated with each other (C). Arbitrary units (AU); Correlation coefficient (R^2); Cutaneous T-cell lymphoma (CTCL); Kilobases (kb); Quantitative real-time polymerase chain reaction (qPCR); Terminal restriction fragment (TRF)

FIGURE 3 Influence of DNA quality on telomere length measurement. (A) Sézary (Sz) patients' (a) relative qPCR telomere length measurement and (b) patient samples marked in colored triangles DNA quality analysis by agarose gel electrophoresis. (B) Two cell lines (one with short telomeres and another with long telomeres) (a) DNA heat degradation confirmed by agarose gel electrophoresis and (b) their relative qPCR telomere length measurement. Telomere length of both cell lines significantly increased following DNA degradation ($P = .0001$ for short telomere cell line and $P = .0037$ for long telomere cell line). Arbitrary units (AU); Sézary (Sz); ** $P < .01$; *** $P < .001$



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).

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
	heated	23.89		24.13		
	heated	26.87	26.83	23.38	23.40	
		26.79		23.41		
Long telomere	not heated	22.07	22.14	16.18	16.09	66.16
	heated	22.20		16		
	heated	24.06	24.17	15.33	15.26	
		24.28		15.18		

4.3 | Sample' tumor cell percentage

We observed that the telomere length of our Sz patient cohort (Figure 4A) was significantly shorter when compared with that of healthy lymphocytes ($P = .0238$). We then compared

Sample' tumor cell percentage

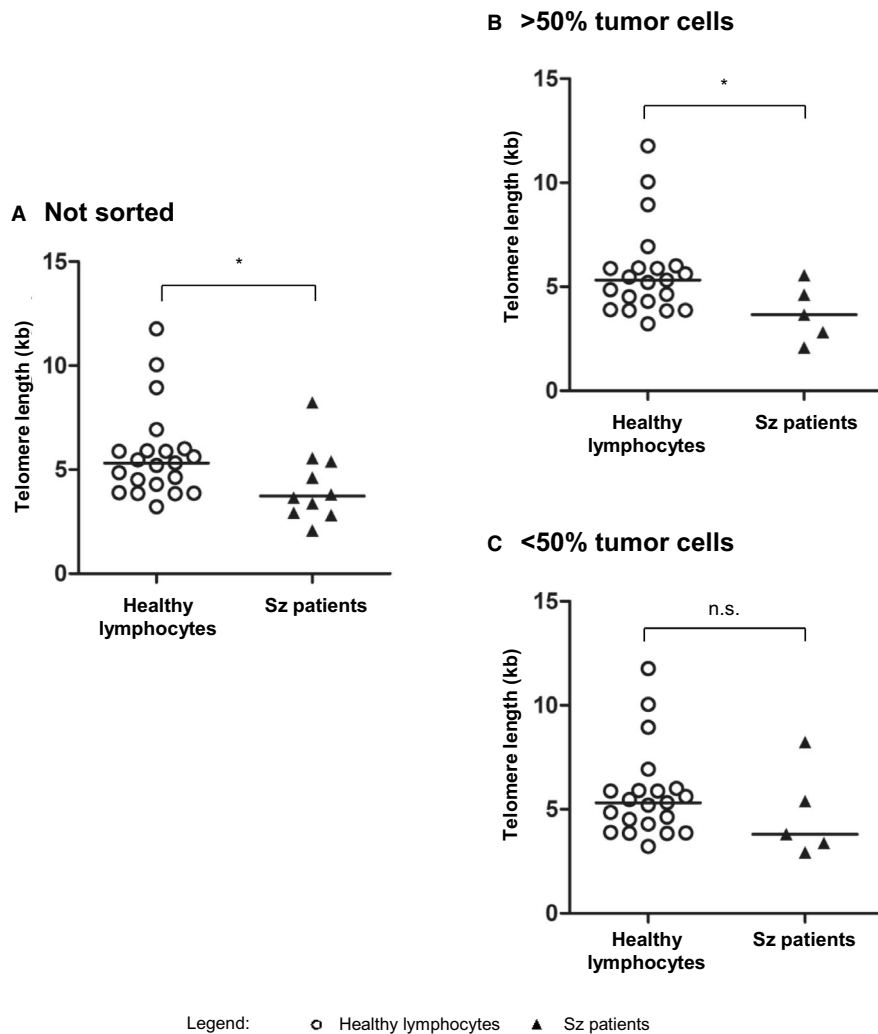


FIGURE 4 Influence of samples' tumor cell percentage on telomere length in comparison with healthy donors. (A.) Telomere length of Sz patients' samples not sorted were significantly shorter when compared with that of healthy lymphocytes ($P = .0238$). (B.) Telomere length of Sz patients' samples with more than 50% of tumor cells have significantly shorter telomeres than those of healthy lymphocytes ($P = .0374$). (C.) Telomere lengths of Sz patients' samples with less than 50% of tumor cells were not statistically different from those of lymphocytes from healthy donors ($P = .1719$). Kilobases (kb); Nonstatistically significant (n.s.); Sézary (Sz); $*P < .05$

