



Telomere Dysfunction And Chromosomal Instability In Hodgkin Lymphoma

Corina Cuceu

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Corina Cuceu. Telomere Dysfunction And Chromosomal Instability In Hodgkin Lymphoma. Cancer. Université Paris Saclay (COMUE), 2015. English. NNT : 2015SACLS210 . tel-01424178

HAL Id: tel-01424178

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NNT : 2015SACLS210

THESE DE DOCTORAT
DE
L'UNIVERSITE PARIS-SACLAY
PREPAREE A
“ L'UNIVERSITE PARIS-SUD ”

ECOLE DOCTORALE N° 582
Cancérologie, Biologie, Médecine, Santé

Spécialité de doctorat : Sciences de la vie et de la santé

Par

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Dysfonctionnement des télomères
et instabilité chromosomique dans le lymphome de Hodgkin

Thèse présentée et soutenue à l'Institut Gustave Roussy le : 15 Décembre 2015

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Titre : Dysfonctionnement des télomères et instabilité chromosomique dans le lymphome de Hodgkin

Mots clés : Lymphome de Hodgkin, instabilité chromosomique, télomères,

Résumé : Le lymphome de Hodgkin est caractérisé, d'un point de vue histologique, par la présence de rares cellules tumorales nommées cellules de Reed et Sternberg, au sein d'un infiltrat cellulaire polymorphe, inflammatoire et réactionnel. Cette dernière résulte de la transformation tumorale de cellules lymphocytaires B qui acquièrent des propriétés d'échappement au système immunitaire, de prolifération, de résistance à l'apoptose et une instabilité chromosomique. Néanmoins, la rareté des cellules tumorales, impliquant des problèmes techniques mais aussi de caractérisation des événements primaires dans l'initiation de cette instabilité chromosomique, a été bien débattue dans la littérature. Mais les mécanismes impliqués dans l'instabilité chromosomique dans le lymphome de Hodgkin demeurent obscurs.

La première partie de cette thèse a été consacrée à l'étude des mécanismes impliqués dans l'instabilité génomique du lymphome de Hodgkin via l'instabilité des microsatellites et l'instabilité chromosomique en utilisant 7 lignées de lymphome de Hodgkin. Nous avons montré pour la première fois l'implication des microsatellites dans l'instabilité génomique des lymphomes de Hodgkin (MSI-H (microsatellite instability-high) dans 3/7 lignées). De plus, nous avons montré que deux mécanismes favorisent l'émergence d'une instabilité chromosomique: le premier implique une instabilité télomérique qui est présente essentiellement dans les petites cellules tumorales induisant la formation des chromosomes dicentriques, des amplifications des gènes (Jak2 comme exemple) et des arrangements chromosomiques complexes. Le deuxième mécanisme est lié essentiellement à un défaut de réparation des cassures double-brin avec l'apparition des chromosomes dicentriques sporadiques et une fréquence importante des micronoyaux avec la formation des ponts anaphasiques.

La deuxième partie de cette thèse a été consacrée à l'étude des mécanismes de maintenance des télomères dans les ganglions tumoraux du lymphome de Hodgkin (50 patients) comme dans les lignées tumorales. Nous avons montré qu'il existe une cohabitation entre les deux mécanismes importants de maintenance des télomères, l'activation de la télomérase d'une part et le mécanisme ALT (alternative lengthening of télomères) d'autre part. Nous avons identifié la présence de petites cellules dans les ganglions hodgkiniens comme dans les lignées tumorales avec une forte activité de la télomérase par contre la cellule de Reed Sternberg est caractérisée par un profil ALT avec la présence des corps PML et une très faible activité de télomérase. La fréquence des cellules télomérase ou ALT varie d'un ganglion à un autre et d'une lignée à une autre. Un drastique raccourcissement télomérique a été observé dans les cellules exprimant la télomérase. Pour les cellules ALT, une grande hétérogénéité de la taille des télomères ainsi que la présence des chromosomes dicentriques sporadiques ont été détectées. Le suivi des patients à long terme pendant 10 ans, nous a permis d'établir une corrélation entre le profil ALT et la survenue de mortalités et de morbidités. De plus, l'étude de la radiosensibilité des lignées tumorales a montré que les lignées ALT sont plus résistantes que les lignées télomérases.

La troisième partie de cette thèse a été consacrée à la validation de ces deux concepts d'instabilité chromosomique via l'instabilité télomérique et à celle des mécanismes de maintenance des télomères, en utilisant un modèle de lymphome de Hodgkin établi dans le laboratoire à partir de la lignée L428.

Ces données auront une retombée clinique importante non seulement dans la compréhension et le traitement des lymphomes de Hodgkin mais aussi dans d'autres pathologies malignes.

Title : Telomere dysfunction and chromosomal instability in Hodgkin Lymphoma

Keywords : Hodgkin lymphoma, chromosomal instability, Telomeres

Abstract: The study of Hodgkin lymphoma (HL), with its unique microenvironment and long clinical outcomes, has provided exceptional insights into several areas of tumour biology. Findings in HL have not only improved our understanding of human carcinogenesis, but have also pioneered its translation into the clinic.

Tumoral cells in HL, called Hodgkin and reed Sternberg cells (HRS), are characterized by a highly altered genomic landscape with a wide spectrum of genomic alterations, including somatic mutations, copy number alterations, complex chromosomal rearrangements, and aneuploidy. Moreover, the scarcity of HRS cells and the resulting technical problems of their in situ characterization, the primary cytogenetic events and the clonality of these possible aberrations has been a matter of debate in the past. As a consequence, a few accepted and established HL cell lines are widely used in the majority of research projects conducted worldwide.

In this thesis, first we have first investigated the possible mechanisms underlying genomic instability including microsatellite and chromosomal instability in HL cell lines. We provide the first evidence that the genomic instability observed in HL is related to microsatellite instability and chromosomal instability related to two major mechanisms: first, telomere fusion leading to dicentric chromosomes formation and breakage/fusion/bridge (B/F/B) cycles involving the repeated fusion and breakage of chromosomes following the loss of telomeres in small cells associated with the lower expression of TRF2, as well as an elevated copy number of the Jak2 gene and the presence of nucleoplasmic bridges containing telomere and centromere sequences. The second mechanism is related to defective DNA repair via non homologous end-joining (NHEJ) repair with the presence of nucleoplasmic bridges without telomere or centromere sequences, accompanied by the micronucleus without centromere sequences and a higher frequency of sporadic dicentric chromosomes.

The second part of this thesis has focused on investigating telomere maintenance mechanisms (TMMs) not only in HL cell lines but also in lymph nodes of HL patients. A telomerase-independent mechanism for TMM in HL has been proposed in the absence of detectable telomerase activity (TA) in some cases. The major finding of this work has been the demonstration of the presence of both telomerase and ALT mechanism in lymph nodes of HL patients as well as in HL cell lines. We have identified a subset of tumors with some small cells expressing telomerase and Reed Sternberg cells containing ALT-associated PML bodies. A significant correlation was observed between telomere length and TMMs. Drastic telomere shortening was observed in cells with telomerase expression and elevated heterogeneity of telomere length was found in ALT profile cells. Interestingly, complex chromosomal rearrangements, included sporadic dicentric formation, were observed in ALT profile cell lines. Interestingly, the relationship between TMMs and all-cause mortality and morbidity during 10 years of follow-up of HL patients using cox proportion hazard models demonstrated a poor clinical outcome for HL patients exhibiting primarily ALT mechanisms. Similarly, higher radiation sensitivity was observed for cell lines with high telomerase activity compared to cell lines with the ALT profile.

The third part of this thesis aims to validate firstly, the mechanism underling chromosomal instability via telomere dysfunction, and secondly, the presence of both TMMs using a xenograft HL animal model.

These insights can be exploited for the development of novel prognostic biomarkers and innovative therapies not only in HL but also in other hematological diseases.

ACKNOWLEDGMENTS

I am grateful to Pr. Sylvie Rogez and Pr. Patrice Carde, to accept the hard task of the evaluation of this thesis. I also acknowledge Pr. Annelise Bennaceur-Griscelli and Dr. Pierre Busson for their participation in the jury.

I address my respectful thank you to Dr. Laure Sabatier, director of my host laboratory and the co-director of my thesis, not only for accepting me to be a part of her team LRO, providing me with all the equipment and the tools needed throughout this training, but also for sharing her knowledge with me and always leading me in the right direction through useful discussions and the encouragement that she gave me during my work.

I also would like to thank Dr. Radhia M'kacher, the leader of my scientific work, for the professionalism with which she guided me on my way to achieving the title of doctor in science, for the competence and the permanent scientific guidance, for the real support during the entire time of my PhD and the development of the thesis.

A huge thank you of course to Michelle Ricoul, who was always there for me during the entire time of my PhD to teach me, help me and guide me in all the experiments that I made in the lab. With your kindness, your joy of living and your unconditional help you were the person who contributed the most in giving me confidence and support all the time spent in the lab since the first day of my arrival in France.

Thanks to all the colleagues who were a part of the LRO. I thank Monika, for being my best friend and colleague, for all our discussions concerning work, but not only, also to Laurette and Annie for our French lessons and distressful "pause pomme". A special thanks to Mustafa, you inspired me with your determination of solving any difficulties that come in your way. Thank you Luc for our long and useful scientific discussions, William for always being there and helping me in correcting my English, Aude and Marion for their technical and moral help, to Asmorom, for his informatics skills and to all the people who were my colleagues for a short period of time; Pamela who first introduced me to cytogenetics, Grace and Marie for their pleasure of working and joyful spirit, Geraldine who was always easy to talk to, and all the trainees for their scientific advice and good times spent together.

Thank you to Pr. Hugue de Thé, the director of the Department of Biochemistry of Saint Louis Hospital and to François Plassa for their help and support.

I am grateful to Leonhard Heidingsfelder for his exquisite skills in microscopy and for his help, to Elie El Maalouf and Bruno Colchcio of MIPS laboratory (University of Mulhouse) for their informatics support and knowledge.

Thank you to Francis Finot and André Essahli from the Genetic Toxicology Department from Covance for their technical support. Thank you to Françoise Pflumio, and to the entire laboratory LRD from CEA and also to Dr. Nadira Delhem and the entire M3T laboratory from the Institut Pasteur de Lille for their collaboration.

Thank you to all the colleagues and personnel from the University Paris Sud for their technical and moral support

I would like to give my sincere respect and acknowledge to my family who was there for me with all their heart, to my parents and my brother: I dedicate this success to them. I give my thanks and my love to my dear husband and our baby boy Dorian; you are the most priceless things in my life. Vă iubesc!

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ALL	Acute Lymphoblastic Leukemia
ALT	Alternative Lengthening Of Telomeres
AML	Acute Myeloid Leukaemia
ATM	Ataxia-Telangiectasia Mutated
ATR	Ataxia-Telangiectasia And Rad Related
B/F/B	Breakage/Fusion/Bridge
B-ALL	Pre-B-Cell Acute Lymphoblastic Leukemia
BCL3	B-Cell Lymphoma 3-Encoded Protein
BCL-xL	B-Cell Lymphoma-Extra Large
CCL20	Chemokine (C-C Motif) Ligand 20
CCR	Complex Chromosomal Rearrangements
CGH	Comparative Genomic Hybridization
cHL	Classical Hodgkin Lymphoma
CIHHV-6	Chromosomally Integrated HHV-6
CT	Computerized Tomography
CXCL10	C-X-C Motif Chemokine 10
CYLD	Cylindromatosis
DNA	Deoxyribonucleic Acid
DSBs	Double-Strand Breaks
EBNA-1	Epstein–Barr Nuclear Antigen 1
EBV	Epstein-Barr Virus
FACS	Fluorescence-Activated Cell Sorting
FAS	Tumor Necrosis Factor Receptor
FI	Fluorescence Intensity
FISH	Fluorescent <i>in situ</i> Hybridization
FOX	Forkhead Box Transcription Factor Family
FOXC1	Forkhead Box Protein C1
FOXD1	Forkhead Box Protein D1
FOXM1	Forkhead Box Protein M1
FOXN3	Forkhead Box Protein N3
FOXO1	Forkhead Box Protein O1
FOXP1	Forkhead Box Protein P1
GC	Germinal Center
gH2Ax	Gamma-H2A Histone Family, Member X
HDACi	Alkylating Histone-Deacetylase Inhibitor
HHV6	Human Herpesvirus 6
HL	Hodgkin Lymphoma
HNPCC	Hereditary Non-Polyposis Colon Cancer
HRS	Hodgkin Reed-Sternberg
IF-FISH	Immuno-Fluorescence <i>in situ</i> Hybridization
IHC	Immunohistochemistry
IκB	Inhibitor Of Kb
IKK	Iκb Kinase

IL21	Interleukin 21
JAK	Janus Kinase
JAK2	Janus Kinase 2
JCV	John Cunningham Virus
JUNB	Transcription Factor Jun-B
KLHDC8B	Kelch-Like Domain-Containing Protein 8B
LCM	Laser Capture Microdissection
LDcHL	Lymphocyte Depleted Classical Hodgkin Lymphoma
LMP	Latent Membrane Protein
LMP1	Latent Membrane Protein 1
LMP2	Latent Membrane Protein 2
LRcHL	Lymphocyte Rich Classical Hodgkin Lymphoma
MAPK	Mitogen-Activated Protein Kinases
MCcHL	Mixed Cellularity Classical Hodgkin Lymphoma
M-FISH	Multicolor Fluorescence <i>in situ</i> Hybridization
MMR	Mismatch Repair
MMR	Mismatch Repair
MN	Micronuclei
MR11	Double-Strand Break Repair Protein MRE11A
MSI	Microsatellite Instability
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBUD	Nuclear Buds
NF-κB	Nuclear Factor-KappaB
NFKBIA	NF-Kappa-B Inhibitor Alpha
NFKBIE,	NF-Kappa-B Inhibitor Epsilon
NHEJ	Non Homologous End-Joining
NIK	NF-κB-Inducing Kinase
NLPHL	Nodular Lymphocyte Predominant Hodgkin Lymphoma
NOTCH1	Notch Homolog 1, Translocation-Associated
NPB	Nucleoplasmic Bridges
NScHL	Nodular Sclerosis Classical Hodgkin Lymphoma
OS	Overall Survival
PBL	Peripheral Blood Lymphocytes
PBMC	Peripheral Blood Mononuclear Cemm
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death-Ligand 1
PET	Positron Emission Tomography
PFA	Paraformaldehyde
PFS	Progression-Free Survival
PI3K	Phosphoinositide 3-Kinase
PML	Promyelocytic Leukaemia
PNA	Peptidic Nucleic Acid
POT1	Protection Of Telomeres Protein 1
PRDM1a	PR Domain Zinc Finger Protein 1
Q-FISH	Quantitative Fluorescent <i>in situ</i> Hybridization

RANK	Receptor Activator Of NF- κ B
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RS	Reed-Sternberg
RSC	Reed-Sternberg Cells
SOCS1	Suppressor Of Cytokine Signaling 1
SSB	Single-Strand Breaks
SSC	Saline Sodium Citrate
STAT	Signal Transducers And Activators Of Transcription
TA	Telomerase Activity
TIF	Telomere Dysfunction-Induced Focus
TMM	Telomere Maintenance Mechanisms
TNFAIP3	Tumor Necrosis Factor, Alpha-Induced Protein 3
TP53	Tumor Protein 53
TRAF3	TNF Receptor-Associated Factor 3
TRF1	Telomeric Repeat Factor 1
TRF2	Telomeric Repeat Factor 2
WB	Western Blot
WHO	World Health Organization

Introduction

Hodgkin lymphoma (HL) is one of the most common lymphoma in the developed world, with the incidence of approximately 3 per 100 000 person-year (Swerdlow 2008). HL involves peripheral lymph nodes and can affect organs such as liver, lung, and bone marrow.

In 1832 Thomas Hodgkin published the first article on malignant lymphomas called “On some morbid appearances of the absorbent glands and spleen” (Hodgkin 1832), where he described seven patients with generalized lymphadenopathy and splenomegaly examined post mortem. The term “Hodgkin’s disease” was proposed for the first time in 1865 by Samuel Wilks nine years after he published a series of 45 cases of “lardaceous disease” which included five of Thomas Hodgkin’s cases. Wilks acknowledged the significance of Hodgkin’s notes and observations of the condition that did not even exist as a disease at that time, and honored him by associating his name with this malignant disease of the lymphoid system. Dorothy **Reed** Mendenhall (1874-1964) and Carl **Sternberg** (1872-1935) are the two other scientists who will always be associated with Hodgkin lymphoma. They have given their names to the Hodgkin and Reed-Sternberg (RS) cells. Carl Sternberg was an Austrian pathologist who, in 1898, described large cells characteristic of HL, however, comprising a minority of the cells in the tumors (Sternberg 1898). His description of the HRS cells was further refined by Dorothy Reed, who in 1902 published the article “On the pathological changes in Hodgkin’s disease, with special reference to its relation to tuberculosis” (Reed 1902) where she made it clear that HL was not a variant of tuberculosis, and provided skilled drawings of the HRS cells. Previously, the high proportion of tuberculosis in HL patients had led pathologists to assume that HL might be a variant of tuberculosis. However, Reed showed that there was no reaction to tuberculin (Reed 1902).

Since that time, RS cells have become the hallmark of classic Hodgkin lymphoma, and their origin has been well established as a subpopulation of transformed germinal center B cells (Kuppers 2002, Kuppers et al 2012, Kuppers et al 1994). The presence of this particular malignant cell represents only 1% of the lymphocytes, macrophages, granulocytes, and eosinophils surrounding the involved samples.

The majority of RS cells are positive for CD30-a tumor necrosis factor receptor involved in tumorigenesis and tumor progression (Molin et al 2001). Approximately

70% of the RS cells in patients express CD15 while CD20 is expressed in approximately 11% -33% of cases (Portlock et al 2004, Tzankov et al 2003). Generally, RS cells do not express B-cell antigens such as CD45, CD19, and CD79A.

It is now recognized as a clonal B cell neoplasma and therefore been renamed Hodgkin lymphoma. The World Health Organization (WHO) 2008 classification recognizes two histological types of Hodgkin lymphoma. The first type is *classical Hodgkin lymphoma (cHL)*, with four subtypes: *nodular sclerosis (NScHL)*, *mixed cellularity (MCcHL)*, *lymphocyte rich (LRcHL)* nodular (common) and *diffuse* (rare) forms, and *lymphocyte depleted (LDcHL)*. The second type is a Nodular lymphocyte predominant Hodgkin lymphoma. These subtypes are defined according to the number of Reed-Sternberg cells, the presence or absence of fibrosis, cell surface markers and characteristics of the inflammatory microenvironment (Steidl et al 2010b). The *Nodular sclerosis cHL* subtype is the most frequent subtype of cHL in developed countries corresponding to 75%–80% of all HL cases and is seen mostly in young patients (median age 28 years). The *mixed cellularity* subtype is the second most common type (15% to 30%) and is seen mostly in older adults.

Typically, HL has a bimodal age-specific incidence pattern with a peak in incidence in patients aged 15-to-34 and another, later peak in patients aged 65-84. HL is ranked as the fourth-most common cancer in patients aged 15-to-34 (after testicular cancer, melanoma and breast cancer) and represents 9% of all cancers in this age group (or almost 20 % of all cancers in 15-to-19 year-old patients alone). However, HL represents only 0.2 % of all cancers in patients aged over 65 years.

In addition, in about 40% of classical HL in Western world, and in more than 90% of pediatric cases of HL in Central America, HRS cells are latently infected by EBV (Kapatai & Murray 2007, Kuppers et al 2012). HRS cells are clonally infected, suggesting that EBV infection is an early event in HL pathogenesis (Anagnostopoulos et al 1989). It was suggested that EBV play a major as an initial event in HL pathogenesis by rescuing crippled germinal center B cells from apoptosis (Brauninger et al 2006, Kuppers et al 2012). While, much attention has been focused on the role of EBV in HL, growing evidence suggests that other viruses may also be associated with HL. Nevertheless, large studies have confirmed differences in clinical characteristics between EBV+ and EBV – HL, with a poorer prognosis for EBV+ HL patients older than 50 year old (Diepstra et al 2009).

The original treatment regimens for HL, developed in the 1960s and 1970s, consisted in the association between chemotherapy regimens and radiation therapy. The type and the number of cycles of chemotherapy, and the additional use of radiotherapy are based on the stage of the HL and the type and number of prognostic factors (Rathore & Kadin 2010).

Survival following childhood Hodgkin Lymphoma is currently very high and better than for adults. In a study of 57 956 children with cancer performed by EUROCare-5, Gatta, et al. (Gatta et al 2014) showed that in Europe, the 5 year survival rate (95.4%) for Hodgkin lymphoma is the second highest, after retinoblastoma (97.9%), higher than i.e. Burkitt's lymphoma (90.2%) or Acute myeloid leukemia (AML) (62.7%).

Actually, about 15-20% of patients with advanced stage HL still die following relapse or progressive disease and a similar proportion of patients are over-treated, leading to treatment-related late sequelae including solid tumors and organ dysfunction (Armitage 2010) (Engert et al 2010) (van Leeuwen et al 1994). The cohort study of HL patients shows that the survival of these patients who are essentially cured, is much lower than that which would be expected (Castellino et al 2011). The risk of these former patients of dying from a complication of treatment, given in the distant past, is greater than the risk of dying from a recurrence of the disease itself. The extent and nature of these morbidities and late mortalities has been the subject of numerous and detailed studies.

The majority of cases of HL were cured well before we understood the nature of the malignant cell of origin. Recent studies have shown that mortality in Europe due to HL has not changed significantly over the last 20 years (Jemal et al 2009) despite a major progress in our knowledge of the nature of HRS cells and microenvironment of these cells. These data conducted to raise the question on the really tumoral cells in HL. More than 20 years ago, Newcom et al reported that the L428 HL cell line, consisting predominantly of HRS cells, also contained a small population of phenotypic B cells; these phenotypic B cells appeared to be responsible for the generation of the HRS cells and the continuous growth of the cell line (Newcom et al 1988). In addition, it has been well documented that mononuclear Hodgkin cells have a high proliferation potential compared to the poor growth of RS cells (Hsu et al 1988). Recently, Jones et al (Jones et al 2009) demonstrated in HL cell lines, lymph nodes, and in peripheral blood of newly diagnosed HL patients that the presence of B-cell subpopulations (<1%) were responsible for the generation and

maintenance of the predominant HRS cell population. In addition, the presence of lymphoma specific immunoglobulin gene segments was detected in peripheral blood at initial diagnosis or during follow-up using a highly sensitive next-generation sequencing method (Oki et al 2015).

HRS are characterized by a highly altered genomic landscape with a wide spectrum of genomic alterations, including somatic mutations, copy number alterations, complex chromosomal rearrangements, and aneuploidy. Nevertheless, no cytogenetic investigation was performed in clonogenic B cells in HL patients or cell lines. Moreover, the scarcity of HRS cells and clonogenic B cells and the resulting technical problems of their in situ characterization, the primary cytogenetic events and the clonality of these possible aberrations has been a matter of debate in the past. As a consequence, a few accepted and established HL cell lines are widely used in the majority of research projects conducted worldwide.

In light of these findings, this thesis was composed of 4 parts:

1. The first part of this thesis was dedicated to a review on the genomic instability in HL. We described the major progress in the understanding of the pathogenesis of HL in the last two decades. Nevertheless, the mechanisms underlying genomic instability of HL stay obscure as well as the primary transforming events.
2. The second part was dedicated to study the mechanisms underlying genomic instability in 7 HL cell lines potentially involved in the development of this disease by applying combined molecular and cytogenetic approaches. We provide the first evidence that the genomic instability observed in HL is related to microsatellite instability and chromosomal instability related to two major mechanisms: first, telomere fusion leading to dicentric chromosomes formation and breakage/fusion/bridge (B/F/B) cycles involving the repeated fusion and breakage of chromosomes following the loss of telomeres in small cells associated with the lower expression of TRF2, as well as an elevated copy number of the Jak2 gene and the presence of nucleoplasmic bridges containing telomere and centromere sequences. The second mechanism is related to defective DNA repair via non homologous end-joining (NHEJ) repair with the presence of nucleoplasmic bridges without telomere or centromere sequences, accompanied by the micronucleus without centromere sequences and a higher frequency of sporadic dicentric chromosomes.

3. The third part of this thesis has focused on investigating of telomere maintenance mechanisms (TMMs) not only in HL cell lines but also in lymph nodes of HL patients. A telomerase-independent mechanism for TMM in HL has been proposed in the absence of detectable telomerase activity (TA) in some cases. The major finding of this work has been the demonstration of the presence of both telomerase and ALT mechanism in lymph nodes of HL patients as well as in HL cell lines. We have identified a subset of tumors with some small cells expressing telomerase and Reed Sternberg cells containing ALT-associated PML bodies. The relationship between telomere maintenance mechanisms detected in lymph nodes and the clinical outcomes of patients after 10 years of follow-up was investigated. In addition, the radiation sensitivity of HL cell lines were assessed and corroborated with mechanisms of telomere maintenance.
4. Finally, we try to validate all these findings using HL animal model establish recently in the laboratory from L428 cell line. Cytogenetic investigation and telomere maintenance mechanisms were assessed.

The understand of the cellular origin of tumor cells, the relationship between HRS cells and putative precursor or stem cells in the unique microenvironment of HL could progress not only our knowledge concerning the bridge between non neoplastic cell and neoplastic cells in HL but also the sensibility of these cells to different treatment modalities (Canellos et al 2014) and proposed a personalized treatment.

Part I

The first part of this thesis is dedicated to a review of a major progress on the understanding of the pathogenesis of Hodgkin lymphoma in the last two decades, focusing especially on genomic instability.

In this part we firstly, described the cellular origin of the neoplastic cells and the important molecular pathways (NF- κ B, P53, JAK2/STAT, FOX) supporting its pathogenesis.

Secondly, we investigated the implication of the two distinct mechanisms underlying genomic instability: microsatellite instability and chromosomal instability. We have essentially analyzed the possible role of telomere length and telomere maintenance in Hodgkin lymphoma.

Thirdly, we have detailed the interaction between the tumor cells microenvironment and genomic instability in Hodgkin lymphoma. The important role of viral infection in the initiation or the progression of the pathogenesis of Hodgkin lymphoma was also described. We have focused especially on the implication of three viruses in Hodgkin lymphoma: EBV, HHV6 and JC virus.

Finally, the impact of these mechanisms in the clinical outcomes of Hodgkin lymphoma patients, underling the therapeutic implications was discussed.

We concluded that the mechanisms underlying genomic instability in HL stay obscure as well as their prognostic factors. The understanding of the passage between malignant and non-malignant cells in the tumor microenvironment of Hodgkin lymphoma could elucidate some events and mechanisms in the initiation of this disease.

Genomic instability in Hodgkin lymphoma: the past, the present and future

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Abstract

The study of Hodgkin lymphoma (HL), with its unique microenvironment and long clinical outcomes, has provided exceptional insights into several areas of tumor biology. Findings in HL have not only improved our understanding of human carcinogenesis, but have also pioneered its translation into the clinic.

HL can be considered to be a successful paradigm of modern treatment strategies. Nonetheless, about 15-20% of patients with advanced stage HL still die following relapse or progressive disease and a similar proportion of patients are over-treated, leading to treatment-related late sequelae including solid tumors and organ dysfunction.

Tumoral cells in HL are characterized by a highly altered genomic landscape with a wide spectrum of genomic alterations, including somatic mutations, copy number alterations, complex chromosomal rearrangements, and aneuploidy.

In this article, we review the genomic instability mechanisms in HL starting with the cellular origin of neoplastic cells and the mechanisms supporting its pathogenesis, focusing especially on the role of the microenvironment in the induction of genomic instability in HL. The emerging possibilities to exploit these aberrations as prognostic biomarkers and guides for personalized patient management will be discussed.

Introduction

HL is classified into 2 large distinct entities, classical Hodgkin lymphoma (cHL) representing the majority of the cases (more than 95%) and nodular lymphocyte predominant HL (NLPHL). cHL is divided into four subtypes: nodular sclerosis (NS) (70%), mixed cellularity (MC) (20-25%), lymphocyte-rich (LR) (5%) and lymphocyte-depleted (LD) (1%). These subtypes differ in their clinical and histopathological features, especially micro-environmental features. However, all subtypes are characterized by the presence of large mono-nucleated or multinucleated cells with prominent nucleoli called Hodgkin/Reed-Sterneberg (HRS) cells. In the absence of recurrent chromosomal rearrangements that serve both as informative diagnostic/prognostic markers and as clues to deregulated gene targets located at chromosomal breakpoints (Kuppers & Hansmann 2005), the cell surface marker antigens CD15 and CD30 are used to confirm the diagnosis after immunohistochemistry staining. HRS cells represent only 1-2% of the cellular infiltration which includes lymphocytes, macrophages, granulocytes, and eosinophils. HL represents a unique model of the interaction of a tumor cell with its microenvironment.

HL accounts for approximately 10% of all lymphomas and 1% of all cancers in industrial countries with an estimated annual incidence in France of 1840. Current treatment of HL provides a very high cure rate (80%). Because of this success, the first and amongst one of the best examples in oncology, and because this disease affects young patients, it is the object of intense interest amongst Oncologists, Cytogeneticists and Radiobiologists, insofar as lessons learned from the study of this disease and its treatment can serve as a basis for novel therapeutics and the follow-up of other cancers. Nevertheless, cohort studies of Hodgkin's disease patients show that the survival of these patients who are essentially cured, is much lower than that which would be expected (Castellino et al 2011). The risk of these former patients of dying from a complication of treatment, given in the distant past, is greater than the risk of dying from a recurrence of the disease itself. The extent and nature of these morbidities and late mortalities has been the subject of numerous and detailed studies. In addition, a substantial proportion of HL patients (20%) still experience relapsing or refractory disease eventually progressing to death (Keller et al 2012).

The majority of cases of HL were cured well before we understood the nature of the malignant cell of origin (Canellos et al 2014). Indeed, there has been much confusion and debate on this point. Early speculation hypothesized derivation from a macrophage or histiocyte, or even a hybridized cell of uncertain origin. Using micro dissection of the malignant cells and the polymerase chain reaction, Kuppers has demonstrated that these cells derived from a pre-apoptotic B cell of germinal center origin with multiple somatic mutations in the clonal immunoglobulin genes (Kuppers et al 1994).

Importantly, Reed-Sternberg (RS) cells represent the most prominent HRS-cell subtype in biopsy specimens and were defined as differentiated end state of HRS cells playing a pivotal role in the interaction with the tumor microenvironment in situ (Skinnider & Mak 2002, Steidl et al 2011, van den Berg et al 1999). However, the development of these giant tumor cells was controversially discussed for a long time (Drexler et al 1989, Kuppers et al 2001).

Recently Rengstl et al (Rengstl et al 2013, Rengstl et al 2014) has demonstrated, using long-term time-lapse microscopy, that re-fusion of daughter cells is the main route to giant HRS cell formation and RS cells neither develop by endomitosis nor by acytokinetic mitosis. One of major characteristics of these cells is the occurrence of complex and hyperdiploid chromosomal aberrations that reflect chromosomal instability. Nevertheless, these abnormalities have been observed not only in H/RS cells but also in a subset of morphologically normal cells, in proximity to the H/RS cells (Jansen et al 1998). In addition, a high frequency of chromosomal aberrations and the presence of complex chromosomal rearrangements have been detected in circulating lymphocytes before treatment (Barrios et al 1989, M'Kacher et al 2003c). However, the relationship between this subset of morphologically normal cells and H/RS cells is not clear. The cytogenetic analysis of HL has been hampered by two major barriers, the first being the inability to grow lymph nodes in vitro, and the second, the lack of an efficient animal model which can be used to understand the mechanisms underlying chromosomal instability of HL.

In this review, we discuss the important known mechanisms underlying genomic instability in HL lymphoma, and analyze different possible multistep processes in the ongoing genesis of H/RS chromosomal instability. Finally, we analyze the

relationship between different mechanisms underlying genomic instability and the clinical outcome of patients.

Origin of tumor cells in cHL

HRS cells co-express the activation marker antigens of various hematopoietic lineages including B, T, dendritic, NK, and myeloid cells. The activation marker antigens CD15 and CD30 are established markers for the immunohistological detection of the malignant cells in HL (Gorczyca et al 2003). The rarity of these cells has made difficult the establishment of their origin. Until 1994, the origin and clonality of HRS cells remained obscure and many hypotheses were proposed and debated (Otto et al 2012). Using microdissection and single-cell PCR techniques to detect the rearrangement of immunoglobulin (Ig) genes, Kuppers demonstrated a potential origin from B lineage cells (Kuppers et al 1994, Kuppers et al 2002), and concluded that germinal center (GC) B-cells are the origin of HRS cells in most cases. However, in a few cases, clonal T cell receptor rearrangement has also been detected, suggesting a rare subpopulation (1-2%) of HL of T cell origin (Bargou et al 1997, Seitz et al 2000)

Actually, the detection of HRS cells using immunohistochemistry was used as a clinical marker of HL, nevertheless, some studies suggested the existence of “HL cancer stem cell” or the clonotypic B cells responsible for the growth and the maintenance of HRS (Jones et al 2009) and represent HL-initiation cells. Recently, using a highly sensitive next-generation sequencing method, lymphoma specific immunoglobulin gene segments were detected in peripheral blood at initial diagnosis or during follow-up (Oki et al 2015). It has been well documented that mononuclear Hodgkin cells have a high proliferation potential compared to RS cells (Hsu et al 1988). It will be important to investigate using HL cell lines the proliferative and clonogenic potential of CD30 and CD15 negative cells and their potential to restore the parental cell line. The investigation of the relationship between these clonotypic or negative B cells and HRS cells in HL can provide not only more information about the origin of the tumor cell in HL but may also define a new therapeutic strategy targeting the cancer stem cells in HL. In addition, the mechanism involved in the transformation from a germinal center B cell into HRS cells is still unknown.

Advances in the molecular understanding of the transforming events in cHL

The poor in vitro growth of HRS cells has considerably limited the ability to carry out cytogenetic studies which have been mostly carried out in the few existing HL cell lines. As a consequence, the vast majority of studies in HL have focused on unraveling the molecular pathways which may play a role in the transformative events in HRS cells. In this review, we focus on five important molecular pathways.

1- NF- κ B pathway

The constitutive activation of the NF- κ B pathway plays an important role in HRS cells. NF- κ B is a transcription family composed of homo and heterodimers of p50, p52, p65 (RelA), RelB, and c-Rel. It is inactivated by the binding of the I κ B protein in the cytoplasm. Phosphorylation of I κ B by the I κ B kinase (IKK) complex leads to its degradation, releasing NF- κ B p65p50 heterodimers which then transfer to the nucleus where they regulate the transcription of various target genes including a variety of pro-inflammatory cytokines and anti-apoptotic factors (Bcl-xL, cIAP2, FLIP).

In HRS cells, signal transduction through CD30, CD40 and receptor activator of NF- κ B (RANK) have been shown to activate the IKK complex maintaining NF- κ B signaling (Horie et al 2002). Various mechanisms downstream of IKK have also been shown in HL to be abnormal. Mutations in the genes of the NF- κ B inhibitors, I κ B α and I κ B ϵ (encoded by NFKBIA and NFKBIE, respectively) are found in about 10–20% of cases (Lake et al 2009); (Liu et al 2010). Genomic gains of REL are present in about 30% of HL (Joos et al 2002); (Martin-Subero et al 2002). A20, which is encoded by the TNFAIP3 gene, is an inhibitor of NF- κ B activity, and is inactivated in about 40% of CHL cases (Malynn & Ma 2009). Of note, TNFAIP3 mutation is almost always associated with EBV infection in CHL, suggesting that TNFAIP3 inactivation and EBV infection are complementary for CHL pathogenesis (Schmitz et al 2009).

Another mutation which leads to NF- κ B pathway activation is the gain of NF- κ B-inducing kinase (NIK) which is seen in approximately 25% of cases (Saitoh et al 2008); (Otto et al 2012). Constitutive activation of NIK leads to sustained RelB signaling in HL cell lines (Ranuncolo et al 2012). Other regulators of NF- κ B, such as BCL3, CYLD and TRAF3 have also been shown to be mutated, but only in rare cases (Martin-Subero et al 2006, Otto et al 2012, Schumacher et al 2010)

2- JAK/STAT pathway

The JAK/STAT pathway is another important pathway in HRS cell pathogenesis. In humans there are four JAK and seven STAT proteins, which mediate signaling through cytokine receptors. Cytokine stimulation activates JAKs, leading to the phosphorylation of STATs. Phosphorylated STATs dimerize and translocate to the nucleus where they induce expression of target genes (Vainchenker & Constantinescu 2013). STAT3, STAT5A, STAT5B and STAT6 have been shown to be activated in HRS cells (Hinz et al 2002, Scheeren et al 2008, Skinnider et al 2002, Vrzalikova et al 2011). Expression and activation of STAT5A and STAT5B is also increased by NF- κ B activity in HRS cells (Hinz et al 2002). In addition, IL21 is expressed in HL leading to activation of STAT5A, and STAT5B as well as STAT3 (Scheeren et al 2008). JAK2 chromosomal gains are seen in about 20% of HL, with occasional cases of translocation ((Joos et al 2000, Van Roosbroeck et al 2011). Finally, SOCS1, an important inhibitor of STAT activity presents inactivating mutations in about 40% of classical HL cases (Weniger et al 2006).

A recent clinical study highlights the importance of the JAK/STAT pathway in HL by demonstrating the efficacy of the blockade of the PD-1 pathway, which may be used in HL to evade immune detection. In classical HL, alterations in chromosome 9p24.1 increase the abundance of the PD-1 ligands, PD-L1 and PD-L2, and promote their induction through JAK/STAT signaling. Treatment of relapsing or refractory HL patients with the PD-1 blocking antibody Nivolumab resulted in an objective response for 87% of the patients with 17% and 70% showing a full or partial response, respectively (Ansell et al 2015).

3- P53 pathway

The TP53 tumor suppressor gene has two main functions: one as a gene-specific transcription factor and one as a specific inhibitor of the transcription of certain genes. One important function of p53 is its ability to activate apoptosis, and the disruption of this process can promote tumor progression and resistance to treatment (Fridman & Lowe 2003). For a long time it was believed that inactivation of p53 function is usually the result of point mutations in exons 5 to 8 also known as hot

spots (98% of all p53 mutations). As a consequence, the majority of studies have only superficially addressed the other regions of the gene. Alterations have been found in virtually every region of the protein but only a handful of the most frequently occurring mutations have been studied in depth to understand their contribution to cancer progression (Leroy et al 2013). The status of the p53 pathway in cHL remains unclear, and a lack of proven TP53 mutations contrasts with often high expression levels of p53 protein (Janz et al 2007). It has been proposed that mutations in the TP53 gene in biopsy material as well as cell lines derived from cHL are rare and not notably involved in the pathogenesis of HRS cells. The analysis of point mutations of the P53 tumor suppressor gene in HL confronts a technical problem due to the minority of HRS cells in the tumor tissues and the use of only primer sequences of exons 5 to 8. Interestingly, a reevaluation of the P53 status in HL cell lines demonstrated the presence of deletions within exon 4 in the L428 cell line and nearly a complete loss of exons 10-11 in L1236 and exons 8-11 in HDLM-2. This reevaluation of the P53 status in HL using a sensitive technique reinforces the concept that mutation of TP53 may be involved in the pathology of some cases of HL (Robles & Harris 2010) and perhaps the genomic instability observed in this disease as well as the occurrence of late complications such as secondary cancer following HL treatment (Schulz et al 2015) or HL as a secondary event (Schneider et al 2014). The correlation between TP53 status and high-grade progression in B-cell lymphoma has been previously established (M'Kacher et al 2003a, Sanchez-Beato et al 2003). In summary it remains unclear whether the influence of p53 plays a role in the malignant transformation of RS cells. A mutated TP53 gene would most likely facilitate the observed aggressive genetic instability following profound aneuploidy and complex cytogenetic rearrangements, as well as the inter-individual heterogeneity that characterize this malignancy.

4- ATM and ATR pathways

In mammalian cells, ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases, members of the phosphatidylinositol 3-kinase-related kinase (PI3KK) family, play an important role in DNA damage recognition and the initial phosphorylation events (Tibbetts et al 2000, Zhou & Elledge 2000).

ATM gene is responsible for Ataxia-telangiectasia disorder characterized by radiosensitivity, immunodeficiency and increased genomic instability (Shiloh 1995).

ATM gene is located on chromosome 11q22-23 and plays a major role in signaling pathways activation by DNA damage. Missense and truncation mutations in the ATM gene have been demonstrated in adult leukemia (Bullrich et al 1999, Schaffner et al 1999, Stoppa-Lyonnet et al 1998, Yuille & Coignet 1998), as well as in Mantle cell lymphoma patients (Camacho et al 2002, M'Kacher et al 2003a, M'Kacher et al 2003b, Schaffner et al 2000, Stilgenbauer et al 1999). Some studies have investigated the possible involvement of ATM gene in the pathogenesis of pediatric HL (Offit et al 2002), (Liberzon et al 2004). The rare polymorphic variant of the ATM gene were observed in two series of children HL: 5 of 14 children (35%) (Takagi et al 2004) and 2 of 23 patients (9%) (Liberzon et al 2004) and associated in this series with a more aggressive clinical course of the disease. Nevertheless, ATM expression and function are impaired in many HL cases (Starczynski et al 2003) and in several HL-derived cell lines (Dutton et al 2006, Takagi et al 2004). Using L428 cell line, characterized for the aberrant down-regulation of ATM activity (Dutton et al 2006) and up-regulation of FLIP protein levels (Dutton et al 2004, Mathas et al 2002) transient transfection of ATM is sufficient to decrease FLIP levels and sensitive L428 cells to FAS s induced apoptosis (Stagni et al 2008). The study demonstrated that targeting of ATM kinase activity significantly contributes to death receptor resistance of HL cell lines. The validation of this approach needs supplementary investigation. Of note, the screening of 52 survivors of HL, adults and children, who developed secondary malignancies, demonstrate the absence of ATM mutation (Nichols et al 1999).

The ataxia telangiectasia and Rad3-related (ATR) plays an important role in maintaining genome integrity during DNA replication through the phosphorylation and activation of Chk1 and regulation of the DNA damage response.

Only one paper (Liu et al 2008) examined ATR gene alterations in 8 HL cell lines and 7 clinical cases after HRSC isolated from tissues affected by HL using laser capture microdissection (LCM) microscopy. ATR alterations were detected in 6 out of 8 HL cell lines and in 3 out of 7 clinical cases. Three alterations, deletion of exon 4, deletion of exons 29-34 and insertion of 137 bp in exon 46/47 were commonly observed in both cell lines and clinical samples. Interestingly, HL cells with ATR alterations except for deletion of exon 4 showed a delay/abrogation in the repair of DNA double-strand breaks (DSBs) and single-strand breaks (SSB) as well as

exhibiting a defect in p53 accumulation. These studies demonstrate the possible role of ATR alteration in the accumulation of genomic instability in HRS cells.

5- FOX pathway

FOX genes encode transcription factors which regulate basic developmental processes during embryogenesis and in the adult (Nagel et al 2014). Several FOX genes show deregulated expression in particular malignancies, representing oncogenes or tumor suppressors (Benayoun et al 2011, Hannenhalli & Kaestner 2009). FOXP1 and FOXM1 play contrasting role in the pathogenesis of B-cell lymphoma including Hodgkin lymphoma. The screening of six Hodgkin lymphoma cell lines for FOX gene activity by comparative microarray profiling, revealed overexpression of FOXC1 and FOXD1, and reduced transcription of FOXN3, FOXO1, and FOXP1 (Nagel et al 2014). The analysis of FOX genes in HL patient's samples supported these findings. The authors proposed that the amplification of FOXC1 at 6p25 and a gain of the FOXR2 locus at Xp11 were the results of genomic aberrations and the implementation of the TGF β - and WNT-signaling pathways in the deregulation of FOXD1 and FOXN3. In addition, Vogel MJ et al has provided a link between FOX1 repression and PRDM1a downregulation in HL and proposed PRDM1a as a tumor suppression in HL (Vogel et al 2014).

Genomic instability mechanisms in Hodgkin lymphoma

Two distinct major mechanisms have been described for genetic instability. The first is a distinct DNA mutation (microsatellite instability) and the second, the accumulation of numerical and structural aberrations implying gain and loss of chromosome regions (chromosomal instability).

1- Microsatellite instability

Microsatellite instability (MSI) is characterized by very high mutation rates at small DNA repeat sequences (1-6 base pairs in length). This phenotype is caused by abnormal functioning of DNA mismatch repair (MMR) genes. MSI is the most prevalent cause of hereditary non-polyposis colon cancer (HNPCC) (Aaltonen et al 1993, Ionov et al 1993, Thibodeau et al 1993), but may also result in gastric cancer, endometrial cancer (Duval et al 2002), ovarian cancer, hepato-biliary tract cancer, urinary tract cancer, brain cancer and skin cancers. In addition MSI was a feature of

immunodeficiency- related non-Hodgkin lymphomas and HIV-related lymphomas (Borie et al 2009, Duval et al 2004). However, no evidence of HL involvement has been observed. The role of MSI in HL has not been extensively investigated. The few studies that have been carried out were far from conclusive; one study reported the presence of three out of four microsatellites exhibiting interstitial rearrangements in one HL cell line (MacLeod et al 2000), while a second study showed the absence of MSI in lymph nodes of patients indicating that the MMR system is unlikely to contribute to the genomic instability in HL (Re et al 2002a).

2- Chromosomal instability

The absence of known specific cytogenetic abnormalities represents a significant challenge for the early treatment and prevention of HL. The lack of a cytogenetic signature is due to a high level of inter-individual variation of chromosomal aberrations in HL patients, suggesting chromosomal instability. The mechanisms of chromosomal instability in HL and the evolution of the Reed-Sternberg cell are completely unknown, keeping Hodgkin Lymphoma an enigmatic disease. Moreover, the scarcity of HRS cells and the resulting technical problems of their *in situ* characterization, the primary cytogenetic events and the clonality of these possible aberrations has been a matter of debate in the past. As a consequence, a few accepted and established cHL cell lines are widely used in the majority of research projects conducted worldwide (Drexler & Minowada 1992, Kanzler et al 1996).

2-1 Chromosomal aberrations were not restricted to H-RS cells

HRS cells represented only a minority of 0.1% to 1% of the total cell population in affected lymphatic tissue. Small mononucleated cells were characterized by a higher proliferation potential compared to bi-or polynucleated cells. Su-Ming Hsu(Hsu et al 1988) demonstrated the lack of bromodeoxyuridine (BrdUrd), an analogue of thymidine that is incorporated into the nuclei of replication cells, uptake by multinuclear cells in culture. Many years after, Kuppers confirmed the very low proliferation capacity of these cells (Rengstl et al 2013). These data have been confirmed in our laboratory using Cytocalasin B, an agent that has been most widely used to block cytokinesis and the separation of daughter cells after mitosis. No proliferation of binucleated cells was observed after 5 days of culture in the presence of cytochalasin B as well as in single-cell culture in 7 HL cell lines. The lack of

proliferation of binucleated cells could show that these cells are likely to be end-stage cells, as it has been suggested already (Peckham & Cooper 1969, Peckham & Cooper 1973).

On another hand, using *in situ* hybridization, Jansen et al (Jansen et al 1998) demonstrated the presence of numerical aberrations with a range of 1-12% with trisomy occurring frequently in freshly frozen or paraffin-embedded biopsy samples of HL patients. The abnormal nuclei differed significantly in size of HRS cells but did not differ in morphology or size from normal cells leading to the possible role of this subset of morphological normal cells in malignance HL cells. The presence of higher frequency of chromosomal aberrations in circulating lymphocytes of HL patients prior to treatment have been demonstrated previously (Barrios et al 1988, Falzetti et al 1999, M'Kacher et al 2003c, Wolf et al 1996).

2-2 Centrosomes, micronuclei and aneuploidy

Centrosome amplification is an early event in the development of cancer. Indeed, an increase in centrosome size and number correlates with chromosomal instability (Lingle et al 2002). Aneuploidy is a common characteristic of cancer cells, and it has been established that centrosome amplification can significantly contribute to aneuploidy by favoring chromosome mis-segregation during mitosis (Vitre & Cleveland 2012). Another consequence of chromosomal amplification and defects in the spindle mechanism is the formation of micronuclei, which arise due to DNA damage or mis-segregated chromosomes (Fenech 2007). Together with aneuploidy, micronuclei play a proven role in cancer risk.

Krem et al. showed that a potential candidate responsible for the induction of centrosomal amplification is the Kelch protein KLHDC8B (Kelch-Like Domain-Containing protein 8B) (Krem et al 2012). This protein, which is expressed during mitosis, was found to be mutated in a subset of familial and sporadic HL (Timms & Horwitz 2010). He then showed that KLHDC8B is responsible for the maintenance of chromosomal stability thus establishing for the first time an association between a specific gene and centrosomal amplification, aneuploidy and micronucleus formation in HL (Krem & Horwitz 2013). However, the precise role of centrosome amplification or duplication, micronuclei formation and other nuclear abnormalities such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs which are related to aneuploidy and chromosomal instability, remain unclear for HL.

Of note, it has been observed that lymphocyte radiosensitivity correlates with the frequency of micronucleated cells prior to treatment suggesting that high MN frequency in HL patient lymphocytes before treatment can serve as a prognostic factor for the effectiveness of radio and chemo therapy (Pelevina et al 2012).

2-3 Chromosomal aberrations and gene amplification in HL

Despite the multitude of studies performed with the goal to find a specific cytogenetic marker, it is disappointing that no significant translocation indicating a possible gene or genes involved in the etiology of Hodgkin lymphoma has been found. However, these studies provided important information by unequivocally demonstrating the presence of a clonal population of cells with malignant characteristics, frequently with complex chromosomal rearrangements, (M'Kacher et al 2003c, Salas et al 2012), as well as considerable aneuploidy in not only Hodgkin and Reed-Sternberg cells but also normal lymphocytes in HL, which was highly variable from case to case.

Furthermore, it was shown that a number of nonrandom changes, including several that are also common in other malignancies, including the non-Hodgkin's lymphomas, are frequently present, e.g. deletions of del(4q), del(6q), and del(7q), translocations such as t(2;5), t(14;18) leading to overexpression of BCL2 (Yoshida et al 2012), or t(14;19), overexpressing BCL3 (Martin-Subero et al 2006). While these are common for specific types of non-Hodgkin lymphomas; they may show some specificity for HL, but until now, no study has shown their systematic occurrence in HL.

The amplitude of aberrations in cells of HL patients is indicated by the significant presence of complex chromosomal rearrangements.

Several studies analyzing copy number changes in micro-dissected Hodgkin RS cells, using comparative genomic hybridization (CGH), have provided evidence of genomic imbalance involving several regions of either chromosome gains including dup(2p), dup(9p), dup(17q), dup(19q), or dup(20q), or chromosome losses including del(6q) and del(13q) (Steidl et al 2010c) (Hartmann et al 2008). Notably, several duplicated regions include genes known to be constitutively expressed in cHL. Amongst these, gains of STAT6 (12q13), NOTCH1 (9q34), and JUNB (19p13) were confirmed (Hartmann et al 2008).

In a recent study, a novel Hodgkin lymphoma susceptibility locus on chromosome 19p13.3 was identified, which includes the transcription factor 3 gene (known as TCF3 or E2A immunoglobulin enhancer-binding factors E12/E47), which has been shown to be associated with pre-B-cell acute lymphoblastic leukemia (B-ALL) (Cozen et al 2014).

Another candidate gene possibly involved in the malignant transformation of HRS cells is Janus Kinase 2 (JAK2), a non-receptor tyrosine kinase located on chromosome 9p24. An increasing body of evidence shows that JAK2 gene rearrangements and amplifications occur in cHL. A recent study documented the association between 9p24.1 copy number and PD-1 ligand expression in cHL and the frequent involvement of the JAK2 locus (Green et al 2010). Other studies, using either Fluorescence in situ hybridization (FISH) (Martin-Subero et al 2003, Van Roosbroeck et al 2011), CGH array (Joos et al 2000), Western blot (Navarro et al 2009) or chemical inhibition (Green et al 2010), indicate the interaction of JAK 2 with different genes as a susceptibility locus in cHL patients and HL cell lines.

Telomere dysfunction in Hodgkin Lymphoma

Telomeres are dynamic nucleoprotein structures that protect the ends of chromosomes from degradation and activation of the DNA damage response. Many studies have demonstrated the important role that telomeres play in the maintenance of genome integrity and stability. When telomeres become too short, and before genes are affected, or chromosomes fuse together, cells stop dividing and undergo senescence. It is now well documented that telomere dysfunction is an important biomarker of aging and can be used in the prognosis of several diseases (Chin et al 2004, Meeker & Argani 2004, Tanaka et al 2012). In HL lymph nodes and established cell lines, HRS cells were characterized by disturbed nuclear architecture, disruption of the sheltering complex and erosion of telomeres (Knecht et al 2010a, Knecht et al 2009, Mai 2010), with the presence of telomere-poor 'ghost' nuclei (Knecht et al 2010b). The significant difference was observed between Hodgkin cells and Reed Sternberg cells suggesting the loss of telomere sequences during the transition between Hodgkin and Reed Sternberg cells. The organization of telomeres in Hodgkin tumor cells and Reed-Sternberg cells can predict treatment outcome. Knecht, H. et al. 2012 showed that telomere organization in aggregates,

i.e. clusters of telomeres in nuclei, can predict a poor response of patients to conventional treatment (Knecht et al 2012).

The mechanism of telomere maintenance in HL lymph node and established HL cell lines was performed only by few papers and has been a subject of debated. Norrback et al (Norrback et al 1998) has demonstrated the presence of telomerase activity in 31 of 77 HL lymph nodes at decreased levels and a high levels of telomerase expression in HL cell lines. Nevertheless, Brousset et al after demonstrated the presence of telomerase activity in HL lymph nodes (Brousset et al 1997) and using a large series of samples, confirmed that the majority of cases (only 2 cases positive out of 20) lack telomerase activity. A telomerase-independent mechanism for telomere maintenance in HL has been proposed in the absence of detectable telomerase activity.

There is solid evidence that telomere length in peripheral blood lymphocytes can aid in the prediction of the development of a secondary cancer following treatment for Hodgkin lymphoma (M'Kacher et al 2007) and it represents a risk factor for the occurrence of secondary diseases, i.e. cardiovascular disease (Girinsky et al 2014).

All these findings help in understanding the influence that telomeres have, first on the occurrence of the disease, second, on the treatment outcome, and last, on the long term effects after the treatment. Telomeres represent a possible marker for individual chromosomal instability and provide a first hint for an eventual individualized telomere-dependent treatment.

Viral infection and chromosomal instability in HL

Viruses have been shown to be responsible for 10%-15% of cancer cases. The epidemiological features of Hodgkin Lymphoma suggest that an infectious agent may be involved in its pathogenesis. The role of viral infection in malignant transformation and chromosomal instability is still debatable. Furthermore, numerous studies are focused on the importance of the time of first infection, for example of the uterus, at a very young age or as an adult. Later exposure to common infections may predispose certain individuals to the development of lymphomas (Michos et al 2009).

1- EBV

Epstein–Barr virus (EBV) is a human gamma-herpes virus that establishes latent infections in B lymphocytes, where only a subset of viral genes is expressed and

virus replication is suppressed (Murata 2014). The prevalence of EBV in HRS cells varies according to the histological subtype and epidemiological factors. The highest frequency is found in MC HL and the lowest incidence in NS HL. Approximately 40% to 60% of cHL in the Western world are EBV positive. The pathogenesis role of EBV in HL has been suggested as well as the contribution to the survival of HRS cells (Vockerodt et al 2015). EBV-infected RS cells were shown to stimulate the stroma production of particular chemokines such as CXCL10 (Teichmann et al 2005) and to produce CCL20, capable of attracting regulatory T-cells (Treg) to the disease site (Baumforth et al 2008, Flavell et al 2008). Latent membrane protein (LMP) 1 and 2 are expressed in HRS cells. LMP1 aggregates on the cell membrane, mimicking and activating CD40 receptor, leading to the constitutive activation of NF- κ B pathway (Kilger et al 1998). Also, it has been suggested that immunologic reaction against EBV can occur in the peripheral blood of some HL patients (Khan 2006). The oncogenic role of EBV in HL or other neoplasm such as Burkitt lymphoma has been investigated. EBNA-1 and LMP-1, independently promote genomic instability by inducing DNA damage, the first by inducing reactive oxygen species (ROS) via transcriptional activation of the catalytic subunit of the leukocyte NADPH oxidase, NOX2/gp91phox (Kamranvar & Masucci 2011) and the second by inhibiting DNA repair through down-regulation of the DNA damage-sensing kinase, ataxia telangiectasia mutated (ATM) and inactivation of the G2 checkpoint (Gruhne et al 2009). Short telomeres were associated to LMP1 expression in HRS cells, even in young patients (Knecht et al 2012) and the EBV nuclear antigen-1 (EBNA1) induces loss or gain of telomere signals and promotes telomere fusion (Kamranvar & Masucci 2011). Recently, Lajoie et al (Lajoie et al 2015) demonstrated that LMP1-dependent deregulation of telomere stability and nuclear organization via shelterin downregulation, in particular TRF2, favors chromosomal rearrangements. The hypothesis that telomeric aggregates and ongoing breakage-bridge-fusion cycles lead to disturbed cytokinesis and finally to multinuclearity, as proposed in EBV-associated HL (Lajoie et al 2015).

Moreover, it has already been shown that patients positive for LMP-1 expression have a poor prognosis, suggesting LMP-1 as a new prognostic marker for HL patients (Ping et al 2014). While EBV is identified as an independent prognostic factor for poor outcome in countries where EBV infection prevails, (Elsayed et al 2014), studies using pooled data obtained from different regions including developed and underdeveloped countries show that the presence of EBV in cHL has little effect on overall or event-free survivals (Lee et al 2014).

2- HHV6

While much attention has been focused on the role of EBV in HL, growing evidence suggests that other viruses may also be associated with HL. A relevant herpesvirus often linked to HL, from the same family of Herpesviridae as EBV, but from the **Betaherpesvirinae** subfamily, is Human Herpes Virus type 6 (HHV-6) (Braun et al 1997, De Bolle et al 2005). HHV-6 was isolated in 1986 from patients with lymphoproliferative diseases and HIV (Salahuddin et al 1986), demonstrating a high affinity for CD4 positive T lymphocytes (Lusso et al 1988, Takahashi et al 1989). Two types of HHV6 were classified: HHV-6A and HHV-6B (Dominguez et al 1999).

HHV-6 was found in various lymphomas, particularly in Hodgkin lymphoma (HL).

The association between HHV-6 and HL was suggested by Torelli et al. (Torelli et al 1992). Since then there is a growing number of publications on the association between HHV-6 and Hodgkin lymphoma.

Marasca R. et al reported (by PCR) the presence of HHV-6 in samples obtained from peripheral blood and lymph nodes in HD patients (Marasca et al 1990).

Anti-HHV-6 antibodies were detected in the serum of patients suffering from Hodgkin lymphoma (Berrington de Gonzalez et al 2006, Shanavas et al 1992, Torelli et al 1992). HHV-6 DNA was also detected by quantitative PCR in lymph nodes (Secchiero et al 1995) and in lymph nodes and saliva by quantitative real time PCR (Collot et al 2002).

The HHV-6 virus type B is the most often associated with HL (Lacroix et al 2007, Schmidt et al 2000) although in the absence of EBV (Lacroix et al 2007).and it is frequently detected especially in Nodular Sclerosis HL subtype (Collot et al 2002, Lacroix et al 2007, Torelli et al 1991).

Several studies have reported the oncogene characteristic of DR7 (ORF-1) protein of the HHV-6 type A (DR7A) (Kashanchi et al 1997) and of DR7B, showing its ability to bind to p53 and leading to an increase of NF-kB expression (Lacroix et al 2010).

All these studies suggest that the HHV-6 may have an oncogene role in the Hodgkin lymphoma.

Among the members of the Herpesviridae family, HHV-6 is certainly one whose integration into the host genome is the most regularly observed (CIHHV-6). On average, the prevalence of CIHHV-6 is estimated of 1 to 2% of the world population.

Luppi et al were the first to report the integration of the viral genome of HHV in the PBMC cellular genome of one patient suffering from multiple sclerosis and two other patients suffering from lymphoproliferative disease (Luppi et al 1994, Luppi et al 1993).

Both types of HHV6 have the ability to integrate into the human genome. Nevertheless the CIHHV-6B is about 2 times more common than CIHHV-6A (Morissette & Flamand 2010, Nacheva et al 2008). There appears to be no preferential locus of chromosomal integration of the virus in the human genome. However chromosome 17p13.3 seems to be frequently targeted (Morissette & Flamand 2010).

The consequences of acquisition of CIHHV-6 are still unknown. However, Huang et al. reported that a telomere which harbors CIHHV-6 is subject to truncations, which results in the shortening of the telomere (Huang et al 2014).

3- JC virus

JCV, a human Polyomavirus, is known to establish latent or persistent infections in humans, to undergo periodic reactivation, and to cause disease mostly in cases of immunodeficiency, such as in AIDS or during treatment with drugs intended to induce a state of immunosuppression (Ferenczy et al 2012). It is a ubiquitous virus commonly found in the general population with a seroprevalence in adults of 39-81% (Dalianis & Hirsch 2013). Most people acquire JCV in childhood or adolescence.

The etiologic contribution of polyomaviruses is suggested to represent a “driver” role in cancer by one of three mechanisms. The first mechanism is the “hit-and-run”; where viral infection contributes at an early stage to oncogenic progression, by promoting chromosomal instability; but the virus is no longer detected at the time of diagnosis. This mechanism is the most relevant for virus-cancer interaction. The second mechanism called “passenger”, is where the virus acts in an already transformed cell, but it doesn’t contribute to the acquisition of oncogenic characteristics, and the third mechanism is the “by-stander”, where it infects the neighboring cells, or is detected in other neighboring anatomic compartments but is not directly related to the malignancy. Several studies have demonstrated the chromosomal instability induced by JCV infection (Cougot et al 2005, Vilkin & Niv 2011) (Cougot et al 2005). However, the etiological role of JCV in Lymphoma is still controversial (Del Valle et al 2004, Engels et al 2005, Gellrich et al 2005, MacKenzie

et al 2003, Vilchez et al 2002). A role for JCV as a factor in Hodgkin lymphoma was first suggested by Carde et al. in 2006, who associated the co-detection of JCV and EBV genomes in HRS cells and peripheral blood lymphocytes (PBL) of relapsing HL patients, especially young patients (Carde P 2006.). Moreover, M'kacher et al. demonstrated an association between JCV and chromosomal instability in PBL of HL patients. The presence of rogue cells, with multiple chromosomal aberrations including dicentric formation, was documented (Neel 1998). Interestingly, JCV and EBV coactivation was correlated to shorter progression free survival and the mechanism of “hit and run” was proposed in HL lymphoma (M'Kacher et al 2010).

Interaction between genomic instability and clinical outcomes of HL patients

Correlation between chromosomal aberrations and histopathological subgroups in HL patients as well as the clinical outcomes of these patients, have never been reported. Classical cytogenetic studied has not contributed much in the past, mainly because of difficulties in obtaining a sufficient number of mitoses from Reed-Sternberg cells. Nevertheless, the presence of complex chromosomal rearrangement in HL karyotype was founded in advanced stages of the disease (Falzetti et al 1999).

Using new methods to evaluate genetic lesion such as gene expression profiling (Sanchez-Aguilera et al 2006), comparative genomic hybridization analysis (Giefing et al 2008), micro-RNA expression profiling (Greaves et al 2013) and viral oncogene sequencing (Goodrich & Duesberg 1988), and clinical outcomes of HL patients were performed and possible therapeutic targets in refractory or relapsed patients were proposed. The major role of microenvironment of HRS in tumor progression was demonstrated previously and the number of tumor-associated macrophages in the tumor microenvironment of CHL has been shown to be associated with shortened PFS and OS (Steidl et al 2011, Steidl et al 2010b).

The telomere shortening and the presence of telomere aggregate in HL lymph nodes were correlated age dependent to the higher risk of relapse in HL patients (Knecht et al 2012). Also telomere shortening in peripheral blood lymphocytes assessed in retrospective cohorts of HL patients has been correlated to the higher occurrence of second cancer (M'kacher et al 2007) and cardiovascular disease (M'kacher et al 2014).

Microenvironment-targeted biomarkers of treatment outcome have become an increasing interest. Histopathological markers as well as serum markers and gene expression analysis have been extensively studied.

Conclusion

After a major progress in the understanding on the pathogenesis of HL in the last two decades, the mechanisms underlying genomic instability of HL stay obscure as well as the prognostic factors of chromosomal instability. Despite the identification of the cellular origin of tumors cells and various cellular and molecular pathways in the pathogenesis of HL, the primary transforming events of HL still remain unknown. Taken all together these alterations are probably secondary and may reflect genetic instability of HRS cells. However, the cytogenetic characterization of clonotypic cell suggested by Jones et al (Jones et al 2009) and approved their presence by Oki Yasukiro et al (Oki et al 2015) in peripheral blood of HL patients will represent the first step in the determination of primary transforming events.

The lack of reliable animal model of Hodgkin lymphoma presents a major obstacle for mechanistic studies to understand the pathology as well as the development of novel therapies. The understanding of the interaction between malignant and non-malignant cells in the tumor microenvironment of HL could elucidate some events in this transformation. The monitoring of nuclear morphology in real-time by combining time-lapse microscopy coupled with specific DNA probes could provide some information not only concerning the formation of RS cells (re-fusion or endomitosis) but also the numerical chromosomal aberration and the further of micronucleus cells observed in lymph nodes and HL cell lines. We believe that there is not only one event leading to the initiation of HL but several events, as there is no single cytogenetic entity in the cHL. The characterization of the bridge between all these pathways and the subtype of HL permits us to best understand the mechanisms underlying this pathology.

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Part II

The identity of the possible mechanisms responsible for genomic instability in Hodgkin lymphoma remains unclear largely because of the growing difficulty of Hodgkin lymphoma cells *in vitro* and *in vivo*. The use of Hodgkin lymphoma cell lines represented actually the unique issue to approach this problem. In the second part on this thesis, we have investigated the possible mechanisms underlying genomic instability including microsatellite and chromosomal instability in 7 HL cell lines using different approaches and techniques.

First, we have characterised the immunophenotype of HL cell lines using conventional markers of HRS cells (CD30 and CD15) as well as the p53 status using a functional assay.

Second, we have reevaluated the possible implications of microsatellite instability in Hodgkin cell lines using Revised Bethesda Guidelines (Umar et al 2004). We demonstrated for the first time the presence of high microsatellite instability in 3 HL cell lines and the absence of MSI in one cell line.

Third, we provide the first evidence that chromosomal instability was related to two major mechanisms: first, telomere fusion leading to dicentric formation and breakage/fusion/bridge cycles involving the repeated fusion and breakages of chromosomes following the loss of telomeres in small cells, compared to Reed Sternberg cells. This mechanism was found essentially in Nodular Sclerosis sub group. The second mechanism is related to defective DNA repair via non homologous end-joining (NHEJ) with the presence of nucleoplasmic bridges without telomere or centromere sequences, accompanied by the micronucleus without centromere sequences and a higher frequency of sporadic dicentric chromosomes. High heterogeneity of telomere length in these cells lines was found.

This work is a first step in understanding genomic instability (initiation and progress) allowing us to evaluate the role of small cells in genomic instability.

Multiple mechanisms can lead to genomic instability in Hodgkin lymphoma cell lines: Microsatellite or chromosomal instability related to telomere end fusion or DNA repair defects

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Acknowledgements

This work was supported by a grant from the European Community's Seventh Framework Program (EURATOM) contracts Fission-2011-249689 (DoReMi). We are indebted to Pr H de Thé (Laboratoire de biochimie B, Hopital Saint-Louis, Paris) for p53 and MSI investigations. We also thank Francis Finot and André Essahli (Covance Laboratory Porcheville, France) for micronucleus investigations and Wala Najar for technical help.

Abstract

Background:

Hodgkin lymphoma (HL) is a malignancy characterized by the presence of scarce tumor cells called Reed-Sternberg cells (HRS), derived, in most cases, from germinal center B cells. Genomic instability is a characteristic feature of HRS. In this study, we have investigated the possible mechanisms underlying genomic instability including microsatellite and chromosomal instability in HL cell lines.

Materials and Methods:

Seven established HL cell lines were used: L428; L540; L591; L1236; HDLM-2; KMH2 and SUP-HD1. Microsatellite instability was assessed by PCR and chromosomal instability including telomere status using FISH techniques. Molecular approaches to characterize the p53 status and DNA repair mechanisms were performed.

Results:

The characterization of the HL cells revealed p53 mutations for three cell lines (L428, L1236 and HDLM2). The highest frequency of MSI was observed for L428 (4/5), KMH2 and HDLM2 (3/5), and the lowest for L540, L591 and SUP-HD1 and none for L1236. The analysis of telomere dysfunction revealed a high level of intercellular telomere length heterogeneity associated with nuclear shape alterations and a low level of lamin B as well as the accumulation of BP53 and γ H2Ax foci for all HL cell lines. Telomere dysfunction was associated with the progression of chromosomal instability due to the presence of dicentric chromosomes and breakage/fusion/bridge (B/F/B) cycles involving the repeated fusion and breakage of chromosomes following the loss of telomeres associated with the lower expression of TRF2, as well as an elevated copy number of the Jak2 gene and the presence of nucleoplasmic bridges containing telomere and centromere sequences. This mechanism was observed primarily in HDLM2. The second mechanism which was observed, essentially in L1236 and KMH2, was related to defective DNA repair via non homologous end-joining (NHEJ) repair with the presence of nucleoplasmic bridges without telomere or centromere sequences, accompanied by the micronucleus without centromere sequences and a higher frequency of sporadic dicentric chromosomes. In addition,

the p53 mutations revealed for the three cell lines (L428, L1236 and HDLM2) appear to be independent of the mechanisms underlying chromosomal instability.

Conclusion:

We provide the first evidence that the genomic instability observed in HL is related to MSI and chromosomal instability related to two major mechanisms: telomere fusion and the NHEJ repair pathway. These insights can be exploited for the development of novel prognostic biomarkers and innovative therapies.

Key words: Hodgkin lymphoma, chromosomal instability, telomere dysfunction, MSI, P53

Introduction

Hodgkin lymphoma (HL) is a unique disease in which the neoplastic Hodgkin and Reed–Sternberg (HRS) cells account in most cases for only 1% of the tumor tissue, with the remaining tumor microenvironment composed of various inflammatory cells (Stein et al 2008). Although HRS cells display multiple chromosomal aberrations, few are recurrent and the targeted genes, if they exist, remain unknown. Nevertheless, understanding the pathology of HL and developing rational therapies may well require identifying the mechanisms underlying oncogene alteration. Several aberrantly activated pathways have been identified in HL cells, comprising NF- κ B, JAK/STAT, MAPK, and the PI3K pathways (Matsuki & Younes 2015). As in lymph node tumors, no specific chromosomal rearrangements have been observed (Atkin 1998, Falzetti et al 1999) but the occurrence of complex chromosomal abnormalities and hyperploidy have been observed within HRS cells (Weber-Matthiesen et al 1996). It remains to be shown by what mechanisms somatic chromosome changes occur in HRS cells.

Genomic instability is a feature of HRS cells and plays a crucial role in the initiation and progression of HL. There are two major distinct oncogenic pathways, namely, microsatellite instability (MSI) and chromosomal instability (Al-Sohaily et al 2012). MSI is considered to be the result of a defect in DNA mismatch repair (MMR) genes. The implication of MSI in oncogenic processes and clinical outcomes has been linked not only to colorectal carcinoma (Eveno et al 2010, Weber-Matthiesen et al 1996) but also several other solid tumors (Deschoolmeester et al 2011) and hematological diseases (Borie et al 2009). The only study that investigated the implication of MSI in the L1236 cell line and 7 primary cases of HL highlighted the proficient DNA MRR systems in HRS cells and concluded that MSI was not implicated in the oncogenic process of these cells (Re et al 2002a).

Chromosomal instability is defined as an accumulation of numerical and structural chromosomal aberrations. One important mechanism for chromosome instability is through the loss of telomeres (Blackburn 2001, De Lange 1995, De Lange 2005). Telomeres consist of non-coding DNA at the end of chromosomes, and play an important role in the maintenance of genome integrity and stability. The loss of the protective function of telomeres leads to the formation of chromosomal fusions and

“breakage-fusion-bridges” (B/F/B) cycles, which can lead to chromosome imbalances, gene amplification, non-reciprocal translocation and altered genetic expression. Telomere shortening has been described in HL lymph nodes as well as in peripheral blood lymphocytes in HL patients and has been associated with poor clinical outcome (Guffei et al 2010, Knecht et al 2012, M'Kacher et al 2007, M'Kacher et al 2015). The transition from mononuclear to binuclear cells in HL has been associated with telomere loss (Guffei et al 2010). An additional mechanism that can lead to chromosomal instability is the formation of a dicentric chromosome which has been observed in oncogenic processes, particularly in hematological disorders (Gascoigne & Cheeseman 2013). The presence of dicentric chromosome has not been described briefly for HRS cells, but a high frequency of dicentric chromosomes in circulating lymphocytes has been reported for untreated HL patients (M'Kacher et al 2007, M'Kacher et al 2003c). In addition, aneuploidy or chromosome missegregation is considered to be another major chromosomal instability pathway (Krem & Horwitz 2013). Centrosome amplification associated with aneuploidy has been proposed to drive chromosome instability in HL (Knecht et al 2012). The precise role of telomere shortening, dicentric chromosome formation and aneuploidy in generating chromosomal complexity and ongoing instability remain unclear for HL. Because of the problems encountered in the molecular and cytogenetic analyses of primary Hodgkin's tumors, due essentially to the lack of in vitro growth of tumor cells, cell lines derived from malignant HRS cells are often used (Jansen et al 1998). In the present study, 7 HL cell lines were characterized with respect to HL surface markers and TP53 status. MSI was reevaluated and chromosomal instability including telomere dysfunction, dicentric chromosome and micronucleus formations, as well as the karyotypes, were investigated.

In this paper, we aim to understand the possible mechanisms underlying genomic instability in HL potentially involved in the development of this disease by applying these combined molecular and cytogenetic approaches. We demonstrate, for the first time, the presence of two potential mechanisms for the transformation of HRS cells: MSI and chromosomal instability including telomere dysfunction, dicentric chromosome formation, complex chromosomal rearrangements, B/F/B cycles and aneuploidy. We analyzed these two mechanisms in L1236 and HDLM2 cell lines in more detail. We provide the first evidence of multiple mechanisms for genomic

instability in HL suggesting a potential role for these mechanisms in disease outcome and treatment modalities of HL patients.

Materials

Cell lines used and Culture Conditions

The human HL-derived cell lines L428 (Schaadt et al 1980), L591 (Schaadt et al 1980), SUP-HD1 (Naumovski et al 1989), L540 (Schaadt et al 1980), HDLM2 (Diehl et al 1985), L1236 (Wolf et al 1996) and KMH2 (Kamesaki et al 1986), were obtained from Dr Steffen Junker (Aarhus University Denmark). The origin and the histological characteristics are shown in supplementary data 1A.

The Hodgkin lymphoma cell lines and the in vitro EBV-infected cell lines NAD and REMB were cultured at 37 °C in an atmosphere containing 5 % CO₂ in RPMI 1640 medium (Gibco, Grand Islands, NY) supplemented with 10 % Fetal Bovine Serum (Eurobio, Courtaboeuf, France) and 1% Antibiotic-Antimycotic (Gibco, Grand Island).

Cytogenetic slides (Preparation of Metaphase Spreads)

The cells were exposed to colcemid (0.1 µg/mL) (Gibco KaryoMAX, Cat. no. 15212-012) for 2 hours at 37 °C, 5 % CO₂, in a humid atmosphere in order to block cells in metaphase. After harvesting the cells and centrifugation for 7 min at 1400 RPM at RT, the supernatant was removed, and the cells were re-suspended in a solution of warm (37 °C) 0.075 M potassium chloride (KCl) (Merck) and incubated for 20 min in a 37 °C water bath (hypotonic shock). For the pre-fixation of the cells, around 5 drops of fixative (3:1 ethanol/acetic acid) was added to each tube under permanent agitation, and the tubes were centrifuged for 7 min at 1400 RPM at RT. The supernatant was removed, and the cells were suspended in fixative solution and centrifuged using the same parameters. After twice more repeating these fixative steps, the cells were stored in fixative solution at 4 °C overnight, and metaphases were spread on cold, wet slides the next day. Slides spread with metaphases were dried overnight at RT, and stored in -20 °C until further use.

Methods

CD30 and CD15 detection and cell cycle analysis using Flow Cytometry

Immuno-phenotyping of Hodgkin lymphoma cell lines was performed on a LSRII flow cytometer (BD) using FITC- and APC-conjugated mouse anti-human specific monoclonal antibodies for CD30 (Ber-H83) and CD15 (HI98), respectively. Both antibodies were obtained from BD Biosciences. Staining for CD15 and CD30 was performed according to the manufacturer's instructions. After addition of 1ug/ml Hoechst (BD PharMingen) to discriminate dead cells, the cells were analyzed with FACS as previously described (Baerlocher et al 2006).

Cell-cycle fractions were determined by Hoechst nuclear staining. Briefly, cells were harvested, washed in PBS, and incubated in Hoechst solution for 30 minutes at room temperature. Data were collected using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo Version 7.5.5. The results represent the mean value of 3 independent experiments.

TP53 functional assay for screening of the cell lines

To study the functionality of the TP53 gene, a functional assay was performed in accordance with the method described by Flaman et al (Flaman et al 1995). The assay tests the entire DNA-binding domain (aa 102-292) as the p53 expression vector is linearized at codons 67 and 346.

Briefly, TP53 mRNA is reverse transcribed, amplified by PCR and co-transfected into yeast with a linearized expression vector carrying the 5' and 3' ends of the p53 open reading frame. Gap repair of the plasmid with the PCR product results in the constitutive expression of human TP53 protein. Yeast that has repaired the plasmid are selected on a medium lacking leucine. The medium contains sufficient adenine for growth of Ade⁺ cells, leading to the generation of white colonies. Thus, colonies containing wild-type TP53 are white (Ade²⁺) and colonies containing the TP53 mutation red. After identification of non-functional TP53 samples, red colonies were cloned and sequenced by the Sanger method to characterize the TP53 mutation. Finally, we classified the mutations using the IARC p53 database according to their impact on the protein.

Evaluation of Microsatellite Instability (MSI) in HL cell lines

The Revised Bethesda Guidelines was used to determine the MSI status. Analysis of MSI was performed by 5-plex polymerase chain reaction (PCR) using a panel of 5

quasimonomorphic mononucleotide repeat markers (BAT26, BAT25, NR21, NR22, and NR24) that map to intron 15, intron 16, 5' untranslated region (UTR), 3' UTR, and 3' UTR of the MSH2, c-kit, SLC7A8, transmembrane protein precursor B5, and ZNF2 genes, respectively. A single 5-plex polymerase chain reaction (PCR) allows co-amplification of all 5 markers, which are subsequently analyzed using a genetic analyzer (ABI PRISM 310; Applied Biosystems, Courtaboeuf, France) and computerized fragment analysis. For each of the 5 primer pairs used in the PCR, 1 primer was end labeled with the fluorescent dye FAM, HEX, or NED as follows: BAT26 (5'-TGACTACTTTTGACTTCAGCC-3' and FAM-5'-AACCATTCAACATTTTAAACCC-3'), BAT25 (5'-TCTGCATTTTAACTATGGCTC-3' and NED-5'-TCGCTTCCAAGAATGTAAGT-3'), NR21 (5'-TAAATGTATGTCTCCCCTGG-3' and HEX-5'-ATTCCTACTCCGCATTCACA-3'), NR22 (5'-GAGGCTTGTC AAGGACATAA-3' and FAM-5'-AATTCGGATGCCATCCAGTT-3'), and NR24 (5'-CCATTGCTGAATTTTACCTC-3' and HEX-5'-ATTGTGCCATTGCATTCCAA-3'). The PCR contained 40 to 50 ng of DNA, 200µM deoxyribonucleotide triphosphate, 2.5mM magnesium chloride, 1µM of each primer, and 1 U of DNA polymerase (AmpliTaq Gold, Applied Biosystems) in a total volume of 20 µL. Cycling variables were 7 minutes at 95°C for one cycle, 45 seconds at 95°C, and 1 minute at 60°C for 40 cycles, with a final extension step for 10 minutes at 72°C. Two microliters of the completed PCR was added to 20 µL of HiDi formamide (Applied Biosystems) containing 1 µL of ROX (GeneScan 500, Applied Biosystems), vortexed, denatured for 2 minutes at 95°C, and immediately placed on ice. The denatured PCR products were separated by capillary electrophoresis using the genetic analyzer and were further analyzed using commercially available software (GeneScan, Applied Biosystems). HL cell lines with instability of 3 markers were defined as MSI-H, those with <3 as MSI-L or MSS if there was no instability (Boland et al 1998).

Centrosome Assay

Cells were cytospined onto slides and fixed for 15 min with 3% paraformaldehyde in PBS pH 7.4 at RT, following three washes each for 5 min in ice cold 1X PBS. Permeabilization of cells was performed by incubating slides for 10 min in 1X PBS containing 0.25% Triton X-100, following three washes in 1X PBS. Cells were then incubated with 2% BSA in PBST for 30 min to block nonspecific binding of the antibodies. The primary antibody consisting of monoclonal anti-γ-tubulin (mouse IgG1 isotype Sigma-Aldrich Cat. No. T5326) was added to the slides and they were

left to incubate over night at 4°C. The following day, the primary antibody was decanted and secondary antibody consisting of Cyanine 3-conjugated anti-mouse IgG was added and the slides incubated 1h at 37°C in a dark chamber. After several washes in 1X PBS, slides were counter stained with DAPI, mounted with PPD and stored at 4°C until image acquisition. Images of nuclei were captured using Metasystems/Metacyte software.

Immunofluorescence for DNA repairs proteins and shelterin protein complexes

Cells were cytopspined onto poly-L-lysine-coated glass slides at 700 rpm for 4 min, fixed with 10% formalin for 10 min, and treated with 0.25% Triton X-100 solution for 10 min. After blocking with 5% bovine serum albumin (Sigma), the cells were incubated overnight in 4°C with primary antibody. After washing in PBS, cells were treated with Cyanine 3 anti-mouse IgG (Invitrogen, Carlsbad, CA) or FITC anti rabbit (Sigma Aldrich) secondary antibody at 37°C for 45 min. cells were mounted with in p-phenylene diamine after counterstaining with 4,6-diamidino-2-phenylindole (Sigma). As a negative control, staining was carried out in the absence of primary antibody.

Immunofluorescent-FISH (IF-FISH)

IF-FISH was performed using a protocol similar to one described previously (Ye et al 2010). Cells were centrifuged at 1000g after 5 h of colchicine (0.09 mg/ml) treatment at 37 µC in a humidified atmosphere of 5% CO₂. The pellet was washed in 16PBS at 37uC and re-centrifuged. The cells were subjected to hypertonic shock by resuspension in 34 mM citrate at 37uC to obtain a suspension of cells at a concentration of 60 000 cells/ml and incubated for 1 h at 37uC. 200 ml of the suspension was subsequently applied to polylysine slides by cytospin. Following fixation (PFA 3%, sucrose 2%), cells were immunostained as already described. Prior to telomere hybridization with the PNA probe (CCCTAA)₃- FITC, cells were successively fixed (PFA 4%, 2 min), washed in PBS and dehydrated (50/70/100 ethanol).

Western blot analysis

DNA-PKcs, Ku 86, Ku70, γH2AX, BP53, p53 and phosphospecific p53 expression were tested by Western blotting on lysates of HL cell lines using anti-DNAPKcs (Neomarker, Fremont, CA, USA), anti-Ku70 (Oncogene, Cambridge, MA, USA), anti-p53 (Santa Cruz Biotechnology) and anti-p53 phosphospecific (Novocastra Laboratories) antibodies. Cells were sonicated in 500 µl of a buffer containing 8 mol/urea, 150 mmol/l β-mercaptoethanol, 50 mmol/l Tris-HCl (pH 7.2) and centrifuged for 30 min at 4°C to remove cellular debris. Samples were subjected to electrophoresis on 12% (p53 and Ku) or 6% (DNA-PKcs) SDS–polyacrylamide gels, blotted onto nitrocellulose membranes and developed using the ECL system (Amersham, Uppsala, Sweden). To verify that equivalent amounts of each sample were loaded, the filters were additionally probed with anti-actin antibody (AC74, Sigma). Densitometry was performed to evaluate the intensity of Ku70, p53, DNA-PKcs and actin bands.

Micronucleus assay

The cytokinesis blocking agent cytochalasin B (6 µg/mL, during 24 hours) is the agent that has been most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the formation of binucleate cells (Fenech 2007). However, cytochalasin B was not used in any of the experiments of HL cell lines due to its toxicity and the complexity of the analysis. This improvement of the technique makes it possible to score micronuclei in mononuclear cells in the absence of any confounding factors. Cells were incubated in a humidified atmosphere of 5% (v/v) CO₂ in air at 37 °C until arrest and metaphase spread. The culture medium was discarded and hypotonic shock was induced by incubating the cells with 18mM KCl solution diluted (1:3, v/v). The cells were then fixed with acetic acid/ethanol (1:3, v/v), and the cell suspension was dropped onto the slides. The slides were stored at -20 °C until further use.

Telomere quantification

Telomere quantification was performed using the Q-FISH technique with a Cy-3-labelled PNA probe specific for (TTAGGG) (Panagene). Two approaches were developed. The first approach consisted of the quantification of telomere length in interphase cells permitting the investigation of intercellular variation in a large number of scored cells. Quantitative image acquisition and analysis were performed using

Metacyte software (Metasystem, version 3.9.1). The mean fluorescence intensity (FI) of telomeres was automatically quantified in 10 000 nuclei for each slide. Settings for exposure and gain remained constant between captures. The experiment was performed in triplicate. The second approach consisted of quantifying telomere length in metaphases using automated acquisition module Autocapt software (MetaSystems, version 3.9.1) and a ZEISS Plan-Apochromat 63x/1.40 oil and CoolCube 1 Digital High Resolution CCD Camera with constant settings for exposure and gain. The mean telomere length was measured and telomere loss and telomere doublet scoring were performed. This approach allows the study of intra-cellular variation of telomere length. The experiment was performed in triplicate. Telomere length measured as mean fluorescence intensity (FI) was strongly correlated with telomere length measured by Southern blot analysis using the telomeric restriction fragment (TRF) (data not shown). The mean telomere length is expressed in kb.

Telomere-Centromere staining

Cytogenetic slides with metaphases and micronucleus slides were subject to telomere and centromere staining.

Telomeres and centromeres were stained using the Q-FISH technique with a Cy-3-labelled PNA probe specific for TTAGGG for telomeres and a FITC-labeled PNA probe specific for centromere sequences (both from Panagene, Daejeon, South Korea) as described (M'kacher et al 2014). Briefly, slides were washed with 1X PBS for 5 min at room temperature. Fixation was performed with 4% formaldehyde for 2 min at room temperature, and the slides washed 3 times with PBS for 5 min. After 7 min of pepsin treatment (0.5mg/ml) at 37°C, the slides were washed in PBS and refixed in 4% formaldehyde for 2 min. After 3 PBS washes during 5min, slides were sequentially dehydrated with 50%, 70%, 100% ethanol and air-dried. The probe solution consisted of 50µl of PNA probe (0.3µg/ml telomere and centromere). The slides with the probe solution were denatured on a hot plate at 80°C for 3 min and incubated in the dark for 1h at room temperature. After hybridization, the slides were washed with 70% formamide /10mM Tris pH 7.2 3 times during 15min and then in 50mM Tris pH 7.2/150mM NaCl pH 7.5/0.05% Tween_20 (3x 5 min). After washing with PBS, the slides were counterstained with DAPI and mounted with PPD. Quantification image acquisition and analysis were performed using Isis software (version 3.9.1, MetaSystems, Newton, MA).

M-FISH

Multicolor fluorescence in situ hybridization (FISH) assays are indispensable for a precise description of complex chromosomal rearrangements. Using the combination of five labeled chromosome-specific paints, each chromosome is marked and even small structural chromosomal aberrations are detectable.

Staining was performed using multicolor FISH probes (MFISH 24XCyte, Metasystems) according to the protocol provided from the manufacturers on the same slides used for telomere and centromere staining.

Slides were washed in 2X SSC (Life Technologies) buffer at 70 °C and allowed to cool to 37°C. The chromosomes were then denatured by incubating the slides in a basic solution of 0.07 N sodium hydroxide (NaOH) (Sigma). After washes in 0.1X; 2X SSC buffer for 1min, the slides were dehydrated in increasing concentrations of ethanol, then allowed to air dry. Slides were incubated with the probe mixture in an atmosphere of 50 % formamide (Sigma) at 37 °C for 4 days. After a series of post-hybridization washes, slides were counterstained with 6'-diamidino-2-phenylindole (DAPI) (Sigma), and mounted with p-phenylenediamine (PPD) (Sigma). Metaphases were captured using the Metasystems/AutoCapt software.

Micronucleus and chromosomal aberration scoring

Micronucleus scoring was performed after telomere and centromere staining. Automated scoring of mononucleated cells was performed using Metafer 4 image analyser software (MetaSystems) and a Zeiss Axioplan 2 imager. Ten thousand mononucleated cells per culture (two cultures per cell line, i.e. 20 000 cells per cell line) were processed in the image analyser. Following telomere and centromere staining, MN were classified based on the detection of centromeric regions and telomeric sequences. We detected MN with telomeric and centromeric sequences (mal segregation of whole chromosomes), MN with only telomeric sequences (acentric chromosome or chromatid fragments) and MN without any sequences (interstitial deletions). In addition, nucleoplasmic bridges (NPBs) were assessed following telomere and centromere staining (dicentric chromosomes caused by telomere end fusion) or without (dicentric chromosomes resulting from misrepair of DNA strand breaks). Nuclear buds (NBUDs) were also assessed in order to study the process of elimination of amplified DNA possibly generated via BFB cycles (with telomere sequences or without) or the process of elimination of excess chromosomes may occur in polyploidy cells to facilitate aneuploidy rescue.

The scoring of chromosomal aberrations was performed first after telomere and centromere staining to score dicentric chromosomes and different kinds of acentric fragments with 4 telomeres (resulting from the fusion event), 2 telomeres representing terminal deletions and acentric fragments without any telomere sequence representing interstitial deletions. Second, karyotype analysis was performed using the M-FISH technique on the same slide. The introduction of telomere and centromere staining in the establishment of complex karyotypes allows easy detection and robust classification of chromosomal abnormalities. Complex chromosomal rearrangements were defined as those chromosomes implicated in three or more chromosomal exchanges.

Similarly Break-Fusion-Bridge were defined as those chromosomes that were involved in multiple cycles of repetitive fusions and breakage following the loss of a telomere (Marotta et al 2013).

Images of metaphase cells were acquired using automated acquisition module Autocapt software (MetaSystems, version 3.9.1) using a ZEISS Plan-Apochromat 63x/1.40 oil and CoolCube 1 Digital High Resolution CCD Camera. The analysis was carried out using Isis software (MetaSystems, version 5.5). Automatic scoring of MN following TC staining was performed using MN-score software (MetaSystems, version 5.5).

Spectral karyotype analysis was performed using Isis software (version 3.9.1, MetaSystems, Newton, MA).

Construction of the karyotype evolutionary tree

We manually constructed a "karyotype evolutionary tree" combining data from the M-FISH results, telomere/centromere staining and Micronucleus assay. The normal karyotype "species" (46, XY) was taken as the root/ancestor for all of the evolved karyotypes.

Results

1-Phenotype characterization of HL cell lines

Immunophenotype analysis of all cell lines using specific surface markers for HL (CD30 and CD15) revealed a high level of heterogeneity regarding the expression of

these two markers and reflects the heterogeneity observed in the lymph nodes of HL patients. While the L428 cell line (B cell origin) demonstrated a strong signal for CD30 and CD15, the L540 cell line (T cell origin) presented multiple (two) subpopulations with respect to the expression of these surface markers. Interestingly, the two mixed cellularity cell lines (KMH2 and L1236) showed a similar phenotype, all cells were positive for CD15 and only a subpopulation was positive for CD30 as detected by flow cytometry (supplementary data 1B) and confirmed by immunohistochemistry (supplementary data 1C).

2-Identification of P53 mutations in HL cell lines

The functional status of TP53, a well-known tumor suppressor gene that encodes p53, was performed using a functional yeast assay as previously described (Flaman et al 1995). This analysis showed the presence of 100% red colonies for three cell lines (L428, L1236 and HDLM2), indicating a nonfunctional status of TP53, and also clonal homogeneity of both cell populations (Figure 1A). Sequencing of the p53 cDNA confirmed the presence of the mutations in L428 (exon4), L1236 (exon 10-11) and HDLM2 (exon 8-11) in agreement with a previously published study (Feuerborn et al 2006). Interestingly, FISH analysis for the TP53 gene also revealed a deletion of one locus of TP53 for HDLM2 (Figure 1A-B). TP53 protein expression was detectable by immunofluorescence for all cell lines at different levels. L1236 cells expressed high levels of p53 as well as KMH2. In contrast the HDLM2 and L428 cell lines expressed only low levels of p53 (Figure 7E), for HDLM2 possibly due to deletion of one locus of the p53 gene. In summary, these results demonstrate the absence of p53 function in three of the cell lines, due to mutations and, in one case, deletion of one allele (HDLM2).

A

Cell line	TP53				TP53 gene copy number	Copies of chromosome 17
	FASAY-RED	SPLIT 5-RED	SPLIT 3-RED	RESULTS		
L428	100	100	0	MUT	6/7 genes	6 copies
L591	3	ND	ND	WT	2 genes	2 copies
SUP-HD1	3	ND	ND	WT	ND	ND
L540	5	ND	ND	WT	3 genes	4 copies
HDLM-2	100	1	94	MUT	1 gene	2 copies
L1236	100	3	30	MUT	2/3 genes	3 copies
KM-H2	2	ND	ND	WT	2 genes	3 copies

B

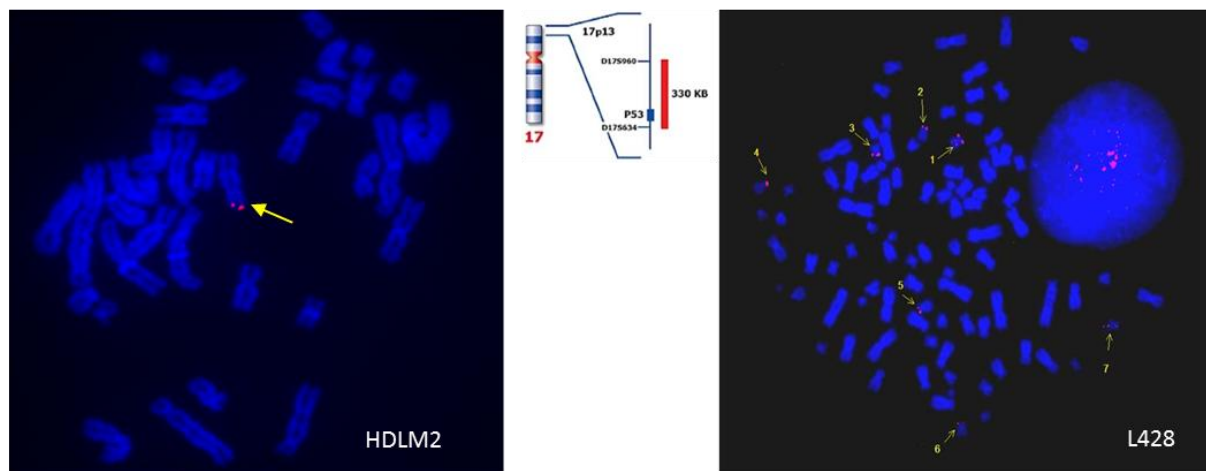


Figure 1: P53 status in HL cell lines, (A) TP53 mutations were detected by a functional assay that tests the transcriptional competence of human TP53 expressed in yeast. Copy number of P53 gene was performed using FISH technique as well as the copy number of chromosome 17 (B) illustration of P53 copy number and chromosome 17 in HDLM2 cell line showing the presence of only one allele and in L428 cell line with the presence of higher copy number compared to the copy of chromosome 17.

3-Genomic instability

3-1-Genomic instability in HL cell lines via microsatellite instability

MSI is another form of genomic instability but its role in the pathogenesis of HL has not been extensively investigated. Table 1 shows the results obtained after the screening of MSI using five quasimonomorphic mononucleotide repeats. These results demonstrate the absence of MSI in L1236 (0/5), low MSI (MSI-L) (1/5) in L591, SUP-HD1 and L540, and high MSI (MSI-H) (more than 3/5) in HDLM2, KMH2 and L428. Interestingly, a correlation between MSI and the coexpression of CD30 and CD15 was found with the HL cell lines exhibiting high MSI also exhibiting a high number of cells expressing both CD15 and CD30 (95,5% for L428, 95.3% for KMH2, and 92.3% for HDLM2) . However, there appears to be no link between TP53 status and MSI.

Table 1 : The status of 5 used quasimonomorphic mononucleotide repeat markers to study MSI in HL cell lines

Cell line	Microsatellite (MSI)*					RATIO*
	PE83 NR21	PE31 BAT26	PE47 BAT25	PE85 NR24	PE84 NR2	
L428	MSI	MSI	MSI	AIA	MSI	4/5
L591	AIA	AIA	AIA	AIA	MSI	1/5
SUP-HD1	AIA	AIA	AIA	AIA	MSI	1/5
L540	MSI	AIA	AIA	AIA	AIA	1/5
HDLM-2	MSI	MSI	MSI	AIA	AIA	3/5
L1236	AIA	AIA	AIA	AIA	AIA	0/5
KMH2	AIA	MSI	MSI	AIA	MSI	3/5

* 0/5= stable; 4/5= unstable

3-2 Genomic instability via chromosomal instability in HL cell lines

3-2-1-Telomere dysfunction in HL cell lines

3-2-1-1-HL cell lines exhibit high telomere length heterogeneity

Figure 2 shows the mean telomere length of the 7 HL cell lines and its distribution depending on the size and irregularity of the cells. Drastic telomere shortening (less than 6kb) was observed for 3 cell lines (HDLM2, L428 and L591). The L540, L1236 and SUP-HD1 cell lines also exhibited short telomeres (7.5 -9.1kb). Only KMH2 cells exhibited a high mean telomere length (21kb) (Figure2A). High intercellular heterogeneity of telomere length was observed in KMH2, L1236, SUP-HD1 and L540 cells. A significant correlation was observed between the size of the cells and telomere length, as illustrated in Figure 2B showing the normalized fluorescence intensity of telomeres as a function of the size of the cells, clearly demonstrating the increase of telomere length in small cells compared to large cells. This intercellular heterogeneity in telomere length was associated with the irregularity of the cell nuclei of the HL cell lines (supplementary data 2A) implying that cells with higher nuclear irregularity have short telomeres (supplementary data 2B).

In order to elucidate the cause for the high level of nuclear irregularity of HL cell lines, the expression of lamin B1, a protein responsible for the regularity and the maintenance of the nucleus, was quantified. The expression level of this protein was very low in all the 7 HL cell lines (figure 2C) compared to controls. Similar to the repartition of telomere length, the small cells in HL cell lines showed a higher intensity of lamin B1 staining compared to that of large cells (supplementary data 3) linking the relationship between lamin B1 expression and telomere dysfunction.

3-2-1-2-HL cell lines exhibit high telomere instability

The quantification of telomere length in metaphases of the cell lines allows the confirmation of not only the data concerning mean telomere length and its heterogeneity following quantification of the nuclei, but also permits the study of intracellular telomere dysfunction such as telomere loss and telomere doublet formation.

Figure 2D shows the frequency of telomere loss and doublet formation in the 7 HL cell lines compared to that of two controls (healthy donors and two lymphoblastic cell lines NAD and REMB). HL cell lines with drastic telomere shortening exhibited a higher rate of telomere loss (HDLM2). Of note, small cells showed a high rate of telomere loss compared to larger cells (figure 2E). In addition, telomere doublets were observed essentially in HL cell lines with long telomeres (KMH2) in particular in the smaller cells, as well as in L1236 and HDLM2 (figure 2F). These data show that telomere dysfunction is more associated with small cells than with large cells. This telomere dysfunction in small cells of HL lines could be the first step in the process of telomere instability and the progression of chromosomal instability.

3-2-1-3-Telomere dysfunction leads to telomere induced foci (TIFs) in HL cell lines

To determine if the telomere dysfunction in the HL cell lines is associated with DNA damage, the quantification of nuclear foci formed following H2AX phosphorylation by immunofluorescence was performed for all cell lines. γ H2AX foci were observed in the nucleus of all HL cell lines. The total intensity is represented in Figure 2G. These foci were highly present in large cells showing the accumulation of DNA damage in these cells. HDLM2 cells characterized by drastic telomere shortening and higher telomere loss exhibited higher γ H2AX foci intensity.

In order to position the induced γ H2AX foci on the chromosomes, we adapted the Immunofluorescence (IF)-FISH method (Pottier et al 2013) to allow simultaneous telomere hybridization and immunofluorescent staining of γ H2AX on the same metaphase chromosomes (Figure 2H). Using this approach, it is possible to determine if the γ H2AX foci colocalise with telomeres or are found in the intra-chromosomal region. Interestingly, the KMH2 and L1236 cell lines exhibited a high frequency of intra-chromosomal foci in addition to telomeric foci (figure 2Ha). In contrast, HDLM2 and L428 showed essentially telomeric foci (figure 2Hb). The colocalisation of telomere staining and γ H2AX foci suggested that the loss of

telomeres may be associated with γ H2AX formation in the absence of DSB, a sign of telomere dysfunction. The presence of intra-chromosomal γ H2AX foci for KMH2 and L1236 could reflect the formation of spontaneous DSB in these cell lines.

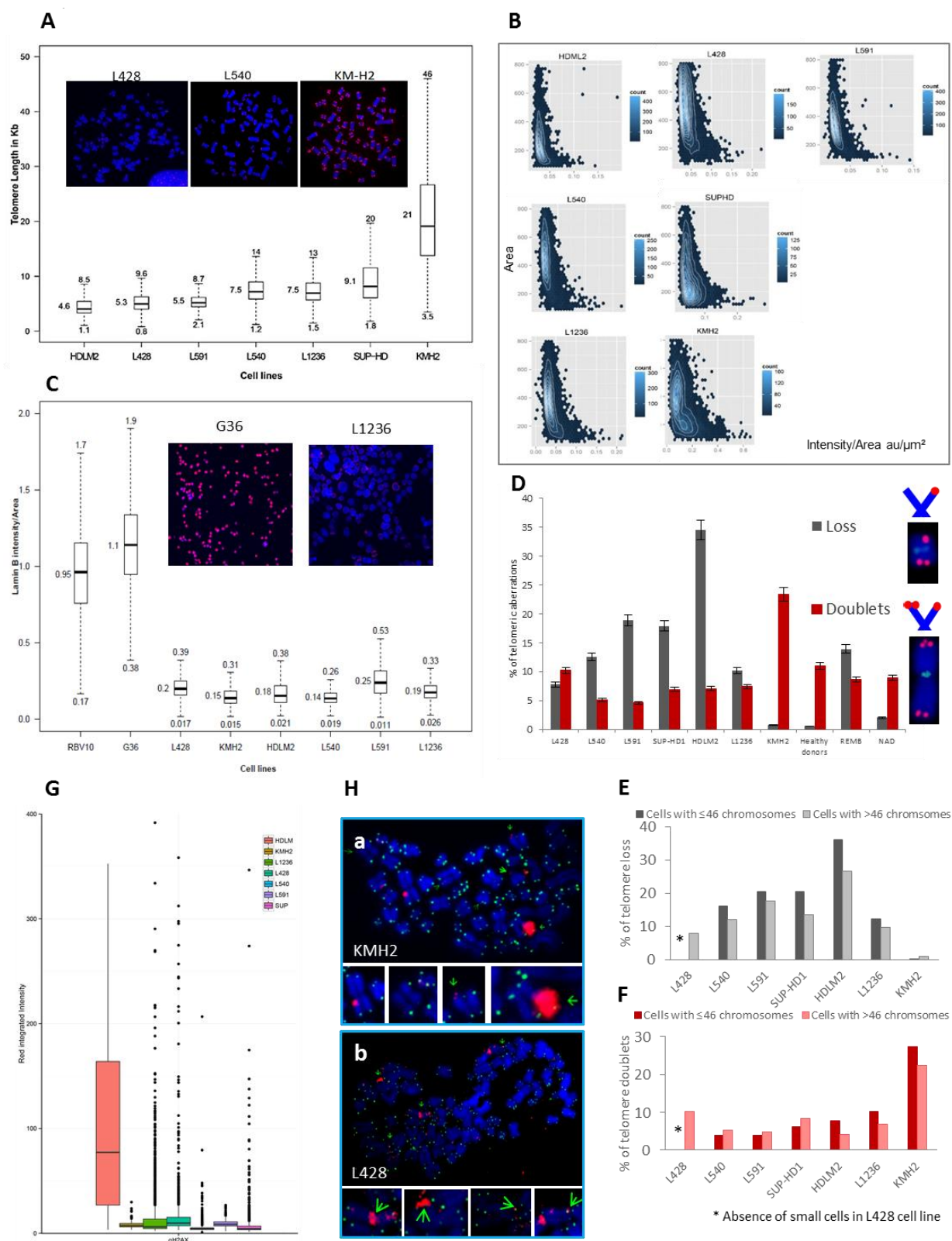


Figure 2: Telomere dysfunction in HL cell lines (A) Quantification of telomere length using teloquant-Q-FISH technique after Cy-3-labelled PNA probe specific for (TTAGGG)_n C3 staining. Telomere length values are given as mean as well as min and max and expressed in kb. The mean fluorescence intensity (FI) of telomeres was automatically quantified in 10 000 nuclei for each slide (B) the intensity of fluorescence normalized by the area of cells was represented in function of the area of

cells. The HRS cells exhibited a significant decrease of intensity of fluorescence of telomeres compared to small cells. KMH2 cell line demonstrate the presence of two populations with significant different telomere length (C) Lamin B1 protein, involved in maintenance of nuclear shape and play a role in telomere integrity, expression is significant different in HL cell lines compared to control. Image of the fluorescent signal of lamin B1 at the nuclear of HL cells and control were represented. (D) Telomere aberrations (telomere loss and doublet formation) was scored in HL cell lines and two lymphoblastic cell lines. (G) Total intensity of spontaneous γ H2AX foci in HL cell lines. (H) Representative images obtained following IF-FISH, TIFs (yellow) represent co-localization of γ H2AX (red) and telomere (green) signals. (E-F) telomere dysfunction in the small and big cells in HL cell lines (E) high frequency of telomere loss was observed in the small cells compared to big cells. Nevertheless, the frequency of telomere doublets in small cells was less than that observed in the big cells (F).