the telomere lengths based on samples' tumor cell percentage (Figure 4B,C). We observed that samples with more than 50% of tumor cells (Figure 4B) had significantly shorter telomeres than those of healthy lymphocytes ($P = .0374$), while telomere lengths of samples with less than 50% of tumor cells (Figure 4C) were not statistically different from those of lymphocytes from healthy donors ($P = .1719$).

5 | 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 reliability, sensitivity, and reproducibility, and to reduce intra and interassays

discrepancies.³⁷ Furthermore, it allows obtaining telomere length in absolute kilobases, otherwise only possible by TRF analysis. TRF, although considered as the "gold standard" for telomere length evaluation, requires large DNA quantities which constraints its applicability to cancer study. We often do not have access to large amount of cells or genetic material, so qPCR presents an advantageous tool.²⁷

The main hurdle in using qPCR-based techniques to explore 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.³⁸ Cytogenetic data allowed us to investigate chromosome 17 status of CTCL cells, and this information was important since the qPCR kit uses a 100 base pair-long region on this chromosome as a reference. By cytogenetic data, we guaranteed (under the resolution limit of 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. Hence, the correct telomere length calculation is extremely important to assess and discover associations between telomere length and a certain disease. In this work, the majority of CTCL cell lines and Sz patients were 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 sequences, are concordant with each other (Figure 2C). TRF analysis, on the other hand, measures the telomeres including their subtelomeric region, which generally overestimates telomere length of around 1 kb.¹⁸ Indeed, mean cell lines' telomere length estimated by TRF (5.652 kb) is around 1 kb greater than that estimated by qPCR (4.320 ± 0.143 kb) (Figure 1Ca).

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, we verified that 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, occurred 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 3Bb). 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 observations that short telomere length is a characteristic of Sz tumor cells and that the surrounding nontumor cells present longer telomeres.³⁰ Therefore, the analysis of samples with high tumor cell 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 (Figure S1). Hence, we discriminated between natural telomere shortening and a pathological decrease, which is a hallmark of Sz cells.³⁰

In conclusion, the increased utility of telomere length assessment as a biomarker of cancer cells emphasized the importance of accurate telomere length estimation.

Cancer cells accumulate genetic and chromosomal abnormalities and we do not always have access to a large amount of cells or genetic material to work with. The qPCR-based techniques used to assess telomere length can overcome these problems. Our results, limited by being performed in an uncommon disease which did not allow statistical power calculation, indicate that accurate measurements can only be obtained, with high tumor cell representation samples, undegraded DNA, well-defined cell ploidy, and a known chromosomal status.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Joana Ropio and Alain Chebly wrote the original draft, Paula Soares, Jean-Philippe Merlio, and Edith Chevret edited. All authors provided substantial contributions, conception and design, acquisition of data, or data analysis and interpretation. Edith Chevret supervised all the research. All authors gave final approval of the submitted version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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L'épigénétique comme modulateur de l'expression de *hTERT* dans les lymphomes T cutanés

Résumé :