3-2-2-Aneuploidy and dicentric chromosomes lead to chromosomal instability in HL cell lines

Aneuploidy and the formation of dicentric chromosomes with two functional centromeres lead to genomic instability and tumoral progression (Gascoigne & Cheeseman 2013). The introduction of telomere and centromere staining in the assessment of aneuploidy and dicentric chromosomes in cancer cell lines not only makes it easier to detect dicentric chromosomes and centric rings, but also renders the scoring of acentric chromosomes with 4, 2 or no telomere signals more reliable and robust (M'kacher et al 2014).

The level of aneuploidy as well as unstable chromosomal aberrations of HL cell lines was examined in 200 metaphases of each HL cell line. All HL cell lines demonstrated numerical chromosomal aberrations as shown in Figure 3A. Only L591 cells demonstrated 40% of diploid cells without the loss or gain of chromosomes. The results obtained using cell cycle flow cytometry confirmed the aneuploidy with the presence of an important sub-G1 population in some cell lines (HDLM2 and L1236) (Figure 3B). These findings demonstrate that aneuploidy is the first common cytogenetic characteristic of HL cell lines.

The second common characteristic appears to be the presence of dicentric chromosomes in all HL cell lines (Figure 3C). HDLM2, L1236, KHM2 and L428 cells exhibited a higher frequency of dicentric chromosomes compared to L591, L540 and SUP-HD cells. Interestingly, this higher frequency was not associated with a higher frequency of acentric chromosomes (Figure 3Da). L1236 and KHM2 cells exhibited a higher frequency of acentric chromosomes compared to the other cell lines. The majority of acentric chromosomes consisted of terminal acentric chromosomes (Figure 3Db) with the exception of KMH2 which exhibited a higher frequency of fusion acentric chromosomes (Figure 3Dd). Of note, the presence of interstitial acentric chromosomes (Figure 3Dc) demonstrates the loss of chromosome regions caused by two double strand breaks. For all the cell lines, few dicentric chromosomes were accompanied by a fusion acentric fragment with 4 telomeres.

Normally, the formation of dicentric chromosomes following exposure to genotoxic agents, such as radiation, is associated with the presence of an acentric fragment consisting of the fusion product of the two chromosome ends called the accompanying fragment. Only in the KMH2 cell line have we observed dicentric chromosomes associated with the accompanying acentric chromosome. The higher frequency of dicentric chromosomes in HDLM2 was associated with drastic telomere shortening, higher telomere loss, higher telomere deletion (Figure 3E), and the presence of γ H2AX foci (Figure 2G) colocalized to the telomere region and fewer acentric chromosomes (Figure 3Da). Western blot analysis shows the low level of TRF2 and POT1 proteins (Figure 3F-G). These data underline the link between telomere dysfunction and dicentric chromosome formation which could be related to telomere end fusion.

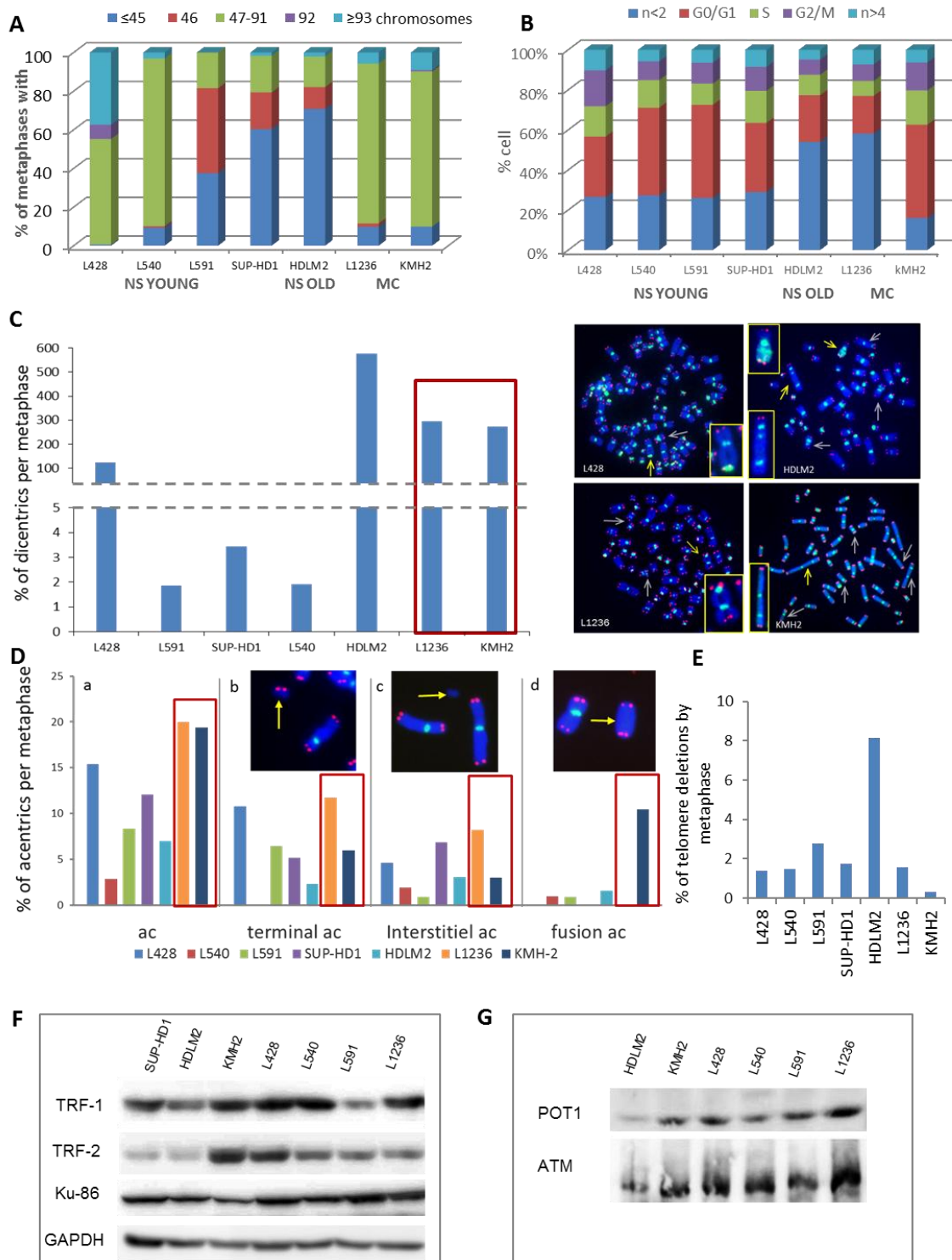


Figure 3 : Chromosome aberrations in HL cell lines (A) numerical chromosomal aberrations were performed after the scoring of 200 metaphases. (B) Cell cycle checkpoints were analyzed by flow cytometry following Hoechst staining revealing the presence of subG1, s-phase and > 4N. The increased of the Sub-G1 population was observed in HDLM2 and L1236. (C) The frequency of dicentric chromosomes detected using telomere and centromere staining in HL cell lines with some illustration of the presence of clonal dicentric chromosomes in L428, HDLM2, L1236 and KMH2 (D) the frequency of different type of acentric chromosomes in HL cell line (Da) the frequency of all acentric chromosomes in HL cell line detected using telomere and centromere staining (Db) the frequency of terminal acentric chromosomes with only two telomeres (Dc) interstitial deletion without any telomere sequences (Dd) acentric chromosome with 4 telomeres resulting for the fusion of two terminal deletions. (E) Telomere deletion resulting two double strand breaks were scored in all HL cell lines showing a higher frequency in SN HL cell lines compared to MC HL cell lines. (F-G) Western blot analysis of protein implicates in telomere maintenance (F) decreased levels of TRF2 in HDLM2 and SUP-HD cell lines. (G) decreased level of Pot1 in HDLM2 cell lines. GAPDH provided a loading control.

The presence of the higher frequency of dicentric chromosomes in L1236 and KMH2 was independent of telomere length, telomere loss, and telomere deletion but can be correlated with a higher level of BP531 protein expression (figure 7C), the presence of γ H2AX intra-chromosomal foci (figure 2Ha) and higher level of TRF2 and POT1 proteins (figure 3F-G). These data suggest that the formation of dicentric chromosomes is not related to telomere dysfunction in the KMH2 and L1236 cell lines but to the presence of spontaneous DSB in these cell lines.

3-2-3 Micronucleus and centrosome amplification in HL cell lines related to chromosomal instability

The aneuploidy and the high frequency of dicentric and acentric chromosomes in HL cell lines led us to study the frequency of micronuclei (MN) and other nuclear abnormalities such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) which are related to aneuploidy and chromosomal instability. The use of automatic scoring of MN allowed the scoring of five thousand cells for each cell line, providing greater sensitivity and furthermore, the introduction of telomere and centromere staining brought evidence of the nature of the MN. Figure 4Aa shows the frequency of MN after uniform staining (DAPI) for the HL cell lines. Telomere and centromere staining makes it possible to distinguish between MN with telomere and centromere sequences (mis-segregation of whole chromosomes) (Figure 4Ab), MN with only telomere sequences (DNA breaks) (figure 4Ac) and MN without any telomere or centromere sequences (interstitial deletions) (Figure 4Ad). The repartition of the number of micronuclei per cell is shown in supplementary data 4. A large part of MN contained telomere and centromere sequences with a highly variable number of centromeres per MN (supplementary data 5). In addition, MN related to terminal DNA breaks or interstitial acentric chromosomes (with only telomere sequences or no sequence) was observed for all cell lines with the highest frequency for L1236 (figure 4Ac-d). The higher frequency of MN in L1236 cells could play a major role in the process of chromosomal instability in this cell line.

The NBUDs are characterized by having the same morphology as an MN with the exception that they are connected to the nucleus by a narrow or wide stalk of nucleoplasmic material depending on the stage of the budding process (Fenech et al 2011). Figure 4B shows the frequency of NBUD in HL cell lines demonstrating that HDLM2, L428 and KMH2 exhibited a high frequency of NBUDs compared to L591

and SUP-HD. In contrast to the frequency of MN, L1236 showed a low frequency of NBUDs mostly composed of those containing telomere and centromere sequences (Figure 4B-b). KMH2 cell lines exhibited a higher frequency of NBUDs compared to the frequency of MN. This data shows the possible role of NBUDs in the oncogenic process in KMH2.

Nucleoplasmic bridges (NPBs) were observed primarily in HDLM2 and L428, and less in L1236 and KMH2 (Figure4Ca). Interestingly, the NPB's observed in HDLM2 and L428 contained telomere and centromere sequences (Figure4Cb-c). In contrast, the NPB's observed in L1236 and KMH2 were without telomere and centromere sequences and accompanied by MN with only telomere sequences (figure 4C-d). These data confirm not only the higher frequency of dicentric chromosomes observed in these cell lines but also the mechanisms underlying their formation. If the formation of dicentric chromosomes in HDLM2 and L428 are related to telomere erosion, the presence of dicentric chromosomes in KMH2 and L1236 is possibly due to a defect in DNA damage repair.

Multiple centrosomes are known to cause multipolar mitoses and chromosome miss-segregation. We hypothesize that the amplification and number of centrosomes is related to the aneuploidy of the HL cell lines and the presence of dicentric chromosomes. Data from the measurement of centrosome size and number by immunofluorescence for each cell line are presented in supplementary data 6, indicating a high degree of variability among HL cell lines. L1236 demonstrated an increase in the number of centrosomes whereas HDLM2, L428 and KMH2 exhibited an increase in the centrosome volume.

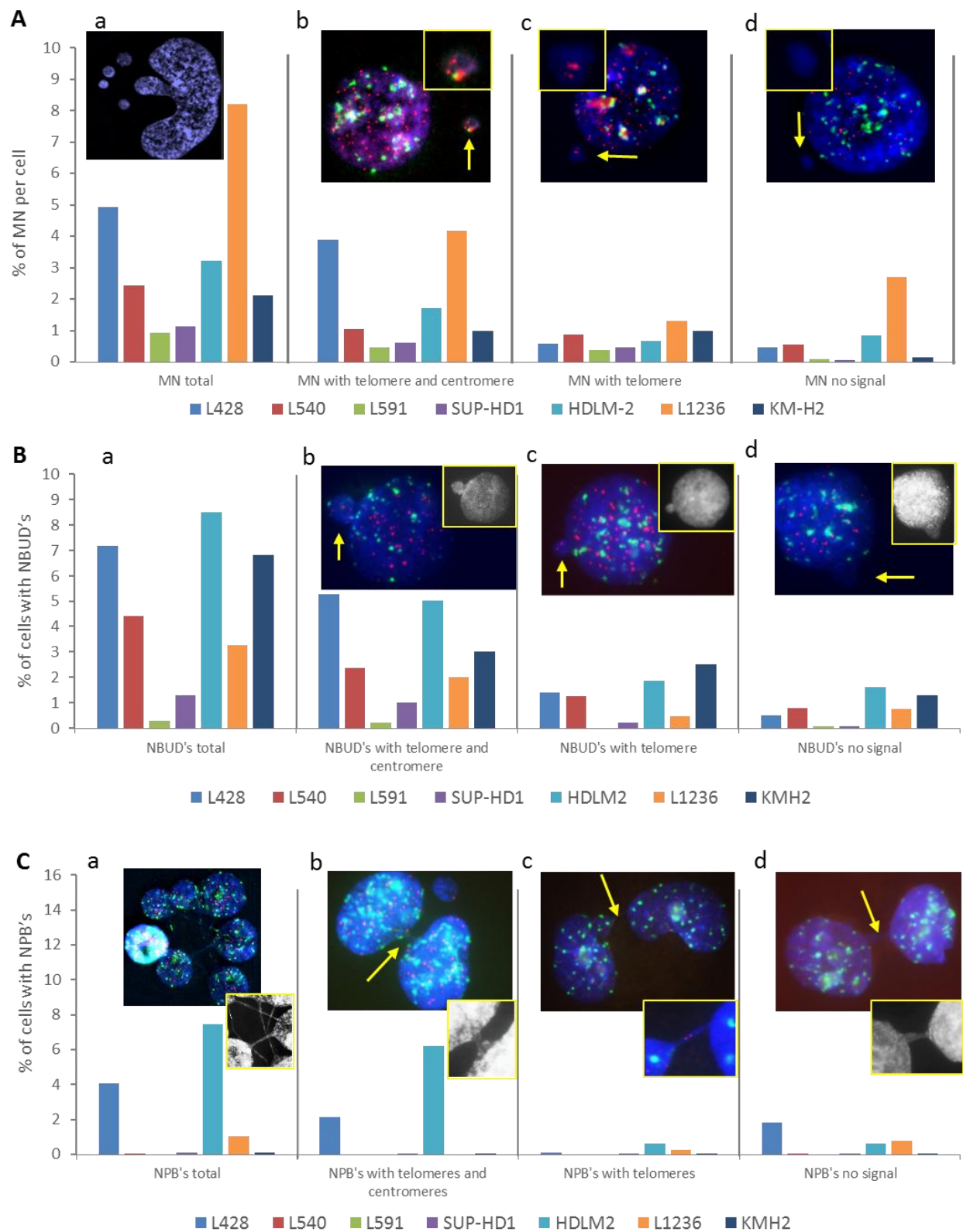


Figure 4: the frequency of micronucleus (MN), nuclear buds (NBUDs) and nucleoplasmic bridges (NPBs) assessed following telomere and centromere staining (Aa) the frequency of MN after homogeneous staining (DAPI) (Ab) the MN with telomere and centromere staining (Ac) MN with only telomere sequences (Ad) MN without any staining. (Ba) The frequency of NBUDs after homogeneous staining (Bb) the frequency of NBUDs with telomere and centromere staining (Bc) NBUDs with telomere staining (Bd) NBUDs without telomere and centromere staining. (Ca) the frequency of NPBs after homogeneous staining (Cb) NPBs with centromere and telomere staining (Cc) NPBs with only centromere staining (Cd) NPBs without any staining.

4-Structural chromosome aberrations

Successive telomere and centromere staining and the M-FISH technique were performed to reliably establish the karyotype for the HL cell lines. Karyotypes were determined for 30 metaphases from each cell line. Table 2 summarizes the numerical and structural chromosomal aberrations found for each cell line with supplementary data 7 showing the derivative chromosomes.

Numerical chromosomal aberrations including gain and loss of whole chromosomes were observed in all HL cell lines and confirmed the results obtained using telomere and centromere staining. Gains were observed more frequently than losses in all HL cell lines, with the exception of chromosomes Y (4/4), 13 (5/7), 15 (2/7) and 8 (2/7). Gains were found for chromosomes 19 (6/7), 9(6/7), 5 (5/7), 11 (5/7), 18 (5/7), and 17 (4/7) (supplementary data 8).

Karyotyping of 30 metaphases for each cell line allowed not only the detection of all chromosomal aberrations and all resulting breakpoints, but also the possibility to search for common chromosomal aberrations shared by the HL cell lines. The total frequency of breakpoints resulting from all aberrations detected by M-FISH and terminal deletions of telomeres detected by telomere and centromere staining are presented in (Figure 5A). It was not possible to precisely identify the exact region of the breakpoints due to the complexity of the aberrations. No common structural aberration was found for all HL cell lines. Nevertheless, for all nodular sclerosis HL cell lines, only t(2;8) was found without the fusion gene of c-myc (supplementary data 9A) and its amplification was observed mostly in L428 and HDLM2. In addition, L428 and L591 cells exhibited t(9;14). On the other hand, in mixed cellularity HL cell lines, t(7;19), t(16;22), t(10;16), t(13;21) and t(11;19) were present for both KMH2 and L1236 cells. Interestingly, an elevated number of copies of Jak2 was observed in 6/7 cell lines. HDLM2 exhibited a particularly high copy number for Jak2 (8 copies) (supplementary data 9B) including 3 copies on chromosome 9 alone.

In addition, M-FISH analysis revealed that six HL cell lines exhibited a high frequency of complex chromosomal rearrangements (CCRs) and break fusion bridge (B/F/B) aberrations (Figure 5 B) associated with a high frequency of total breakpoints (supplementary data 10). Only L591, characterized by the unique staining of CD30, did not show complex chromosomal and B/F/B aberrations. Nevertheless, a significant correlation was observed between the frequency of CCRs and B/F/B and the total number of translocations. However, there is no correlation between these

aberrations and the presence of unstable chromosomal aberrations (supplementary data 10).

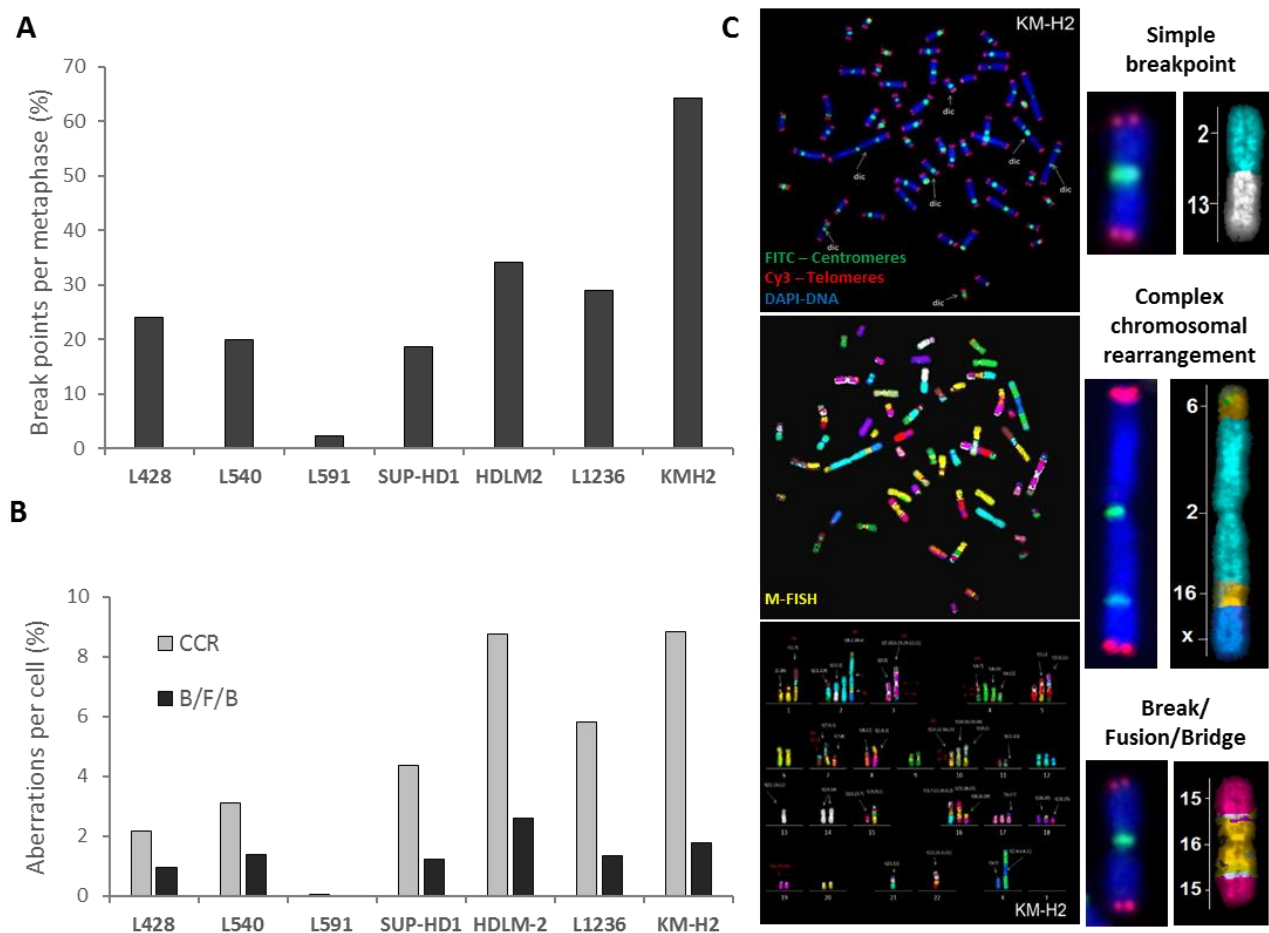


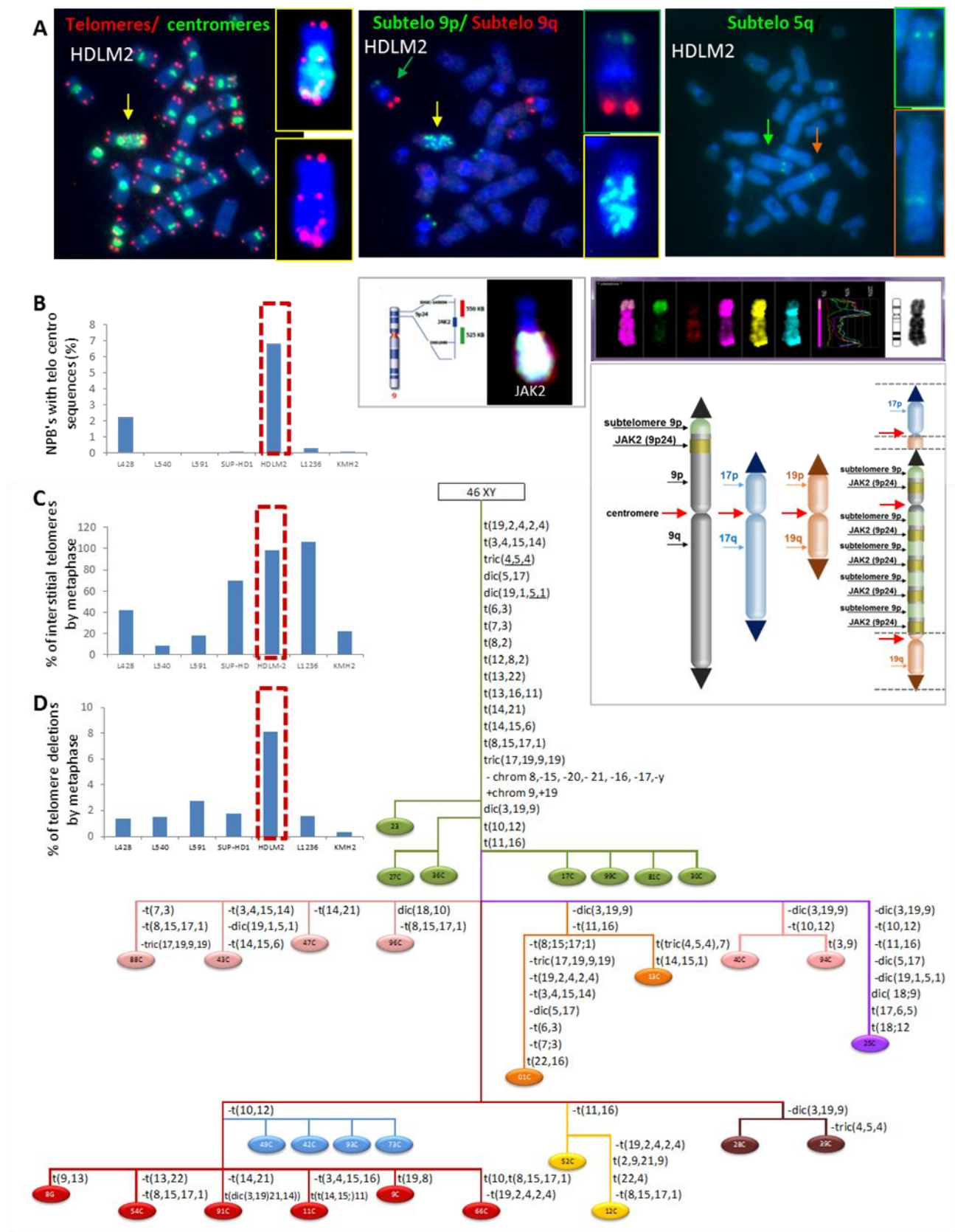
Figure 5 Chromosomal instability detected using M-FISH technique (A) frequency of total breakpoint in HL cell lines (B) the frequency of complex chromosomal aberrations (CCRs) and break fusion Bridge (B/F/B). (C) Illustration of metaphase stained first with telomere and centromere PNA probes and second with M-FISH probe permitting to classify the chromosomes and establish the karyotype. This metaphase contained simple chromosomal aberration, CCRs and B/F/B.

Table 2: Origin, sub type, numerical and structural chromosomal aberrations in HL cell lines.

HL subtype	Origin	Cell line	Numerical Aberrations		Structural Chromosome Aberrations					
					Clonal				Spontaneous	
			mean nr of chromosomes (min-max)	Gain and loss of whole chromosome	common translocation	deletions and amplifications	additional translocations	additional dicentrics	translocations	dicentrics
Nodular sclerosis derived from young patients	B-cell	L428	94,25 (71-103)	gain chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, 19, 20, 21, 22, x, loss chromosome 13	t(2;8)	del(6p); iso(6p), del(12p), iso(12p), del(17p)	t(5;5), t(6;5), t(9;11;21), t(13;5), t(9;14), t(14;9), t(18;7), t(12;7), t(17;2), t(1;x), t(7;15), t(21;x), t(16;9;16;9;1), t(14;17), t(8;18)	dic(3;15)	t(10;8), t(8;x), t(20;12), t(10;x), t(17;4), t(19;14), t(18;16), t(21;19), t(9;5), t(5;9;6), t(5;14), t(6;9), t(16;9), t(3;x), t(11;19), t(16;22), t(17;x), t(6;14), t(3;3), t(18;2), t(9;2), t(9;4), t(3[14;9]), t(8;2;5), t(3;19), t(9;6;14;4)	dic(10;11)
	B-cell	L591	45,52 (29-49)				t(8;11), t(14;9), t(12;7) frag 16			dic(13;6), dic(2;8)
	B-cell	SUP-HD1	41,63 (10-95)	gain chromosomes 8,9, 19 loss chromosome 13,y		del (5p), amplif (1) iso 5p, iso 14,	t(4;16), t(4;18), t(6;6;9), t(9;8;12), t(8;18), t(18;8), t(9;12), t(12;9), t(15;2), t(15;5), t(22;13), t(11;t(-4;16)), t(19;12), t(t(2;8);2)), t(8;t(18;8),		t(16;x), t(x;16), t(20;3), t(1;17), t(2;17), t(5;4), t(3;2), t(19;12), t(6;8), t(5;4)	dic(11;11), dic(9;9), t(5;4)
	T-cell	L540	64,15 (33-118)	gain chromosomes 5,9,11,12,19, loss chromosomes 8 ,10, 13, X		del (18)	t(1;15), t(11;14), t(1;5), t(5;1;5), t(22;11;14), t(2;12), t(1;12), t(7;19), t(8;20;6), t(16;18), t(16;x), t(21;17;15), t(21;20), t(x;2)		t(8;2;8), frag 12, del (4) ac 4, t(3;9), frag 17,	dic(7;3), dic (17;21)
	T-cell	HDLM-2	35,8 (24-78)	gain chromosomes 5, 9,11, 12, 19, loss chromosomes 8, 15, 16, 20, 21,22, y			t(19;2;4;2;4), t(3;4;15;14), t(6;3) , t(7;3), t(8;2), t(12;8;2), t(13;22), t(13;16;11), t(14;21), t(14;15;6), t(8;15;17;1), t(10;12), t(11;16),	dic(5;17), dic(19;1;5;1) , dic(3;19;9), tric(17;19;9;19), tric(4;5;4)	t(17;6;5), t(18;12), t(3;9), t(tric(4;5;4;7)); t(14;15;1), t(22;16), t(2;9;21;9), t(22;4), t(10;t(8;15;17;1), t(19;8), t(t(14;15;11)); t(dic(3;19);21;14), t(9;13)	dic(18;9), dic(18;10)
Mixed cellularity	B-cell	KM-H2	57,07 (45-90)	Gain chromosomes 1,2,4,5,7,8,10,11,12,15,16,17,19,22,x loss chromosomes 13, y,	t(7;19), t(16;22), t(10;16), t(13;21), t(11;19),		t(1;16), t(13;2), t(4;16), t(11;12), t(1;8;3), t(11;2;9), t(2;8;16;21;3;7), t(3;5;11), t(5;1), t(7;9;1), t(8;1;19), t(10;16;10;16), t(10;1), t(12;21), t(15;18), t(15;9;1), t(17;x),t(22;16;3;10), t(x;5), t(3;8), t(15;t(16;15)); t(18;13), t(3;dic(2;16;x)), t(dic(10;22;16); 22;6)), t(12;t(13;21)), t(5;t(15;7)), t(4;12), t(8;12), t(18;20), t(6;dic(2;16;x)), t(dic(10;22;16);10), t(13;21), t(11;t(15;7)), t(2;4;t(x;4;2)), t(18;t(16;15)), t(6;t(x;4;2)), t(7;dic(10;3;15;19;12;11)),	dic(11;7), dic(10;3;15;19;12;11), dic(1;7), dic(7;7), dic(19;19), dic(10;22;16), dic(2;16;x), dic(x;4;2),	t(7;9;14), t(18;20), t(9;2), t(4;10), t(2;9), t(16;19), t(9;8;10), t(5;20), t(22;16), t(1;9;7;1;16), t(4;10),	dic(2;4;x;4;2;4;x;4;2),
	B-cell	L1236	61,2 (33-87)	Gain chromosomes 1,2,3,4,5,6,7,9,10, 11,12,15,16,17,18, 19,20,21,x loss chromosomes 8, 13, 14, y			t(1;14), t(14;1), t(4;8), t(11;21), t(22;15), t(22;6), t(7;17), t(20;15), t(3;16), t(10;14;16;19), t(1;8), t(6;1), t(1;18), t(18;1), t(18;t(7;17), t(6;9;6;8), t(8;1;14), t(16;19), t(15;7), t(12;19), t(3;9), t(1;9), t(12;16),	dic(2;21), dic(12;12), dic(21;t(7;17)), dic(12;17 ;y), dic(9;16)	t(10;16), t(19;9), t(1;17), t(9;21), t(12;1), t(15;8), t(16;19), t(2;2), t(21;5;11), t(14;9), t(x;14), t(3;9), t(18;8), t(4;1;9), t(4;1), t(2;18), t(19;14;1), t(9;19;4), t(19;9), t(x;x), t(3;18), t(15;7), t(11;12), t(12;13), t(13;12), t(15;20), t(10;20), t(15;2), t(20;5), t(14;2), t(1;9), t(3;14), t(22;8), t(2;x), t(17;t(7;17)), t(20;t(7;17), t(10;20), t(15;2), t(7;8), t(10;7;11), t(7;9), t(16;20), t(3;14;1), t(13;21), t(22;20;15)	dic(3;3), dic(8;9), dic(12;16), tric(14;1;8;21;19;21;1 9), tric(12;21;7;17), dic(12;7;1), dic(2;2), dic(4;14;1); dic(19;5); dic(x;15),

Identification of the cytogenetic pathway of chromosomal instability in HL cell lines using HDLM2 and L1236

To obtain an overall picture of the oncogenic pathways, the cell lines HDLM2 and L1236 were used to assess two possible mechanisms leading to chromosomal instability in HL. HDLM2 and L1236 both exhibit the same TP53 mutation (table1), p53-dependant G1 cycle arrest (figure 2B), and a high frequency of dicentric chromosomes, CCRs and B-F-B. Nevertheless, HDLM2 cells exhibited a high level of MSI (3/5 markers) while L1236 exhibited none. Also, HDLM2 showed a higher frequency of clonal chromosome dicentrics accompanied by a few acentric chromosomes (terminal and interstitial acentric chromosomes) and a higher frequency of telomere deletions (Figure 3E) compared to L1236 which contained fewer telomere deletions and more acentric chromosomes (terminal and interstitial acentric chromosomes) and fewer clonal and more sporadic dicentric chromosomes. Subtelomere staining was performed following telomere and centromere staining showing that the dicentric chromosomes observed in HDLM2 were the result of telomere erosion followed by telomere fusion (Figure 6) and are associated with the presence of NPB's with telomere and/or centromere sequences (Figure 6) and a high frequency of interstitial telomere and telomere deletions (Figure 6). All together, these data show that the dicentric chromosomes observed in HDLM2 arise from telomere end fusions caused by telomere dysfunction with telomere sequence loss possibly as a result of defects in the assembly of telomere capping proteins confirmed by Western blot (Figure 3F-G). The painting of the sub-telomere of chromosome 9 and 5 involved in dicentric chromosomes showed the presence of sub telomere sequences at the breakpoints for HDLM2. However, the lack of subtelomere sequences in dicentric chromosome breakpoints of L1236, the absence of telomere and centromeres sequences in NPB's and the low frequency of telomere deletions indicate that the formation of dicentric chromosomes was not related to telomere erosion but results from misrepair of DNA double strand breaks (Figure 7). The quantification of protein expression of Ku86, DNA-PKcs, BP53, ATM and MR11 by Western blot (Figure 3F-G) demonstrated the overexpression the defect in the DNA repair pathway in this cell line.



Discussion

Genomic instability has been described as a driving force for the aggressiveness of HL (Re et al 2002b). Chromosomal instability related to telomere dysfunction and multiple structural and numerical chromosomal rearrangements have been consistently reported for HL patients and cell lines (M'Kacher et al 2007). Nevertheless, the mechanisms underlying this genomic instability remain unclear (Knecht et al 2013). Few cytogenetic studies in HL patients have been performed primarily due to the lack of in vitro growth of tumor cells as well as the absence of a reliable animal model (von Kalle et al 1992). On the other hand, molecular studies are confronted with the problem of high heterogeneity in HL lymph nodes reflecting the unique microenvironment of the tumor cells in HL (Liu et al 2014). By performing cytogenetics and molecular studies on a panel of seven HL cell lines reflecting the heterogeneity of HL patients and two types of HL disease, we are able to propose possible mechanisms underlying genomic instability in nodular sclerosis (NS) and mixed cellularity (MC) HL cell lines.

The detailed characterization of the HL cell lines used in this study can pave the way in understanding the mechanisms involved in the oncogenesis and genomic instability of HL. The phenotype of the seven cell lines clearly demonstrates their heterogeneity reflecting that of patients with respect to the clinical markers of HL (CD30 and CD15). It is important to take into account that the cell lines were derived from diverse sources including high-grade, refractory tumors as well as lymphoma cells disseminated into the blood (L1236) or pleural effusions (L428 and HDLM2). The TP53 status of HL has been the subject of debate and remains controversial (Feuerborn et al 2006) and the lack of proven TP53 mutations in lymph nodes contrasts with the often high levels of its expression (Schneider et al 2014). In this study, functional yeast assays revealed the non-functionality of TP53 in three cell lines and DNA sequencing revealed the same mutations previously described for L1236, HDLM2 and L428 (Feuerborn et al 2006). In addition, the HDLM2 cell line showed a deletion of one P53 allele revealed by the FISH technique. However, the other cell lines harbor wild type TP53. This re-evaluation of the P53 status in HL using a sensitive technique reinforces the concept that mutation of TP53 may be involved in the pathology of some cases of HL (Robles & Harris 2010) and perhaps the genomic instability observed in this disease as well as the occurrence of late complications such as secondary cancer following HL treatment (Schulz et al 2015).

or HL as a secondary event (Schneider et al 2014). Given that some of the derived HL cell lines have mutated TP53, it would be important to investigate the TP53 status in lymph nodes derived from relapsing or refractory HL patients. The correlation between TP53 status and high-grade progression in B-cell lymphoma has been previously established (M'Kacher et al 2003a, Sanchez-Beato et al 2003).

Following immunophenotype and TP53 characterization, the aim of this study was to investigate the mechanisms underlying genomic instability in HL cell lines. Two important models have been implicated in the genomic instability observed in cancer cells; microsatellite instability (MSI) and chromosomal instability, which is inversely correlated with MSI in hereditary non-polyposis colorectal cancer (Goel et al 2007) and determines the specific therapeutic strategy (Buecher et al 2013).

MSI analysis has previously been performed on the lymph nodes of patients as well as on the L1236 cell line and the unique publication on this topic demonstrates that MSI is rare and therefore not notably involved in the genomic instability of HRS cells in HL (Re et al 2002a). Re-evaluating MSI in HL-derived cell lines, we confirmed the results obtained for L1236 with respect to the absence of MSI and demonstrated, however, that a high frequency of MSI occurs in L428, KMH2 and HDLM2 cells and propose MSI as another form of genomic instability for some HL cell lines. Altogether, this is the first report describing a high frequency of MSI in HL cell lines. Similar to L1236, the other cell lines (3/7) also exhibit a low level of MSI (Borie et al 2009).

Chromosomal instability, a second form of genomic instability, has been defined as an accumulation of point mutations, translocations, chromosomal gains and losses, telomere shortening and defects in the nuclear architecture, known as laminopathies that can cause genome instability (Barascu et al 2012).

Genomic stability systems also include specific mechanisms for maintaining the appropriate length and functionality of telomeres (Blackburn et al 2006). Telomere shortening has been previously reported in HRS cells as well as in HL cell lines and has been shown to be associated with the presence of t-stump and poor clinical outcome. We have previously demonstrated, by examining the circulating lymphocytes of HL patients, the presence of a subgroup with an elevated risk of developing late complications (secondary cancer and cardiovascular disease characterized by drastic telomere shortening associated with the presence of complex chromosomal rearrangements (M'Kacher et al 2007, M'kacher et al 2014). In the current work on the 7 HL cell lines we clearly observe a high level of telomere

length heterogeneity reflecting previous observations of circulating lymphocytes from HL patients, and progressive telomere loss between mononuclear and binuclear cells. All NS cell lines presented telomere shortening associated with a higher frequency of telomere aberrations (loss of one or two telomere and the presence of telomere doublets). Interestingly, mononuclear cells had longer telomeres associated with a higher frequency of telomere loss compared to binuclear cells. Telomere loss observed for mononuclear cells was associated with the appearance of γ H2AX foci at the telomeres. This specific alteration of telomeres may have drastic consequences for the induction and transmission of chromosomal rearrangements (Reddel et al 1997). The loss of telomere integrity of mononuclear cells in NS-HL cell lines may represent the first step in the ongoing process of chromosomal instability. The HDLM2 cell line presented drastic telomere shortening and higher telomere loss and deletion associated with the presence of a higher frequency of γ H2AX foci at telomeres compared to other cell lines. The telomere profile of the HDLM2 cell line may be related to the age of the patient (74 years) from which this cell line was derived as well as the poor clinical outcome of older HL patients with drastic telomere shortening. In contrast, in the two MC HL cell lines, telomere length was higher than that observed in the SN HL cell lines, and KMH2 in particular, presented a higher level of telomere length heterogeneity as well as the presence of two subpopulations with significantly different telomere lengths. In addition, the MC cell lines presented a significant lower frequency of telomere aberrations. Interestingly, telomere and chromosome localization of γ H2AX foci were identified indicating the presence of spontaneous DSB in these cell lines. We therefore suggest that for the NS HL cell lines there is the induction of an acute DNA damage response at telomeres that leads to unprotection of the 3 telomere overhang (Longhese 2008). The ensuing telomere dysfunction may play a major role in chromosomal instability in SN HL cell lines. The implication of telomere dysfunction in the chromosomal instability of CM cell lines is not clear and requires further investigation.

Telomere dysfunction in HL cell lines was associated with a defect in the nuclear architecture. All HL cell lines presented a low level of lamin B1 and highly irregular nuclei. The presence of lamins in hematopoietic cells has been a matter of debate (Rocha-Perugini & González-Granado 2014). Only one study has been performed in NS HL lymph nodes and demonstrated the expression of lamin B1 and the absence of lamin B2 (Jansen et al 1997). However, the importance of these proteins in the regulation of the function of the immune system warrants further investigation

in HL cell lines and lymph nodes, in particular in relationship with telomere dysfunction and nuclear irregularity (Camps et al 2014).

The second major mechanism that can lead to chromosomal instability is the formation of a dicentric chromosome (Gisselsson et al 2000, MacKinnon & Campbell 2011, Yamamoto et al 2011). The incidence of recurrent and clonal dicentric chromosomes seems vary in different types of hematological malignancy (Berger & Busson-Le Coniat 1999) and their incidence has been underestimated due to the limitations of the technique used for their detection. Dicentric chromosomes are considered as unstable chromosomal aberrations and disappear with successive cell divisions with the natural occurrence of this aberration being very low. In rare cases, dicentric chromosomes can be stably maintained by inactivation of one of the centromeres, but the mechanisms of this inactivation remain poorly understood (Stimpson et al 2010). In this study, we describe, for the first time, the presence of clonal and sporadic dicentric chromosomes in HL cell lines using telomere and centromere staining followed by M-FISH to identify the implicated chromosomes. In this study, we detected several unexpected dicentric and pseudo-dicentric chromosomes occurring as clonal abnormalities in the L428, HDLM2, L1236 and KMH2 cell lines. Two different mechanisms may underlie the formation of these clonal dicentric chromosomes.

First, in the NS HL cell lines (L428 and HDLM2), the presence of clonal dicentric chromosomes is related to telomere erosion, leading to the chromosome uncapping followed by fusion of two chromosomes and dicentric chromosome formation. In the L428 cell line, dic(3;15) was observed for all analyzed metaphases, and using subtelomere probes of chromosome 15p, we demonstrate the presence of the sub telomere region of chromosome 15 at the breakpoint. Similarly, in the HDLM2 cell line, characterized by drastic telomere shortening, higher frequency of telomere loss and a low level of the TRF2 protein, dic(5;17), dic (19,1,5,1), tric(17;19;9;19) and tric(4;5;4) were observed for all analyzed metaphases and the use of sub-telomeric probes of chromosomes 5 and 19 showed the presence of subtelomeric sequences at the breakpoint regions. However, the clonal dicentric chromosomes observed in all metaphases may be quite stable, usually due to the fact that one centromere is functionally silenced. The data obtained using the micronucleus assay confirms these findings. Bridge formation was not observed for all cells and the observed bridges were related to sporadic dicentric chromosome formation and contained telomere sequences clearly demonstrating that, in this case, dicentric chromosome formation

was related to telomere erosion (Fenech et al 2011). Of note, dic(5;17) has also been observed in myeloid cell proliferation (Berger & Busson-Le Coniat 1999). Further investigation using centromere-binding proteins (CENPA, CENPC and CENPE), necessary components of an active centromere, could be performed to determine whether that clonal dicentric chromosomes carry an inactive centromere. In addition, sporadic dicentric chromosome formation was observed in all NS cell lines. Second, In the MC cell lines (L1236 and KMH2), were observed mostly clonal dicentric and isodicentric chromosomes. These cell lines are characterized by a low level of telomere loss and high heterogeneity of telomere length as well as a high level of TRF2 protein. The hypothesis that telomere erosion induced the formation of dicentric chromosome in these cell lines was quickly abandoned due to the lack of subtelomeric sequences at the breakpoints. It appears that the breakpoints were localized at the heterochromatin. Future studies could be undertaken to determine the precise location of the chromosomal breakpoints in these cell lines. Several factors may favor rearrangements of heterochromatin and illegitimate recombination resulting in acquired translocations. They are somatic pairing, DNA sequence homologies between centromeric DNA, methylation status that can stretch DNA and favor chromosomal breakage in the corresponding area, the presence of transposons within heterochromatin DNA, and even heterochromatin polymorphisms that have been suspected to favor an abnormal recombination rate (Berger 2000).

This reevaluation of chromosomal instability in HL cell lines using different cytogenetic approaches does not only confirm the karyotype published previously (Berglund et al 2003, Naumovski et al 1989, Wolf et al 1996) but also permits to distinguish two different mechanisms underlying genomic instability in HL cell lines. In NS HL cell lines, we showed that high frequency of loss of telomeres in small cells compared to HRS cells, occurred early in the transformation process, leading to the formation of dicentric chromosomes, gene amplification and BFB rearrangement. In this study, we proposed a model that explain routes through which telomere loss may induce tumor initiation and progression in NS HL. Concerning MC HL cell lines, we demonstrate that chromosomal instability was not related to telomere dysfunction and the dicentric formation was induced due to non-homologous end joining (NHEJ) pathway of double-strand break (DSB) repair. Future investigations in the localization of breakpoints in these cell lines could permit to clarify this process.

Conclusion

This is the first study that demonstrates the implication of MSI in genomic instability in HL cell lines. It will be important to study the sensitivity of HL cell lines with higher MSI to alkaline agent or ionizing radiation in order to define a new therapeutic strategy as well as to colorectal carcinoma. The second main finding in this work is the existence of two different mechanisms in HL with respect to chromosomal instability reflecting the clinical differences observed in terms of remission and survival of NS and MC HL. In NS HL cell lines, telomere dysfunction could be a major event in the initiation and the progression of chromosomal instability. Also, in MC HL cell line, the defect of NHEJ of DSB repair appeared a major mechanism in the chromosomal instability in these cell lines. This study provides evidence for a genomic mechanism of centromere inactivation that occurs in some dicentric chromosome. Future studies will be important for determining the molecular basis for the choice of the inactivation pathway (genomic versus epigenetics) is taken (Stimpson et al 2010). We believed that our work is an important step in understanding genomic instability (initiation and progress) and may give rise to new therapeutic possibilities by targeting the cause or the consequences of specific chromosomal aberrations.

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Supplementary data

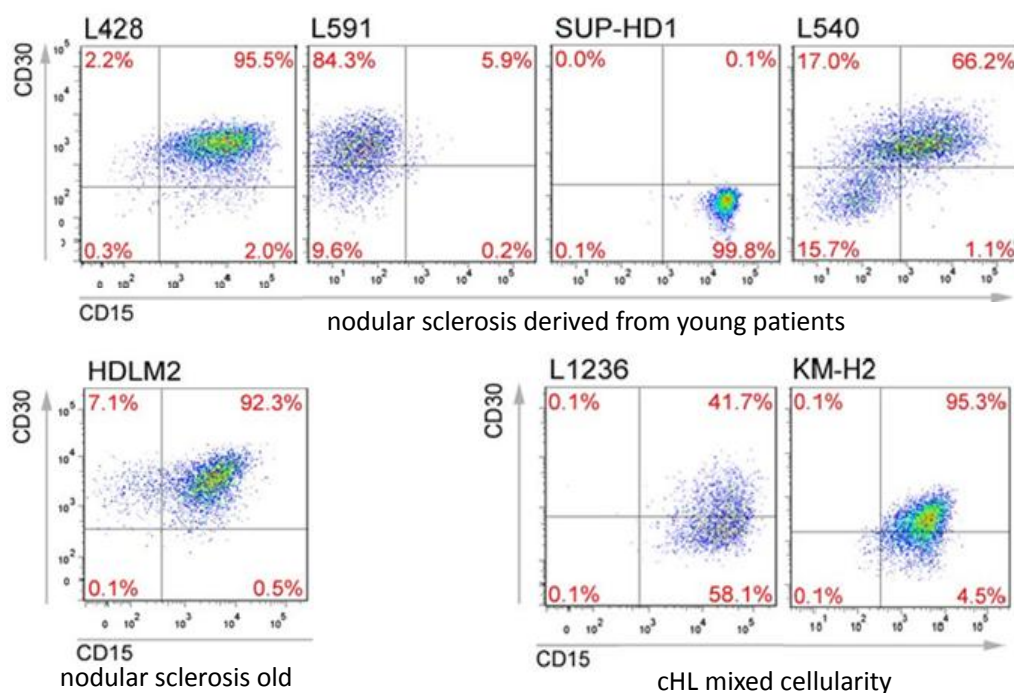
Supplementary data 1

Origins and immunophenotype Characterization of HL cell lines

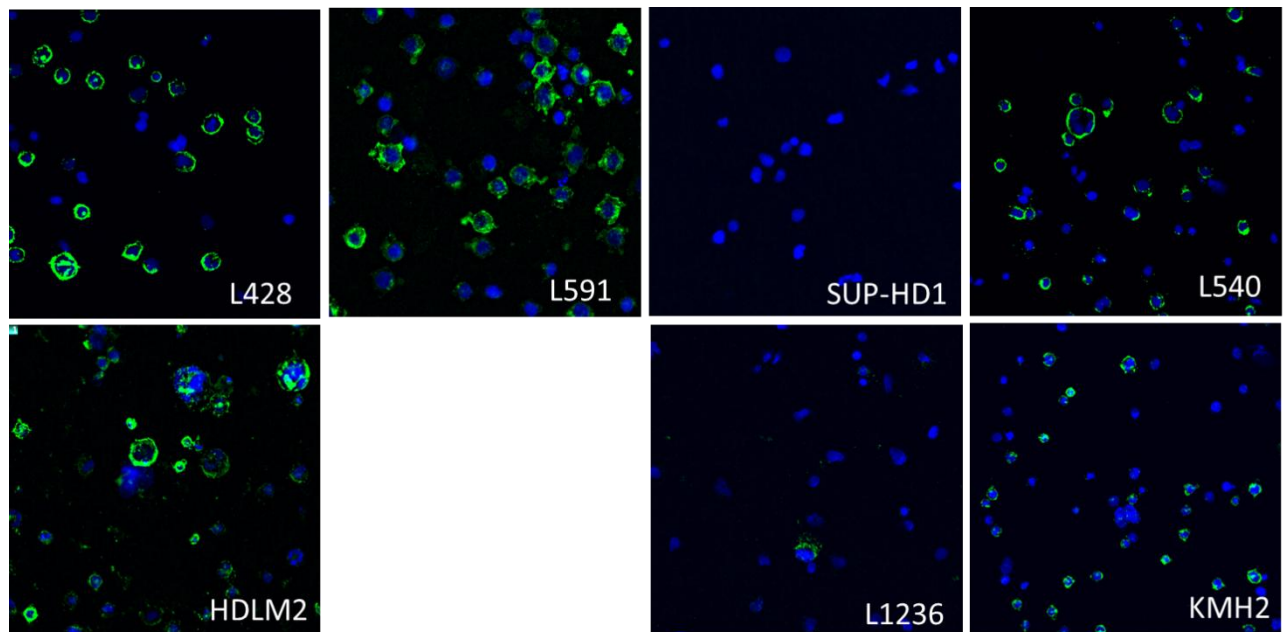
A : Different origins, histologic types and EBV status of used HL cell lines.

Cell line	Origin	Histology Type	EBV status	Gender (age)
L428	B-cell	nodular sclerosis young	-	Female (37)
L591	B-cell	nodular sclerosis young	+	Female (31)
SUP-HD1	B-cell	nodular sclerosis young	-	Male (33)
L540	T-cell	nodular sclerosis young	-	Female (20)
HDLM2	T-cell	nodular sclerosis old	-	Male (74)
L1236	B-cell	mixed cellularity	-	Male (34)
KMH2	B-cell	mixed cellularity	-	Male (37)

B : Immunophenotype characterization using CD30 and CD15, the clinical markers of HL cell lines, demonstrate the heterogeneity in the expression of these two markers.

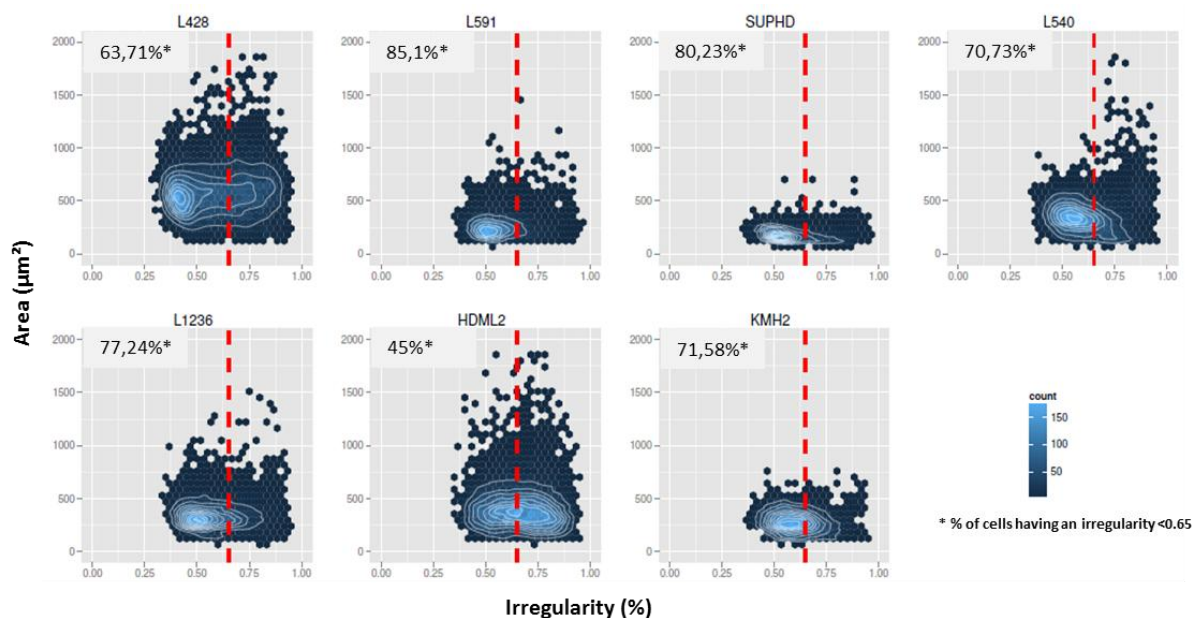


C : Immunofluorescence staining with CD30 antibody on HL cell lines demonstrate the high heterogeneity of CD30 expression.

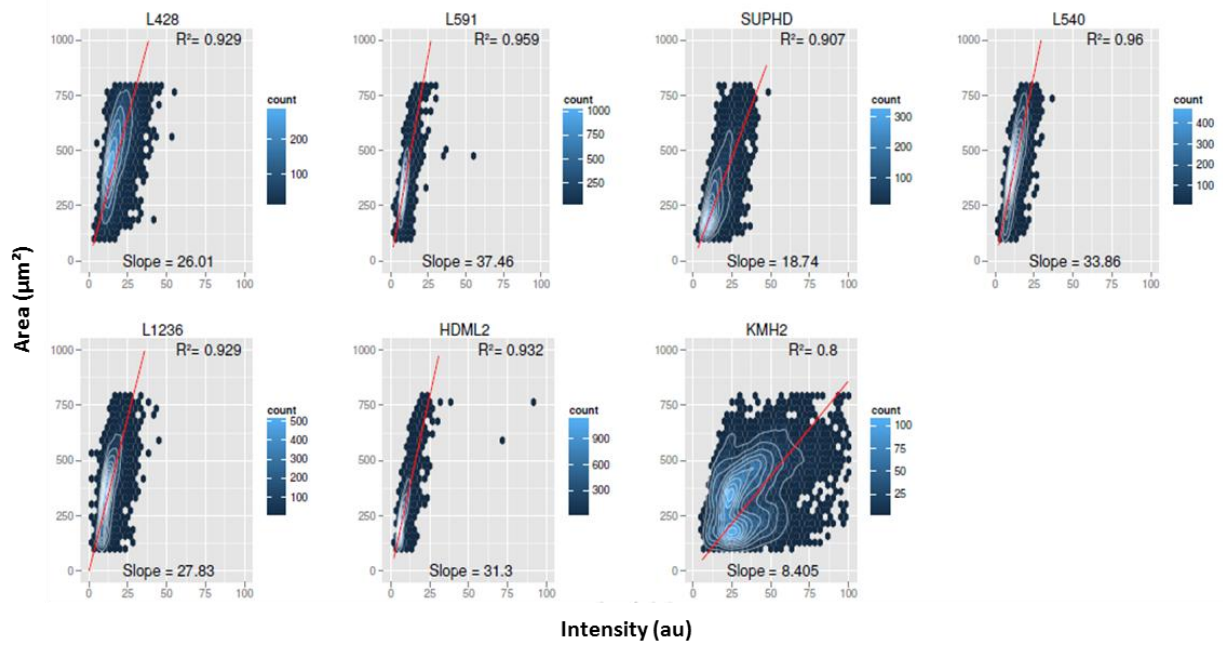


Supplementary data 2

A : HL cell lines were characterized by nuclear irregularity. More HL cell lines exhibited more than 70% of irregular nucleus.

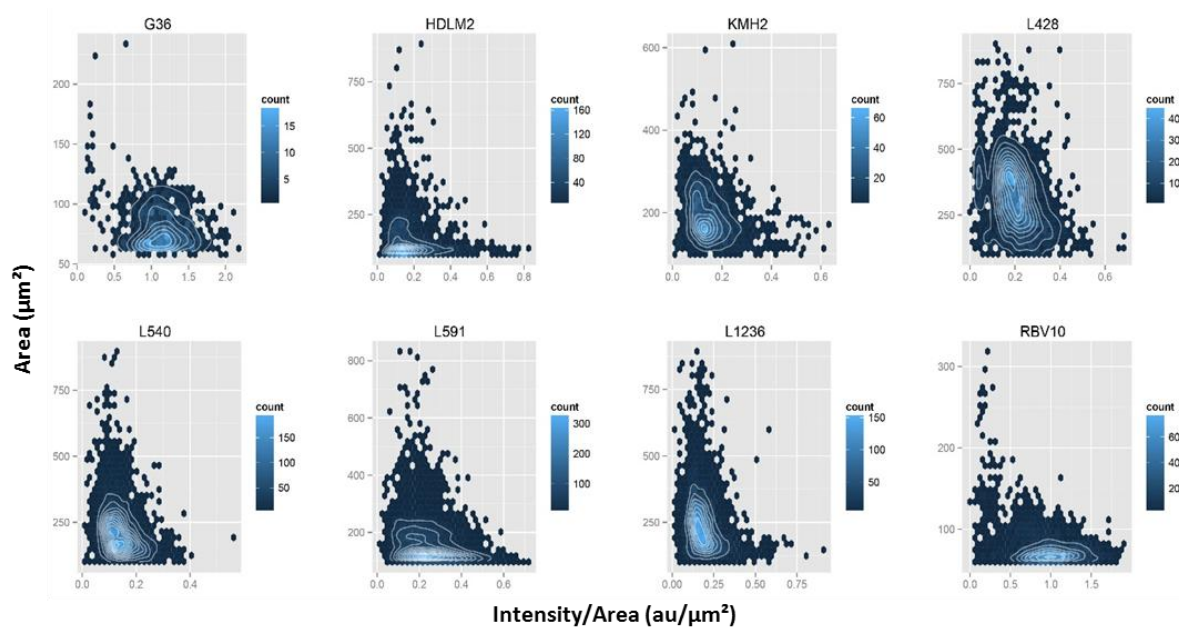


B: Telomere length in HL cell lines according the area of the cells. The slope of the curve was less than 30 demonstrating that big cells have a short telomere than small cells. KMH2 cell line presented two populations: small cells with a long telomeres and big cells with short telomeres



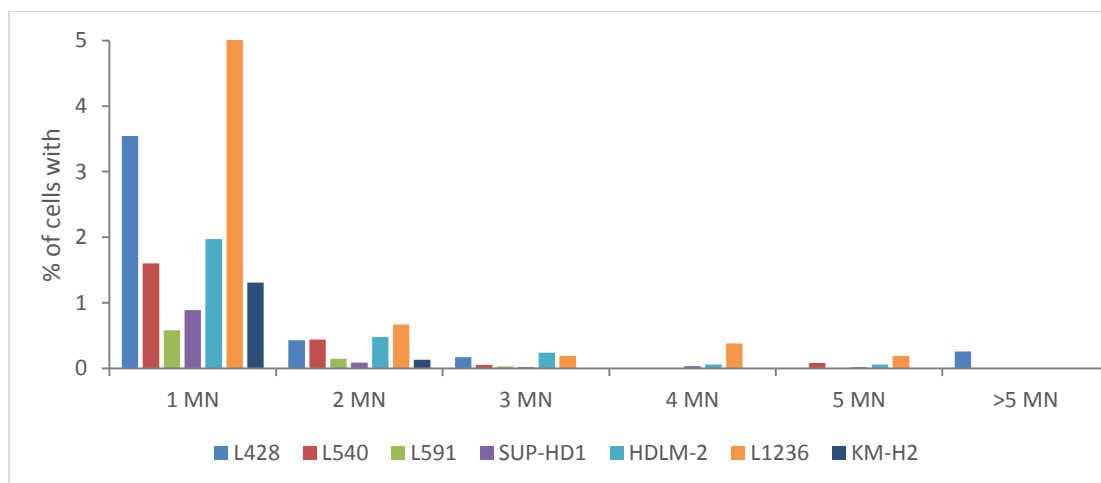
Supplementary data 3

The intensity of Lamin B according the area of the cells demonstrated the low intensity of lamin B1 in big HL cell. G36 and RBV10 were a lymphoblastic cell lines and served as control



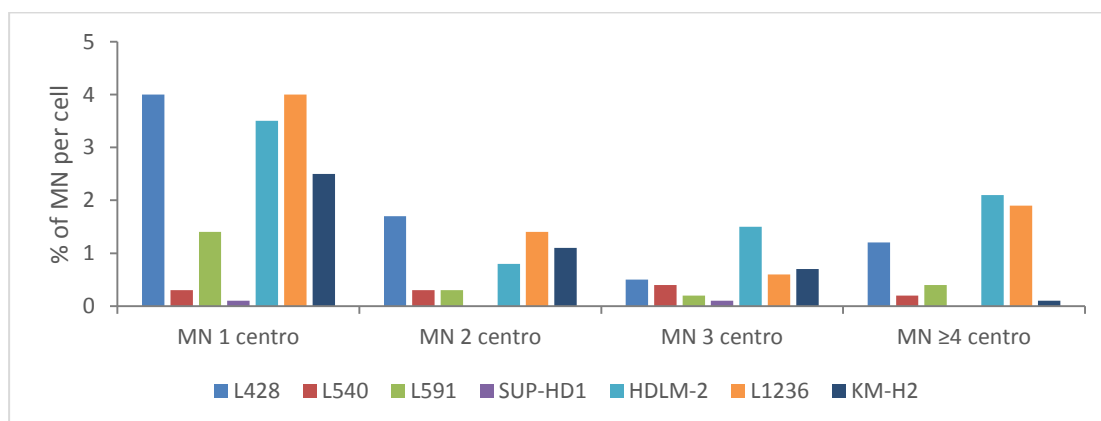
Supplementary data 4

Spontaneous micronuclei formation in HL cell lines; the repartition of cells having one and more micronuclei was presented.



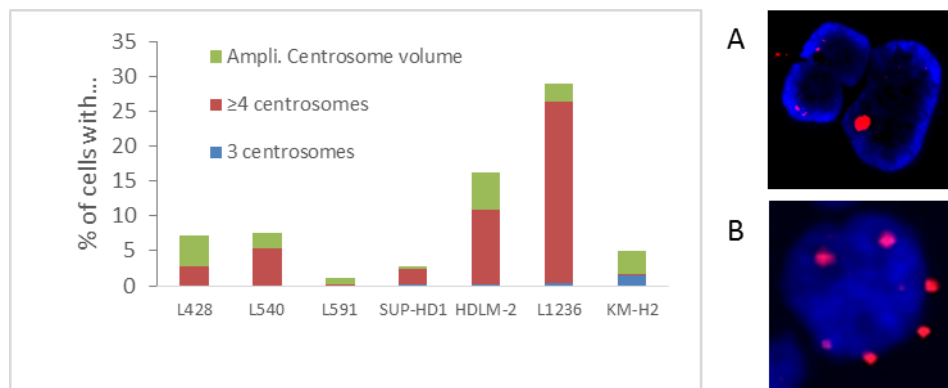
Supplementary data 5

MN contained telomere and centromere sequences; amount of centromeres per micronucleus.



Supplementary data 6

Immunofluorescence using Anti-gamma Tubulin antibody centrosome marker;
Measurement of centrosome size (image A) and number (image B) for each cell line



Supplementary data 7

Using M-FISH technique after telomere and centromere staining, structural chromosomal aberrations were assessed in all HL cell lines.

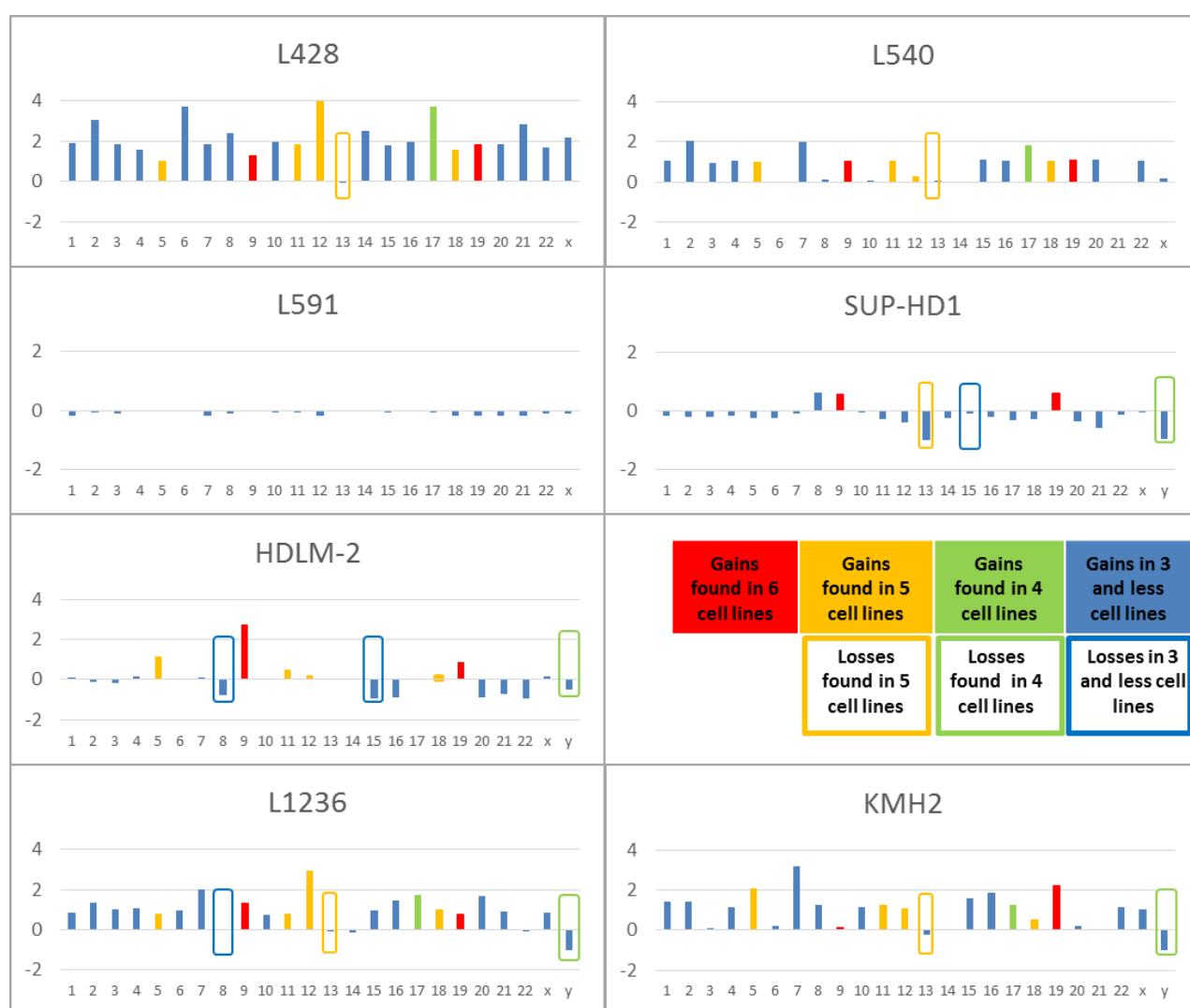
L428 (30) 2909		L540 (30) 1962		L591(30) 1403		SUP-HD1 (30)	
						amplification 1	
der 1	t(1,x) [29]	der 1	t(1,5) [29]	der 8	t(8,11) [30]	der 1	[29]
der 2	t(2,8) [62]		t(1,12) [30]	der 12	t(12,7) [5]		t(1,13) [1]
der 3	t(3,19) [1]	der 2	t(2,12) [30]	der 14	t(14,9) [30]		t(1,17) [1]
	t(3,3) [1]		t(2,8) [29]	dic	dic(13,16) [1]	der 2	t(2,8) [3]
	t(3,x) [1]		t(8,2,8) [1]		dic(2,8) [1]		t(2,8,2) [26]
der 5	t(5,5) [23]	der 3	t(3,9) [1]		dic(7,7) [1]		t(2,17) [2]
	t(5,5,6) [1]	der 5	t(5,1,5) [29]			der 3	t(3,2) [1]
	t(5,6) [59]	der 7	t(7,19) [30]			der 4	t(4,16) [26]
	t(5,9,6) [2]	der 8	t(8,20,6) [30]				t(4,16,5) [3]
	t(5,14) [1]	der 14	t(11,14) [29]				t(11,4,16) [26]
der 6	t(6,14) [1]	der 15	t(15,1) [63]				t(4,18) [27]
	t(6,9) [1]	der 16	t(16,18) [30]			der 5	t(5,4) [3]
der 7	t(7,15) [29]		t(16,x) [29]			der 6	t(6,6,9) [28]
der 8	t(8,x) [2]	der 21	t(21,17,15) [30]				t(6,8) [1]
	t(8,18) [2]		t(21,20) [30]			der 8	t(9,8,12) [31]
	t(8,2,5) [1]	der 22	t(22,11,14) [30]				t(8,18) [25]
der 9	t(9,14) [44]	der X	t(x,2) [30]				t(8,18,12) [1]
	t(9,11,21) [32]	Dic	dic(17,21) [1]			der 9	t(9,21) [25]
	t(9,5) [1]		dic(7,3) [1]			der 12	t(21,9) [28]
	t(9,6,14,1) [1]					der 13	t(13,1) [1]
	t(9,11) [1]					der 15	t(15,2) [31]
	t(9,4) [1]						t(15,5) [31]
	t(9,2) [1]					der 16	t(16,19) [1]
der 10	t(10,8) [1]						t(16,x) [1]
	t(10,x) [1]					der 18	t(18,8) [1]
der 11	t(11,19) [3]						t(8,18,8) [19]
der 13	t(13,5) [58]					der 19	t(19,12) [21]
der 14	t(14,9) [31]					der 20	t(20,3) [1]
	t(t(14,9,4)) [19]					der 22	t(22,13) [30]
	t(14,17) [16]					der x	t(x,16) [1]
	t(3,14,9) [1]					Dic	dic(11,11) [2]
der 16	t(16,22) [2]						
	t(16,9,16,9,1) [30]						
	t(16,9) [1]						
der 17	t(17,2) [45]						
	t(17,11) [1]						
	t(17,4) [2]						
	t(17,x) [1]						
der 18	t(18,7) [12]						
	t(18,2) [1]						
	t(18,16) [1]						
der 19	t(19,14) [1]						
der 21	t(21,x) [29]						
	t(21,19) [1]						
Dic	dic(3,15) [17]						
	dic (10,11) [1]						

HDLM2 (30) 1125		L1236 (30) 1817	
der 2	t(19,2,4,2,4) [27]	der 1	t(1,8) [14]
der 3	t(3,4,15,14) [27]		t(1,14) [26]
	t(3,9) [1]		t(1,18) [27]
der 6	t(6,3) [29]		t(9,1,18) [1]
der 7	t(7,3) [28]		t(1,9) [3]
der 8	t(8,2) [31]		t(1,17) [1]
	t(12,8,2) [30]	der 2	t(2,2) [1]
der 9	t(9,13) [1]		t(2,18) [1]
	t(2,9,21,9) [1]		t(2,x) [1]
der 10	t(10,12) [15]	der 3	t(3,16) [26]
der 11	t(11,16) [20]		t(3,9) [13]
der 13	t(13,16,11) [30]		t(3,18) [1]
	t(13,22) [29]		t(3,14,1) [1]
der 14	t(14,15,6) [29]		t(3,14) [1]
	t(14,21) [28]	der 4	t(4,8) [29]
	t(14,15,1) [1]		t(1,14,t(4,8)) [2]
	t(14,15,11) [1]		t(4,1) [1]
der 15	t(8,15,17,1) [25]		t(4,1,9) [1]
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			t(16,20) [1]
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			t(15,20,9) [1]
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		der 22	t(22,15) [25]
			t(22,6) [28]
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KMH2 (30) 1866			
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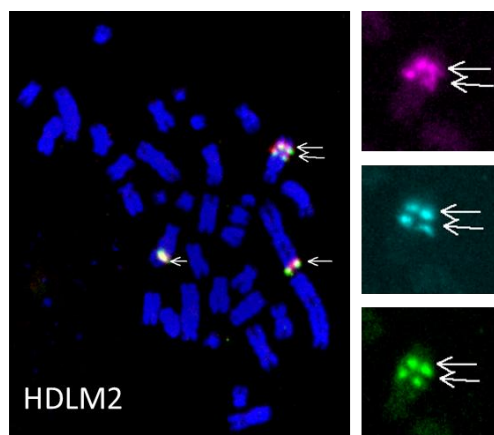
Supplementary data 8

Numerical chromosomal aberrations including gain and loss of whole chromosomes. Gains of chromosomes 9 and 19- marked in red- were found in 6/7 cell lines; gains of chromosomes 5,11,12,and 18 -marked in yellow- were found in 5/7 cell lines; and gain of chromosome 17 –marked in green- was found in 4/7 cell lines. Gain of other chromosomes was observed in at least one cell line. Loss of chromosome 13 – framed in yellow- was found in 5/7 cell lines; chromosome y -framed in green was found in 4/4 cell lines and loss of chromosomes 8 and 15 -framed in blue- were found in two/7 cell lines. Loss or gains of more copies of the same chromosome are shown on the y axis, value 0 representing the normal number of chromosomes.

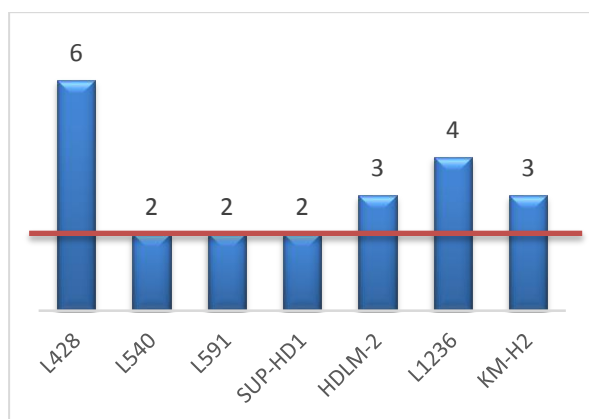
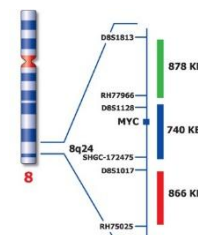


Supplementary data 9

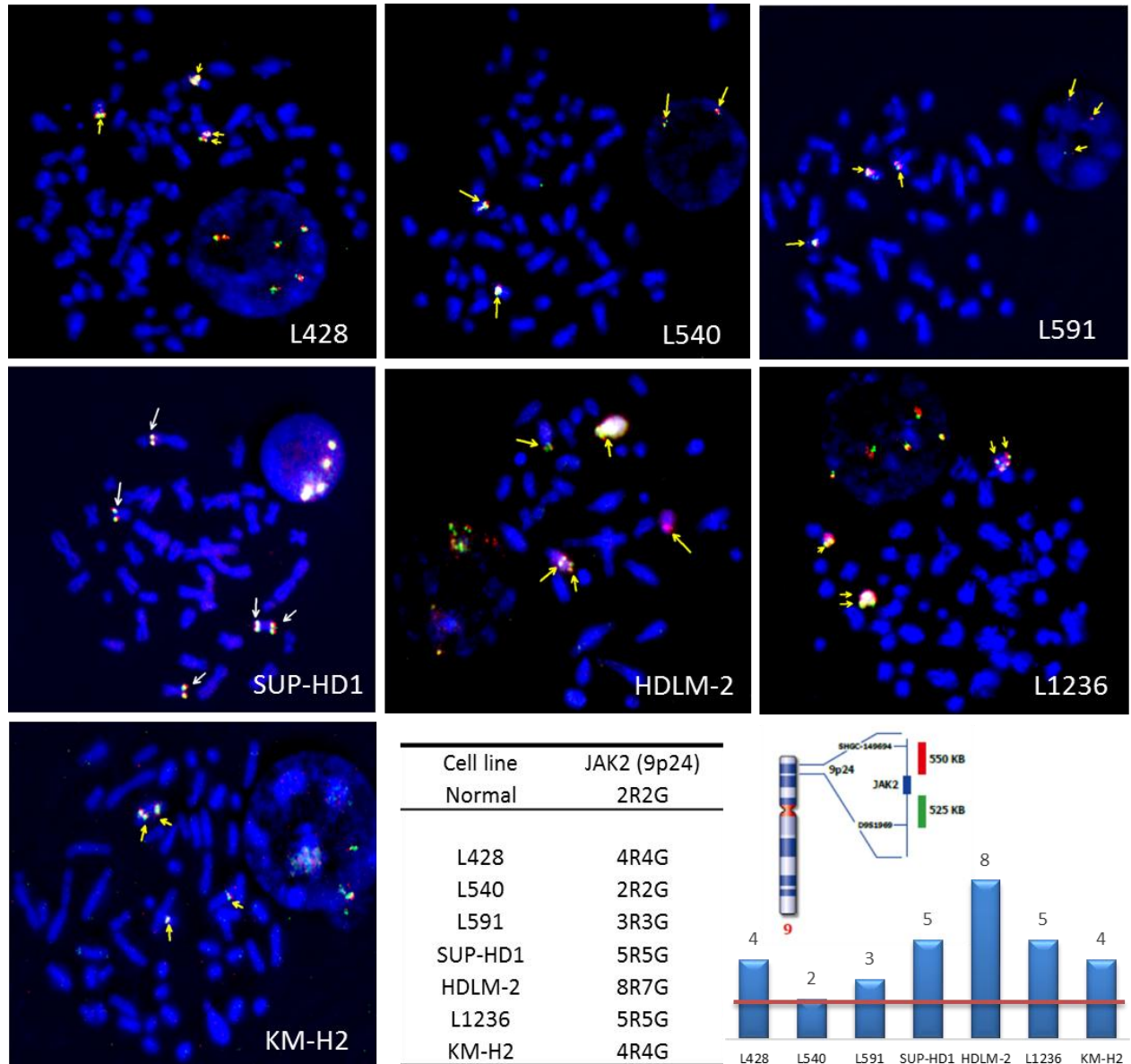
A: c-MYC gene in HL cell lines was assessed using FISH painting. The amplification in the region containing c-MYC gene was found in L428 cell lines (6 copies), L1236 (4 copies), HDLM2 and KM-H2 (3 copies), without the mutation in the gene c-MYC



Cell line	cMYC (8q24)
Normal	2R2G
L428	6R6G
L540	2R2G
L591	2R2G
SUP-HD1	2R2G
HDLM-2	3R3G
L1236	4R4G
KM-H2	3R3G

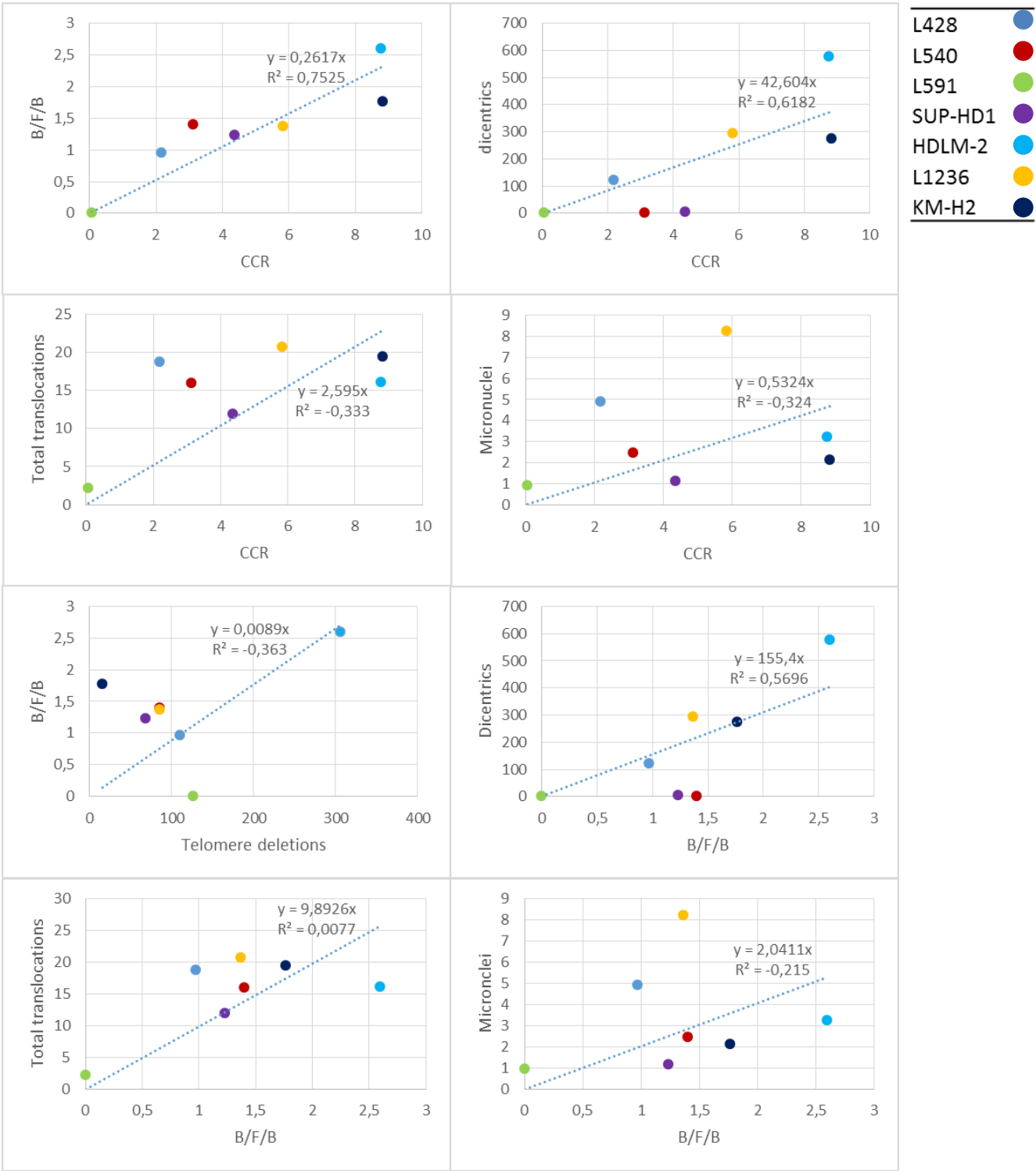


B: Immunofluorescence of JAK 2 gene in HL cell lines. High copies number of the gene were found in 6/7 cell lines; without the mutation of the JAK2 gene.



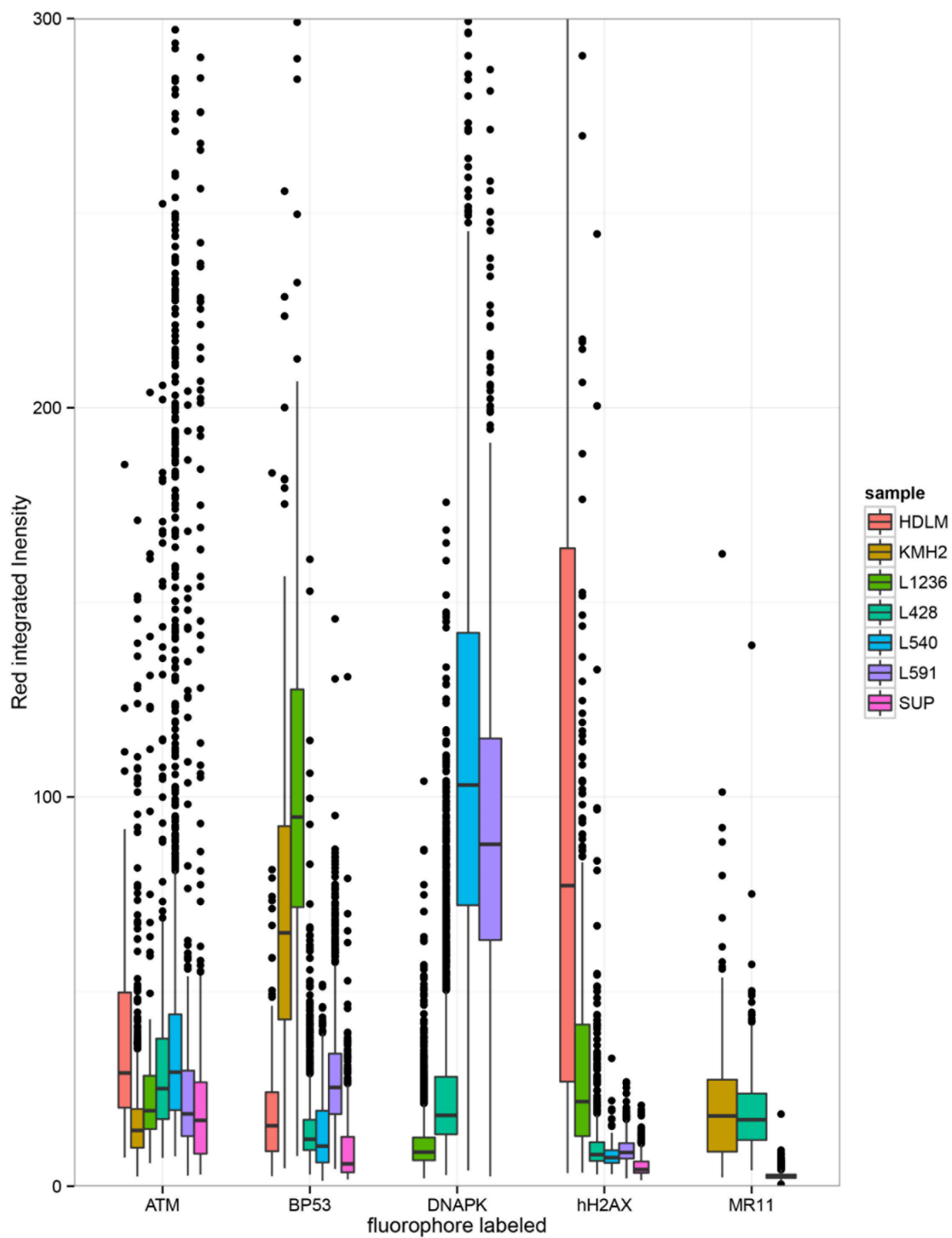
Supplementary data 10

Correlation between CCR and B/F/B and correlation of CCR and B/F/B with the number of dicentric chromosomes, micronuclei and total translocations respectively.



Supplementary data 11

Base line expression of proteins implicated in the DNA repair (gH2AX, BP53, DNA-PKcs, ATM, MR11) were assessed using immunofluorescence technique. The mean total intensity of the foci of different proteins was represented as well as the confidence intervals.



Part III

In this part, we exposed the establishment of a reliable xenograft model of Hodgkin lymphoma suitable not only in the exploration and discovery of new therapy protocols but also in the study of the origin of the HL cells which grew in the mice and the different steps in the transformation from “clonogenic cells”.

The main finding in this work was to demonstrate the diploid origin of the cells growing in the mice as well as in methylcellulose. Negative profile of these cells regarding Hodgkin lymphoma markers (CD30 and CD15) was found. Cell sorting of negative cells in HL cell lines was performed in order to validate this hypothesis and demonstrate their clonogenic potential to re-establish the parental cell line.

My contribution in this work, was to focus on the cytogenetic characterization of injected cells into NSG mice and the cells derived from tumors obtained from different organs of mice. Telomere dysfunction and chromosomal instability were performed using the same techniques described in the last part of this thesis.

Characterization of a reliable xenograft model of Hodgkin lymphoma suitable for the study of tumor origin and the design of new therapies

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Number of text pages: 19

Tables: 3

Figures: 5

Running head: HL animal model

Grant: This work was supported by a grant from Mundipharma (MUND-CEA: 01087).

Abstract

Background:

The enigmatic disease Hodgkin lymphoma (HL) is the subject of extensive ongoing research. The limited accessibility of biopsies, heterogeneity between patients and poor *in vitro* growth of tumor cells have necessitated the development of animal models to decipher immune escape mechanisms and design new therapies. We have successfully determined the conditions for the *in vitro* amplification and characterisation of “clonogenic B cells” in HL cell lines and their transplantation into immunodeficient NOD-SCID-gammac^{-/-} (NSG) mice.

Methodology:

Seven HL cell lines were cultured in semi-solid medium and clones were isolated and amplified prior to transplantation by the intravenous route into NSG mice. Immunohistochemistry, immunofluorescence, flow cytometry and cytogenetics studies were performed to characterize the original cell lines as well as injected and graft cells. PET/CT scan was used to quantify tumor cell infiltration in NSG mice.

Results:

Following *in vitro* amplification, cells of the L428 cell line showed a high cloning efficiency, increased proliferation and reliable reproducibility of HL growth in xenografted NSG mice compared to several other HL cell lines. Four to eleven weeks following injection (10^3 to 10^6 cells), infiltration of the lymph nodes, liver, bone marrow, and spleen by tumor cells was observed. Mice injected with 10^3 cells showed a high survival rate (more than 28 weeks) without late effects, offering a large time window for drug testing. Significant tumor cell infiltration was observed 7 weeks after injection. Immunophenotype characterization of the first cells harvested from the mice demonstrated a CD30, CD15 and CD14 negative profile. Over time, the cells acquired CD14 followed by CD15 and finally CD30 surface markers. Cytogenetic analysis confirmed the diploid origin of the cells growing in the mice. Cell sorting of negative cells in HL cell lines was performed to validate this hypothesis and demonstrating their clonogenic potential to recover the parental cell line.

Conclusion:

The establishment of this reliable animal model of HL is a major advance, not only for the development of novel therapeutic strategies for relapse/refractory HL patients, but also for understanding the pathology of the disease itself and creating the possibility to translate this methodology to HL patients.

Introduction

Hodgkin lymphoma (HL) is a malignancy of the immune system, characterized by the presence of scarce tumor cells called Hodgkin and Reed-Sternberg cells (HRS), derived, in most cases, from germinal center B cells (Kuppers et al 1994). Nevertheless, the poor *in vitro* growth of HL lymph nodes and the lack of reliable animal models present a major obstacle for mechanistic studies to understand the pathology and the development of novel therapies. Cell lines derived from malignant HRS cells are, therefore, of particular importance (Nagel et al 2013).

Even though all established HL cell lines give rise to solid tumors following subcutaneous or intraperitoneal injection of untreated SCID mice at the site of injection (Dewan et al 2005b, von Kalle et al 1992), few reproducible and systemic models of HL have been described. The xenografted HL cell lines, as well as human tumor cells, display a low efficiency (50% and 23% respectively) (Jones et al 2009, Kapp et al 1993) for the induction of mediastinal tumors and tumor dissemination. Only, one approach, using the L540 and L540cy, T-cell lines, has been successfully used for the standardization of new molecules for the treatment of HL after the induction of mediastinal tumors and tumor dissemination (Borchmann et al 2003). Despite the fact that L428 and KMH2, the most studied cell lines, originated from B cells, consisting predominantly of HRS cells, it has not been possible to successfully and reproducibly establish xenografts with immunodeficient NOD-SCID-gammac^{-/-} (NSG) mice (von Kalle et al 1992). While a previously published study demonstrates successful engraftment of these HL cells in NSG mice, it required a large number of cells, was only performed on a limited number of animals, and no further validation has been reported (Dewan et al 2005b). More than 20 years ago, Newcom et al. reported that the L428 HL cell line contained a small population of clonogenic B cells which appeared to be responsible for the generation of the HRS cells and the continuous growth of the cell line (Newcom et al 1988). More recently, Jones et al (Jones et al 2009) demonstrated in HL cell lines (L428 and KMH2), the lymph nodes of patients, and the peripheral blood of newly diagnosed HL patients, that the presence of B-cell subpopulations (<1%) was responsible for the generation and maintenance of the predominant HRS cell population (Jones et al 2009). However, the low frequency of these cells in HL patients and the importance of the specific microenvironment necessary for the growth of HL cells (Sanchez-Aguilera et al

2006), have precluded the generation of an HL animal model via the direct injection of these cells from patients (Jones et al 2009) whereas it has been possible to generate a multiple myeloma animal model from patient material (Matsui et al 2008). In this work, we propose that L428 would be the best candidate to establish a xenograft HL animal model. We have successfully established conditions for the *in vitro* amplification of these cells and their transplantation into NSG mice leading to a tumor model characterized by long-term survival without side effects. We have investigated the infiltration of the cells into various organs, as well as the reproducibility of the model. Immunophenotype analysis of the first cells to arise in the NSG mice demonstrated the absence of HL surface markers with the acquisition first of CD14 followed by CD15 and finally CD30 antigen over time. Cytogenetic analyses demonstrated the diploid character of the tumor cells isolated from the mice. We provide the first proof of concept for the transformation from B-cells to HRS cells suggesting a potential role for this mechanism in the treatment modalities of HL patients.

Materials and methods:

Materials

Cell lines and culture conditions

The human HL-derived cell lines L428, KMH2, L591, HDLM2, L540, SUP-HD and L1236 were provided by Dr Stefen (ATCC, MD, USA) and were confirmed to be free of Mycoplasma infection. The cells were grown in Gibco RPMI 1640 medium supplemented with Glutamax (Gibco-BRL, Grand Island, NY) and 10%FBS (Eurobio, Courtaboeuf, France) and 1% antibiotics (Gibco-BRL) at 37°C. The different cell lines exhibited varying division rates.

Xenograft models

Immunodeficient NOD-SCID-gammac^{-/-} (NSG) mice aged 5 to 6 weeks were purchased from Charles River (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ). The mice arrived two weeks before the beginning of the experiments and were housed in micro-isolator cages during the entire course of the study. Immediately prior to the injection of HL cells, mice were exposed to 3Gy gamma irradiation using an IBL637 ¹³⁷Cs irradiator at a dose rate of 0.61Gy/min. From 10³ to 10⁶ cells, either from the

original cell lines or following amplification, were injected intravenously via the retro-orbital sinus or the tail.

The mice were sacrificed by cervical dislocation and different organs were analysed and tumoral infiltration examined using multiple techniques (supplementary data 1).

Animal experiments were performed according to French law and were approved by the institutional Ethics Committee for animal Experimentation.

Methods

Clonogenic assays

To assess the clonogenic potential of the HL cell lines, from 10^3 to 10^5 cells (depending on the growth of the cells) were placed into 1 mL of 1.2% methylcellulose (Stem cell Technologies) in RPMI 1640 medium supplemented with Glutamax containing 10% FBS (GIBCO-BRL) and 1% Hepes (GIBCO-BRL). Colonies consisting of more than 50 cells were scored approximately 14 days after plating using an inverted microscope. The larger colonies were selected, re-suspended, and amplified in 24 well microtiter plates. New cell lines were established from the cells which grew in the methylcellulose. The clonogenic index was established and various cell surface markers were measured in order to characterize the cell lines. Three independent experiments were performed in triplicate.

Tissue and slide preparation

Following dissection of different organs, including tumors, the first portion was fixed in 4% PFA for Immunohistochemistry (IHC) while the second was used for FACS and cytogenetic analyses. To obtain a single cell suspension, the tissues were first cut into pieces. Thereafter, the cells were physically disaggregated using an 80 μ m nylon cell strainer and filtered a second time through a 70 μ m nylon cell strainer (BD Biosciences, Erembodegem, Belgium).

The injected L428-c cell line served as a control for all experiments.

Immunohistochemistry analysis

Organs were fixed in 4% PFA, trimmed and post-fixed in 70° ethanol. They were then briefly processed using a vacuum inclusion processor and paraffin blocks were prepared. Five micrometer-thick sections were cut from these blocks and the resulting sections were stained with hemotoxylin-eosine (H&E) for histopathological analysis. In addition, IHC was carried out on of the totality of liver and spleen

specimens for confirmation of the spread of engrafted cells. The search for single cells or small groups of grafted HL cells was performed using an anti-CD30 antibody (DAKO, France; 1:40, EDTA pH9 pre-treatment) and the Ventana Discovery XT IHC system. Apoptosis and tumor necrosis were analysed.

All IHC-stained sections were initially evaluated and scored by a hematopathologists. The same slides and the original scores were then reviewed by human pathologist's expert in Hodgkin Lymphoma.

Fluorescence-activated cell sorting and flow cytometry analyses

Cells were gated to exclude apoptotic or necrotic cells and sorted into CD30-/CD15- and CD30+ and CD15+/CD30+ fractions by gating on the lowest and highest 5% PE-expressing cells, respectively. Following sorting, the CD30-/CD15- cell fractions were analyzed using a FACScan flow cytometer (Becton Dickinson) and found to be more than 98% pure (supplementary data 2) . For phenotypic analyses of cell lines or sorted cells, cells were prepared as described, and then stained with mouse anti-human CD30-phycoerthrin (PE), CD15–fluorescein isothiocyanate (FITC), CD20-allophycocyanin (APC), CD14-APC-cyanine 7 (Cy7), CD45-APC (PE) and CD68 (PerCP/Cy5.5) (all antibodies from BD PharMingen, San Diego, CA). Cells were subsequently analysed using a FACS LSR II (Becton Dickinson, Franklin Lakes, NJ). The injected HL cell line served as a control for all experiments.

The separation of human and mouse cells was performed using mouse anti-human CD45. The characterisation of these selected cells was established using anti-human CD14, CD15 and CD30 antibodies. 10000 events were analysed for each sample if possible.

Immunofluorescence for measuring CD30

Cells were cytopsyn onto poly-L-lysine-coated glass slides at 700 rpm for 4 min, fixed with 10% formalin for 10 min, and treated with 0.25% Triton X-100 solution for 10 min. After blocking with 5% bovine serum albumin (Sigma), the cells were incubated with an anti-CD30 antibody (DAKO A/S, Glostrup, Denmark). Then, cells were treated with Cyanine 3 labelled anti-mouse IgG (Invitrogen, Carlsbad, CA). As a negative control, staining was carried out in the absence of primary antibody. The L428-s cell line served as positive control.

Cytogenetic analysis

Separated cells were cultured in the presence of RPMI 1640 supplemented with 10% FCS and antibiotics, colcemid (0.1 µg/ml) added 2h before harvesting, and the slides with metaphase chromosomes prepared following the standard methanol/acetic acid (3/1, v/v) procedure.

Six new cell lines were generated after one month of *in vitro* culture. Cytogenetic analysis was performed first using telomere and centromere staining with PNA probes in order to detect unstable chromosomal aberrations (M'Kacher et al 2014). Secondly, karyotype analysis was performed using multi-FISH probes (Metasystems GmbH, Althusseim, Germany), according to the manufacturer's recommendations on the same slide. Images of hybridized metaphases were captured using a charge coupled device camera (Zeiss, Thornwood, NY) coupled to a Zeiss Axioplan microscope and processed using ISIS software (Metasystems).

PET/CT imaging

[18F]FDG-714 PET was used to image and quantify *in vivo* infiltration of tumoral cells. PET/CT imaging and data analysis were performed using small-animal INVEON (Siemens, Munich, Germany) tomography. This system combines both PET and CT modalities under the control of a unique workstation, including CT-based attenuation correction which allows for superior quality PET images. PET/CT scans were performed using [18F] FDG at different intervals following cell injection. The PET/CT protocol has been previously described (Pottier et al 2014).

Statistical analysis

Results are presented as the mean \pm SEM. Comparisons between groups were performed using a 2-tailed, paired Student *t* test or analysis of variance (ANOVA) as indicated.

Results

1- Distinct phenotypic and functional cell populations are present in HL cell lines

One of the clinical hallmarks of HL is the presence of CD30 and or CD15 positive HRS cells. To ascertain the proportion of cells expressing CD30 and CD15 for the 7 HL cell lines, flow cytometry was used to evaluate the frequencies of these markers. As shown in Figure 1A, the immunophenotype of the HL cell lines was quite heterogeneous with some, such as SUP-HD1 and L591, expressing only CD15 and

CD30, respectively, and containing a very small proportion of cells expressing these markers while at the other end of the spectrum HDLM2, L428 and KM-H2 contained >90% CD30/CD15 positive cells

Recent studies have suggested the presence of clonogenic B cells in HL cell lines that represent approximately 1% of the total cell (Jones et al 2009). Faced with the difficulty of isolating these subpopulations from the HL cell lines by Fluorescence-activated cell sorting, we tested each cell line for their colony formation capacity in methylcellulose medium. To examine the self-renewal potential, colonies were scored after two weeks, collected and then washed and serially re-plated. Table 1 summarizes the colony formation results, the number of colonies generated for each HL cell line and the proliferative rate of the selected colonies. The clonogenic cells were represented by the mononuclear HL cells. Small abortive colonies derived from binucleated RS cells were observed. Using the L428, KMH2 and L540 cell lines, we have successfully isolated and amplified growing clones that are able to proliferate *in vitro* following culture in methylcellulose. Of note, individual clones derived from the same cell line showed highly variable *in vitro* proliferation (supplementary data 2). All other HL cell lines (L1236, HDLM2, L591 and SUP-HD) grew poorly in methylcellulose exhibiting no *in vitro* proliferation.

The cell lines derived from the growing clones of L428, KMH2 and L540, called respectively L428-c, KMH2-c and L540-c, showed a very high cloning efficiency compared to the parental cell lines (Table 1). Not only was the new plating efficiency of the L428-c HL cell line approximately 10 fold higher than that of the parental cell line, but it also had a significantly greater *in vitro* proliferation rate (Figure 1B-C).

Taking into consideration the proliferation rate and the clonogenicity index, L428-c was used to establish a xenograft model. We further characterized the phenotype of the new cell lines by flow cytometry measuring CD14 antigen, expressed by myeloid derived suppressor cells in the microenvironment, as well as CD30 and CD15. Figure 1D shows the immunophenotype of L428/L428-c at 5, 6 and 8 weeks after *in vitro* growth following methylcellulose isolation. As expected, the majority of cells expressed high levels of CD30 and CD15. However, the clones established 5 and 6 weeks after *in vitro* culture showed a high level of CD14 expression and contained distinct sub populations using the FSC parameter compared to the parental L428 cell line and L428-c after 8 weeks of culture. These sub populations were negative for all markers. Indeed, L428 and the clone after 8 weeks of culture shared a similar profile with respect to their surface markers.

Table 1: *In vitro* growth of clonogenic HL cell lines in semi-solid medium (methyl cellulose).