Les lymphomes T-cutanés (CTCL) sont des tumeurs télomérase-positives exprimant *hTERT*, dans lesquelles ni l'amplification, ni les réarrangements, ni les mutations hotspots du promoteur peuvent expliquer la ré-expression du gène. Comme le promoteur de *hTERT* est riche en CpG, nous avons étudié la contribution des mécanismes épigénétiques dans sa ré-expression, puisqu'aucune étude à ce jour n'a été rapportée dans les CTCL. Nous avons analysé le statut de méthylation du promoteur de *hTERT* dans des lignées cellulaires, des cellules de patients ainsi que dans des cellules issues de donneurs sains. Nous avons également étudié la présence sur le promoteur de *hTERT* des histones H3K27ac et H3K27me3. Les analyses de méthylation spécifiques des cellules CTCL ont révélé un profil de méthylation caractéristique limité aux cellules tumorales, englobant une région distale hyperméthylée de -650 pb à -150 pb et une région proximale hypométhylée de -150 pb à +150 pb, à partir du TSS. Ce double profil de méthylation observé sur le promoteur de *hTERT* est identique à celui observé dans d'autres types de tumeurs. La région distale hyperméthylée identifiée dans les cellules tumorales CTCL correspond à la région nommée récemment « région TERT oncogénique hyperméthylée » (THOR) et qui est rapportée associée à la réactivation de la télomérase dans les tumeurs, mais jusqu'à présent non rapportée dans les lymphomes. Nous avons évalué l'effet sur THOR de deux inhibiteurs d'histone désacétylases (HDACi), la romidepsine et le vorinostat, tous deux approuvés pour le traitement des CTCL ainsi que d'un inhibiteur de l'ADN méthyltransférase (DNMTi) 5-azacytidine, non approuvé pour les CTCL. Nos résultats obtenus à partir d'une cohorte limitée semblent suggérer, que la 5-azacytidine ne provoque pas la déméthylation de la région hyperméthylée du promoteur de *hTERT* alors que ce traitement s'accompagne d'une diminution de l'expression de *hTERT* et, fonctionnellement d'une baisse des capacités clonogènes des cellules. La romidepsine et le vorinostat modifient peu les marques d'histones H3K27ac et H3K27me3 présentes au niveau du promoteur *hTERT*. En conclusion, les résultats obtenus dans les cellules CTCL comparées à ceux de cellules saines confirment que la méthylation du promoteur de *hTERT* dans les cellules tumorales est particulière et spécifique à ces cellules, faisant de cette méthylation un biomarqueur de la cellule tumorale. De plus, ils révèlent que la méthylation du promoteur *hTERT* est relativement stable même sous la pression de thérapies épigénétiques, suggérant que la régulation de *hTERT* par ces thérapies s'opère en priorité de manière indirecte.

Mots clés : Lymphomes cutanés, Syndrome de Sézary, Epigénétique, Méthylation de l'ADN, Histones, HDACi.

Epigenetic modulation of *hTERT* expression in cutaneous T-cell lymphomas

Abstract:

Cutaneous T-lymphomas (CTCL) are telomerase-positive tumors expressing *hTERT*, in which neither amplification, nor rearrangement, nor promoter hotspot mutations can explain the re-expression of the gene. As the *hTERT* promoter is rich in CpG, we investigated the contribution of epigenetic mechanisms in its re-expression, since no studies to date have been reported in CTCL. We analyzed the methylation status of the *hTERT* promoter in cell lines, patients' cells and in cells from healthy donors. We also studied the presence, on the *hTERT* promoter, of histones H3K27ac and H3K27me3. Methylation analyzes in CTCL cells revealed a characteristic methylation profile specific to tumor cells, encompassing a distal hypermethylated region from -650 bp to -150 bp and a proximal hypomethylated region from -150 bp to +150 bp, relatively to the TSS. This dual methylation profile on *hTERT* promoter is identical to the profile seen in other types of tumors. The hypermethylated distal region identified in CTCL tumor cells corresponds to the region recently named "TERT hypermethylated oncogenic region" (THOR) and which is reported to be associated with telomerase reactivation in several tumors, but so far not reported in lymphomas. We evaluated the effect on THOR of two histone deacetylases inhibitors (HDACi), romidepsin and vorinostat, both approved for the treatment of CTCL as well as a DNA methyltransferase inhibitor (DNMTi) 5-azacytidine, not approved for CTCL. Our results, obtained from a limited cohort, seem to suggest that 5-azacytidine does not cause a demethylation of the hypermethylated region on *hTERT* promoter, while this treatment is accompanied by a decrease in the expression of *hTERT* and, functionally with a decrease in the clonogenic capacities of tumor cells. Romidepsin and vorinostat can slightly modify the H3K27ac and H3K27me3 histone marks present on *hTERT* promoter. In conclusion, the results obtained in CTCL cells compared with those of healthy cells confirm that *hTERT* promoter methylation is specific to CTCL cells, making this methylation a biomarker of tumor cells. Furthermore, they reveal that the methylation of *hTERT* promoter is relatively stable even under the pressure of epigenetic therapies, suggesting that the regulation of *hTERT* by these therapies can happen indirectly.

Keywords: Cutaneous lymphomas, Sézary syndrome, Epigenetics, DNA methylation, Histones, HDACi.

Unités de recherche

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