HL Cell lines	Colony formation, yes/no	No of colonies/ 10^5 cells, mean (range)	Proliferation of colony/No of colonies
L428	+++	188,5 \pm 18	923 \pm 13
KMH2	+++	169 \pm 20	680 \pm 14
L540	+++	103 \pm 15	420 \pm 19
L1236	+	6 \pm 2	-
L591	-	-	-
SUP-HD1	+	10 \pm 3	-
HDLM2	+	5 \pm 2	-

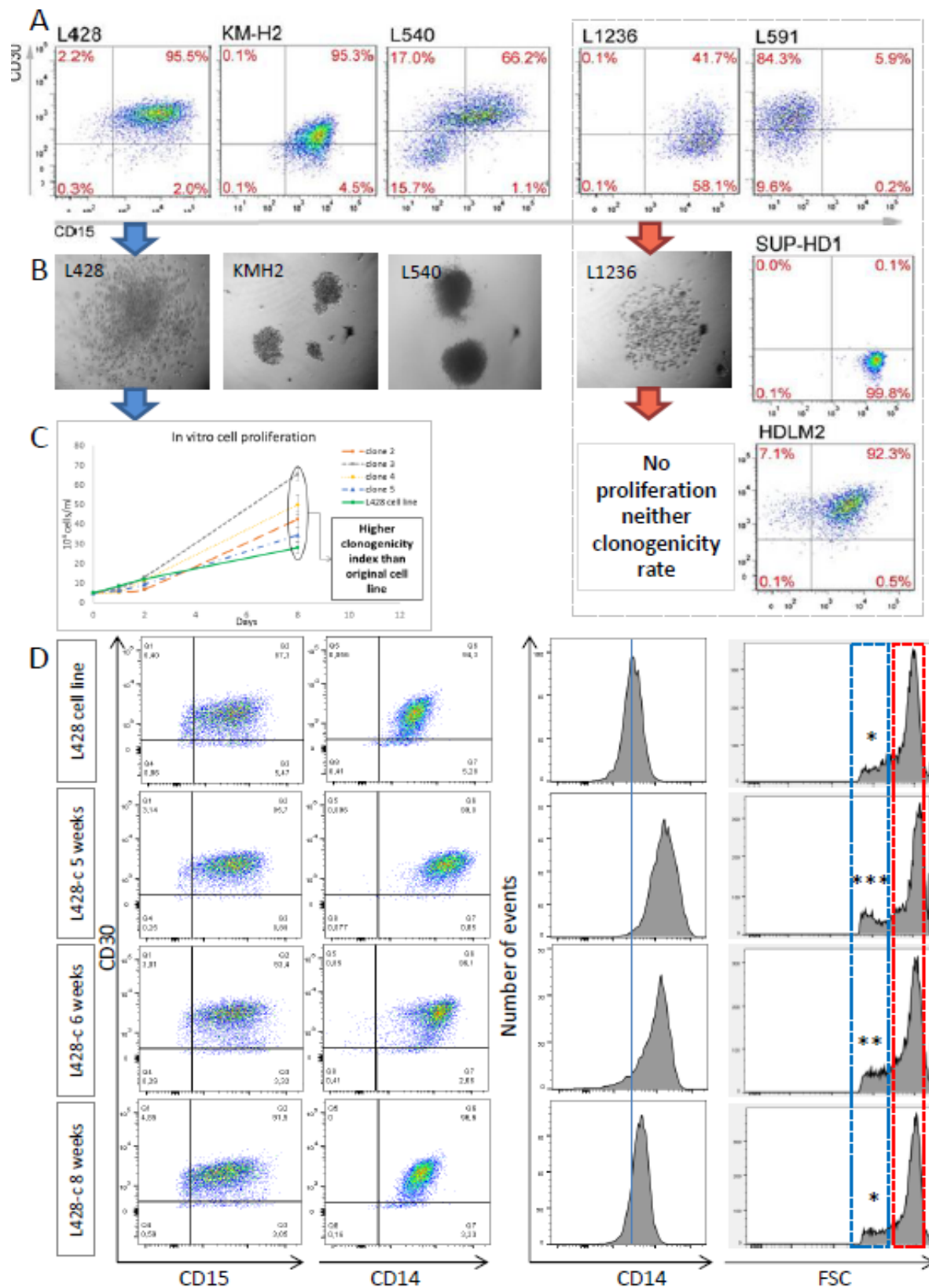


Figure 1 : Immunophenotype and cell proliferation of HL cell lines. (A) Staining for specific surface markers showing the heterogeneity for the expression of CD30 and CD15 surface markers in different HL cell lines. The frequency of cells with different surface markers is represented. (B) Morphology of the clones obtained from L428, KM-H2, L540 and L1236 after growth in methylcellulose. (C) Higher in vitro proliferation of different clones obtained from L428 cell lines compared to the parental cell line. No in vitro cell proliferation was observed for clones derived from L1236. (D) Characterization of the surface markers CD30, CD15 and CD14 of the parental L428 cell line and derived clone (L428-c) at different time intervals: 5, 6 and 8 weeks after establishment. A stronger signal for CD14 was observed in L428-c immediately after establishment compared to the parental cell line or L428-c after several weeks. Forward scattering (FSC) shows the presence of a subpopulation of small cells for L428 and L428-c. The frequency of this subpopulation was higher in L428-c immediately after establishment.

2-Establishment of the xenograph model using the cell line L428-c

After standardization of the *in vitro* culture conditions for the growth of the HL cell lines, the first step of the *in vivo* study consisted of the injection of 10^6 cells of L428-c, KMH2-c, L591 and L1236 per NSG mouse. Of note, while 100% of the mice injected with L428-c displayed tumor infiltration, this was true for only 66% of those injected with KMH2-c (supplementary data 3). No tumor infiltration was observed for mice injected with L591 and all mice died within 5 weeks. For L1236, tumor infiltration was observed at the site of injection in the absence of high proliferation, and tissue necrosis was observed (supplementary data). Table 2 summarizes the data on the infiltration of L428-c HL cells into various organs of the mice. Significant infiltration was observed in liver, spleen and bone marrow (Figure 2) in addition to parotid salivary glands, the eyes and harderian gland related to the site of injection of the cells. Histological analyses of tumors grown in NSG mice showed the presence of large multinucleate CD30 positive cells (Figure 2B-C) as observed for human HL. Of note, the survival of the mice did not exceed 6 weeks using L428-c.

The monitoring of the infiltration of cells using imaging is an important tool for the study of new molecules. [18F]FDG PET/CT is commonly used to evaluate the response of HL to chemotherapy regimens using physiological liver uptake as a reference. Nevertheless, no significant FDG up-take was observed by the large liver tumors of the injected mice (Figure 2E). This result is in line with the lack of liver uptake observed in some HL patients (Rubello et al 2015).

Table 2 : infiltration of L428-c HL cell lines into various organs of NSG mice.

Organ Exam	Histology Exam
Mandibular and parotid salivary glands	
Mandibular lymph node	Tumoral infiltration: mandibular LN, parotide salivary gland
Lung	Tumoral cells in capillaries
Heart	-
Liver	Tumoral cells in sinusoids
Spleen, pancreas	Tumoral infiltration
Stomach	-
Jejunum, colon	-
Kidney	-
Eye and harderian gland	Orbital ocular and conjonctive tissues, harderian glands: bilateral tumoral infiltration
Brain	Meninges: tumoral infiltration and multifocal brain infiltration
Femur and sternum with bone marrow	Femur/sternum: tumoral infiltration
Ovaries or testis	Ovaries: no, Uterus: tumoral infiltration

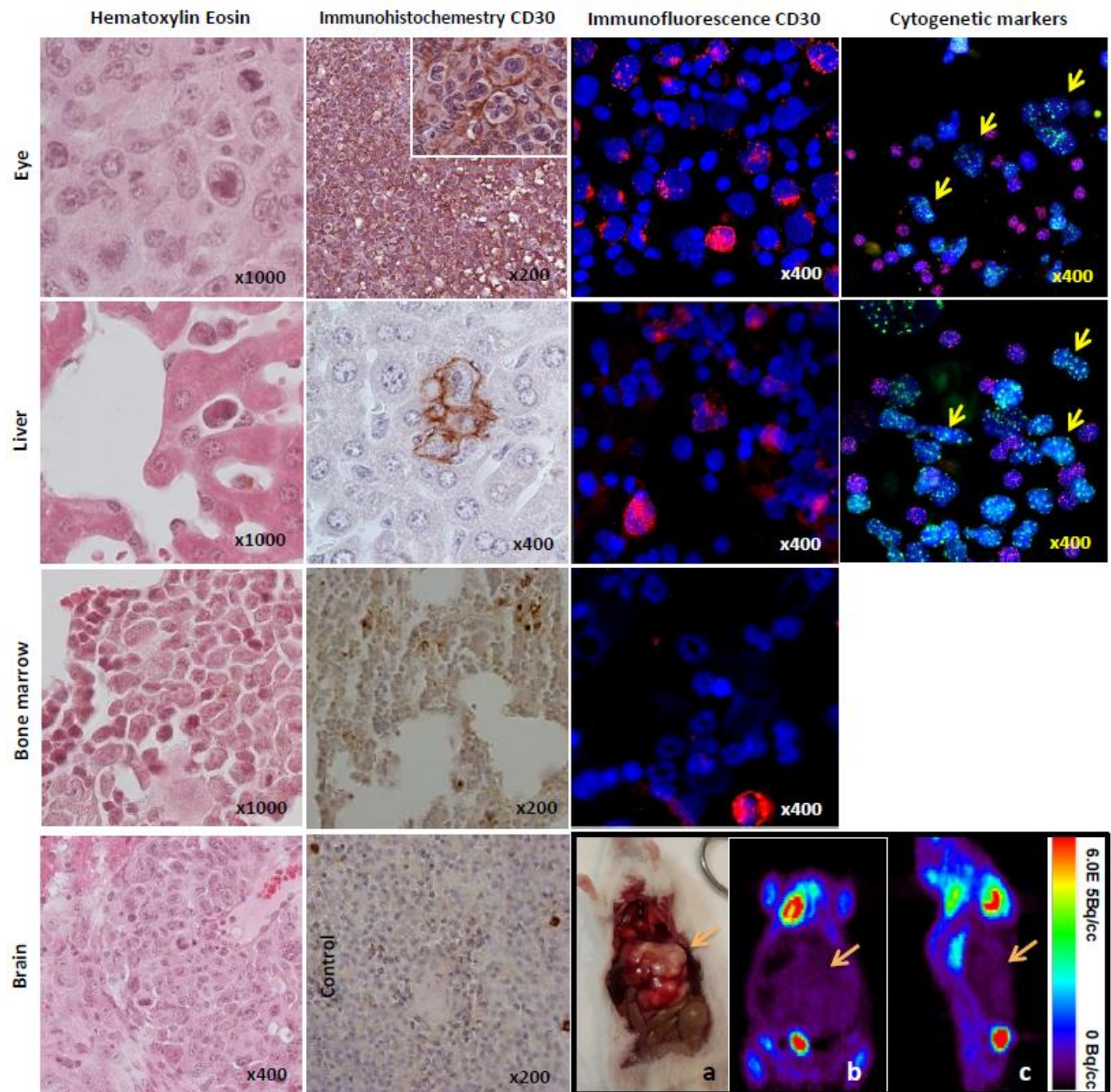


Figure 2 : Infiltration and tumor markers of the L428-c cell line in NOD/SCID/gamma (-/-) (NSG) mice. Hematoxylin and eosine, immunohistochemical and immunofluorescence staining using anti-human CD30 and staining of cytogenetic markers using human centromere probes (green) associated with telomere probes (red) common to humans and mice with a higher intensity for mouse telomeres are shown. The infiltration of tumor cells in the orbit/ocular tissues (site of injection) (a), the liver (b), bone marrow (c), brain (A-d), and the positive control for immunohistochemistry CD30 staining (B-d). Whole body PET images using [^{18}F]FDG on NSG mice developing a severe liver tumor without any significant FDG uptake (E)(a) localization of tumor in liver (b) PET/CT dorsal view and (c) lateral view.

3- Survival of mice and tumor cell infiltration as a function of the number of injected cells using L428-c

The first step towards standardizing the model consisted of determining the optimal number of injected cells to achieve tumor infiltration with a limited effect on viability. A correlation between the number of cells injected (from 10^3 to 10^6) and mortality in the mouse was established. As shown in Figure 3A, the mortality of mice injected with a high number of cells was elevated compared to that observed for mice injected with a lower number of cells. All mice injected with 10^3 cells remained alive, without a significant decrease in weight, out to more than 28 weeks. Due to the low mortality rate for mice injected with 10^3 injected cells, the kinetics of tumoral infiltration was established using this number of cells. A series of 30 mice were followed for a period of 7 months and an evaluation of tumoral infiltration and morphological characterization was performed every week. The weight of the mice was evaluated during the entire period. Weight loss following injection of HL cells followed by weight gain was observed out to 18 weeks (Figure 3B).

The second step was to determine the timing of tumor infiltration, important for the standardization of this animal model for the testing of new molecules. Starting from the fifth week following injection, tumoral infiltration was observed in liver, bone marrow and spleen. Infiltration was clearly detected in a majority of the mice seven weeks after injection as shown in Figure 3C showing a high number of CD45, CD14, CD15 and CD30 positive cells at 7 weeks compared to 5 and 6 weeks (Figure 3C) and tumor progression was time-dependent (Figure 3E) (supplementary data 4).

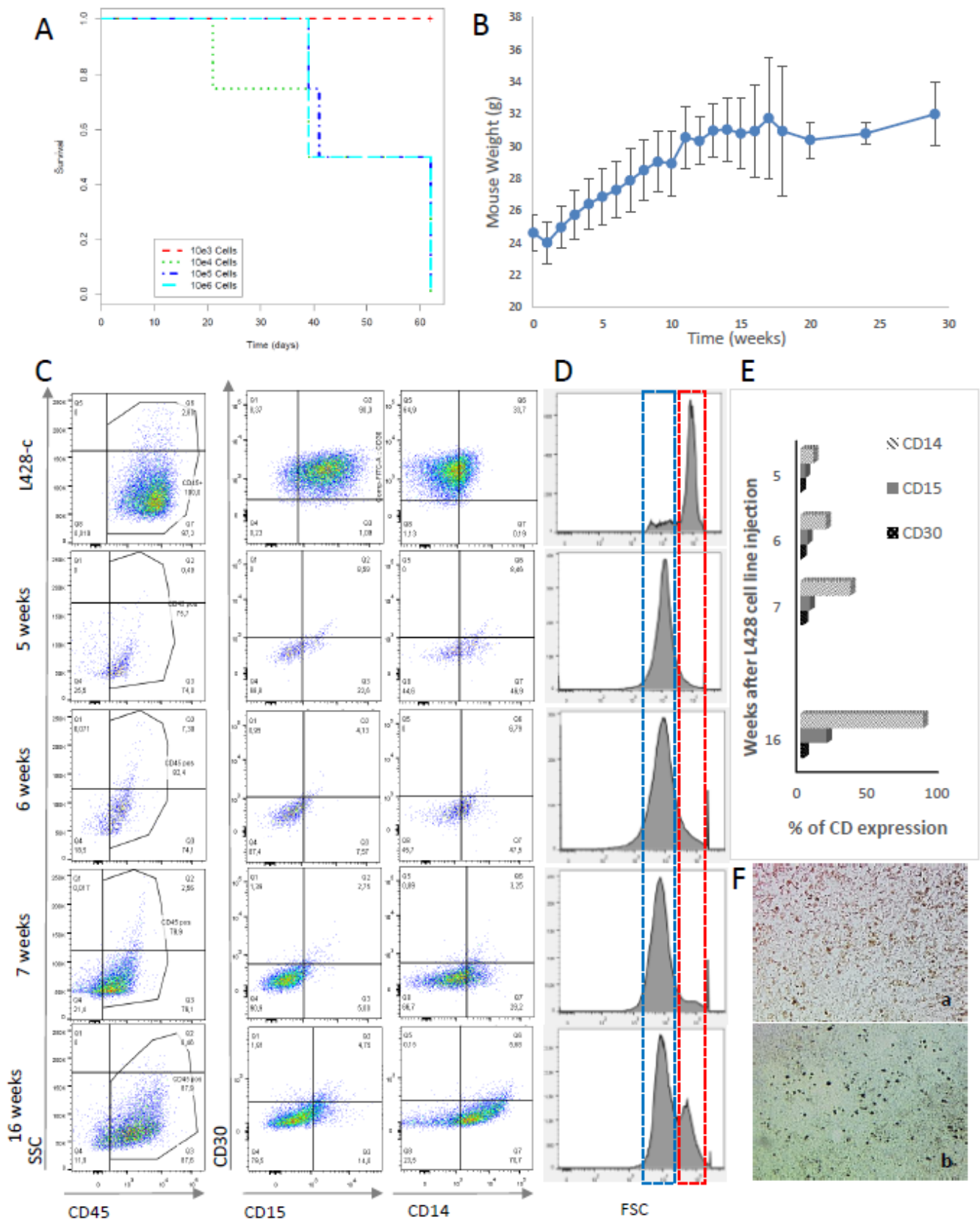


Figure 3 : The follow-up of injected mice and tumor progression in NSG mice. (A) Kaplan Meyer presentation of mouse survival after injection with different cell doses. Mice injected with 10³ cell showed a better tolerability and a higher survival compared to other doses. (B) Mouse weight during the course of the experiment of animals injected with 10³ cells to establish tumor cell infiltration, demonstrating a decrease immediately after injection followed by recovery. (C) Immunophenotype using flow cytometry of tumor cells derived from NSG mice using the surface marker CD45 to select for human cells and CD15, CD30 as specific markers for HL and CD14, a specific marker for myeloid cells. The kinetics of infiltration of tumor cells in the liver of mice at several time intervals after injection show that the best detection of infiltration is at 7 weeks post-injection (D) The forward scattering (FSC) is represented for all derived cells and compared to the injected cell line. The red box represents the mean FSC of L428-c cells and the blue box represents the mean FSC of human tumor cells derived from the NSG mice at different times after injection. The progression from the FSC of L428-c to the human derived cells from mice was observed, demonstrating the progression of human tumor cells derived from mice to the aneuploidy stage. (E) The progression of the immunophenotype of tumor cells derived from NSG mice, demonstrating a high frequency of CD14 positive cells compared to CD15 and CD30 cells. (F) Immunohistochemistry and immunofluorescence using CD68 showing the presence of macrophages in the liver of two mice with different degrees of macrophage infiltration (a, b).

4- Immunophenotype and cytogenetic characterization of tumor cells derived from mice

Immunophenotype analysis of cells derived from the mouse biopsies showed that the initial or primary infiltrating tumor cells growing in mice were negative for CD14, CD15 and CD30 (5 weeks after injection). The progression of tumor infiltration was associated first with the appearance of CD14 positive cells followed by that of cells positive for CD15 and finally CD30 (6, 7, 16 and 32 weeks after injection) (Figure 3C) (supplementary data 4), demonstrating a phenotype similar to that of the L428-c HL cell line by 32 weeks after injection. Nevertheless, these cells showed a higher level of expression of CD14, and CD15 antigens in addition to similar expression of CD30 as the injected L428-c cells (Figure 3C). The level of CD14 expression and the frequency of CD15 and CD30 positive cells were dependent on the degree of tumor cell dissemination (supplementary data 4). Of note, the frequency of triple negative cells in the tumor cell cultures derived from mice was higher than that observed in L428-c or L428 (5% vs 1% and <1%, respectively).

As shown in Figure 3C, the phenotypic characteristics of the initially growing cells were different from those that were injected. We observed that the cells growing in mice were similar to the small subpopulation present in L428-c (Figure 3C). Cells demonstrating aneuploidy were first detectable at 7 weeks after injection and steadily progressed becoming easily distinguishable at 16 and 32 weeks post-injection (supplementary data 4) in liver tumors as seen by the presence of two peaks, the first reflecting the small cells and the second the large cells observed in the parental cell line. Of note, the frequency of CD30 and CD15 positive cells did not exceed 5% of the total cell number at 6 weeks, similar to their observed frequency in Hodgkin lymphoma in men (Figure 3E). Nevertheless, 32 weeks after injection, tumor cells showed a higher frequency of CD30 and CD15 positive cells (more than 50% of the total). Interestingly, the presence of macrophages was confirmed using frozen tissue following CD68 staining and was related to tumor infiltration (Figure 3Fa-b).

Cytogenetic analysis including telomere and centromere staining followed by the M-FISH technique was performed on the parental L428 cell line, L428-c and 6 cell lines derived from the liver tumors of injected mice. Figure 4 shows a tetraploid cell from the parental cell line and the hyper-diploid karyotype of the cell line derived from methylcellulose and injected into the mice. A high frequency of structural chromosomal aberrations was observed in the parental cell line compared to L428-c and cells derived from the mice. Of note, the frequency of dicentric chromosomes

(Table 3) was higher in the cell lines derived from the mice than those observed in L428-c or the L428 parental cell line. The details of the cytogenetic findings can be found in (table 3).

Table 3: Summary of cytogenetic aberrations detected using the M-FISH technique on L428, L428-c* immediately after establishment, L428-c** two months late and on different cell lines derived from mice.

Cell line	Numerical Aberrations		Structural Chromosome Aberrations						
			Clonal			Spontaneous			
	mean no of chromosomes (min-max)	loss and gain of whole chromosomes	Common translocations	deletions and amplifications	additional translocations	translocations	dicentrics		
L428	94,25 (71-103)	gain chromo- somes: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, 19, 20, 21, 22, x,	t(2;8), (5;5), t(6;5), t(9;11;21), t(13;5), t(9;14), t(14;9), t(18;7), t(12;7), t(17;2), t(1;x), t(7;15), t(21;x), dic(3;15)	del(6p), iso(6p), del(12p), iso(12p), del(17p)	t(16;9;16;9;1), t(14;17), t(8;18)	t(10;8), t(8;x), t(20;12), t(10;x), t(17;4), t(19;14), t(18;16), t(21;19), t(9;5), t(5;9;6), t(5;14), t(6;9), t(16;9), t(3;x), t(11;19), t(16;22), t(17;x), t(6;14), t(3;3), t(18;2), t(9;2), t(9;4), t[3(14;9)], t(8;2;5), t(3;19), t(9;6;14;4)	dic(10;11)		
L428-c*	47,12 (19-93)				t(16;9;16;9;1), t(14;17), t(10;2),	t(12;x), t(22;10), t(11;19), t(9;18), t(11;17), t(10;22), t(12;12), t(16;17)			
L428-c**	87,6 (74-92)				t(16;9;16;9;1), t(14;17), t(12;6), t(16;6), t(11;x), t(20;x), t(x;13), t(16;22)	t(1;16), t(11;1), t(12;x), (x;13), t(16;22), t(5;6;7;12), t(22;12), del(12q)	dic(19;17), dic(2;22)		
Mouse 1	72,5 (35-83)	loss chromo- some: 13					t(5;1;5;x), t(11;x), t[2;t(14;9)], dic(11;19), t(16;6), t(6;2), t(6;8;21)	frag 3, t(1;8)	
Mouse 2	83,6 (36-92)						t(16;9;16;9;1), t(14;17), t(6;8), t(10;6), t(15;14), t(x;20), dic(11;19)	t(7;19), t(9;6;12), t(9;6), t(11;4), t(10;6), frag 7, t(7;12), frag x, t(11;x), t(16;4)	dic(11;11;13), dic(19;18), dic(12;17;8), dic(8;11)
Mouse 3	71,36 (45-88)						t(5;1;5;x), t(8;17), t(11;x), t(14;17), t[2;t(14;9)], dic(11;19), t(12;16), t(16;6), t(x;20), frag 2	frag 3, t(17;4), t(3;11;21), t(2;17;2;8), pulverised chrom 6, frag x, t[2;t(14;9)], frag 16, frag 13, frag 15, frag 20, frag 21, frag 7	
Mouse 4	84,26 (55-96)						t(5;1;5;x), t(11;x), t(14;17), t[2(14;9)], dic(11;19), t(16;6), t(x;20), del(3p)	t(16;4), t(4;11), frag 6, frag 2, t(3;11); t(6;19), t(17;4),	dic(2;19), dic[2;t(5;1;5;x)]
Mouse 5	88 (68-96)						t(5;1;5;x), t(11;x), t[2;t(14;9)], dic(11;19), t(16;6), t(x;20)	t(3;10), t(12;x;19), frag 20	dic(22;22), dic(19;8), dic(3;21;7), dic(4;11), dic[t(5;1;5;x);20], dic(6;6), dic(8;8), dic(19;19), dic(8;2;10)
Mouse 6	91,7 (84-97)						t(16;9;16;9;1), t(14;17), t(2;12), t(16;8), tric(14;15;3;15;14)	t[2;(14;9)], t(5;x), del(xq), ring 4, frag 12, frag 15, frag 16, frag 2, frag 18, t[6;t(9;14)], t(7;3)	dic(6;6), dic(11;11), dic[t(9;14);3]

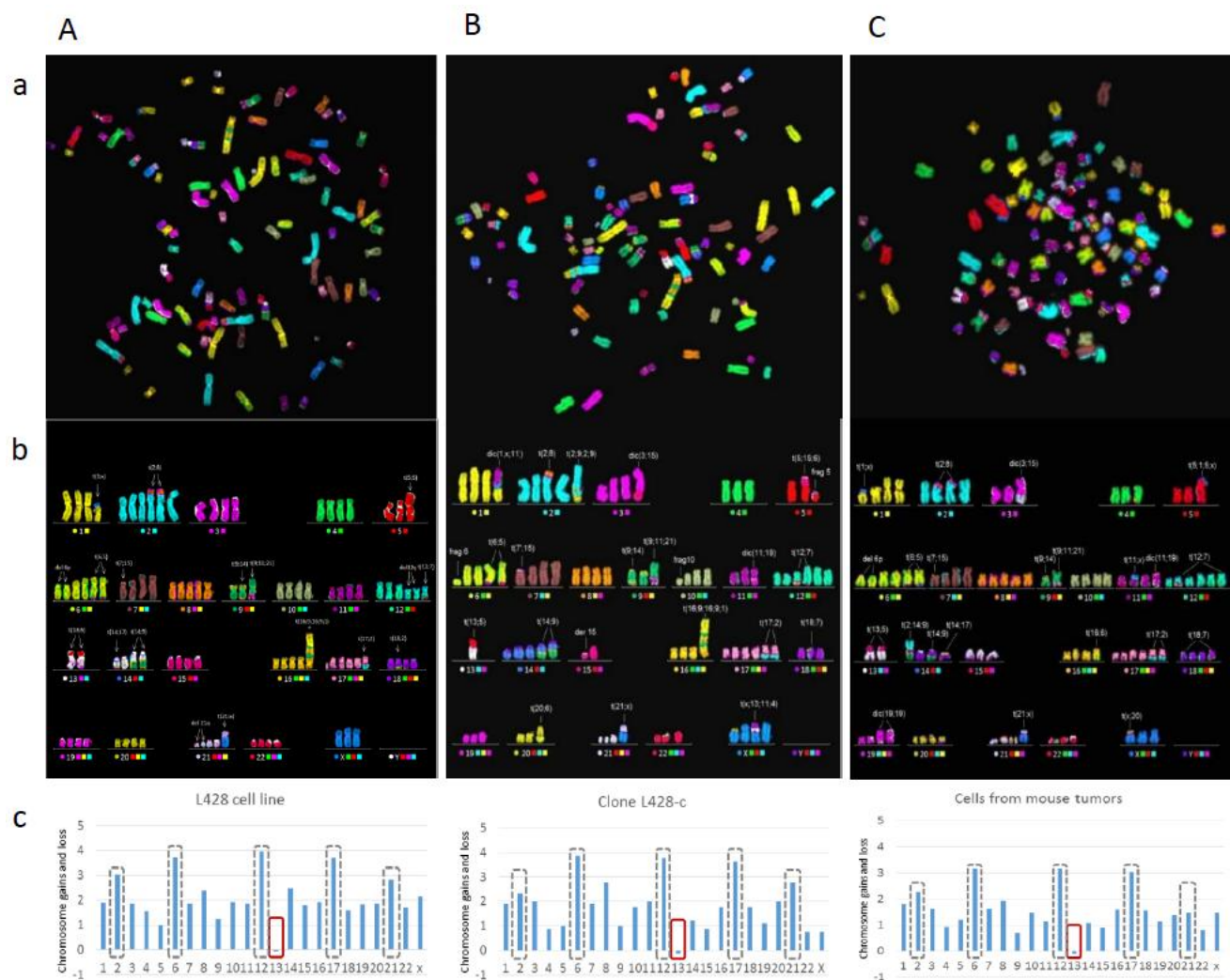


Figure 4 : Cytogenetic analyses of L428 (A), L428-c (B) and a cell line derived from a mouse liver tumor (C). (a) Metaphase staining with chromosome specific probes. (b) Spectral karyotype analysis using multi-FISH. (c) Graphical representation of chromosome gains and losses.

5- Clonogenic potential of CD30⁻ and CD15⁻ cells in HL cell lines

In addition to the establishment of this HL animal model, the main results obtained in this work consist of demonstrating the characteristics of the cells isolated from the mice. In order to validate this finding and to demonstrate the clonogenic growth of cells lacking specific surface markers related to HL, we examined the ability of CD30⁻/CD15⁻ cells to grow *in vitro*. CD30⁻/CD15⁻ cells were isolated from the L428-c and L540 cell lines by Fluorescence-activated cell sorting and analyzed at various time points following culturing (Figure 5). The CD30⁻/CD15⁻ negative cells were similarly negative for CD14. The *in vitro* amplification of these cells was very slow compared to that observed for the cells expressing CD30 or CD30 and CD15 (supplementary data 5). After two weeks of culture of the negative L428-c cells, the cells acquired a similar phenotype to that of the parental cell lines with an elevated level of CD14. The presence of CD30⁻/CD15⁻ negative cells was more frequent in the newly established cell lines than observed for the parental cell line. A similar phenomenon was observed for CD30⁻/CD15⁻ negative cells of the L540 cell line but the growth of these cells was much slower than that of the cells derived from L428-c (Figure 5B). Of note, the cells which remained CD30⁻/CD15⁻ demonstrated very slow *in vitro* proliferation reflecting that observed in the mice.

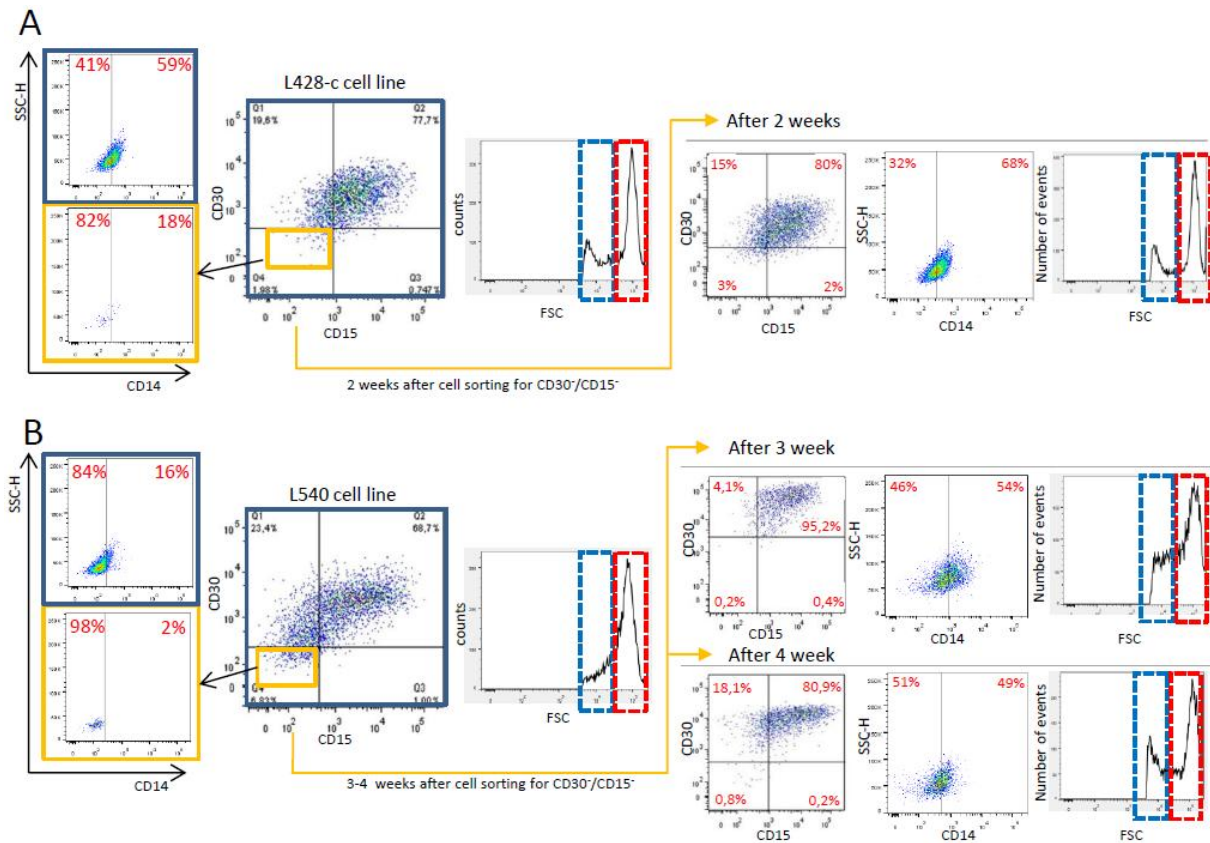


Figure 5: Cell sorting after CD15/CD30 staining for L428-c and L540 cell lines. (A) L428-c cells were stained for CD15 and CD30 (2nd panel from the left) and CD30⁺/CD15⁻ (yellow inset of 2nd panel from the left) were sorted and stained for CD14 (yellow box of left most panel as well as the unsorted cells (blue box of leftmost panel). The third panel from the left shows a histogram of FSC of the unsorted L428-c cells. The right 3 panels show the CD15/CD30 staining, CD14 staining and a histogram of the FSC of the CD15/CD30⁺ cells following two weeks in culture showing a similar profile to the parental cells. (B) The same as in A for the L540 cells with the right panels showing the flow cytometry data following 3 and 4 weeks of culture of the CD15/CD30⁺ cells.

Discussion

Hodgkin lymphoma is a malignant hematologic disease with a very high cure rate. Due to the success achieved in the treatment of this disease, the first and among the most exemplary of oncology, and because it affects younger patients, Hodgkin's lymphoma is the subject of attention of oncologists and hematologists, to the extent that the lessons learned from the study of this disease are the basis for therapy of other cancers. Knowledge of not only the tumor cell, but also its environment is essential to the development of better-targeted therapeutics. However, studies of the transformation mechanisms of tumor cells in Hodgkin lymphoma as well as the development of new therapeutic strategies for the 20% of patients who relapse or become refractory during treatment is hampered by the lack of a reproducible animal model. The only existing animal model of HL currently studied and validated has been established from the modified cell line L540 which is of T-cell origin (Borchmann et al 2003).

In this work, we clearly demonstrate the concept of the highly efficient establishment of an HL animal model. We have successfully transplanted L428-c cells (a cell line derived to L428) into NSG mice following total body irradiation with resulting in high survival and low toxicity permitting not only the use of this animal model for exploring new therapeutic strategies in HL patients but also to understand the nature of the cells grown in NSG mice and the different steps in the evolution of these cells to HRS cells (Scott & Steidl 2014). This model develops via multiple spontaneous genetic events that will help us to discover novel mechanisms of tumorigenesis of HL while preserving the distinct cellular phenotype of the tumor initiating cells.

The presence of initiating clonogenic -cells or cancer stem cells, in classical HL has been a subject of debate (Jones et al 2009, Kuppers 2009). Indeed, the likelihood that morphologically normal B cells are responsible for the generation of HRS has been very well documented (Jansen et al 1999, Jansen et al 1998, Newcom et al 1988). Faced with the difficulty of isolating clonogenic B cells by fluorescence-activated cell sorting (Irsch et al 1998), the rarity of these cells and the very low or inexistent capacity of these cells to proliferate alone *in vitro* (Jones et al 2009), we sought to determine the appropriate experimental culture conditions for growing and amplifying these cells before transplanting them into NSG mice. We studied the clonogenicity of seven HL cell lines. Only three cell lines gave rise to clones in semi-solid medium (methylcellulose) displaying different coefficients of clonogenicity and reflecting the heterogeneity observed in HL patients. The L428 cell line displayed a higher coefficient of clonogenicity than either KMH2 or L540 cells. The selected clones initially exhibited a low proliferation rate which increased with time in culture and which was associated with a higher coefficient of clonogenicity compared to the parental cell lines. These results confirm and extend the previous reports by Newcom using L428 (Newcom et al 1988) and the number of clonogenic cells detected by Jones for the L428 cell line (Jones et al 2009). The immunophenotype of these clones showed weak staining for CD14 and CD15 and a high frequency of mononucleated cells which decreased during the time of culture along with the clonogenic coefficient. Cytogenetic analyses clearly showed the high frequency of small metaphases compared to that observed in the parental cell line in addition to similar cytogenetic changes involving both numerical and structural aberrations. These data suggest that these cell lines arose from single cells.

The enrichment of clonogenic cells allowed the transplantation of these cells by the intravenous route into immune-deficient NSG mice after total body irradiation. L428-c

demonstrated a high transplantation rate likely due to the high coefficient of clonogenicity necessitating the use of only 10^3 cells. Tumor infiltration was observed, essentially to the lymph nodes, liver, bone marrow, spleen and brain. These results confirm and extend the results observed for the unique attempt to obtain an HL animal model using L428 cells (2×10^6) in three NSG mice without irradiation) (Dewan et al 2005a). All other attempts to obtain an HL animal model using the L428 cell line have been unsuccessful (von Kalle et al 1992) . To our knowledge this is the first report to demonstrate the establishment of an HL animal model using this methodology. The introduction of PET/CT for the quantification of tumor cell infiltration in the liver represents a major advance of this animal model. The results obtained for [^{18}F]FDG PET/CT uptake confirm the high heterogeneity observed for FDG uptake in the liver of HL patients. It is important to progress in the search for an additional marker to complement FDG/PET, especially for PET negative patients. This animal model could be of aid in this endeavor.

Immunophenotype characterisation of cells originating from the clones, as well as cells derived from the mice, clearly demonstrates the progression of cells without the surface markers CD30, CD15, and CD14 to CD14 positive, CD14 and CD15 positive, and finally CD15 and CD30 positive cells with a lower signal for CD14. This phenotypic transition was associated with a shift towards aneuploidy. These findings confirm the observation described previously (Newcom et al 1988) suggesting that proliferation may influence the expression of this antigen. While the surface marker negative cells were diploid, the CD30 and CD15 positive cells were binucleate similar to Reed-Sternberg cells. These data may clarify many questions regarding the mechanistic links between HRS cells and monocytes/macrophages (Sanchez-Aguilera et al 2006). A possible prognostic role for lymphoma associated macrophages has been suggested for several different lymphoproliferative disorders, including classic HL. A tumor promoting role for macrophages in HL has been proposed (Kamper et al 2011, Scott & Steidl 2014, Tan et al 2012). The dissemination of human macrophages in the tumors of mice showed the transition from these surface marker negative cells towards monocytes or macrophages and HRS cells. A relationship between the frequency of macrophages and the presence of large tumors in liver was established in this work (data not shown). This observation may contribute to an improved understanding of the pathogenesis of HL and the role of the microenvironment. With respect to these findings, it will be necessary to investigate the mechanisms for the possible transformation of

“macrophage-like” to HRS cells and to establish the link between these two” different “cell populations. By *in vitro* investigation using methylcellulose, we have clearly observed the presence of macrophages derived from clonogenic surface marker negative cells (data not shown). These data can explain the ongoing controversy in the literature concerning the presence of macrophage markers (CD68 or CD163) and their prognostic factor in survival of HL patients.

These findings concerning the origin and the transformation of growing cells in HL have been validated using Fluorescence-activated cell sorting of CD30-and CD15-cells which gave rise to the same parental cell line. We demonstrated in this work for the first time, the capacity of CD15/CD30 negative cells of an HL cell line to regenerate cells similar to the parental cell line by the enrichment of clonogenic cells or surface marker negative cells.

Although Brentuximab vedotine, a CD30 directed antibody-drug conjugate, has produced encouraging results in the treatment of relapse or refractory disease (Stathis & Younes 2015) we demonstrate, nevertheless, in this work that the clonogenic proliferative cells in HL were CD30- cells. These data could be a first step towards the identification of the oncogenic process in HL and a possible new treatment modality directed essentially at refractory or relapsed HL patients.

The cytogenetic analysis of the tumors established in NSG mice and their re-cultured cell lines has led to a number of interesting observations. The frequency of numerical and structural chromosomal aberrations in tumor cell lines established after the passage of L428-c cells in the NSG mouse is much less than observed in the original L428 cell line. Nevertheless, we observed commonly shared numerical and structural aberrations. Of note, the higher frequency of dicentric chromosomes observed in the established cell lines compared to the injected cells could be a reflection of the ongoing chromosomal instability of these lines. The difference of the aneuploidy status of the cell lines derived from NSG mice compared to that of the injected cells and the original cell line favours the argument that the tumor cells grown in the NSG mice are derived from diploid precursors of malignant cells.

The performance of this animal model in studies of drug efficacy as well as for understanding the mechanisms of oncogenesis during HL progression requires further investigation but it is likely that it will be superior to the HL animal models already published (Dewan et al 2005b)

This established animal model using L428-c was validated using the new alkylating histone-deacetylase inhibitor (HDACi) fusion molecule, EDO-S101 in a large series of

mice after the determination of the in vitro activity of this molecules (AL Jawhari et al submitted).

Conclusion

The establishment of this HL animal model characterized by the need for a small number of injected cells, high survival rate and low toxicity could become an important tool in the exploration and discovery of new therapy protocols. Using this animal model, we have attempted to establish the origin of the cells which grew in the mice and the different steps of transformation from “clonogenic cells” or tumor stem cells to monocytes/macrophages and finally to Hodgkin and HRS cells due to the unique microenvironment of HL. It will be interesting to test this same approach, replacing the L428c cells with amplified cells derived from the lymph nodes or circulating lymphocytes of relapsed or refractory patients in the advanced stage of disease. This model could also help to advance the search for new tracers for PET/CT which may address the problem of negative FDG uptake seen for some HL patients.

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Supplementary data

Supplementary data 1

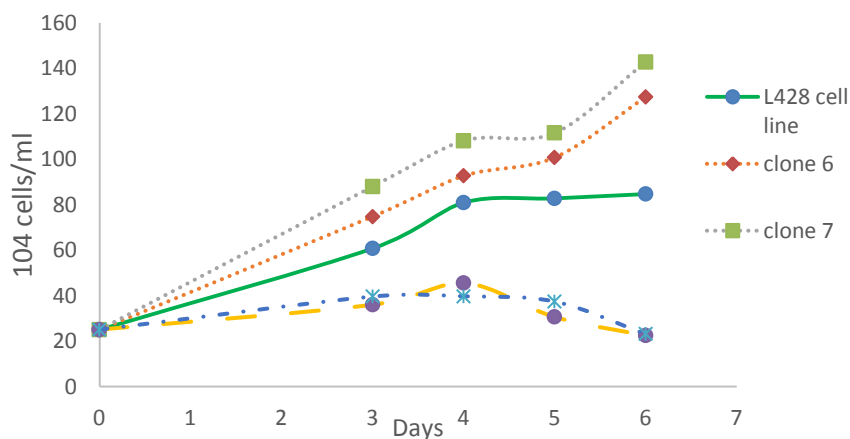
Enumeration of different examined organs and techniques used to study tumor infiltration in NSG mice.

Examined organs	Techniques				
	Anatomopathology in parafin blocks		Single cell suspension		
	HE	IHC CD30	Flow-FISH	IF Cytospin	FISH
Mandibular and parotid salivary glands	✓	✓			
Mandibular lymph node	✓	✓			
Lung	✓	✓			
Heart	✓	✓			
Liver	✓	✓	✓	✓	✓
Spleen, pancreas	✓	✓	✓	✓	✓
Stomach	✓	✓			
Jejunum, colon	✓	✓			
Kidney	✓	✓			
Eye and harderian gland	✓	✓	✓	✓	✓
Brain	✓	✓			
Femur and sternum with bone marrow	✓	✓	✓	✓	✓
Ovaries or testis	✓	✓			

HE= Hematoxylin Eosin, IHC= immunohistochemistry, FISH= Fluorescent in situ hybridization using human PNA probes and alfa satellites probes, IF= Immunofluorescence using CD30

Supplementary data 2

In vitro growth of derived clones of L428 compared to the parental cell line demonstrating the variability of cell proliferation with no in vitro growth of clones 8 and 9.



Supplementary data 3

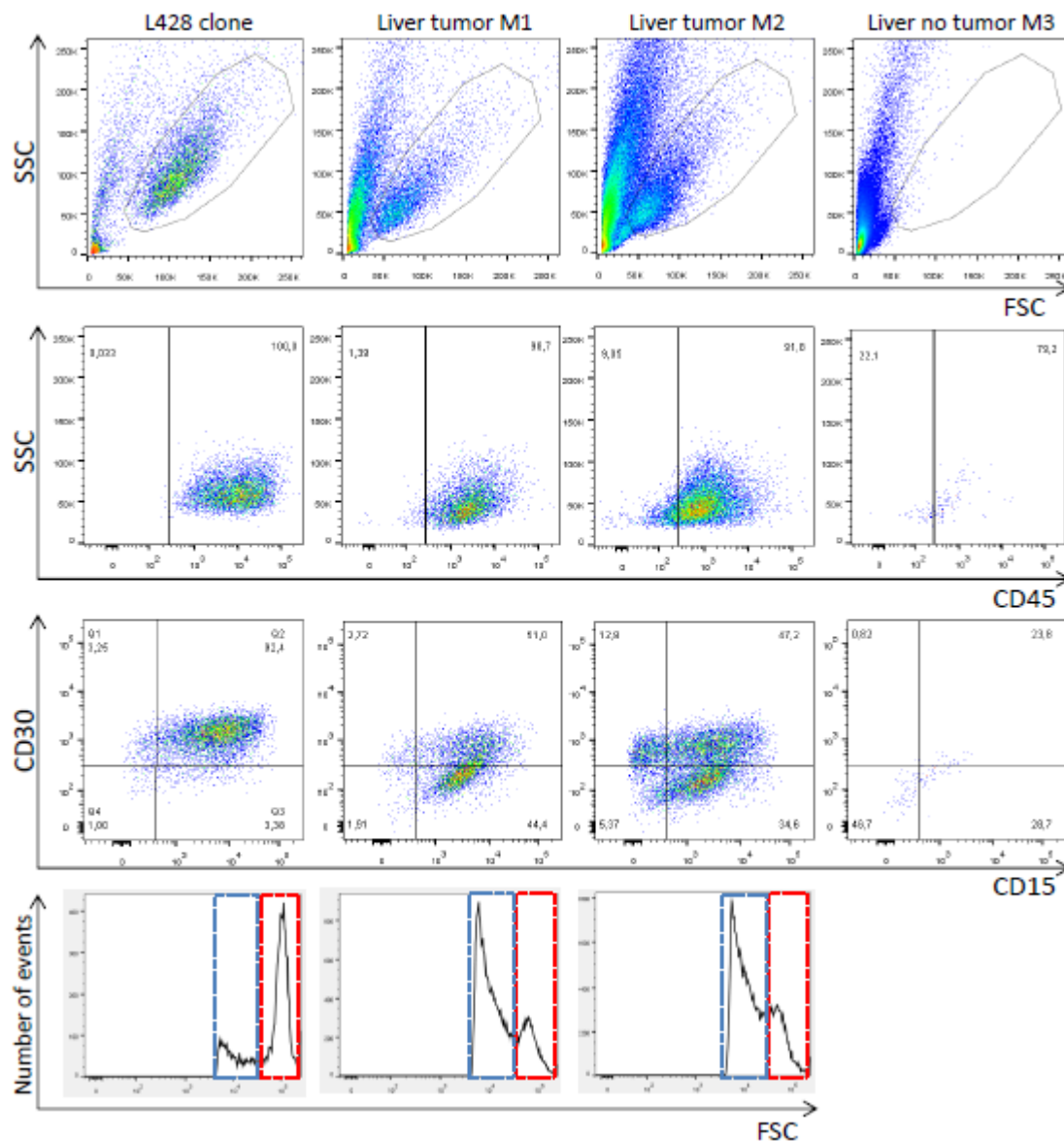
In vivo infiltration of HL cell lines into NSG mice.

Cell line	No of cells inoculated / mouse(10^6)	Time of killing (days after inoculation)	N° of mice with tumor/no of mice inoculated	Infiltration of HL cells into:			
				Live r	Spleen	Bone Marrow	Eye*
L428	10^6	35	12/12	+++	++	++	+++
KMH 2	10^6	35	6/9	+++	++	++	+++
L1236	10^6	120	1/4	-	-	-	++
L591	10^6	60	0/4	-	-	-	-

*Site of injection

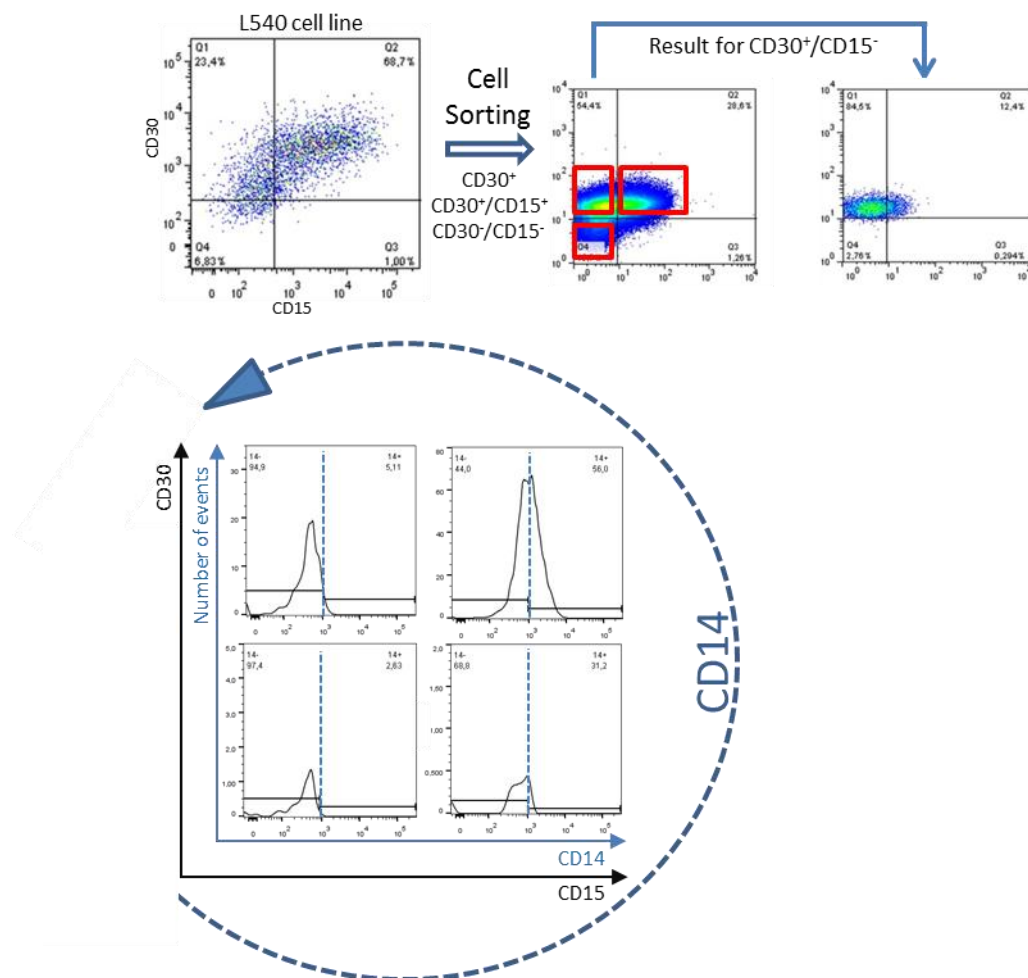
Supplementary data 4

Flow cytometry analysis of cells recovered from liver tumors of 2 mice injected with 103 L428-c cells and a third which did not develop a tumor analysed 16 weeks after injection. The frequency of each sub-population regarding with respect to the CD30 and CD15 markers are represented. The FSC demonstrates the presence of two populations, both for the injected cells and for the liver tumor cells which are found at different frequencies. Liver tumor cells for mouse 2 demonstrate a higher frequency of negative cells (CD30 and CD15) (5% vs 1% for L428-c and 2% for mouse 1) and the presence of cells with only CD30.



Supplementary data 5

Cell sorting of the L540 cell line demonstrates the governance role CD14 in the proliferation of negative cell to the same phenotype of parental cell line



Part IV

We have demonstrated in Part II and Part III the presence of telomere dysfunction and chromosomal aberration in Hodgkin lymphoma as well as the nature of growing cells in this disease. This dysfunction needs the activation of a telomere maintenance mechanism by the cell, in order to support immortalization.

In this part, we demonstrate the presence of both telomerase activity (TA) and alternative lengthening of telomere (ALT) profile mechanisms in HL cell lines as well as in Hodgkin lymph nodes. Telomerase activity was detected in all HL cell lines and in the majority of lymph nodes with different levels. Interestingly, the presence of CD30 negative cells with higher expression of telomerase was observed in HL cell lines and lymph nodes. Nevertheless, the Hodgkin and Reed Sternberg cells showed a lower telomerase expression. Small cells, exhibiting the coexistence of telomerase activity and ALT profile were detected in HL cell lines and in lymph. Perhaps this coexistence of both telomere maintenance mechanisms was due to the hypermutability of these cells. Of note, HRS cells have a very low level of telomerase activity and lack APBs but show a high PML expression.

Significant correlation was found between the activation of mechanisms of telomere maintenance and radiation sensitivity in HL cell lines. ALT HL cell lines appear to be resistant to ionizing radiation. A significant correlation was found between the presence of APBs (ALT profile) in HL lymph node and Event-Free Survival. At multivariate level, higher expression of ABPs and CD68 in EBV positive patients had a significant predictive impact on OS ($p=0.002$), FFP ($p=0.002$) and EFS ($p=0.0003$).

Telomere maintenance mechanisms and Tumor microenvironment are key factors in the outcome of classic Hodgkin lymphoma

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Abstract

Background:

Hodgkin lymphoma (HL) can be considered to be a successful paradigm of modern treatment strategies. Nonetheless, about 15-20% of patients with advanced stage HL still die following relapse or progressive disease and a similar proportion of patients are over-treated, leading to treatment-related late sequelae including solid tumors and organ dysfunction. We analyzed telomere maintenance mechanisms (TMMs) of HL cell lines as well as the samples from HL patients treated with standard therapy and compared the TMMs with favorable and unfavorable clinical outcome.

Materials and Methods:

Frozen samples obtained from 38 HL patients during diagnostic lymph node biopsy and from 24 patients with lymphadenitis were entered in this study. Seven HL cell lines were used as a positive control.

Telomerase activity (TA) and alternative telomere lengthening (ALT) profile, were assessed as well as EBV status and protein expression levels. The TMMs were correlated to clinical outcomes of patients (10.3 years) as well as to radiation sensitivity of HL cell lines.

Results:

The major finding of this study is the presence of both TA and ALT mechanisms in selected lymph nodes of HL patients. This TMMs heterogeneity was confirmed in HL cell lines. If Hodgkin and Reed Sternberg (HRS) cells have an ALT profile, the small cells exhibited a higher telomerase activity. We identified in the same tumor Hodgkin and Reed Sternberg (HRS) cells containing ALT-associated PML bodies; a hallmark of ALT, and separate small cells expressing telomerase. Similarly, in HL cell lines, a high level of TA was detected in L428 and SUP-HD1. L1236 cell line demonstrated lower TA and presented an ALT profile.

Significant correlation was observed not only between ALT profile, EBV status and the frequencies of macrophage cells (microenvironment tumors) but also with the survival of HL patients. ALT HL cell lines showed lower radiation sensitivity compared to telomerase activity HL cell lines.

Conclusion:

The presence of both TMMs (TA and ALT) in Lymph nodes and cell lines is unique. Also TMMs in HL patients related to tumor microenvironment are associated to treatment response and outcome of HL patients.

Key words: Hodgkin Lymphoma, telomerase, alternative lengthening of telomeres, EBV, DNA repair.

Introduction

Hodgkin lymphoma (HL) is a malignancy characterized by the presence of scarce tumor cells called Reed-Sternberg cells (HRS), derived, in most cases, from germinal center B cells (Kuppers et al 1994) harbouring rearranged and somatically mutated immunoglobulin genes (Kuppers et al 1994, Marafioti et al 2000) and is characterized by a defective B-cell expression program (Schwering et al 2003).

Telomere shortening in tumor cells and peripheral blood lymphocytes of HL patients has been recently documented (Knecht et al 2012, M'Kacher et al 2007, Mai & Garini 2006). This dysfunction needs the activation of a telomere maintenance mechanism (TMM) by the cell, in order to support immortalization. Most tumor cells activate expression of the enzyme telomerase (Kim et al 1994). But some cells elongate telomeres using telomerase-independent mechanisms, known as alternative lengthening of telomeres (ALT) (Cesare & Reddel 2010). Previous studies have demonstrated the presence of telomerase or/and ALT mechanisms in different tumor types. In contrast to cells with active telomerase, cells that use ALT are characterized by intracellular heterogeneity in telomere length, ranging from very short to very long (Dunham et al 2000, Henson et al 2005); ALT-associated PML bodies (APBs); and the presence of extrachromosomal telomeric repeats (Cesare & Reddel 2010, Henson et al 2009). Most tumors exhibit characteristics of one TMM. However, many publications that have classified human tumors have also identified a subset of tumors not definitely ALT or telomerase. These tumors display ambiguous characteristics regarding telomere length, telomerase activity, or the presence of APBs (Costa et al 2006, Johnson et al 2005, Villa et al 2008, Yan et al 2002).

The TMMs in HL tumor cells as well as in established HL cell lines were investigated only in a few papers and have been a subject of debated (Brousset et al 1997, Brousset et al 1998, Norrback et al 1998) Norrback et al (Norrback et al 1998) demonstrated the presence of TA in 31 of 77 HL lymph nodes at decreased levels and a high levels of TA in HL cell lines. Nevertheless, Brousset et al later demonstrated the presence of TA in HL lymph nodes (Brousset et al 1997) and using a large series of samples, confirmed that the majority of cases (only 2/20 positive cases) lack telomerase activity. A telomerase-independent mechanism for telomere maintenance in HL has been proposed in the absence of detectable telomerase activity.

In the study reported here, we reevaluated the TMMs in tumor cells as well as in established HL cell lines. We demonstrated for the first time the presence of both

telomerase and ALT positive tumor cells in samples suggesting that there is not an exclusive mechanism used by the cells to maintain their telomeres. However, the TMMs heterogeneity was related to the progression of tumor cells in HL. Interestingly, a significant correlation was established between the dominance of ALT mechanisms, tumor-infiltrating macrophages, EBV status and poor clinical outcome of HL patients. In addition, very low *in vitro* radiation sensitivity was observed in ALT HL cell lines compared to telomerase HL cell lines. These findings will be validate using an HL animal model established from L428 cell line (telomerase dependent cell line).

Material

Patient samples

Pretreatment samples from 38 patients with a histological diagnosis of Nodular Sclerosis, Mixed Cellularity, Lymphocyte Rich of Classical HL were obtained from the Department of Anatomo-Pathology of the University Hospital Centre of Lille. The study was approved by the Institut de Biologie of Lille (CNRS) and University Hospital Centre of Lille Institutional Review Boards, and an informed consent was obtained in writing from each donor (Table1).

Table 1: Patient's characteristics

Characteristics	N° of patients (N=38)		%
Male	19		50
Age	36,7 y		
<45	32		84,2
>45	6		15,8
Stage			
Stage I	31		81,6
Stage II	7		18,4
Treatment			
Chemotherapy only	0		0
Combined modality	38		100
Histologie sub-type			
Nodular Sclerosis	36		94,7
Mixed cellularity	1		2,6
Classic lymphocyte rich	1		2,6
EBV+(LPM1)	13		34,2
Follow-up			
ACR	30		78,9
AWD	4		10,5
DOD	4		10,5

ACR: Alive in complete remission

AWD: Alive with disease

DOD: Dead of disease

To generate TMMs in HL patients, a group of control samples was included in this study: lymph nodes derived from 24 patients (mean age 37.2 years) with follicular hyperplasia or chronic lymphadenitis and 7 HL-derived cell lines (L-540, L-428, KM-H2, HDLM-2, L1236, L591, and SUP-HD1) obtained from the German Collection of Microorganisms and Cell. Cultures and staining were obtained from 3 independent cultures of each cell lines.

Methods

Telomere Repeat Amplification Protocol

The PCR-based telomere repeat amplification protocol (TRAP) assay of telomerase enzyme activity was carried out as described previously (Kim et al 1994, Wright et al 1995). Briefly, cellular extracts were prepared by homogenizing cells in either

CHAPS extraction buffer or Buffer C [20 mmol/L HEPES (pH 7.9), 420 mmol/L KCl, 5 mmol/L MgCl₂, 25% glycerol, 0.1 mmol/L EDTA, 0.2% NP40]. Total protein concentrations were determined using the detergent compatible with the protein assay (Bio-Rad). Equivalent amounts of extract corresponding to 0.1 to 1 µg of total protein were used for each reaction. At least three independent extracts were made for each cell line. At least three independent TRAP assays were done for each extract.

Reverse Transcription-PCR–Based Detection of Telomerase Components

Detection of hTERT message was done as described below in Taqman assays using the Hs00162669_m1 assay (Applied Biosystems). Expression level was determined using the $\Delta\Delta C_t$ method, normalized to the level of 18S rRNA expression (assay 4308329; Applied Biosystems), and quantified relative to HeLa hTERT message levels. To detect transcripts encoding the hTERT component of telomerase, 1 and 0.1 ng of total RNA were transcribed into cDNA as described for the Taqman assays in the presence and absence of reverse transcriptase. The cDNA was then subjected to PCR using primers designed to amplify the telomerase template RNA (5'-CTAACCCTAACTGAGAAGGGCGTA-3') and (5'-GGCGAACGGGCCAGCAGCTGACATT-3'). Reactions were carried out in a commercially available buffer containing 15 mmol/L MgCl₂ (Applied Biosystems). Reactions were incubated at 94°C for 1 min and then subjected to the following for 30 cycles: 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s. Products were visualized on 6% acrylamide gels.

Immunofluorescence Detection of ALT-Associated PML Nuclear Bodies and shelterin proteins complexes

Five µm frozen tissue sections or cultured cells applied onto polykysine slides by Cytospin® were fixed with 4% formaldehyde (PFA, Sigma–Aldrich) for 10 min, and treated with 0.25% Triton X-100 solution (Sigma–Aldrich) for 10 min. After blocking with 5% bovine serum albumin (BlockingReagent® Sigma), the cells were then stained with a primary antibody overnight at 4°C and counterstained with a secondary antibody. Cell nuclei were stained with 4',6-diamidino-2- phenylindole (DAPI, Sigma–Aldrich). As a negative control, staining was carried out in the absence of primary antibody.

Primary antibodies were as follows: PML (InterBiotech Interchim), hTERT(Sigma Aldrich), TRF1(Santa Cruz Biotechnology), TRF2 (Santa Cruz Biotechnology), gH2AX (ser 139, Millipore), ATM(Sigma Aldrich), CD30 (DAKO), CD15 (BD), CD68 BD), LMP1 and MR11. The secondary antibodies were as follows: anti mouse Cyanine 3 (1/100 in PBS-2%FCS, Sigma–Aldrich) and anti-rabbit FITC (1/100 in PBS-2%FCS, Sigma-Aldrich).

Proximity Ligation Assay (PLA) tests were performed according to the manufacture's protocol (Duolink, Olink Bioscience, Sweden) (Pierre Trifilief 2011). PLA has been used for analyzing TRF2 and PML interaction

Analysis: Images of immunofluorescent stainings were captured with a charge-coupled device camera (Zeiss, Thornwood, NY) coupled to a Zeiss Axioplan microscope using MetaSystems®software that allows automatic image scanning. MetaCyte (metasystem®) was used to quantify the fluorescence of different signals.

Western blotting analysis

Western blotting was performed as described previously (60) The primary antibodies anti-PML (H238, sc-5621, Santa Cruz Biotechnology), anti-TRF2 (clone 4A794, Millipore), anti-p53 (DO-1, sc-126, Santa Cruz Biotechnology), anti-POT1 (C-19, sc-397, Santa Cruz Biotechnology), anti-actin (Santa Cruz Biotechnology) were used to normalize protein content of samples. Images shown in the figures are representative of at least three experiments.

Flow cytometry investigations

Apoptosis was determined using Annexin V–FITC and Hoechst (33342) double staining according to the manufacturer's instructions (BD Biosciences) after 2, 6 and 24 hours of radiation exposure at 6 Gy of all cell lines. Cell-cycle fractions were determined by Hoechst nuclear staining. Briefly, cells were harvested, washed in PBS, incubated with Annexin V-FITC and Hoechst solution for 30 minutes at room temperature. Data were collected using a FACSCalibur flow cytometer (BDBiosciences) and analyzed using FlowJo Version 7.5.5. The results represent the mean value of 3 independent experiments.

Fluorescence-activated cell sorting and flow cytometry analysis

Cells were gated to exclude apoptotic or necrotic cells and sorted into CD30-/CD15-, CD30+ and CD15+/CD30+ fractions by gating on the lowest and highest 5% PE-

expressing cells, respectively. Following sorting, the CD30-/CD15- cell fractions were analyzed using a FACScan flow cytometer (Becton Dickinson) and found to be more than 98% pure (supplementary data 2). For phenotypic analyses of cell lines or sorted cells, cells were prepared as described, and then stained with mouse anti-human CD30-phycoerthrin (PE) and CD15–fluorescein isothiocyanate (FITC), (all antibodies from BD PharMingen, San Diego, CA). Cells were subsequently analyzed using a FACS LSRII (Becton Dickinson, Franklin Lakes, NJ). The injected HL cell line served as a control for all experiments.

Transcriptional response after in vitro irradiation of HL cell lines

Basal and post-irradiation expression of four radiation-responsive genes (CCNG1, PUMA, P21, ZMAT3 and SESN1) was determined by quantitative real-time PCR in HL cell lines. The presented data constituted on the report between the expression after exposure and basal expression. The protocol was previously published (Badie et al 2008). These genes were implicated in DNA repair: PUMA is p53 upregulated modulator of apoptosis; P21 regulator of cell cycle progression at G1; ZMAT3 encodes a protein containing three zinc finger domains and over expression of this gene inhibits tumor cell growth; SESN1: plays a role in the cellular response to DNA damage and oxidative stress over expression inhibits tumor cell growth and the expression of this gene is a potential marker for exposure to radiation; CCNG1: encodes a human homologue of the rat G-type cyclin.

Cytogenetics analysis

FISH technique

FISH was performed using a combination of standard procedures from the recommended protocols for chromosome analysis using specific probes for PML-RAR α and hTERT genes. The copy number and the localization of each gene were assessed and 100 metaphases were scored per cell line. A Q-FISH technique was performed using Cy-3-labelled PNA probe specific for (TTAGGG) telomere sequences (Panagene) described previously (Girinsky et al 2014).

CO-FISH

Cells were incubated for 24-48h in fresh medium containing BrdU (10 μ g/ml). An hour before harvesting, Colcemid was added to the media to accumulate mitotic cells.

Cells were harvested and resuspended in 0.075 M KCl (pre-warmed to 37°C). Ice-cold methanol-acetic acid (3:1 ratio) was added to cell suspension. The cell suspension was spun (5 min at 1000 rpm) and washed twice in methanol-acetic acid. Cells were dropped onto slides and allowed to dry overnight. Slides were rehydrated in 1× PBS for 5 min at room temperature, incubated with 0.5 µg/ml RNase A (in PBS, DNase free) for 10 min at 37°C and stained with 0.5 µg/ml Hoechst 33258 in 2× SSC for 15 min at room temperature. Subsequently, slides were placed in a shallow plastic tray, covered with 2× SSC and exposed to 365 nm ultraviolet light at room temperature for 45 min. The BrdU-substituted DNA strands were digested with at least 10 U/µl of Exonuclease III at room temperature for 30 min. Slides were washed in 1× in PBS, dehydrated in ethanol series 70%, 95%, 100% and air dried. FISH was performed as described above, except that slides were not subjected to DNA denaturation.

Statistical analysis

The follow-up time was defined as the time from biopsies to either last follow-up or a given event. Event-free survival (EFS) was measured from the date of biopsies to either disease progression or discontinuation of treatment for any reason or censoring date. Overall survival (OS) was measured from the date of diagnosis to the date of death for any reason. For survival analysis, related to TRF2 and PML colocalisation expression, CD68, PML, and hTERT, three groups were considered: the quartile of patients with high expression (>15%), intermediate (between 5 to 15%) and lower expression (<5%). This cut point was chosen in concordance with previous reports. Survival was estimated by the Kaplan-Meier method and compared using a log-rank test. Two side p-values <5% were considered statistically significant and p<10% as being borderline significant.

Results

1-Telomerase activity or ALT pathway for telomere maintenance in HL cell lines

Several approaches were used to investigate TMMs in HL cell lines. We initially examined relative telomerase activity using TRAP assay in all HL cell lines. Telomerase activity was detected in all HL cell lines (Figure 1A) but the quantification of relative levels of telomerase activity may vary within each cell lines (Figure 1B). FISH painting of hTERT gene (5p15) and control EGR1/CDC25C gene (5q31)

showed the high copy number of hTERT gene in 3/7 HL cell lines and revealed a deletion of one locus of hTERT gene in L1236 cell line (Figure 1C). hTERT protein expression was detectable by immunofluorescence for all cell lines at different levels (Figure 1D). L428 expressed high levels of hTERT proteins as well as SUP-HD1. In contrast L1236 expressed only low level of hTERT, possible due to deletion of one locus of hTERT gene. Taken together, these results demonstrate that telomerase is active in all HL cell lines, but relative levels may vary within each cell lines.

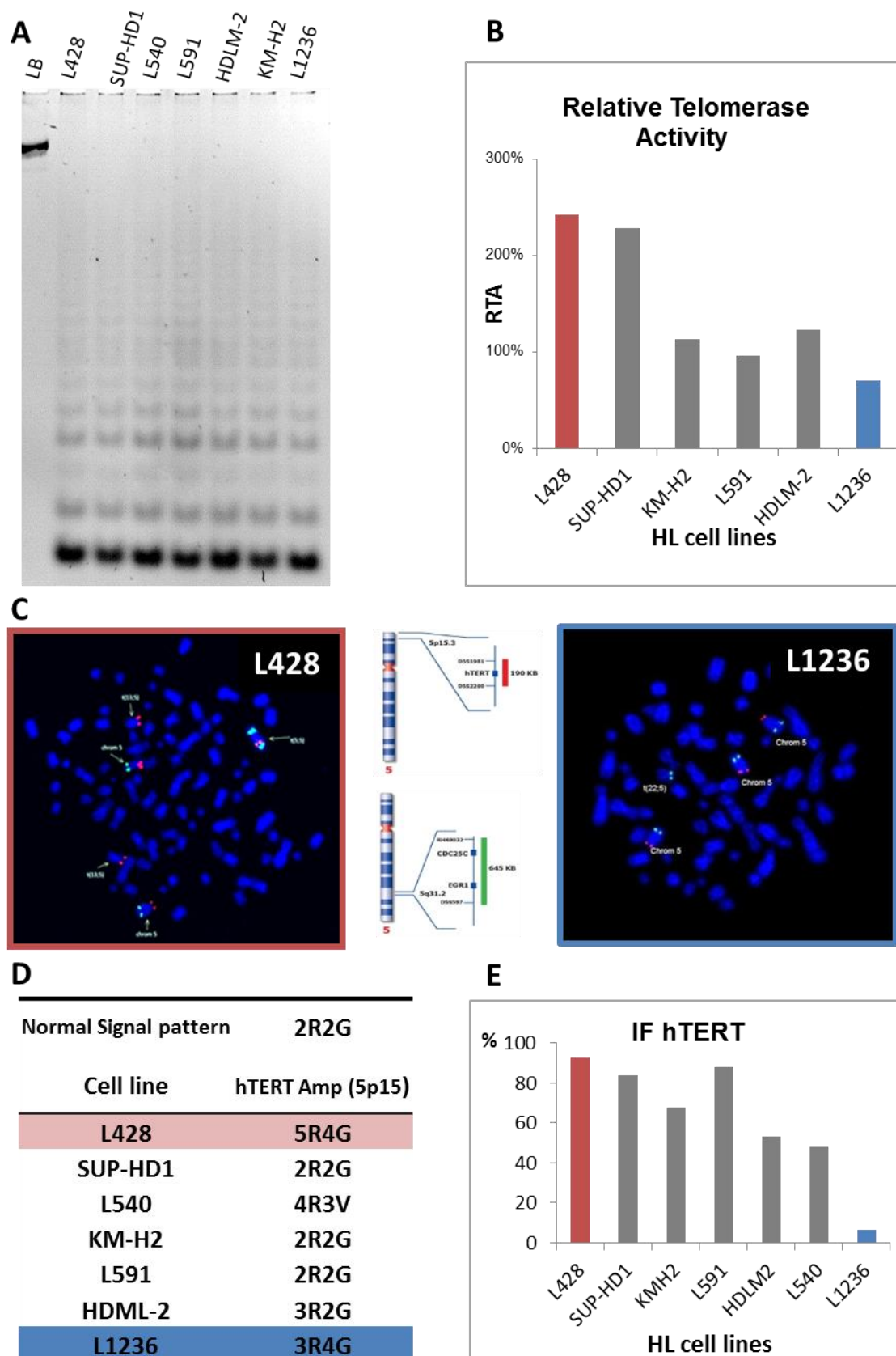


Figure 1 : Telomerase expression in HL cell lines : (A)telomerase activity in HL cell line lysates is measured using the TRAP assay. Negative control band is present (LB). All HL cell lines expressed higher telomerase activity. (B) quantification of relative telomerase activity in HL cell lines demonstrated a high heterogeneous activity (C)(D)using the probes of hTERT gene (5p15) and control EGR1/CDC25C gene (5q31), L428 cell line with high telomerase activity exhibited a higher gene copy number of hTERT and L1236 cell (low telomerase activity) showed a deletion of one locus of hTERT gene (E) quantification of hTERT protein using immunofluorescence technique confirmed the results obtained using TRAP assay and FISH technique.

This high heterogeneity of hTERT expression and the presence of long heterogeneous telomeres, identified by Q-FISH technique previously (Cuceu et al 2015), suggest the presence of ALT mechanisms in HL cell lines. We analyzed ALT characteristics using colocalisation of PML with the telomere/ telomeric proteins to identify APB's. First, the quantification of PML protein signal was performed in HL cell lines using immunofluorescence assay (Figure 2A) and western blot assay (Figure 2B). We further confirmed these data with FISH painting which revealed the high copy number of PML gene in L1236 cell line (Figure 2C). Second, using a proximity ligation assay (PLA), the detection of colocalisation of telomeres, as identified by TRF2 signals and PML was performed. The distribution of the number of PML-TRF2 foci of HL cell lines was represented in figure 2D as well as the manual identification of PML and telomere sequences using telomeric fluorescence *in situ* hybridization (FISH) analysis combined with immunofluorescence for PML (IF-FISH) (Figure 2E). Of note, the CO-FISH technique was performed to quantify the telomeric sister exchanges (T-SCEs), which are rare or absent in non-ALT cells (Londono-Vallejo et al 2004). Figure 2F shows the frequency of T-SCEs in HL cell lines. HDLM2, L591, L540 and L1236 showed a higher frequency of T-SCEs.

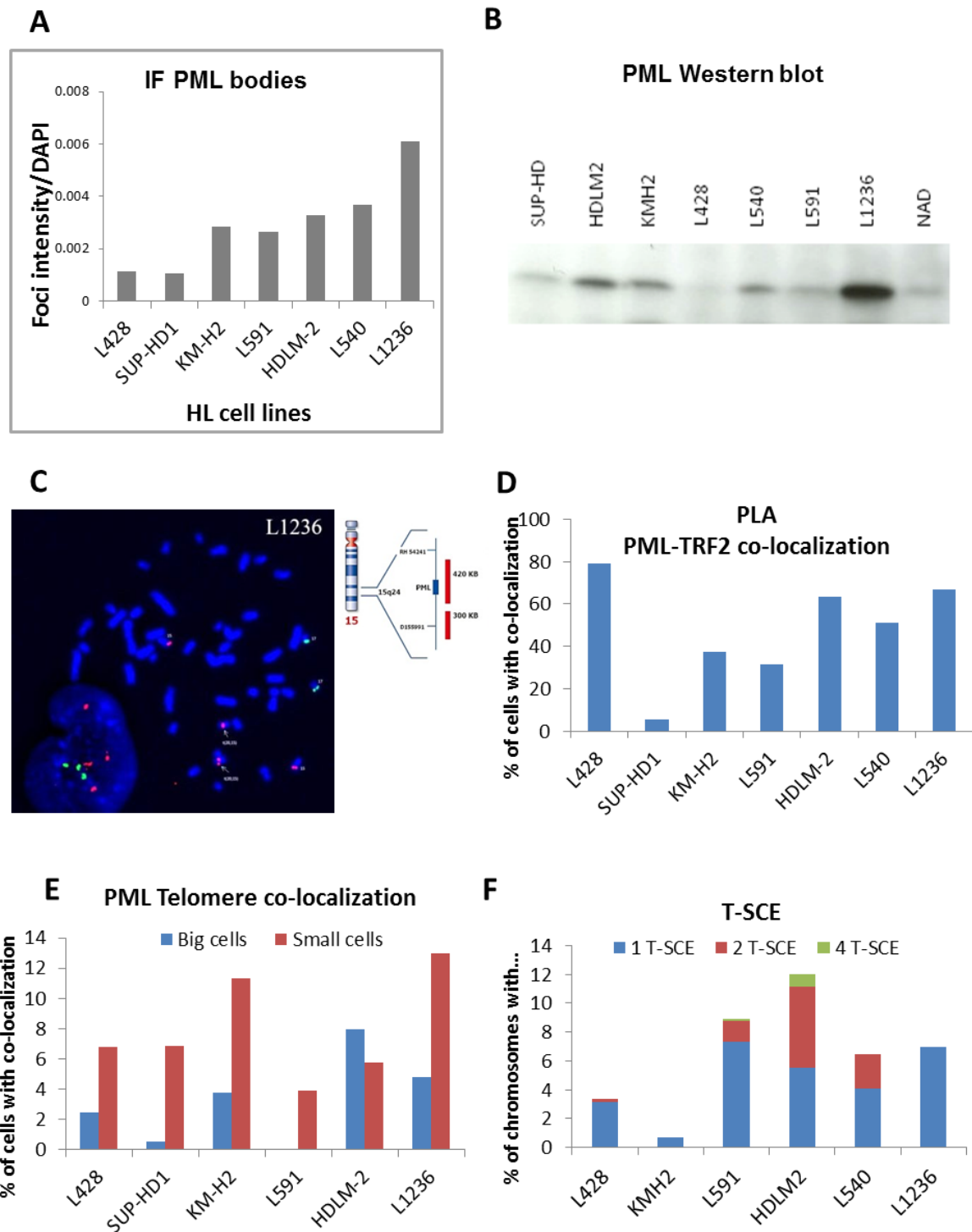


Figure 2 : Alternative telomere lengthling in HL cell lines : (A) quantification of PML protein in HL cell lines using immunofluorescence technique demonstrates a significant difference in the PML level in diffeenent HL cell lines (B) Western blot analysis confirmed the high heterogenity in the PML level in HL cell lines (C)FISH technique using PML-RARa confirmed the higher gene copy number of PML in L1236 compared to other cell lines (D)frequency of the cells with a colocalization of TRF2and PML in HL cell lines demonstrated a lower number of colocalization in SUP-HD (high level of telomerase) (E) using immunofluorescence technique associated to FISH , the mean number of colocalization of TRF2 and PML scored using automated softwear in small and Big cells was represented. This data demonstrates the high number of colocalization foci of TRF2 and PML in small cells (F) Quantification of T-SCE in metaphases of HL cell lines after CO-FISH staining. Cells with one T-SCE event, with two T-SCE events with both leading- and lagging-strand probes simultaneously and with four T-SCE events on both strands and on both p and q arms were assessed.

All these data show the higher heterogeneity in hTERT expression as well as the presence of ABP's. The co-expression of PML bodies and hTERT using immunofluorescence was performed and revealed the presence of cells with only hTERT expression, cells with PML expression and cells exhibited both hTERT with PML expression and the presence of APBs. Figure 3A shows the frequency of the different sub-population and demonstrated a higher heterogeneity in hTERT and PML expression in all HL cell lines. We there after focused on the nature of cells exhibiting PML or/and hTERT proteins and asked whether the PML protein may contribute to telomere surveillance during the transition between the small cells or Hodgkin cells to Reed-Sternberg cells. Considering the higher heterogeneity in the size of cells in HL cell lines, we took advantage of an automated fluorescence image analysis, allowing not only the study of a large number of cells, but also their size determined by the area. The distribution of intensity of fluorescence of PML and hTERT, depending on the area of cells, was significantly correlated to the area of cells. It clearly appears that small cells (1st quartile) exhibited higher intensity of fluorescence of hTERT than a RS cells (4th quartile). The intensity of PML was inversely correlated to the intensity of hTERT (Figure 3B).

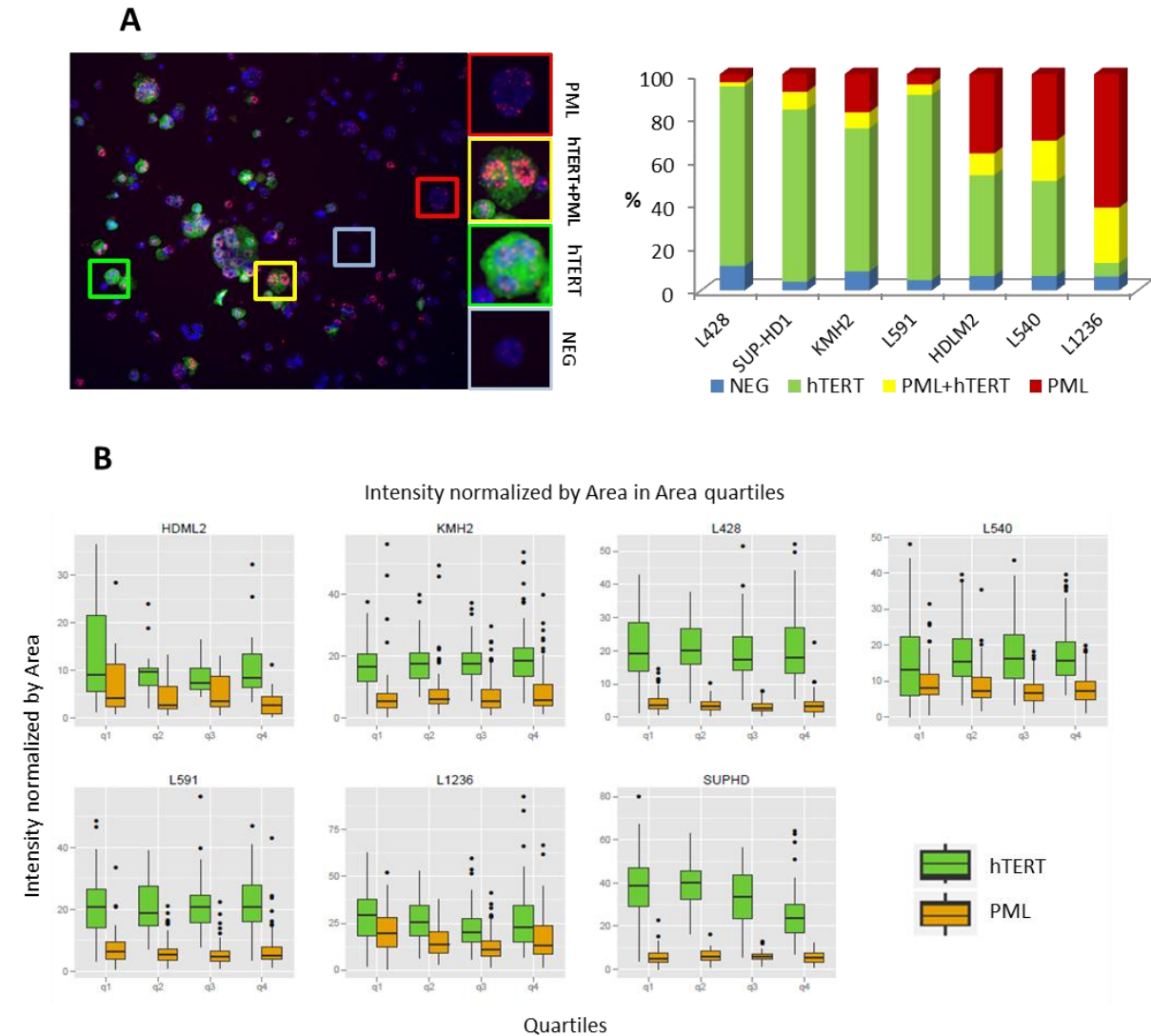


Figure 3 : Interchange between Telomerase and ALT in HL cell lines (A) using simultaneous immunofluorescence staining of hTERT (green signal) and PML (red signal), nuclei of representative HL cell lines demonstrate the presence of cells with only hTERT expression, cells with only PML expression and cell with PML and hTERT expression. The quantification of the number of cells according to this classification demonstrate the coexistence of both Telomerase and ALT mechanisms in the same cell line. L428 and SUP-HD1 (high telomerase activity) exhibited a high frequency of cell with only hTERT expression. However, L1236 cell line (low telomerase activity) exhibited a high frequency of cells with only PML expression (B) using an automated quantification of the signal of hTERT and PML, the intensity of the fluorescence was measured in the different type of cells and the results were represented according to the different quartiles area of cells. The first quartile (small cells) demonstrated high intensity of hTERT signal compared to the fourth quartile (big cells). The intensity of PML in small cells was lower than that observed in big cells except for HL cell lines exhibiting high telomerase activity (SUP-HD and L428).

2-Transition from TMMs according to the phenotype of cells

In order to determine the immunophenotype of cells expressing hTERT or cells with ALT profile, fluorescence activation cell sorting was performed after CD30 and CD15 staining in 5 HL cell lines. Four sub-populations were isolated: negative CD30 and CD15, CD30+CD15-, CD30+CD15+ and only CD30-CD15+. Figure 4 showed the frequency of cell expressing only hTERT, only PML both hTERT and PML and negative cells. Higher frequency of cells with telomerase expression was observed in

CD30 and CD15 negative cells. The decrease of hTERT expression was associated to the increase of PML expression in the transition of HL cells.

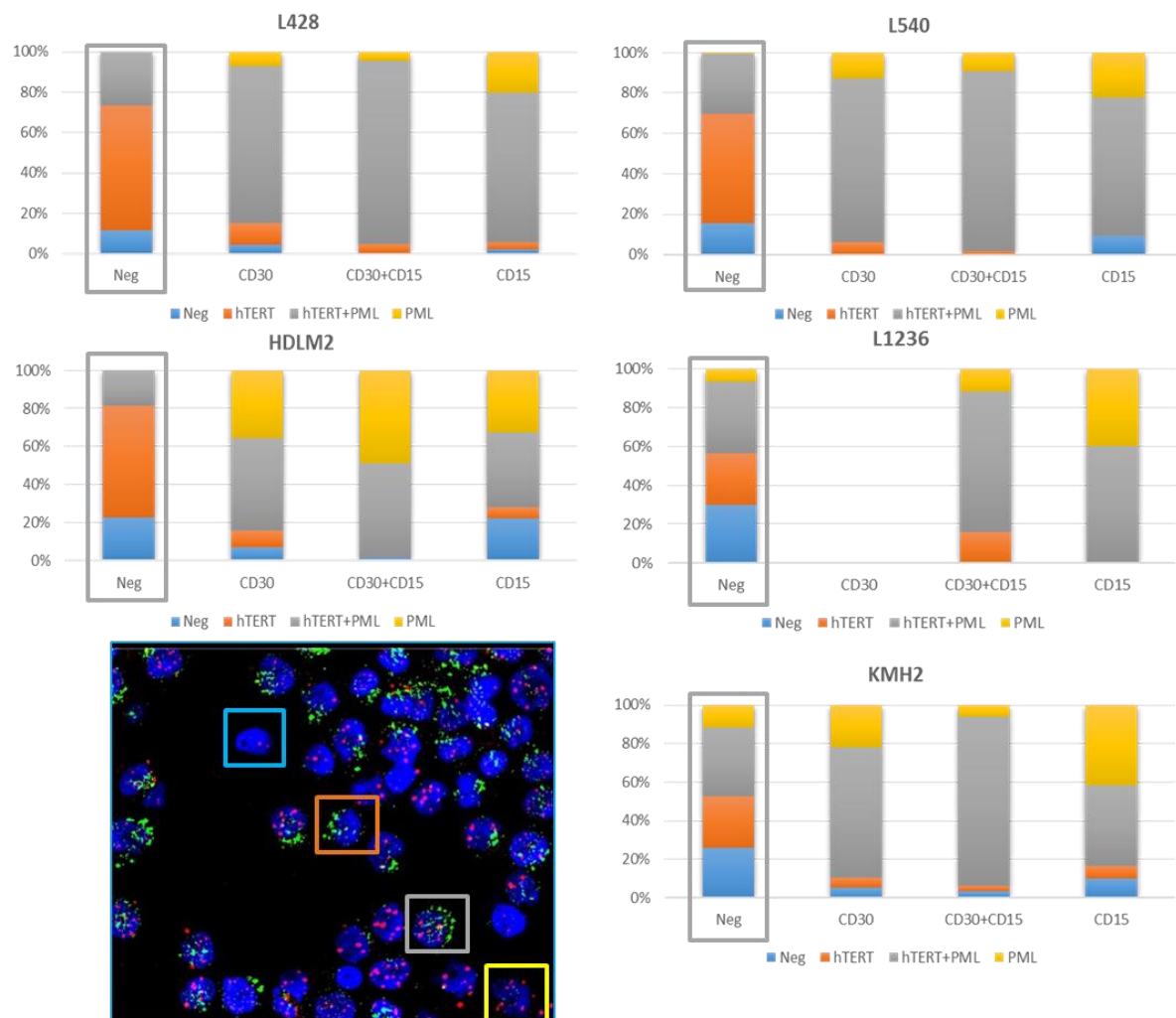


Figure 4 : Presence of telomerase and ALT in different HL cell lines according to their phenotype after fluorescence activation cell sorting. In all NS cell lines, negative population (CD30 and CD15 negative) presented a similar frequency of the different cell types (Negative, only hTERT and hTERT+PML). In contrast, the two MC cell lines presented a similarity and the presence of different cell types was observed. Picture: Nuclei of representative negative CD30 and CD15 of KMH2 cell line.

3-Altered double-strand breaks response in ALT HL cell lines

The telomere dysfunction in Hodgkin tumor cells and peripheral blood lymphocytes demonstrated previously and the presence of mosaic TMMs in tumor cells, suggest that alteration in DNA repair system may be an important biological event influencing treatment resistance in HL. Thus, we decided to explore further the radiation sensitivity of tumor cells of HL cell lines. To this end, HL cell lines and lymphoblastoid B cells were exposed to ionizing radiation and two techniques were used to assess this radiation sensitivity: apoptosis including cell cycle arrest and gene expression.

As depicted in figure 5A, lymphoblastoid B cells displayed a normal response to ionizing radiation characterized by nearly complete mitotic arrest preceding massive apoptosis and a higher response in gene expression (Figure 5B). In contrast, the checkpoint response in HL cell lines was impaired to a variable extent: mitotic arrest was incomplete, induction of apoptosis was weaker, and there was an increase in the hyper-G2/M population at late time points in L428 and, to a lesser extent in HDLM2 and L1236. These results clearly indicate the existence of a common disruption of the mitotic spindle checkpoint in HL cells compared to non-transformed B lymphocytes. Similarly to the apoptosis data, the gene expression demonstrated a significant difference in the response of cell lines compared to lymphoblastoid cell lines and peripheral blood lymphocytes.

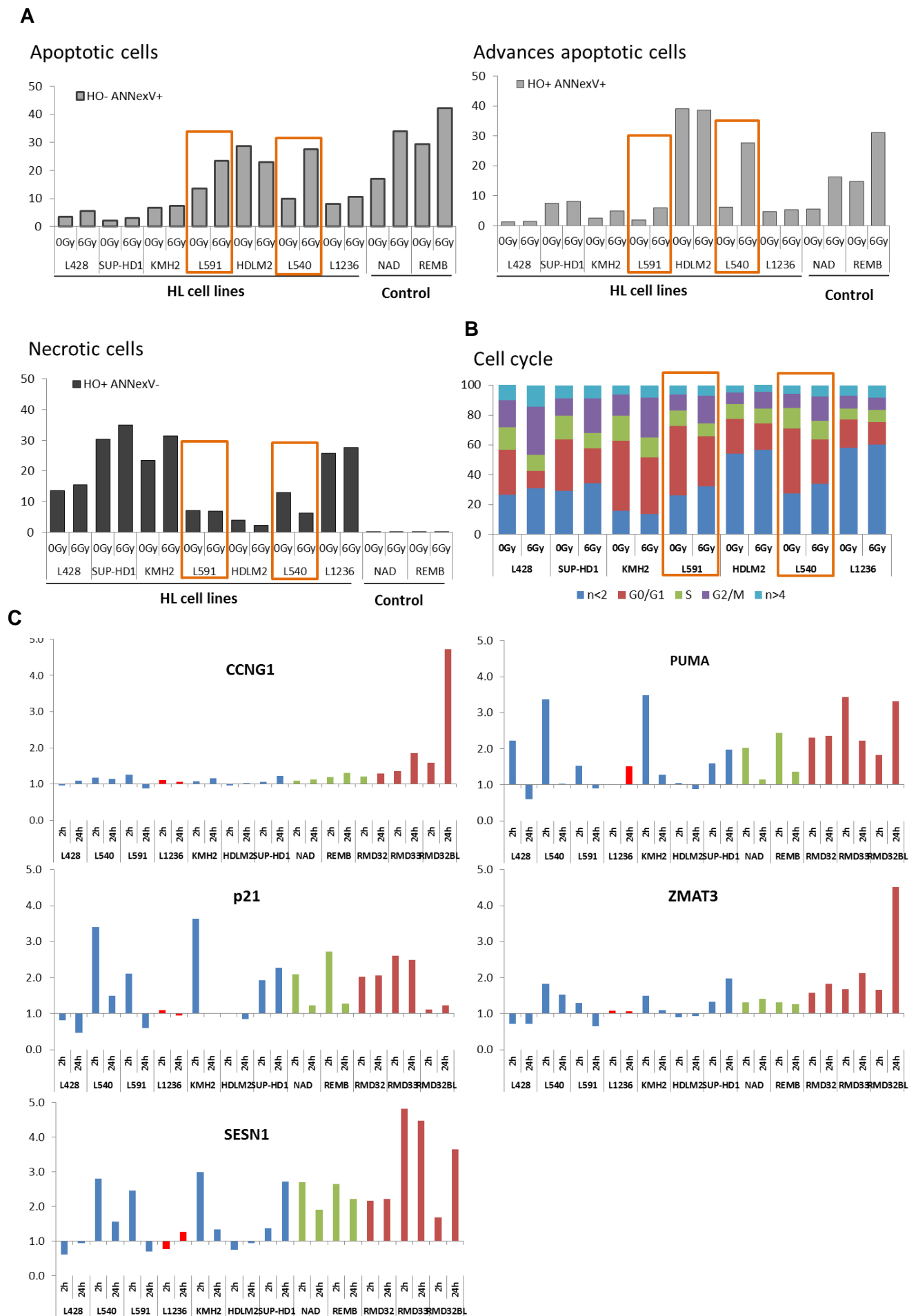


Figure 5 : Alterations in radiation sensitivity of HL cell lines. (A) Cell lines were irradiated at 6 Gy and collected 24h later. Lymphoblastoid B cell lines (REMB and NAD) displayed a normal response to radiation, whereas HL-derived cell lines had

a lower apoptotic rate except for L540 and L59. L1236, KMH2 and SUP-HD cell lines exhibited a higher number of necrotic cells and .L428 and HDLM2 presented no response to irradiation and low number of necrotic cells.(B) Cells show a delay in G2/M phase of the cell cycle (C) Gene expression analysis of P21, CCNG1, PUMA, ZMAT3, SESN1 by quantitative PCR in HL cell lines, lymphoblastoid cell lines and human lymphocytes. Values were standardised to HPRT expression level. The gene expression level ratio of irradiated/mock-treated cell is presented. The mean values of triplicate experiments, each with three reactions, are shown.

4-HL tumor cells activate a switch between telomerase and ALT mechanisms

Following TMMs investigation in HL cell lines the questions that were raised were whether a tumor could contain some cells expressing telomerase and other cells expressing characteristics of ALT, or whether the same cells could express both types of telomere-elongating characteristics. We examined TMMs within 38 HL lymph nodes compared to 24 lymph nodes of follicular hyperplasia or chronic lymphadenitis. To determine the phenotype of cells exhibiting hTERT in HL lymph nodes, immunofluorescence of CD30 antigen, one of the clinical hallmarks of HL tumor cells, and anti-hTERT antibody was performed. Figure 6 shows not only the coexistence of CD30 and hTERT staining in HRS cells but also the presence of small cells expressing very high levels of telomerase without CD30 signal. The suppression of hTERT is accompanied by the appearance of CD30 signal meaning that Hodgkin cells express a higher level of telomerase than Reed-Sternberg cells which express a very weak hTERT signal. These findings are also strengthened by the existence of well-known telomere shortening and chromosomal instability in RS cells compared to Hodgkin cells.

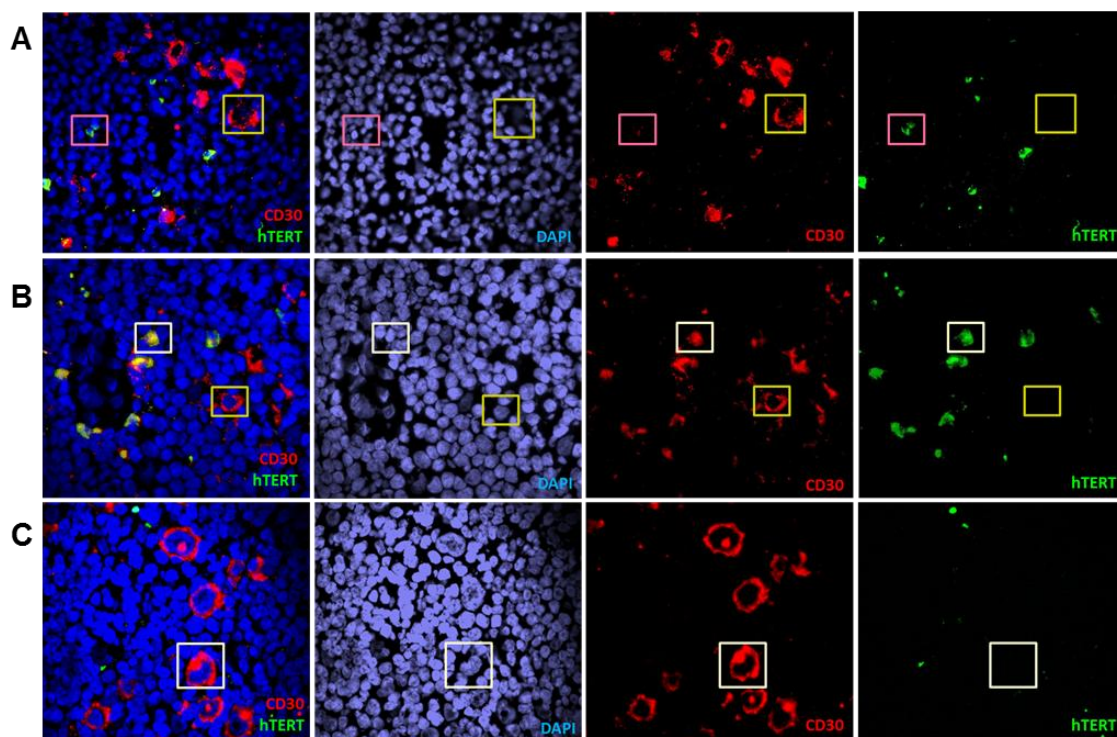
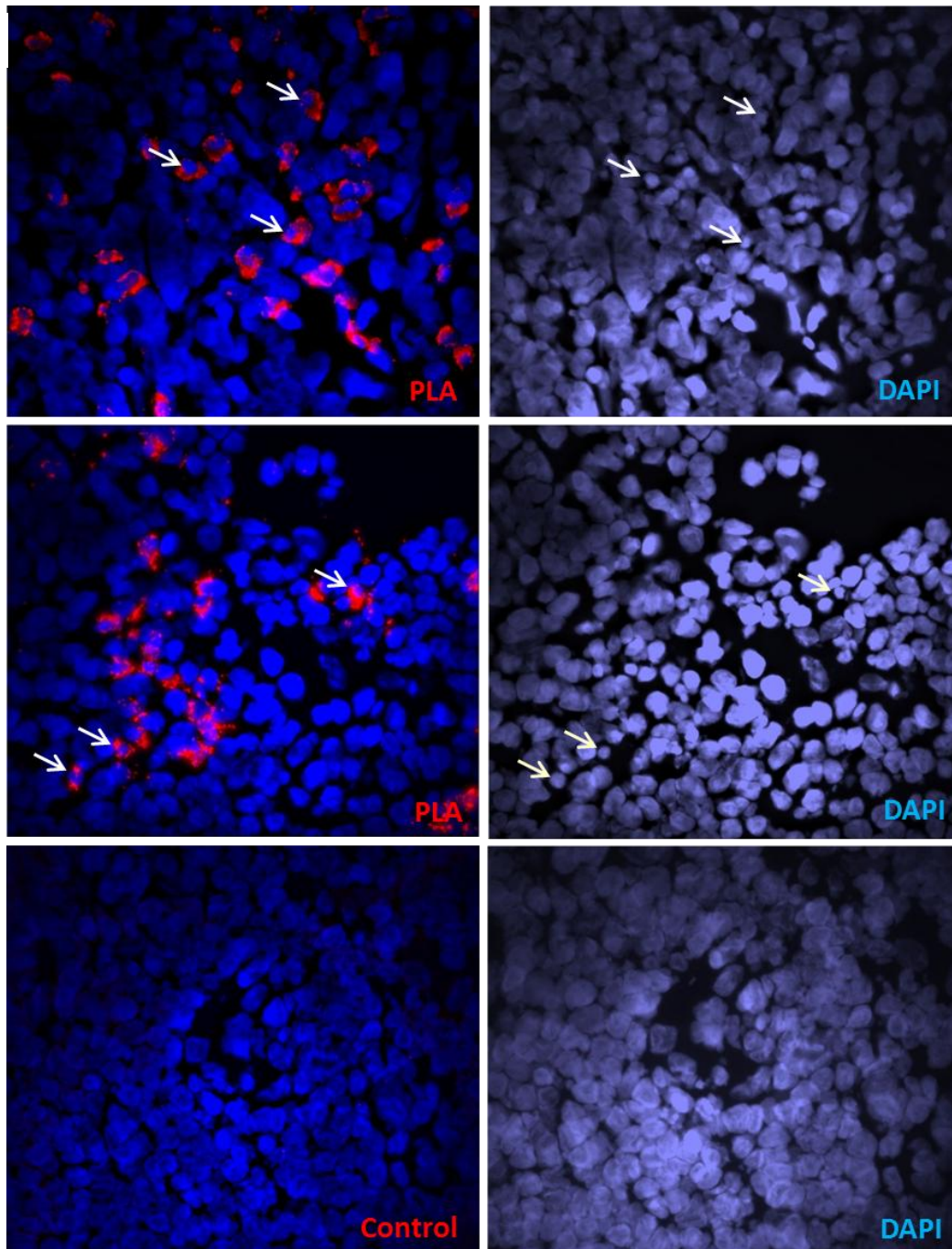


Figure 6 : Immunofluorescence analysis of CD30 monoclonal antibody (in red) and hTERT protein (in green) in lymph nodes from Hodgkin lymphoma patients demonstrate (A) the presence of very low hTERT expression in HR cells and the presence

of small cells with high hTERT expression and very low CD30 expression (B) the colocalisation of hTERT and CD30 expression in Hodgkin cells and (C) the absence of hTERT expression in RS cells.

Next, we sought to study the TMMs of HRS cells expressed low or absence level of hTERT in HL lymph node. As a marker of ALT, we used the colocalisation of PML and TRF2 forming APBs which has been reported to be specific for cells that maintain telomeres by recombination. using the PLA assay test , HL lymph nodes display a wide variability in the proportion of cells with higher frequency of APBs. In control group, only one lymph node displayed the colocalisation of TRF2 and PML signals. In HL lymph nodes, using an automated quantification technique, 3 groups were established: no expression or lower level (<5%), intermediated level (between 5 to 15%) and highest (more than 15%) level of APBs expression as illustrated in Figure 7A. Interestingly, high APB's signal was observed in small cells. HRS cells were characterized by a higher PML expression. To validate the results of PLA assay analysis, we explored the activation of ALT mechanism using a different technical approach: quantification of the colocalisation of PML foci and telomere sequences using IF-FISH technique (PML associated to telomere signal) (Figure 7B).

A



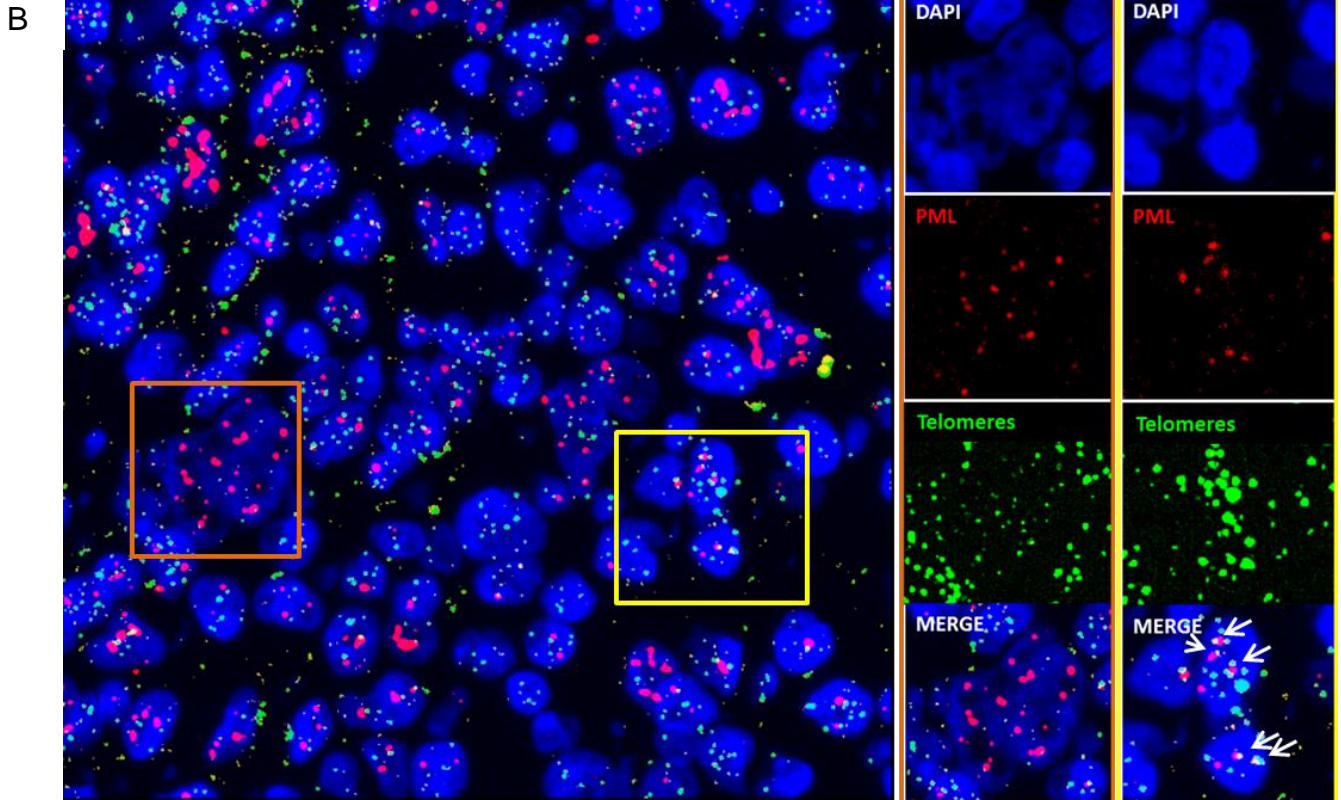


Figure 7 :In situ analysis of the presence of APB's, marker of ALT profile, in HL lymph nodes sections. (A) a proximity ligation assay(PLA) has been used for analyzing PML/TRF2 interactions and demonstrate the presence of many signals in a small cells. Arrows indicate these cells (B) IF-FISH staining of PML and telomeres demonstrate the higher colocalization of PML and telomere in small compared to RS cells from lymph nodes of HL patients.

5-Microenvironment, TMMs and clinical outcome of HL patients

All HL patients entered in this study were stage I (81.6%) or II (18.4%) with mean age of 36.7 years (84.7% <45 years). The follow-up of HL patients and control was performed more than 10 years after treatment. Four patients (10.5%) have died after relapse, refractory or second cancer and four patients (10.5%) have developed a late complication (secondary cancer and cardiovascular disease) and are alive with disease (Table1).

To characterize the microenvironment of tumor cells in HL lymph nodes, two approaches were performed: the first approach consisted in the characterization of the EBV status and the second, in scoring the number of macrophages in HL lymph nodes. The latent membrane protein (LMP)1 staining was performed and revealed the presence of EBV genome in HRS cells in 13 lymph nodes (34,2%). We also investigated the presence of macrophage cells in HL lymph nodes. The number of macrophage in lymph node was scored after anti-CD68 staining using the same nomenclature established by Steidl et al (Steidl et al 2010a).

Three prognostic groups with low, intermediate and high TRF2-PML colocalization (PLA), PML staining only, hTERT staining only, and CD68 density were defined with cut points of less than 5%, 5% to 15%, and more than 15% by using automated software.

The association between different markers and survival as well as the freedom from progression and relapse was estimated by the Kaplan-Meier method and the log-rank test. At univariable level, we found a significant correlation between higher expression of the colocalisation of TRF2 and PML and poorer EFS and OS. Neither the high expression of PML nor the high expression of hTERT correlated significantly to clinical outcome HL patients (supplementary data 2). Interestingly, the presence of LPM1 protein expression was significantly correlated with short OS ($p=0.03$), FFP ($p=0.03$) and EFS ($p=0.009$) (Figure 9).

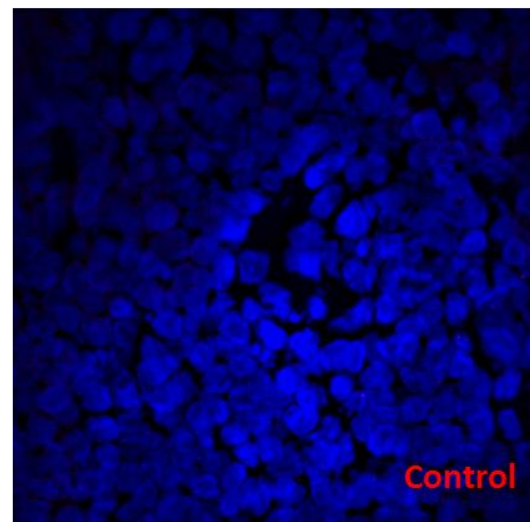
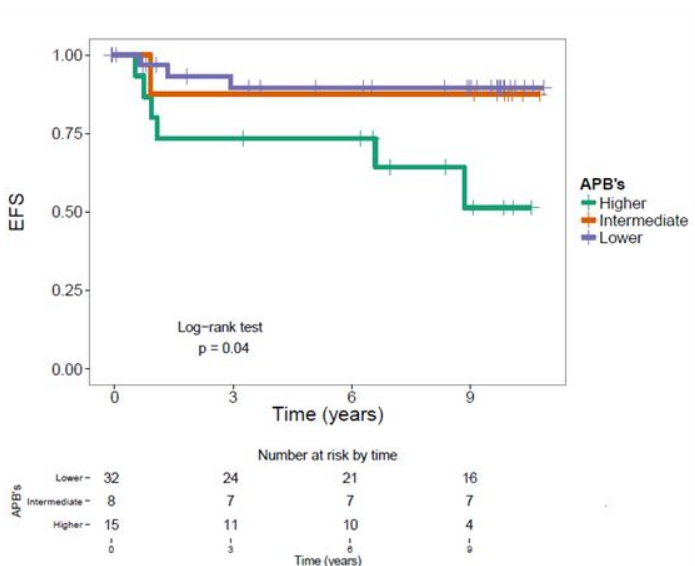
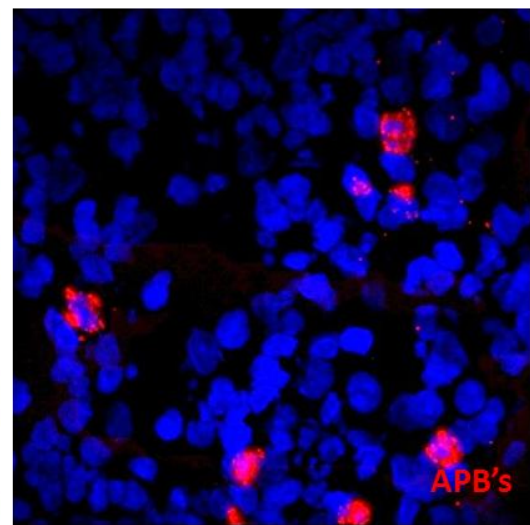
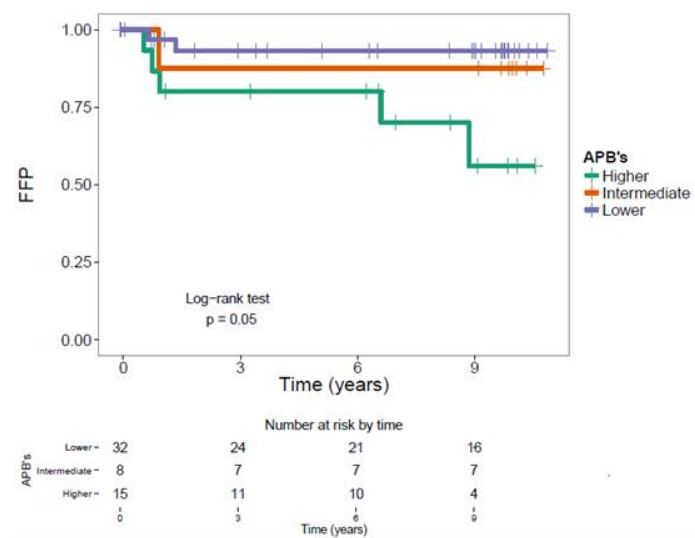
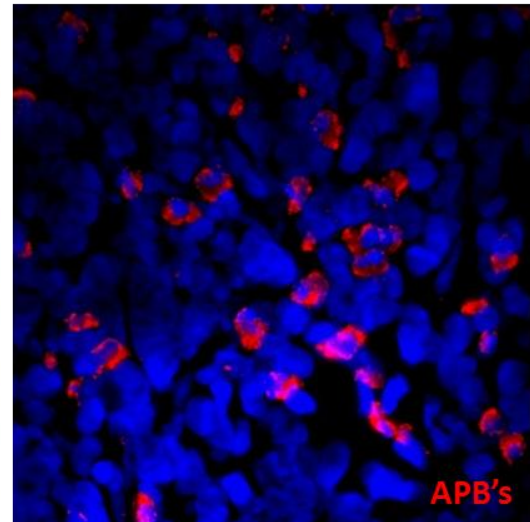
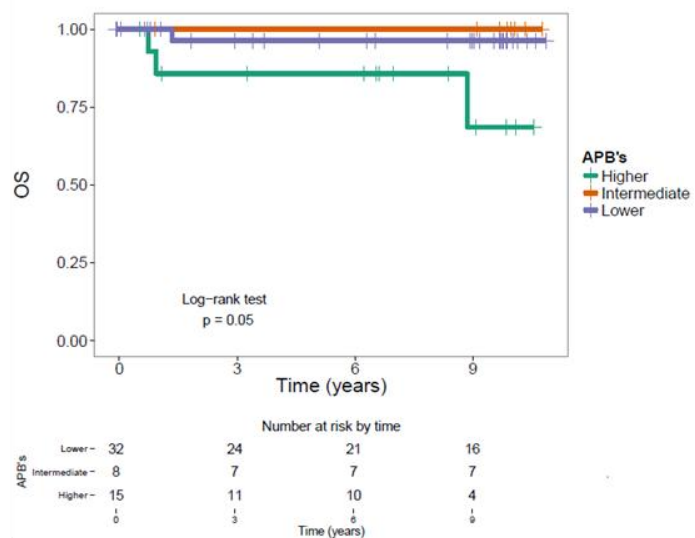


Figure 8: Colocalisation of PML-TRF2 and clinical outcome of HL patients. The patients were grouped into quartiles (quartiles 1 to 3) according to the protein expression level of each marker, and the Kaplan-Meier curves are shown.

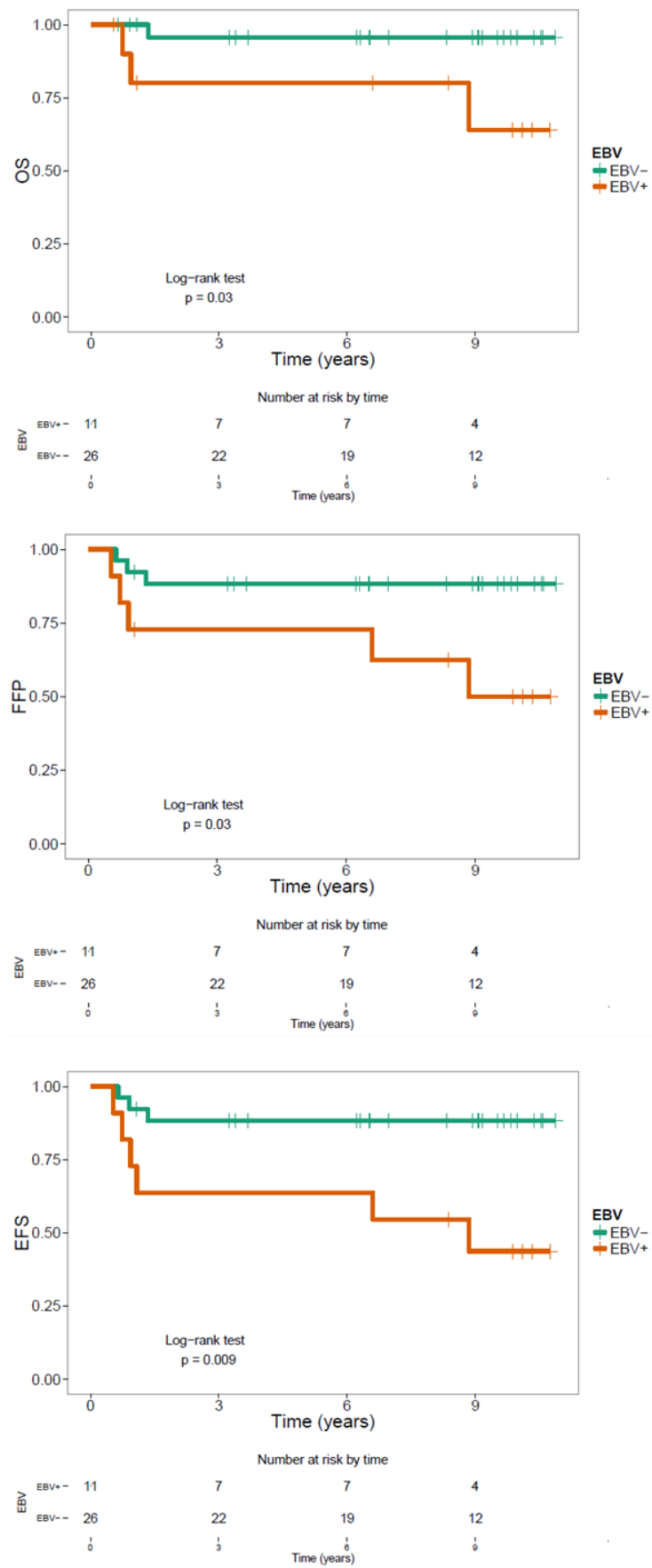


Figure 9 : Analysis of the relationship between EBV (LMP1) status and clinical outcome of HL patients.

At multivariate level, higher expression of TRF2/PML (ABPs) and CD68 in EBV positive patients have a significant predictive impact on OS ($p=0.002$), FFP ($p=0.002$) and EFS ($p=0.0003$) (Figure 10).

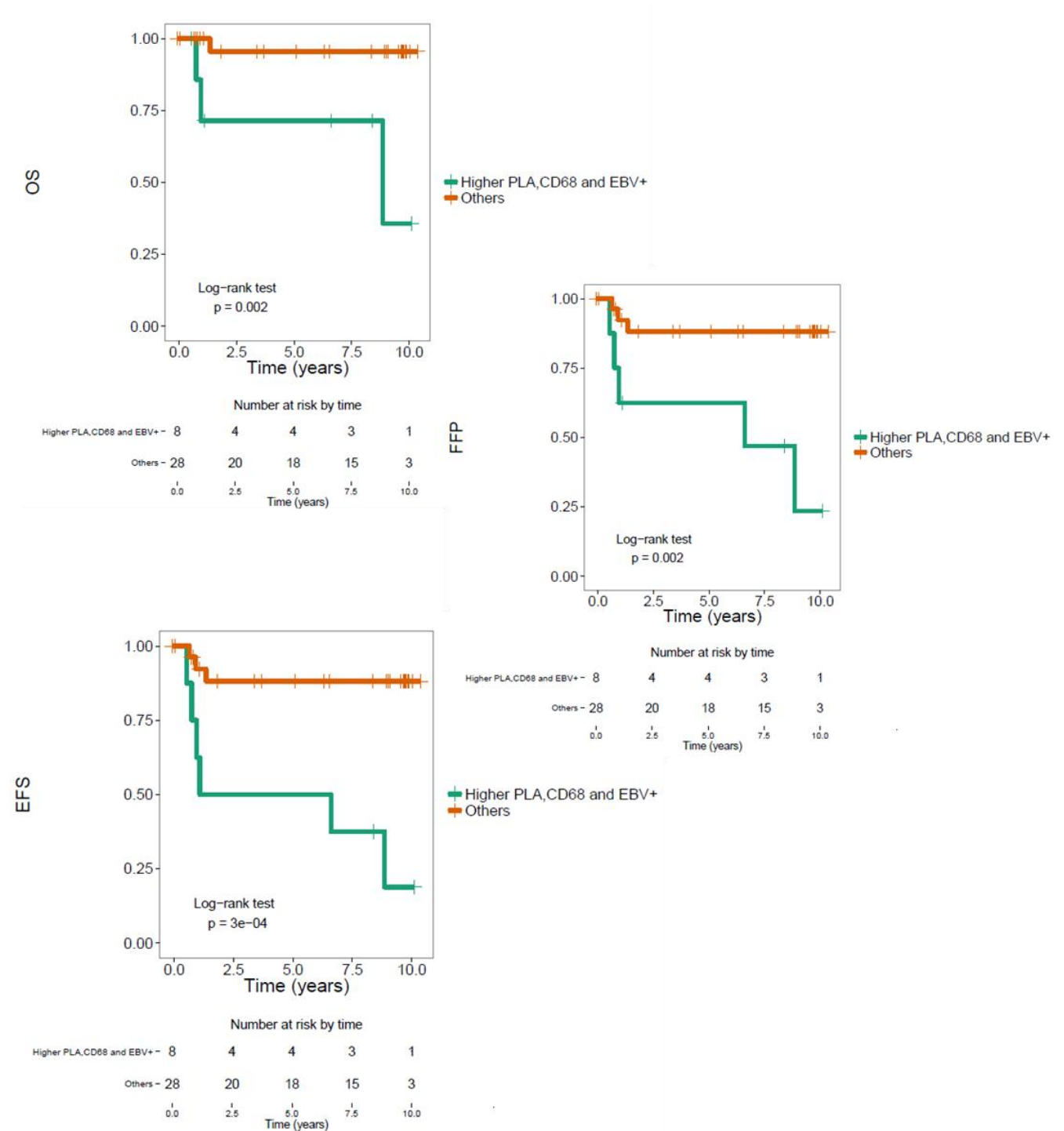


Figure 10 : Analysis of the relationship between higher expression of APB's and CD68 in EBV positive patients and clinical outcome of HL patients.

Discussion

The aim of the present study was to put light on the conflicting results concerning telomere maintenance mechanisms in Hodgkin lymphoma. We investigate the TMMs in 7 HL cell lines and 38 diagnostic lymph-node specimens obtained from HL patients. In contrast to previous studies, we found that HL cells use concomitantly two mechanisms (telomerase and ALT) for telomere maintenance during cell progression reflecting tumor heterogeneity in HL. Furthermore, we speculate that the mosaicism of TMMs in HL has important implication in the therapy of this disease.

Telomere dysfunction related to chromosomal instability has been described in HL patients (Guffei et al 2010, M'Kacher et al 2007). However, in view of the technical difficulties in quantitating telomerase activity and the rarity and heterogeneity of tumor cells in HL lymph nodes, it is perhaps not surprising that the few telomerase studies have yielded divergent results (Brousset et al 1998). The standard method of detecting telomerase in tumors is the Telomere repeated Amplification Protocol (TRAP) assay (Kim et al 1994). This is a highly sensitive semi-quantitative assay. However, the presence of inhibitors of the TRAP in tumor tissues caused false-negative results (Au et al 2011). Immunohistochemistry or immunofluorescence detection of telomerase in tumor tissues is problematic most likely due to the exceptionally low abundance of this molecule, even in tumor cells (Cohen et al 2007). In this study, telomerase activity was performed first in HL cell lines using TRAP assay and the result was detected by PCR. In the same time, immunofluorescence of hTERT protein was performed and a high correlation was obtained between the telomerase activity and the intensity of fluorescence of hTERT protein. This correlation allowed us to use only immunofluorescence assay in the detection of hTERT protein, offering the possibility to investigate the hTERT expression in neoplastic and non-neoplastic cells.

From our knowledge, ALT mechanism was never investigated in HL lymph nodes or in cell lines. The most reliable methods for detecting ALT in tumor samples or cell lines are assays showing APBs formation (Henson et al 2005, Yeager et al 1999), C-circles (Henson et al 2009) or telomere length (Bryan et al 1997). In this study, the presence of APBs was used to investigate ALT cells in HL. Two different techniques were used to detect APBs: the first approach consisted in the use of PLA method

(Trifilieff et al 2011) and the second by immunofluorescence of PML combined with FISH using telomeric probes. ALT may also be detected through the presence of very bright foci telomeric FISH (Heaphy et al 2011). All these approaches were performed with an enhanced imaging system permitting the easy quantification of the number of positive cells and the intensity of fluorescence of each marker as well as the co-expression of two proteins.

The first step for the TMMs investigation in HL consisted in the reevaluation of the TMMs in 7 HL cell lines. In this study, we demonstrate not only the presence of telomerase activity at different levels in all HL cell lines but also, the transition between TMMs of HL cells during the cell progression from small cells to RS cells. Four categories of cells were detected: negative cells for telomerase or ALT, cell exhibiting only telomerase expression, cell with ALT expression and cell with both telomerase and ALT profiles. The small cells exhibited higher telomerase expression than that observed in HRS cells in all cell lines. This data was confirmed using Fluorescence-activated cell sorting of CD30- and CD15- cells which gave rise to the same parental cell line. We demonstrated in this work the heterogeneity of TMMs in CD15/CD30 negative cells of HL cell lines and essentially the presence of cells which have neither telomerase activity nor evidence of ALT profile. The presence of tumor cells which have neither telomerase activity nor evidence of ALT has been reported previously (Hakin-Smith et al 2003) (Costa et al 2006). It is currently unclear and many hypothesis including the presence of another ALT mechanism, or whether these tumor cells do not have any TMMs. This is an important question to address TMM as a therapeutic target.

Recent work has shown that chromosomal aberrations in HL are not restricted to HRS cells, but may also be found in smaller cells with non-HRS cell morphology, proposing the existence of a population of tumor-precursor cells representing a reservoir of cells from which HRS cells develop (Ford et al 2001). In the same way, we have previously demonstrated that telomere aberrations were found in small cells compared to RS cells, even if the telomere length of small cells was significantly higher than those measured in RS cells (Cuceu C. submitted). In this study, we demonstrate that these small cells that are negative for CD30 and CD15 activate firstly telomerase and achieve in their progression an ALT profile. Perhaps HL cells due to their hypermutability may activate both pathways for telomere maintenance in

the progression to HRS cells (Spieker et al 2000). Of note, HRS cells have a very low level of telomerase activity and lack APBs.

In HL lymph nodes, our studies demonstrate that HL lymph node are mosaic for four types of cells: those that use telomerase, those that used ALT, those exhibited both telomerase and ALT and those that are negative. Characteristically, variable numbers of small cell with non-neoplastic morphology and without CD30 expression exhibit higher expression of telomerase in lymph nodes infiltrated by HL. While it has been reported that among 24 control lymph nodes analyzed. It will be interesting to study the nature of these B-cell CD30 negative and their pathogenic role in HL (Spieker et al 2000).

Interestingly, APBs were detected essentially in the small HL cells in the presence of telomerase expression. Nevertheless, the HRS cell exhibited a higher PML body expression with or without telomerase expression. These findings demonstrate for the first time, the coexistence of telomerase and ALT in TMMs in the progression of HL cells. The ability to explore these processes has likewise been limited by the rarity of these cells and the lack of in vitro growth of cells derived from primary HL tumors. Cell lines derived from HL patients in refractory or relapse are the unique possibility actually to understand these processes. The establishment of animal model of HL is a major advance to understand these mechanisms (M'kacher Part III).

Increasing evidence suggests that telomerase is essential for normal stem cell function, and the progressively decreases in more differentiated progeny (Pech et al 2015, Yui et al 1998) suggesting that telomere is the primary factor that regulates normal stem cells function (Flores et al 2008, Masutomi et al 2003, Wright et al 1996). The presence of telomerase expression in negative CD30 cells in HL lymph nodes suggest that clonogenic cells in HL are CD30- with ability to replicate and subsequently differentiate into malignant CD30 and CD15+ cells.

The relationship between TMMs and clinical outcome of patients has been suggested previously. ALT-positive liposarcomas are associated with a worse prognosis (Costa et al 2006) than telomerase positive tumors. Nevertheless, ALT positive glioblastoma multiforme tumors are associated with a better prognosis (Hakin-Smith et al 2003). Interestingly recent clinical trials of telomerase-target therapies also underline the clinical importance of telomere maintenance. The mosaicism of TMM within human tumors may affect the choice of therapeutic approaches (Reddel 2014).

In this study and using a homogeneous cohort (80% stage I, more than 80% SN and young patients) we demonstrate the relationship between clinical outcome and the TMMs in HL lymphoma patients. Lower OS, FFP and EFS were shown in case with high frequency of cells with APB's. Interestingly, despite the small number of patients entered in this study, EBV status appears to be a prognostic factor and confirmed the several reports in the role of EBV in HL clinical outcome (Kanakry & Ambinder 2015). Of note, recent study demonstrates that the clinical outcome of HIV-HL patients, treated with CART and mainly ABVD presented favourable outcomes (Besson et al 2015).

Multivariate levels of APB's and CD68 in EBV positive patients was related to poor clinical outcome of HL patients. The absence of an increases number of CD68+ cells and APB's cells in patients EBV negative and limited stage of disease defines a subgroup of patients for whom the rate of long-term disease-specific survival is 100% with the use of available treatments.

It would be important to confirm these findings in large prospective longitudinal clinical studies in order to understand the TMMs in HL and establish the relationship between these markers and clinical outcome of patients.

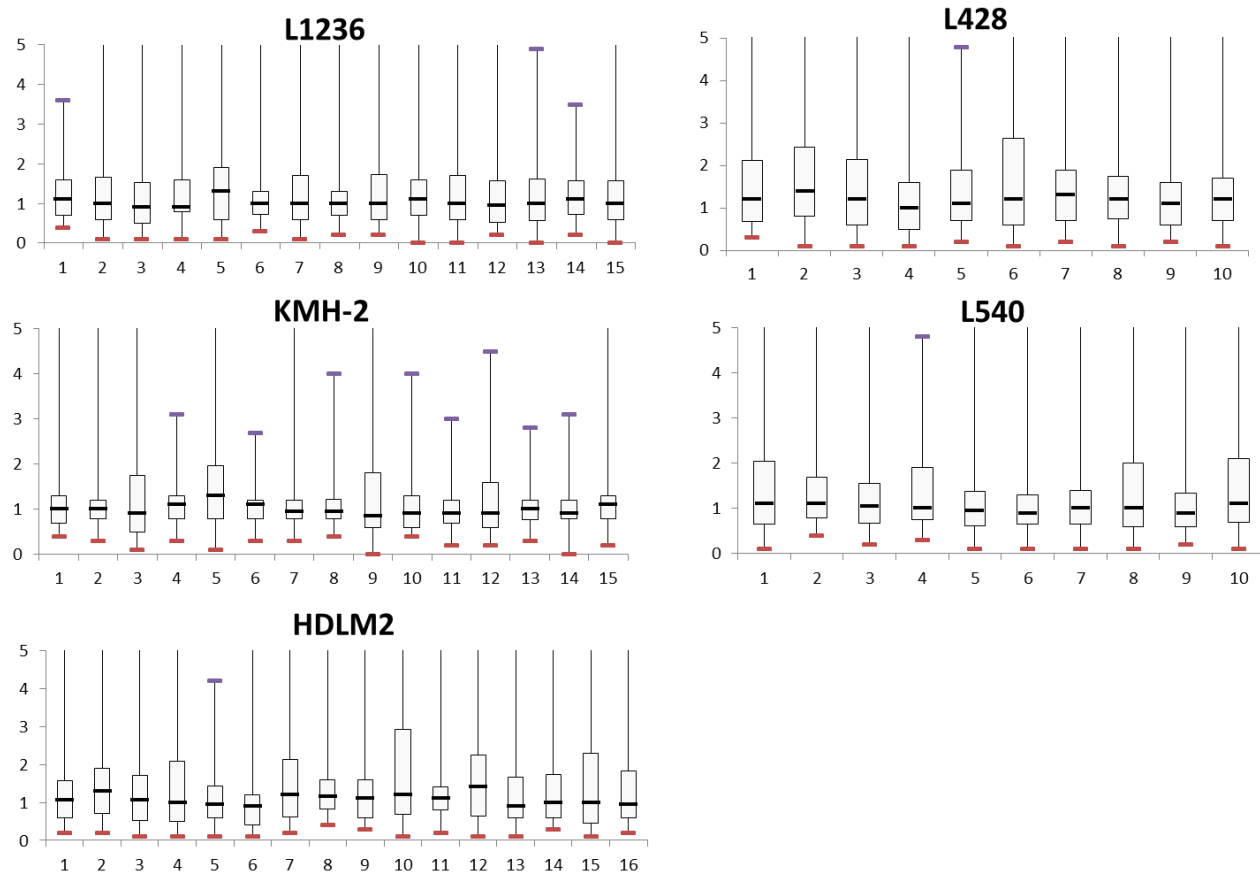
Up to date, good progress has been made in understanding telomere structure and function in normal and cancer cells. It is critically important to develop reliable methods for telomerase and ALT detection, these being likely to become an important prerequisite in the use of treatment targeting one or other of these mechanisms. A striking correlation has been observed between ALT activity in various human cancers and loss of the ATP-dependent helicase ATRX or its binding partner, the H3.3-specific histone chaperone DAXX, both of which are constituents of PML bodies. Recent study has found that ALT cancer cells are hypersensitive to ATR inhibitors (Clynes et al 2015). It will be interestingly to test ATR inhibitors in HL cells (Flynn et al 2015).

Supplementary data

Supplementary data 1

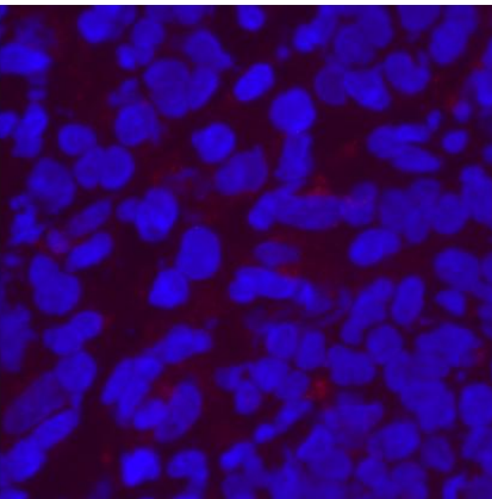
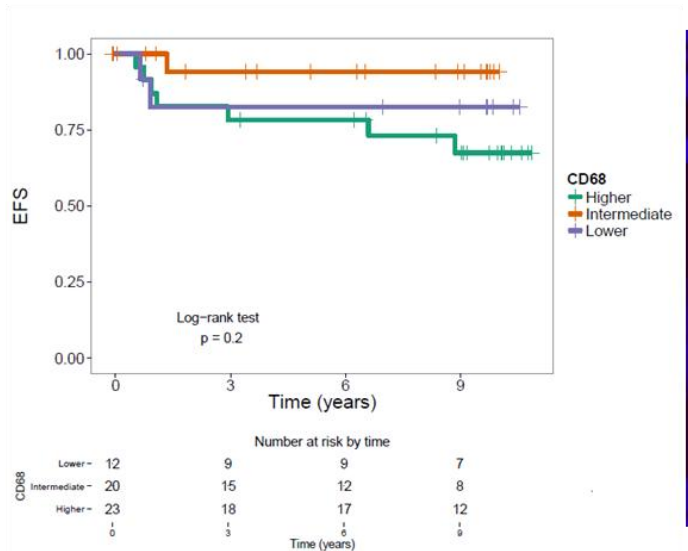
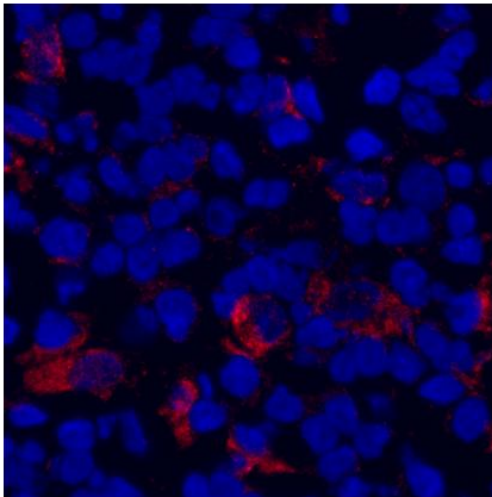
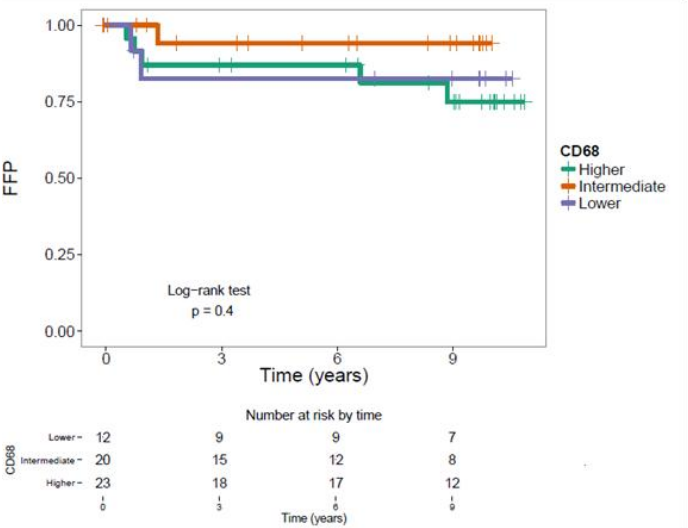
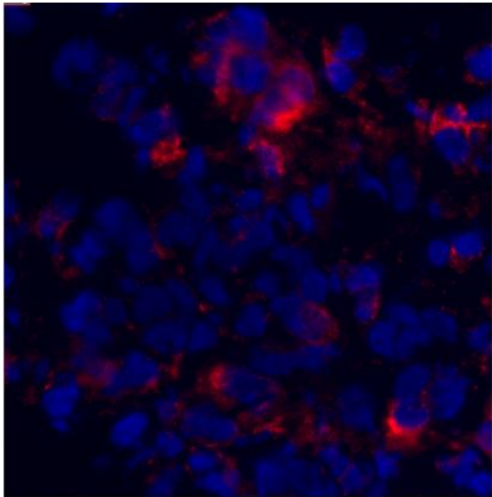
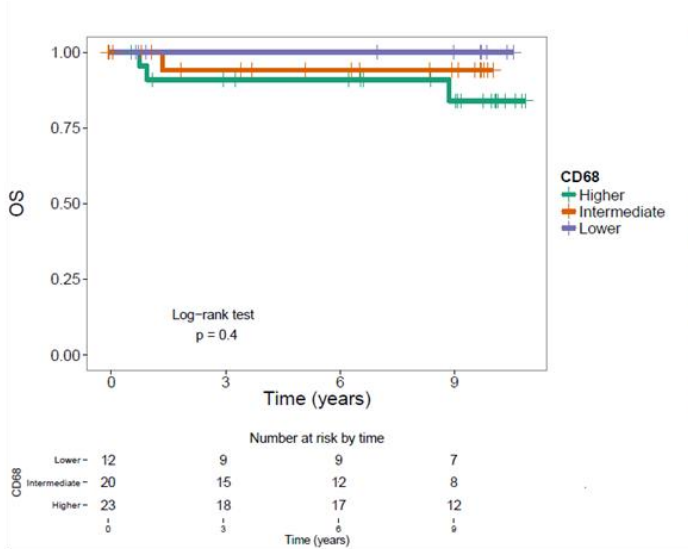
ALT profil was characterized by a higher difference in the intensity of fluorescence in p and q arms. The ratio between p and q arms was presented in each metaphase analyzed.

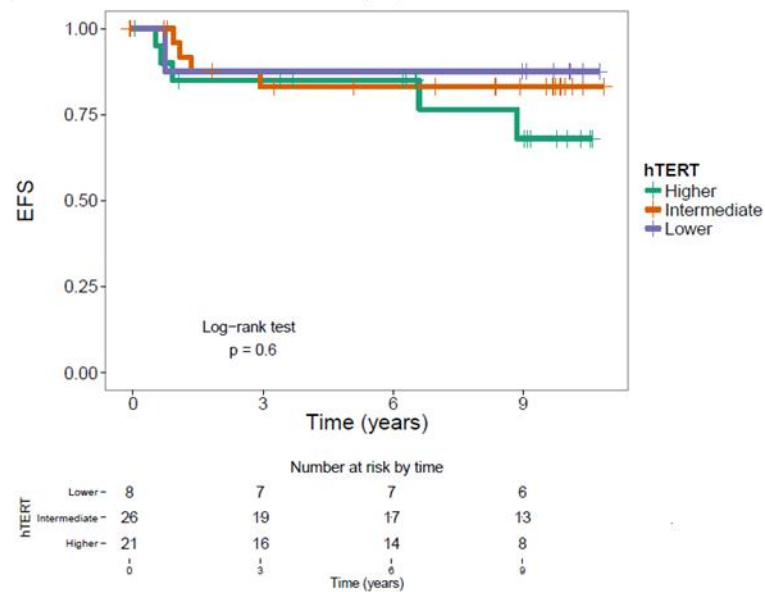
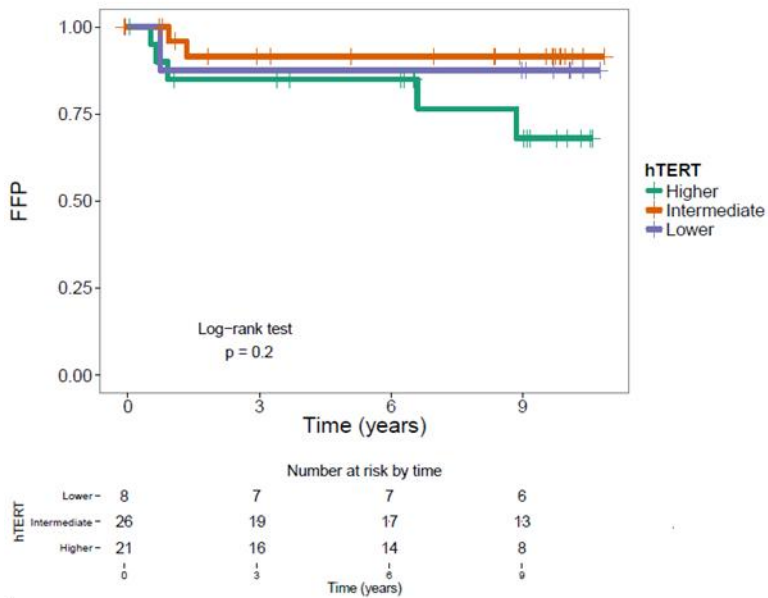
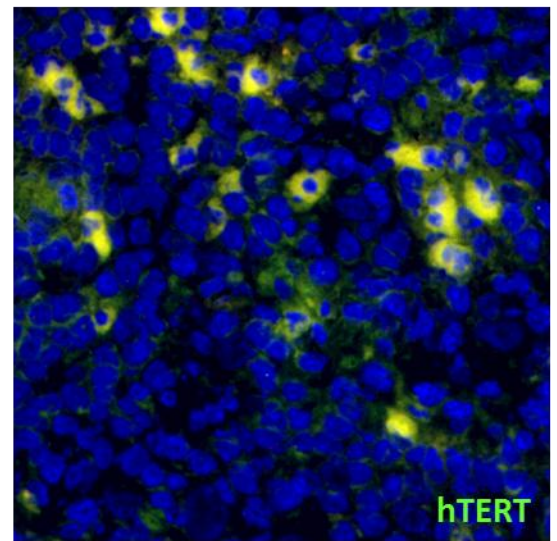
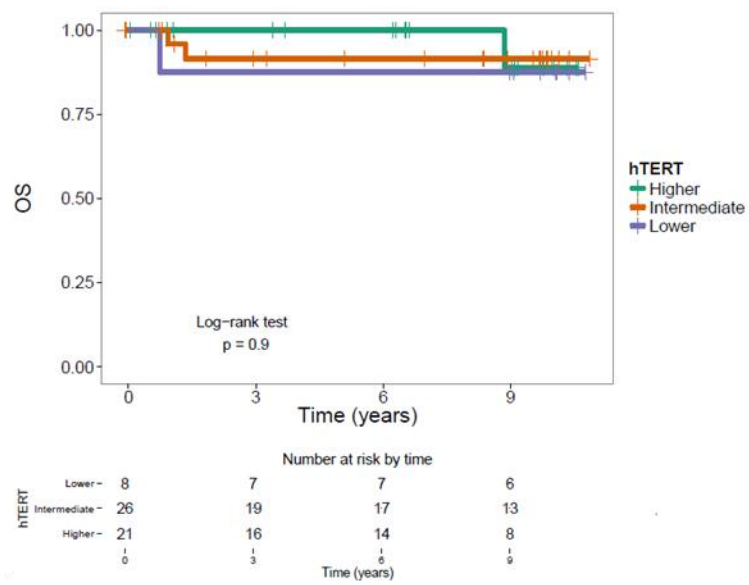
Telomere heterogeneity p/q

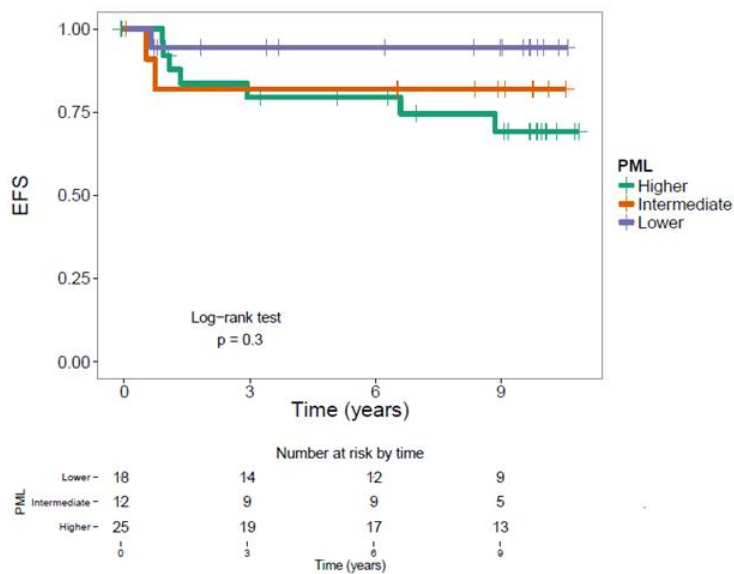
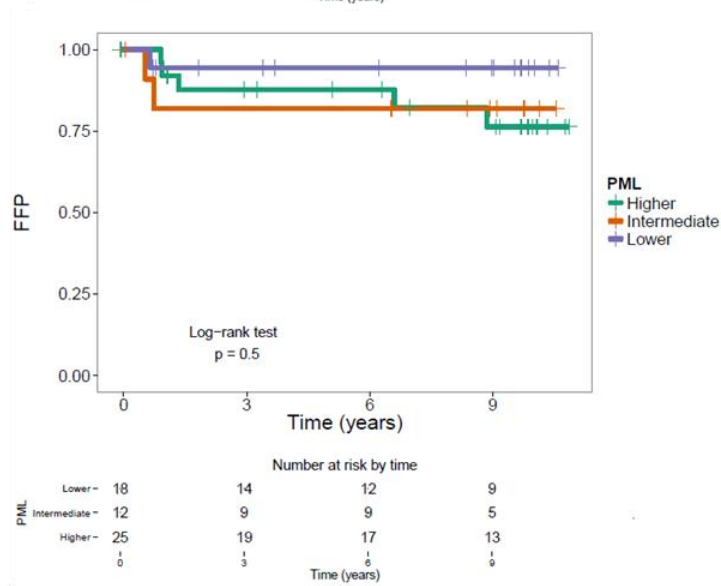
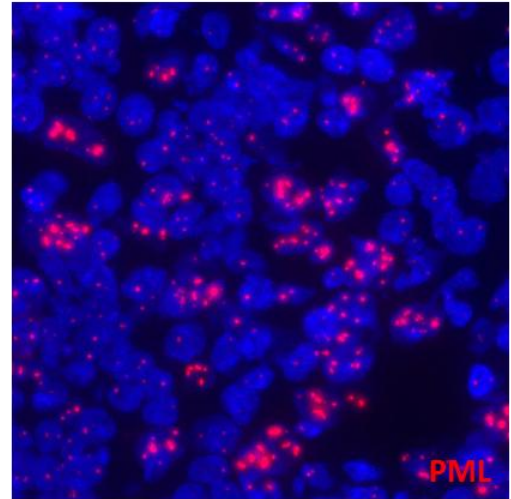
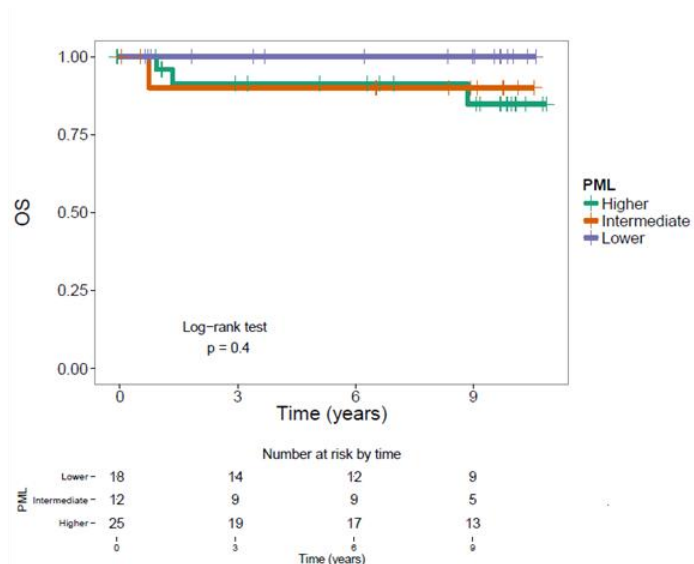


Supplementary data 2

Analysis of the relationship between protein expression and outcome of HL. Immunofluorescence analysis of the expression of CD68, hTERT and PML were performed. The patients were grouped into quartiles according to protein expression level of each marker.







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Part V

This part of this thesis was dedicate to the first characterization of clonogenic Hodgkin lymphoma cells using cell sorting of CD30- cells and HL animal model established previously. We demonstrate the clonogenic ability of these negative cells to produce the similar parental cell line.

This part is an ongoing work and needs more investigation, however, it could open new possibility in the understanding of the lymphomatogenese if HL as well as the standardization of immunotherapy.

Characterization of Hodgkin lymphoma clonogenic cells

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Abstract

The identity of the cells responsible for the initiation and maintenance of Hodgkin and Reed Sternberg cells in Hodgkin lymphoma (HL) remains unclear largely because of the difficulty growing HL cells *in vitro* and *in vivo*. Exploring cell markers and signaling pathway specific to HL clonogenic cells may lead to progress in therapy and improve the prognosis of patients with HL. Telomere dysfunction and chromosomal instability were performed to characterize the clonogenic cells *in vitro* as well as *in vivo*.

Material and methods:

L428 HL cell line and circulating lymphocytes of 50 HL patients were used to characterize the clonogenic cells. Telomere dysfunction was performed using Q-FISH technique and both mechanisms for telomere maintenance, telomerase activity and alternative telomere lengthening (ALT), were used. Chromosomal instability was investigated using telomere and centromere staining followed by M-FISH technique.

Results:

We found that L428 cell line contained small (<2%) subpopulations that lacked CD30 and CD15 expression and had greater clonogenic potential *in vitro* than corresponding CD30+ and CD15+ cells. These cells were similarly clonogenic *in vivo* in NOD/SCID gamma -/- mice. These cells were characterized by a higher telomere instability associated to the presence of higher frequency of dicentric chromosome. High telomerase activity was detected in these cells as well as in circulating lymphocytes of 10 out of 50 HL patients.

Conclusion:

These data suggest that clonogenic cells are CD30- B cells with ability to replicate and subsequently differentiate into malignant CD30+ and CD15+ cells.

Key words: clonogenic cell, Hodgkin lymphoma,

Introduction

Hodgkin lymphoma (HL) is a malignancy of the immune system where the scarce tumor cells called Hodgkin and Reed–Sternberg (HRS) cells are, in most cases, derived from germinal center B cells (Kuppers et al 1994) and characterized by genomic instability (Diehl et al 1985).

Experimental and clinical observations suggested that the malignant phenotype cells of HL are sustained by a subset of cells characterized by the capacity of self-renewal and differentiation (Jones et al 2009, Kuppers et al 2001, Newcom et al 1988, Oki et al 2015). Therefore, it is unclear whether clonogenic cells have the proliferative capacity required for the initiation, maintenance and the progression of HL. On the other hand, these cells have been found in HL lymph nodes and peripheral blood of HL patients as well as HL cell lines. However, the relationship between these clonogenic cells and the HRS cells is unclear. In this study, we hypothesized that if clonogenic B cells represent putative HL stem cells, they should lack CD30 expression. We demonstrate that highly clonogenic cells from HL cell lines do not express CD30. These cells were characterized by the high frequency of telomere loss and high telomerase expression. Less chromosomal aberrations were found compared to that observed in HRS cells. Interestingly telomerase activity was detected in peripheral blood lymphocytes of some patients. The detection of these clonogenic cells can be used as highly specific tumor markers for detection of the disease.

Materials and methods

Cell lines, patient samples, and cell culture

The human HL-derived cell line L428 was cultured in Gibco RPMI 1640 medium supplemented with Glutamax, 10%FBS and antibiotics at 37°C. Peripheral blood lymphocytes samples were obtained from 50 patients with HL and 20 healthy donors granting informed consent and approved by Gustave Roussy Institutes'Institutional review Board.

Mononuclear cells from blood were isolated by density centrifugation (Ficoll-Paque; Life Science) and kept at -80°C. Cytogenetic preparations were performed from HL patients and healthy donors (M'Kacher et al 2007).

To characterize growing cells in L428 HL cell line and circulating lymphocytes, clonogenic growth was evaluated by plating cells (1000 cells/mL for cell lines or 1 ×

10^5 to 5×10^5 cells/mL for lymphocytes) in 1 mL 1.2% methylcellulose (Stem cell Technologies), 10% SVF, 2% mM L-glutamine and complemented with RPMI1640. Samples were plated in quadruplicate onto 35-mm² tissue culture dishes and incubated at 37°C and 5% CO₂. Colonies consisting of more than 50 cells were scored at 14 days for cell lines and 21 days for HL lymphocytes. Serial replating was performed by washing plates RPMI 1640 at 37°C and resuspending cells in the original volume of methylcellulose and concentration as described. Three independent experiments were performed in triplicate.

Fluorescence-activated cell sorting and flow cytometry

Cells were gated to exclude apoptotic or necrotic cells and sorted into CD30-/CD15- fractions by gating on the lowest and highest 5% PE-expressing cells, respectively. Following sorting, the CD30-/CD15- cell fractions were analyzed using a FACScan flow cytometer (Becton Dickinson) and found to be more than 98% pure.

Transplantation of clonogenic cells into NOD/SCID mice

Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice were bred and maintained in the CEA laboratory care facility. Six to 10-week-old mice received 3Gy irradiation (0.62Gy/min using a ¹³⁷Cs γ irradiator). 2 to 4 hours prior to intravenous injection via intravenous route or the dorsal tail vein of 10^3 cells isolated from methylcellulose. Mice were killed at 12 weeks after transplantation. Cells were evaluated by flow cytometry following cell surface staining with mouse antihuman CD45, CD14, CD30, and CD15 antibodies.

Animal experiments were performed according to French law and were approved by the institutional Ethics Committee for animal Experimentation.

Transcriptional response after in vitro irradiation of HL cell lines

Basal and post-irradiation expression of five radiation-responsive genes (CDKN1A, GADD45A, PUMA, CCNB1, and BBC3) was determined by quantitative real-time PCR in HL cell lines. The protocol was previously published (Badie et al 2008).

Cytogenetic analysis

Separated cells were cultured in the presence of RPMI 1640 supplemented with 10% FCS and antibiotics, colcemid (0.1 μ g/ml) added 2h before harvesting, and the slides

with metaphase chromosomes prepared following the standard methanol/acetic acid (3/1, v/v) procedure.

Six new cell lines were generated after one month of in vitro culture.

Chromosomal instability was performed first using telomere and centromere staining with PNA probes in order to detect unstable chromosomal aberrations (M'kacher et al 2014). Secondly, karyotype analysis was performed using multi-FISH probes (Metasystems GmbH, Althusseim, Germany), according to the manufacturer's recommendations on the same slide. Images of hybridized metaphases were captured using a charge coupled device camera (Zeiss, Thornwood, NY) coupled to a Zeiss Axioplan microscope and processed using ISIS software (Metasystems).

Telomere quantification and telomere maintenance mechanisms in clonogenic cells

Telomere length quantification was performed in parental L428 cell line, the isolated clones from methylcellulose and derived cells from mice using Q-FISH technique (M'kacher et al 2014).

Telomerase activity was quantified using a real-time PCR based quantitative telomerase detection kit according to manufacturer's instructions (Allied Biotech Inc., Ijamsville, MD). Equal cell numbers (10^6 cells) were used for each experiment and relative telomerase activity was calculated based on the value of 2 differences in CT value.

Alternative telomere lengthening (ALT) was performed using immunofluorescence detection of PML nuclear bodies and shelterin proteins (TRF2) or telomere staining using PNA probe (M'kacher et al 2014).

Statistical analysis

Results are presented as the mean \pm SEM. Comparisons between groups were performed using a 2-tailed, paired Student t test or analysis of variance (ANOVA) as indicated.

Results

1- Distinct phenotypic and functional cell populations are present in human HL cell lines

More than twenty years ago, Newcom et al identified a small population of phenotype B cells that appeared to be responsible for the propagation of HRS cells within a L428 HL cell line (Newcom et al 1988). Recently, the presence of clonogenic cells in HL was demonstrated in HL cell line, lymph nodes and circulating lymphocytes. In addition, the role of negative cells (CD30-) in HL cell lines was debated. To examine the clonogenic capacity of this subpopulation, we isolated CD30- cells from L428 cell line by FACS and examined each population for colony formation in methylcellulose. After 2 weeks, we characterized the phenotype of CD30- from L428 cell line by flow cytometry and found that CD30- cells expressed CD30 as well as CD15 (Figure 1A). Of note, there is higher frequency of CD30- cells in the obtained cells than in the parental L428 cell line (3% vs 1%). Although the initial plating efficiency of these cells was approximately 10-fold higher than parental L428 cell line (Figure 1B), CD30- cells underwent significantly greater clonogenic expansion than the parental cell line.

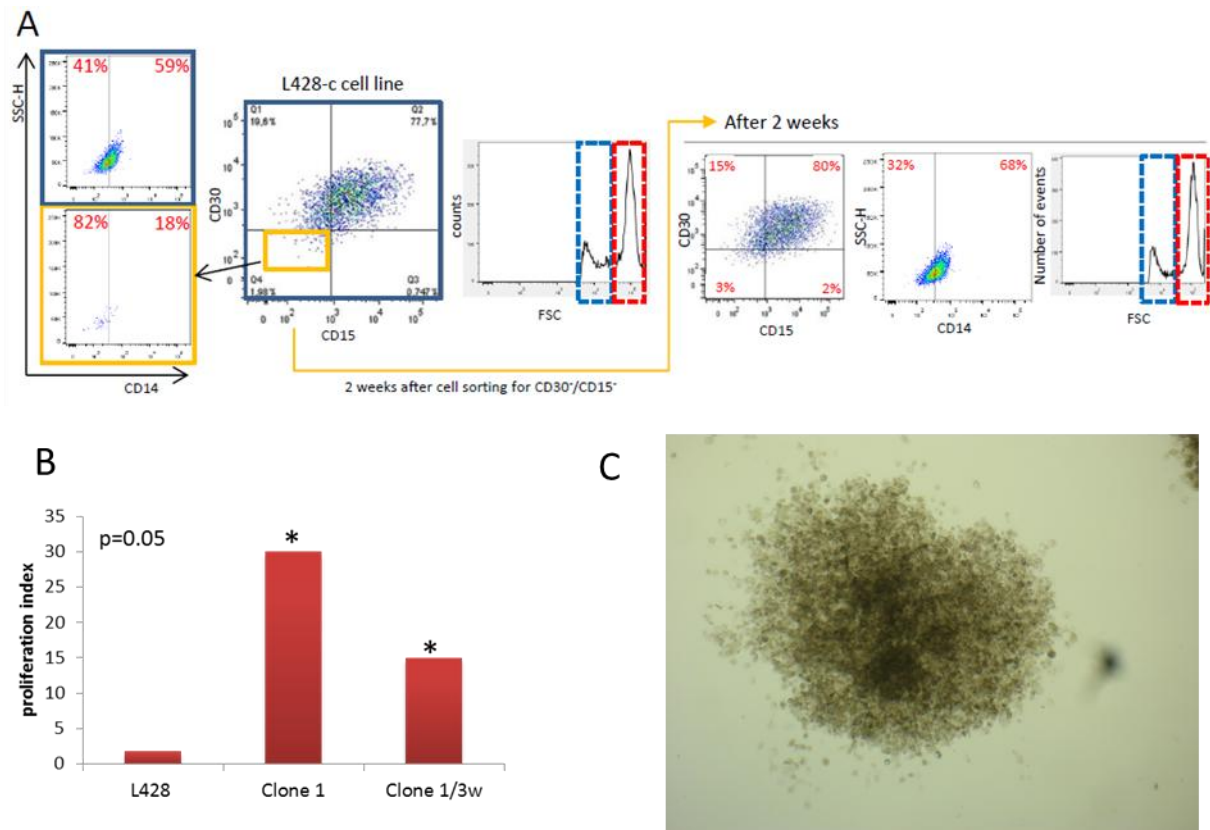


Figure 1 : Clonogenic growth of CD30⁺ cells derived from of L428 cell line. (A) the ability of CD30⁺ cells to recover the parental cell line two week after cell sorting. The phenotype of the new cell line was similar to that observed in parental cell lines excepted in regards to CD30-(B) clonogenic expansion of CD30⁺ cells during serial expansion.(C) Representative picture of a L428 clone.

2- The ability of clone enriched in CD30⁺ cells to be engrafted in NSG mice

The clone obtained after 2 weeks from CD30⁺ cell sorting was injected intravenously into NOD/SCID mice. These cells were engrafted in 80% of animals, as shown by the presence of human CD30 and CD15 (Figure 2). Interestingly, the analysis of cells recovered from liver tumor of 2 mice demonstrated the presence of CD30 and CD15 antigens, as well as negative cells (2% in the 1st mice and 5% in the 2nd mice vs 1% in injected cells).

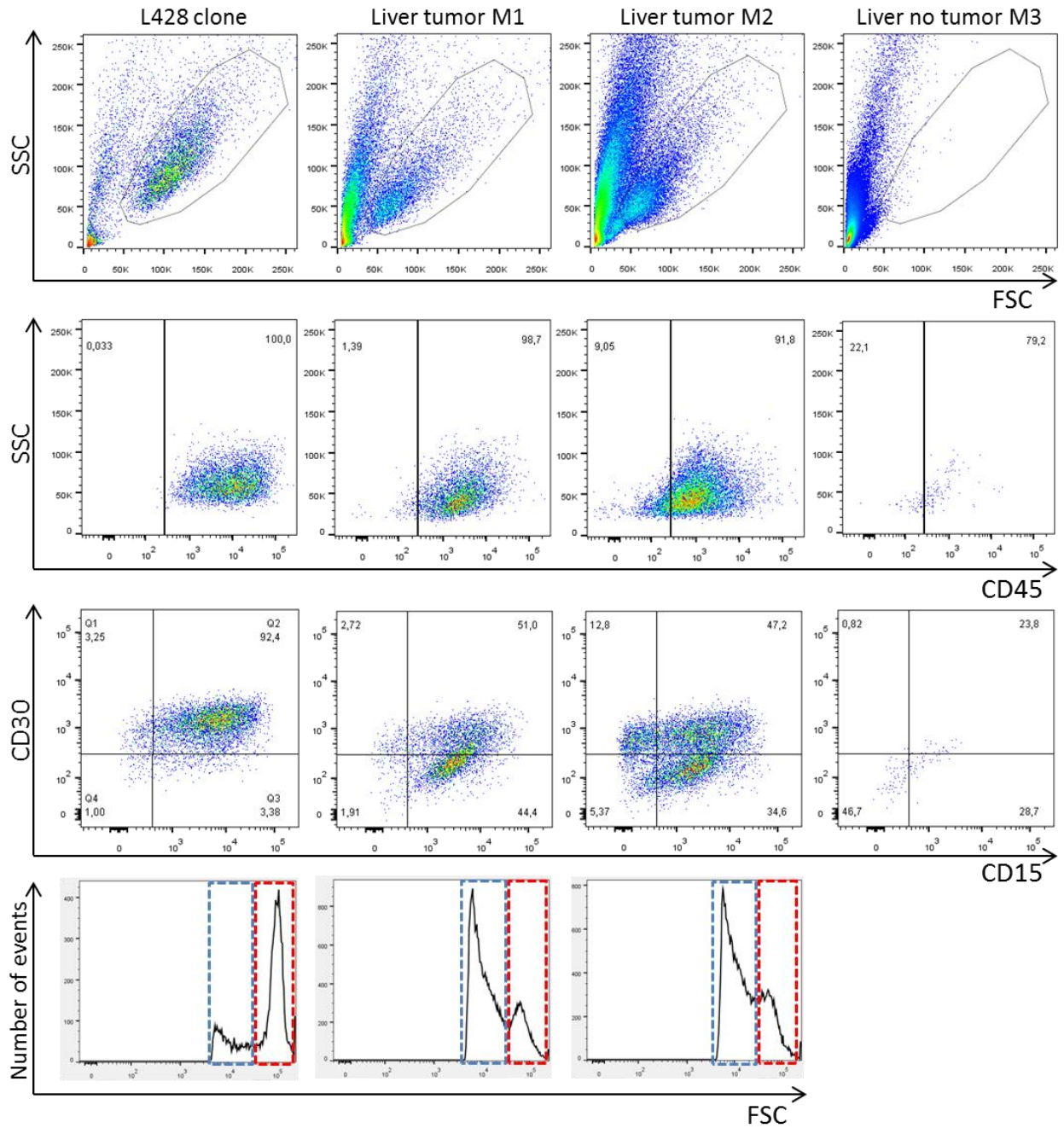


Figure 2 : Flow cytometry analysis of cells recovered from liver tumors of 2 mice injected with 10^3 L428-c cells and a third which did not develop a tumor analyzed 16 weeks after injection. The frequency of each sub-population regarding with respect to the CD30 and CD15 markers are represented. The FSC demonstrates the presence of two populations both for the injected cells and for the liver tumor cells which are found at different frequencies. Liver tumor cells for mouse 2 demonstrate a higher frequency of negative cells (CD30 and CD15) (5% vs 1% for L428-c and 2% for mouse 1).

3- Cytogenetic characterization of HL clonogenic cells

Telomere dysfunction in HL clonogenic cells:

The first step in the characterization of telomere status consisted of quantifying the mean telomere length using the Q-FISH technique considered as the method of choice for high resolution telomere length quantification. This technique makes it possible not only to quantify the mean telomere length at the end of chromosomes but it can also be used to detect the loss of one or two telomeres as well as telomere doublet formation. Figure 3 shows the mean fluorescence intensity of telomeres for the different cells obtained from methylcellulose and derived from mice. Telomere length of HL derived from methyl cellulose or mice were significantly higher than measured in L428 cell line.

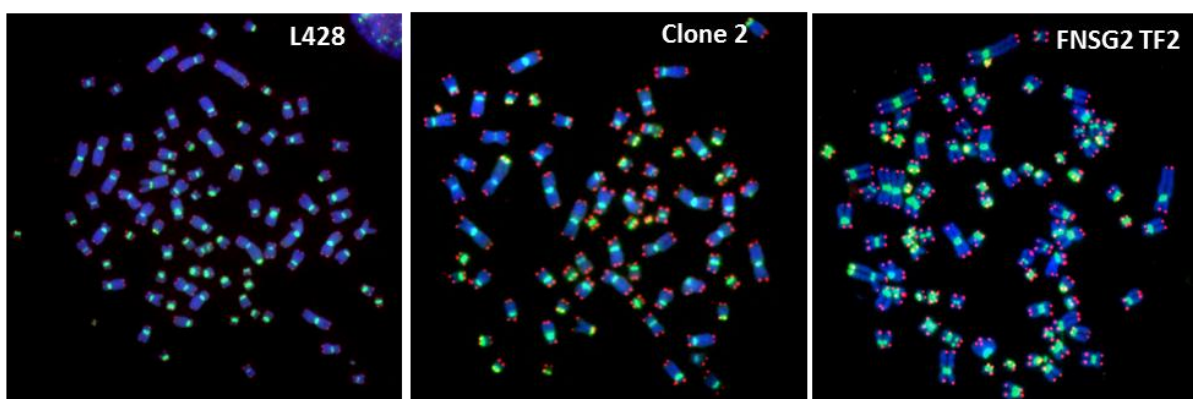
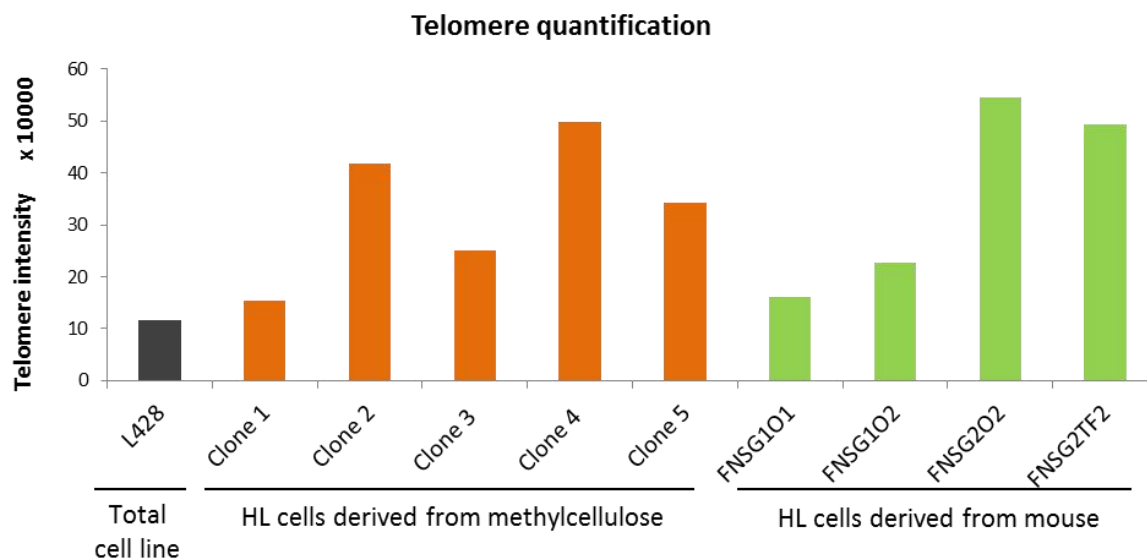


Figure 3: Quantification of telomere length of sorting cell from methylcellulose and cells derived from mice as well as illustration of metaphases derived from each cell line.

The second step of this telomere characterization was the scoring of telomere aberrations consisting in the loss of one or two telomeres or the formation of telomere doublets (Figure 4). Figure 4A shows the significantly higher incidence of

telomere loss in cells derived from methylcellulose and mice compared to that observed in parental cell line. Interestingly, the rate of telomere loss in small cells was much higher than expected for big cells. Nonetheless, cell derived from mice exhibited drastic higher rate of telomere loss.

There was also an increase in telomere doublet formation (Figure 4B) cells derived from methylcellulose and mice cell lines and less in parental cell line. Similarly, telomere deletions rate was high in cells derived from methylcellulose and mice than parental cell line (Figure 4C). Small cell exhibited a high telomere deletion than big cells.

All this data lead to telomere dysfunction in cells derived from methylcellulose and mice than parental cell line. The first cytogenetic characteristic of clonogenic HL cells is the presence of telomere dysfunction without telomere shortening.

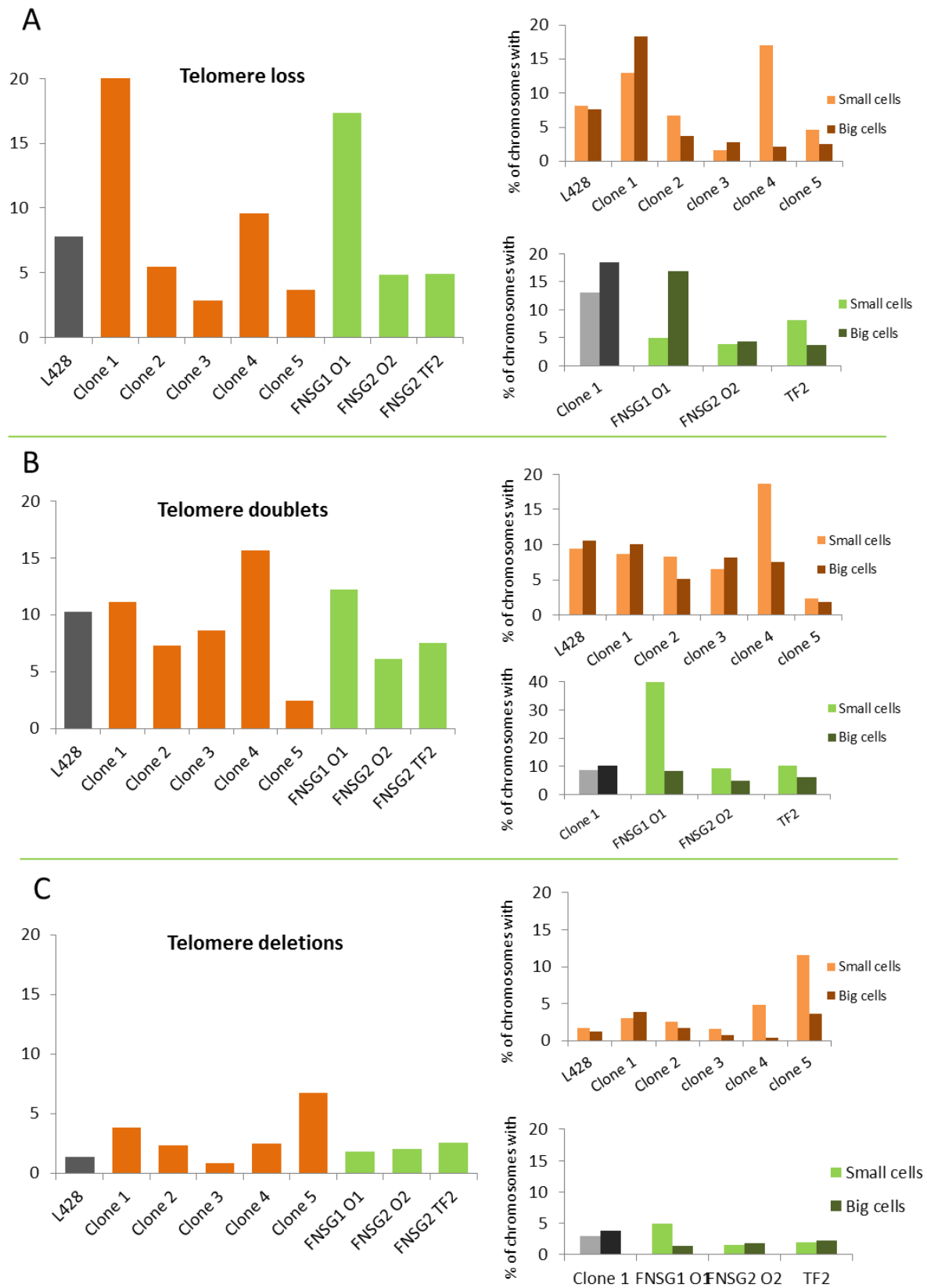


Figure 4 : Telomere aberrations in HL clonogenic cells compared to parental cell line L428.

High incidence of unstable chromosomal aberrations in HL clonogenic cells

Using telomere and centromere staining, dicentric chromosomes and acentric chromosomes were scored in parental cell line and in the cells derived from the clone and from mice. Figure 5 shows the frequency of unstable chromosomal aberrations scored in different derived cell lines. The cell lines derived from methylcellulose and mice showed the presence of higher frequency of dicentric chromosomes. Of note, the dicentric chromosomes observed in these cells were not associated with the presence of acentric chromosomes.

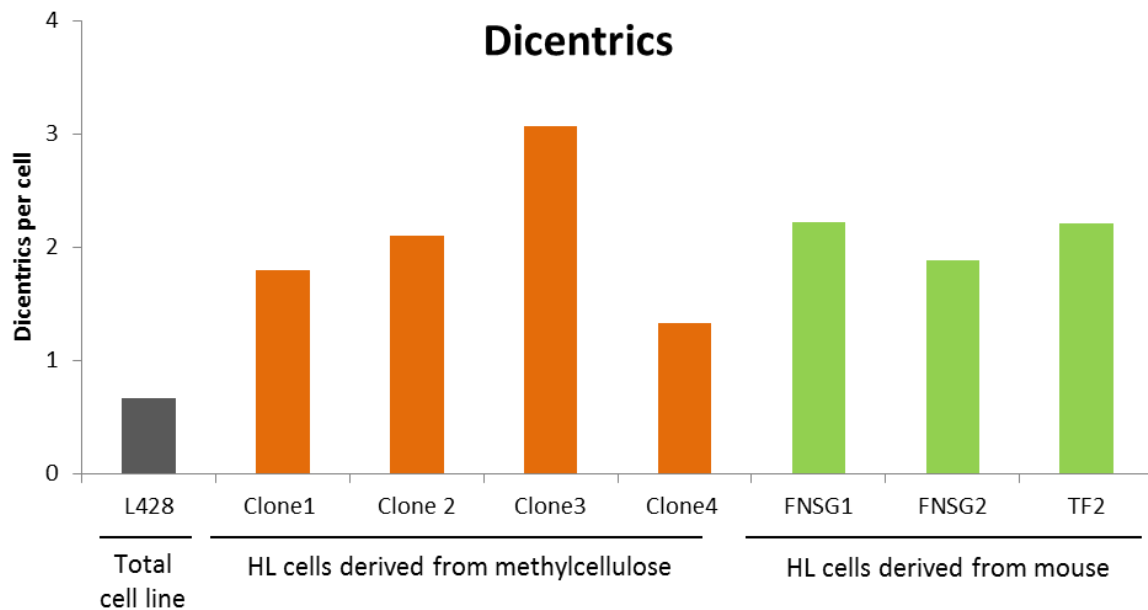


Figure 5 : High frequency of dicentric chromosomes in clonogenic HL cells compared to parental cell line.

Karyotype of HL clonogenic cells

The M-FISH technique was performed following telomere and centromere staining on the same slide stained in order to reliably establish the karyotype for the parental L428 cell line as well as derived cells from methylcellulose and mice. Karyotypes were determined in 30 metaphases. Table 1 summarized numerical and structural chromosomal aberrations. Figure 6 shows the frequency of numerical aberration in L428 cell line, cells derived from methylcellulose and cells derived from mice. Parental L428 cell line presented a higher rate of complex chromosomal rearrangements. The tree of L428 cell line, the tree of cells derived from methylcellulose and the tree of cells derived from mice demonstrate the same origin of these cells (Figure 7).

Table 1 : Cytogenetic aberrations detected using the M-FISH technique on L428, L428 Clone 1 and on different cell lines derived from mice.

Cell line	Numerical Aberrations		Structural Chromosome Aberrations				
			Clonal			Spontaneous	
	mean no of chromosomes (min-max)	loss and gain of whole chromosomes	Common translocations	deletions and amplifications	additional translocations	translocations	dicentrics
L428	94,25 (71-103)	gain chromosomes: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17, 18, 19, 20, 21, 22, x, loss chromosome: 13	t(2;8), (5;5), t(6;5), t(9;11;21), t(13;5), t(9;14), t(14;9), t(18;7), t(12;7), t(17;2), t(1;x), t(7;15), t(21;x), dic(3;15)	del(6p), iso(6p), del(12p), iso(12p), del(17p)	t(16;9;16;9;1), t(14;17), t(8;18)	t(10;8), t(8;x), t(20;12), t(10;x), t(17;4), t(19;14), t(18;16), t(21;19), t(9;5), t(5;9;6), t(5;14), t(6;9), t(16;9), t(3;x), t(11;19), t(16;22), t(17;x), t(6;14), t(3;3), t(18;2), t(9;2), t(9;4), t[3(14;9)], t(8;2;5), t(3;19), t(9;6;14;4)	dic(10;11)
L428-c*	47,12 (19-93)				t(16;9;16;9;1), t(14;17), t(10;2),	t(12;x), t(22;10), t(11;19), t(9;18), t(11;17), t(10;22), t(12;12), t(16;17)	
L428-c**	87,6 (74-92)				t(16;9;16;9;1), t(14;17), t(12;6), t(16;6), t(11;x), t(20;x), t(x;13), t(16;22)	t(1;16), t(11;1), t(12;x), (x;13), t(16;22), t(5;6;7;12), t(22;12), del(12q)	dic(19;17), dic(2;22)
Mouse 1	72,5 (35-83)				t(5;1;5;x), t(11;x), t[2;t(14;9)], dic(11;19), t(16;6), t(6;2), t(6;8;21)	frag 3, t(1;8)	
Mouse 2	83,6 (36-92)				t(16;9;16;9;1), t(14;17), t(6;8), t(10;6), t(15;14), t(x;20), dic(11;19)	t(7;19), t(9;6;12), t(9;6), t(11;4), t(10;6), frag 7, t(7;12), frag x, t(11;x), t(16;4)	dic(11;11;13), dic(19;18), dic(12;17;8), dic(8;11)
Mouse 3	71,36 (45-88)				t(5;1;5;x), t(8;17), t(11;x), t(14;17), t[2;t(14;9)], dic(11;19), t(12;16), t(16;6), t(x;20), frag 2	frag 3, t(17;4), t(3;11;21), t(2;17;2;8), pulverised chrom 6, frag x, t([2;t(14;9)]), frag 16, frag 13, frag 15, frag 20, frag 21, frag 7	
Mouse 4	84,26 (55-96)				t(5;1;5;x), t(11;x), t(14;17), t[2(14;9)], dic(11;19), t(16;6), t(x;20), del(3p)	t(16;4), t(4;11), frag 6, frag 2, t(3;11); t(6;19), t(17;4),	dic(2;19), dic[2;t(5;1;5;x)]
Mouse 5	88 (68-96)				t(5;1;5;x), t(11;x), t[2;t(14;9)], dic(11;19), t(16;6), t(x;20)	t(3;10), t(12;x;19), frag 20	dic(22;22), dic(19;8), dic(3;21;7), dic(4;11), dic[t(5;1;5;x);20], dic(6;6), dic(8;8), dic(19;19), dic(8;2;10)
Mouse 6	91,7 (84-97)				t(16;9;16;9;1), t(14;17), t(2;12), t(16;8), tric(14;15;3;15;14)	t[2;(14;9)], t(5;x), del(xq), ring 4, frag 12, frag 15, frag 16, frag 2, frag 18, t[6;t(9;14)], t(7;3)	dic(6;6), dic(11;11), dic[t(9;14);3]

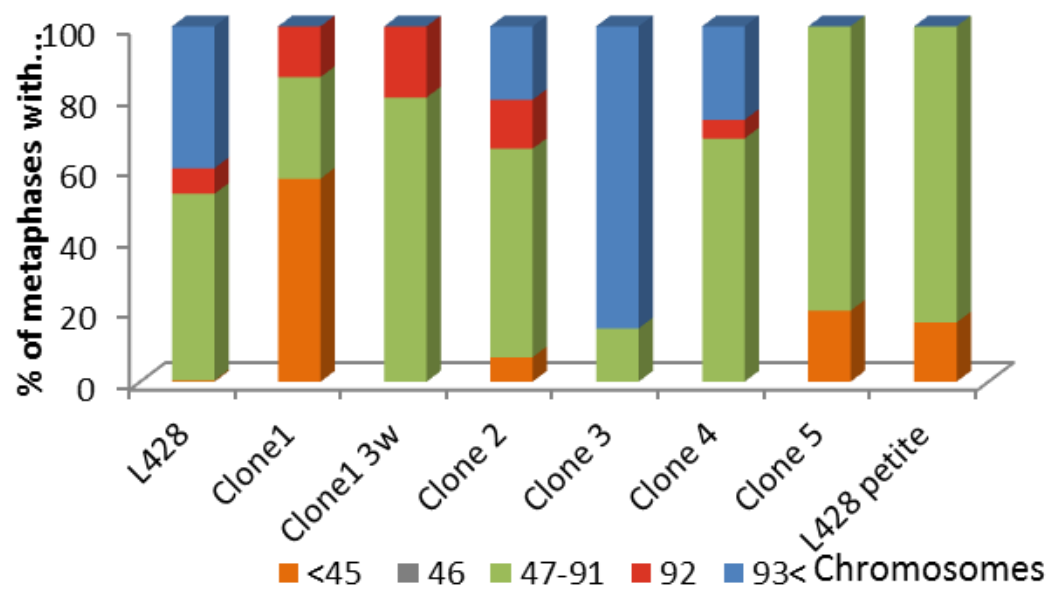


Figure 6: Aneuploidy of clonogenic HL cells demonstrated the diploidy origin of clonogenic HL cells.

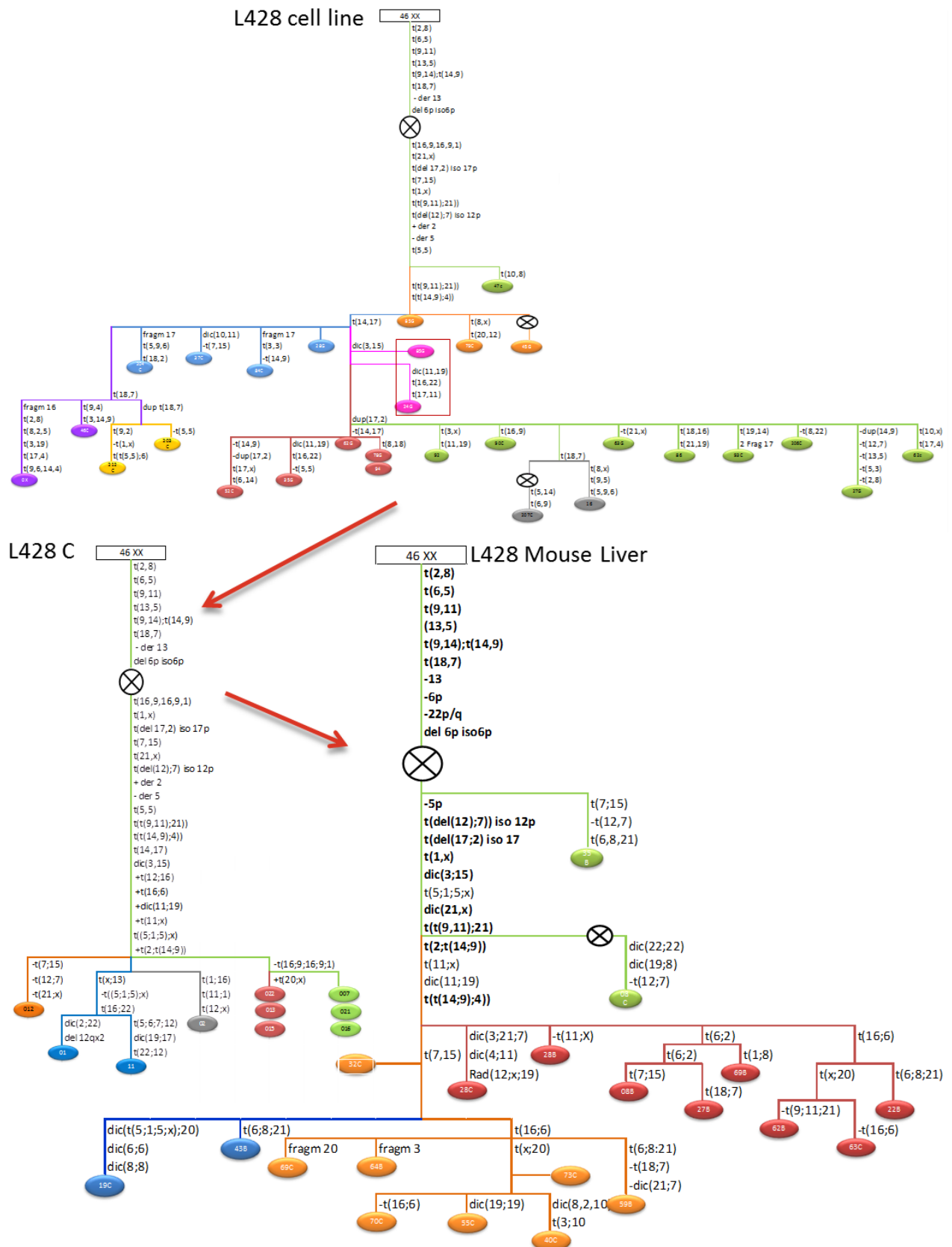


Figure 7: Trees of L428 cell line, cell derived from methylecellulose and cell derived from mice.

Telomere maintenance of HL clonogenic cells

Telomerase activity using TRAP assay was performed on L428 cell line and on cells derived from methylcellulose. Higher telomerase activity was observed in the cells derived from methylcellulose than that observed in L428 cell lines (figure 8A). We confirmed this data using immunofluorescence of hTERT protein associated to PML protein (Figure 8B). On another hand, the telomerase expression in cell derived from mice was assessed using immunofluorescence assay.

Figure 8 B demonstrate the higher telomerase expression in cell CD30- immediately after cell sorting. After 2 weeks of culture, the telomerase expression was lower than observed previously. Interestingly, high telomerase expression was showed in human small cells derived from mice (Figure 8C). In this part, we demonstrate clearly that clonogenic HL cells was characterized by a higher telomerase activity. Whereas, HRS cells exhibited high PML protein expression.

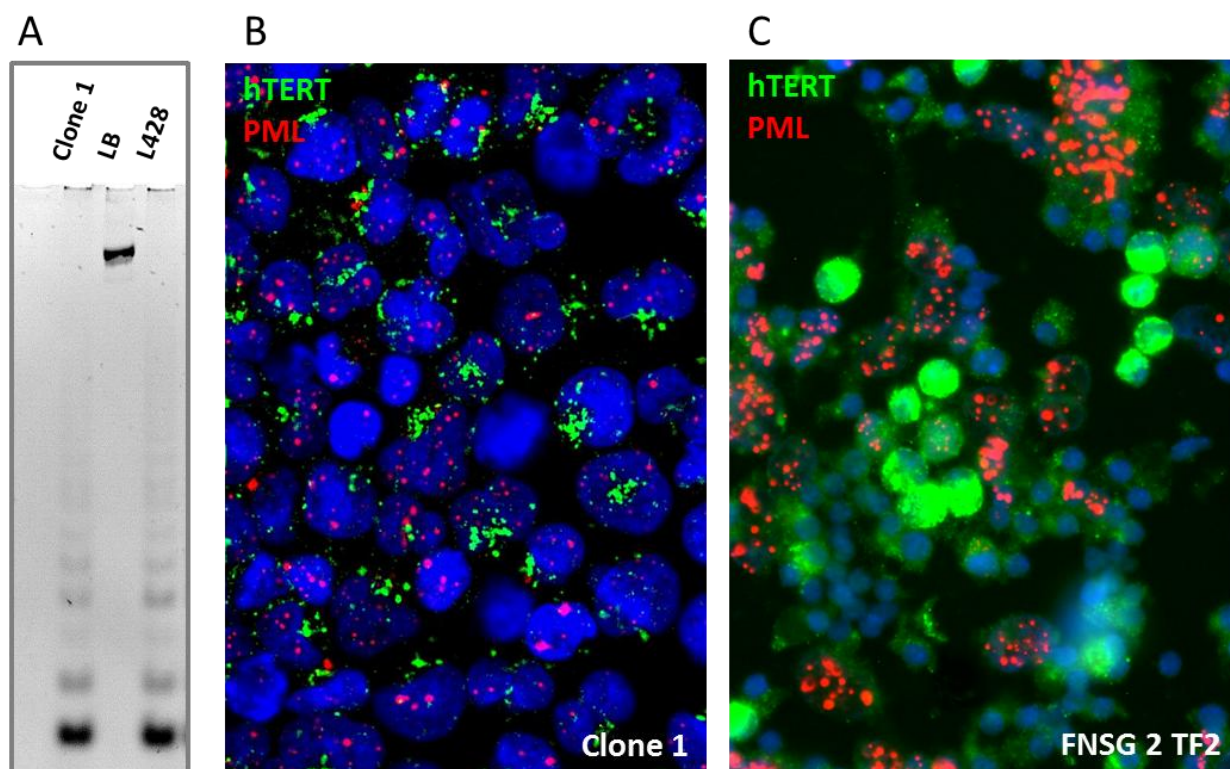


Figure 8: Telomerase expression in clonogenic HL cells, (A) high telomerase activity detected in cells derived from methylcellulose compared to parental cell line (B) immunofluorescence assay with hTERT and PML protein demonstrate higher telomerase expression in CD30- cell immediately after cell sorting (C) the presence of small cells with higher telomerase expression.

4- Clonogenic HL progenitors from clinical samples express telomerase activity

To further define the phenotype of clonogenic HL cells from primary patient samples, we analyzed telomerase activity in circulating lymphocytes of 50 HL patients. High telomerase activity was detected in 10 patients. In healthy donors the telomerase activity was undetectable. Telomere shortening was previously described in these patients (M'kacher 2007) (data not shown).

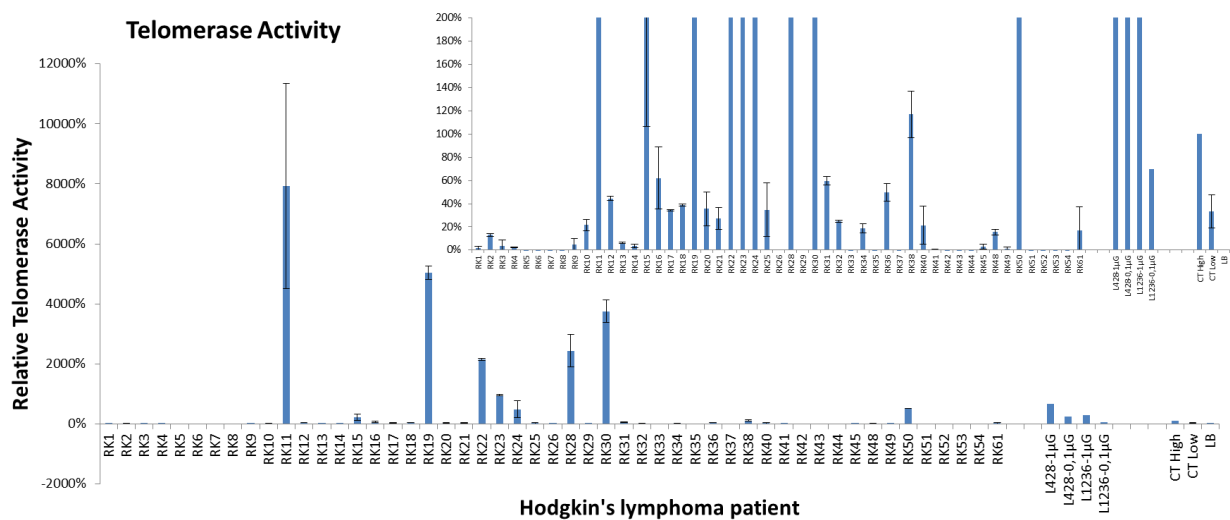


Figure 9: High telomerase activity was detected in circulating lymphocytes of 10 HL patients. L428 and L1236 were used as control cells.

Discussion

Many cancers have now been shown to harbor cells that are phenotypically and biologically similar to normal cells with self-renewal capacity; these so-called cancer stem cells (CSCs) typically constitute only a small fraction of the total tumor (Matsui et al 2004), but are hypothesized to be responsible for relapse after conventional-dose therapy (Ghiaur et al 2012). The CSC concept may also apply to lymphomas (Brennan et al 2010). In HL, The identity of the clonogenic cell of origin has remained controversial (Jones et al 2009, Kuppers 2009). Our data suggest that HL is another example in which cancer stem cells are a rare cell population that is distinct from the differentiated cells that comprise the bulk of the disease. We demonstrated that highly clonogenic HL cells lack CD30 characterized by high clonogenic capacity to generate the parental cell line and able to growth in NSG mice after in vitro amplification.

Telomere dysfunction appears to be a major aberration in these cells associated to high telomerase activity (Masutomi et al 2003). The formation of unstable chromosomal aberration in these cells was a first step in ongoing genomic instability in HL. In circulating HL patient, higher telomerase activity was found and associated to the presence of rogue cells and telomere shortening (data not shown) (M'Kacher et al 2007).

This study is a first step in the characterization of clonogenic HL cell, it will be important to use CGH-array technique high resolution to characterize these cells present in HL cell line and circulating lymphocytes. The animal model established previously can play an important role in this characterization.

In the context of L428 cell line with high telomerase activity, we demonstrate the major role of telomere in the initiation of genomic instability in HL patients.

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General Conclusion

Hodgkin lymphoma (HL) can be considered to be a successful paradigm of modern treatment strategies. The study of HL, with its unique microenvironment and long clinical outcome, has provided exceptional insights into several areas of tumor biology. Findings in HL have not only improved our understanding of human carcinogenesis, but have also pioneered its translation into the clinic. Nonetheless, about 15-20% of patients with advanced stage HL still die following relapse or progressive disease and a similar proportion of patients are over-treated, leading to treatment-related late sequelae including solid tumors and organ dysfunction. The search for new therapies that are specific for tumor cells has focused on differences in gene expression between normal and tumor cells in HL. However, the neoplastic tissue comprises of various inflammation cells with only about 1% of the cellular infiltrate being the neoplastic HRS cells in HL.

The presence of HRS stem cells in peripheral blood lymphocytes and lymph nodes of HL patients is still debated. Nevertheless, the presence of side population cells in several HL cell lines argues against a general role of these cells in the maintenance of HRS cell clones. Using HL cell lines, the aim of this thesis was to understand the mechanisms underlying genomic instability in HL.

In the first part of this thesis, we reviewed the pathogenesis of HL starting with the cellular origin of neoplastic cell, signaling pathways and cytogenetic investigation supporting its pathogenesis, especially focusing on the possible role of telomere dysfunction. We then concluded that the initial event that causes this extensive reprogramming is unknown as well as the mechanisms underlying genomic instability. However, several contributing factors have been identified.

In the second part of the thesis, we focused on the mechanisms leading to genomic instability in HL cell lines. We were able to demonstrate for the first time the possible role of microsatellite instability in genomic instability of HL cell lines. The second main finding in this part, was the existence of two different mechanisms in HL with respect to chromosomal instability reflecting the clinical differences observed in terms of remission and survival of NS and MC HL. In NS HL cell lines, telomere dysfunction could be a major event in the initiation and the progression of chromosomal

instability, essentially in small cells compared to HRS cells. On the other hand, in MC HL cell lines, the defect in NHEJ of DSB repair appeared to be the mechanism leading to chromosomal instability. In addition, we provide evidence of a genomic mechanism of centromere inactivation that occurs in some dicentric chromosomes.

The third part of this thesis consists in the characterization of cells growing in methylcellulose medium, and tumors established after the passage in NSG mice of L428-c cell line. Interestingly, we have confirmed the diploid origin of the cells growing in the methylcellulose and in mice. Cell sorting of side population in HL cell lines (negative cells of all HL markers) validated this hypothesis and demonstrated their clonogenic potential to recover into the parental cell line. The frequency of numerical and structural chromosomal aberrations in infiltrated tumor cells in NSG mouse liver is much less than observed in the parental L428 cell line. Nevertheless, we observed commonly shared numerical and structural aberrations. Higher frequency of dicentric chromosomes was observed in the established cell line coming from mouse compared to the initially injected cells, reflecting the ongoing chromosomal instability of these cell lines. The difference in aneuploidy status of these cell lines derived from NSG mice compared to that of the injected cells and parental cell line respectively, favors the argument that the tumor cells that grew in the NSG mice are derived from diploid precursors of malignant cells.

The tumors established in NSG mice and their re-cultured cell lines have led us to a number of interesting observations. This work is a first step in the characterization of “clonogenic” cells or “cancer stem” cells in HL. Despite that the telomere length of these cells was significantly higher than observed in HRS cells, the frequency of telomere aberrations were significantly higher than observed in HRS cells.

The fourth part of this thesis was dedicated to the telomere maintenance mechanisms in HL. In this part, the TMMs investigation was not restricted to cell lines but also to lymph nodes of HL patients. We have demonstrated the presence of both mechanisms of TMMs in HL lymph nodes as well as HL cell lines: telomerase activity and alternative lengthening of telomeres (ALT). In cell lines, the presence of both cell types: those using telomerase and those using ALT, highlights a novel type of tumor heterogeneity and suggests that telomere maintenance may not be a static characteristic of all tumors. The analysis of TMMs in different sub-populations of HL cell lines after Fluorescence-activated cell sorting demonstrated the presence of higher hTERT expression compared to ALT profile in CD30 and CD15 negative cells. The phenotype transition of these cells was associated to the suppression of hTERT

expression and the appearance of an ALT profile. These findings were also observed in HL lymph nodes. Interestingly, cells with an ALT profile showed a lower *in vitro* radiation sensitivity and higher expression of PML/TRF2. In lymph nodes, these characteristics were correlated to the worse prognosis of HL patient. The inhibition of the transition from telomerase tumor cells to ALT cells could be associated to conventional treatment of HL.

The originality of this work consists in the use of several approaches and techniques. It will open new horizons in the understanding of the mechanisms underlying genomic instability in HL, especially in the precursor role of “clonogenic” or “cancer stem” cells in the progression of the disease. This work allowed us to demonstrate that HRS is only the resultant or the final stage of another cell which is responsible for the cellular proliferation. The use of an animal model and an appropriate technique like CGH-array, could clarify the cytogenetic profile of the growing cells in HL cell lines and identify the primary event in the initiation of the disease.

In 5 out of 7 HL cell lines, we have demonstrated the major role of telomere dysfunction in genomic instability of tumor cells. It would be important to evaluate the genetic control of telomere dysfunction in HL patients. The family survey could be used to investigate the pattern of interfamilial correlation of telomere dysfunction and identification of new pathway in telomere length and maintenance in HL patients. It would also be important to test the inhibitor of ATR in HL cell line as well as in HL animal model.

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Annexes

During my PhD thesis I participated in the writing of an article published in the Enciclopedia of Pathology. I have also participated in the work of three important studies. The first study is describing the development of a new tool for biological dosimetry, the second one places telomere shortening as a prognostic factor for the development of cardiac disease after irradiation, and the third one studies the effect of lead exposure on telomeres of human cells.

Annex 1

Telomere

Metadata of the chapter that will be visualized online

Chapter Title	Telomere	
Copyright Year	2014	
Copyright Holder	Springer-Verlag Berlin Heidelberg	
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2 Telomere

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8 Synonyms

9 Chromosomal extremity; Chromosomal termi-
10 nus; End of chromosome; Shelterin complex;
11 Telomeric DNA

12 Definition

13 Telomeres are specialized nucleoprotein struc-
14 tures located at the ends of linear eukaryotic
15 chromosomes. The name "telomere" was derived
16 from the Greek roots *telos* (τέλος), meaning
17 "end," and *meros* (μέρος, root: μερ-), meaning
18 "part." Telomeres were first described more than
19 70 years ago by the pioneering studies of the
20 geneticists Hermann Joseph Muller and Barbara
21 McClintock in the fruit fly *Drosophila*
22 *melanogaster* and maize, respectively. Since
23 then, telomeres have proven to be critical struc-
24 tures for maintaining genetic stability and integ-
25 rity and have thus been established as guardians
26 of the genome (Fig. 1).

Principle

Telomere Structure and Function

27
28 The structure of telomeres in all vertebrates con-
29 sists of tandem repeats of (TTAGGG/AATCCC)_n
30 DNA sequences, first identified by Moyzis in 1988,
31 and several associated proteins. Together, they
32 form a protective cap called the *shelterin complex*
33 (Fig. 2). The complex is found as a looped structure
34 known as the T-loop, which is formed when the
35 3-prime overhang of the G-rich strand of about
36 50–500 nucleotides folds back and invades the
37 double-stranded region of the telomeric DNA. As
38 the single-stranded telomeric DNA is G-rich, this
39 region may also form G-quadruplexes, which are
40 formed from a series of G-quartets, each containing
41 four guanine bases arranged in a helical fashion.
42

The shelterin complex in humans includes six 43 [Au1]
44 proteins that are associated with telomeric DNA:
45 TRF1, TRF2, TIN2, POT1 [POT1a/b in rodents],
46 TPP1, and RAP1 (reviewed in Palm and de Lange 47 [Au2]
2008). Each of these proteins has evolved specific
48 functions for telomere maintenance, including
49 the regulation of telomerase access and activity
50 as well as the interaction with many DNA repair/
51 recombination factors. TRF1 and TRF2 bind
52 independently (both as homodimers or oligo-
53 mers) to the double-stranded regions of telo-
54 meres, while POT1 binds to the 3' single-
55 stranded G-rich overhang. Interactions between
56 TRF1 and TRF2 with POT1 occur via TIN2 and
57 TPP1. TPP1 contains a telomerase binding
58 domain and may be involved in the process of
59 telomere elongation. Finally, RAP1 is not

essential for the formation of the shelterin complex but stabilizes the structure itself by binding to TRF2.

The protective cap formed by the shelterin complex at the telomeres is known to serve at least three essential functions. First, they protect natural chromosomal DNA ends from being inappropriately recognized as double-strand breaks (DSBs) and therefore prevent unwanted activation of DNA damage response (DDR) pathways. Secondly, telomeres protect chromosomal ends from inappropriate enzymatic degradation. Lastly, they prevent chromosomal end-to-end fusions. Therefore, telomeres play a crucial role in maintaining the stability and integrity of the genome.

Methodology

Telomeres and Replicative Senescence

In 1961, Hayflick and Moorhead demonstrated that human diploid cells have a limited lifespan and cells stop dividing after a certain number of replication cycles. Then in 1990, Harley et al. observed that telomere length naturally decreases with each round of cell division and finally affirmed the hypothesis that telomere length in somatic cells reflects the replicative history of a cellular lineage. More recently, the research on telomere features and the line of evidence that led to the concept that telomerase activity exists has been recompensed in 2009 with a Nobel Prize to Elizabeth H. Blackburn, Carol W. Greider, and Jack W. Szostak (see also Blackburn Nobel Lecture).

Telomere length varies between organisms; in humans, the mean length of the double-stranded end at birth is ~15 kilobases (kb) (Wright et al. 1996). Telomere length also varies on individual chromosome arms, and this inherent heterogeneity is conserved during life. Telomere length in somatic proliferative tissues naturally declines at a rate of approximately 20–300 base pairs per population, doubling (varying with cell type) due to the incomplete replication of telomere ends by conventional DNA polymerases, a situation known as the “DNA end-replication

problem.” After many rounds of cell division, telomeres eventually pass their size “threshold” and become critically short or dysfunctional. In normal cells with intact p53 functions and cell cycle checkpoints, these dysfunctional/uncapped telomeres are sensed as DNA damage and trigger DDR pathways, which prevent the cell from further division, and cells enter a stage of permanent growth arrest called “replicative senescence.” Progressive loss of telomeric DNA therefore acts as an internal “molecular clock” that limits the number of cell divisions and therefore limits the lifespan of normal cells. Since senescent and potentially damaged cells are eliminated or do not give rise to viable progeny, this mechanism works as an important tumor suppressor.

Mechanisms of Telomere Maintenance

The persistent shortening of telomeres can be offset in different cell types (i.e., stem cells, germ cells, or cancer cells) via two mechanisms of telomere elongation: (1) telomerase, a specialized reverse transcriptase that elongates telomeres by adding TTAGGG DNA sequence repeats to the 3' G-strand overhang, and (2) homologous recombination of telomeres via “alternative lengthening of telomeres” (ALT), a pathway independent of telomerase activity.

Telomeres are primarily elongated by telomerase. The enzyme is a ribonucleic protein complex composed of two core components: the telomerase reverse transcriptase (TERT) protein, which reversely transcribes the template region of the second component, the RNA subunit (TERC), onto the 3' end of the telomeric DNA. Telomerase is essential for the long-term proliferation potential of stem cells and germ line cells and for tissue renewal. The enzyme is also constantly expressed in normal somatic cells at low concentrations; however, telomerase is unable to bind to and exert its elongating functions at the telomeres due to the protective function of the shelterin complex, thereby resulting in telomere shortening after each round of cell division. In cell culture, telomerase activation increases the ability of cells to divide continuously and therefore extends their lifespan as shown by Bodnar in 1998.

ALT-positive immortalized cell lines are able to maintain their telomere length throughout many cell doublings in the absence of telomerase activity. ALT involves homologous recombination-mediated DNA replication and requires the activity of the MRE11/RAD50/NBS1 recombination complex. The typical ALT phenotype has only been found in abnormal situations, including immortalized human cell lines, human tumors, and tumors or cell lines derived from telomerase null mice, suggesting that this is an anomalous telomere phenotype.

Quality Aspects

Telomere Dysfunction and Chromosomal Instability

Loss of telomeric functions can arise due to loss of telomeric DNA or from mutations at crucial telomere protein components. Depending on the type of mutation, the uncapping process differs and telomeres undergo progressive shortening. Excessive telomere shortening and/or telomere loss enables gene amplification and chromosomal imbalances and may eventually lead to chromosomal instability, characterized by increased levels of chromosomal aberrations that danger the integrity of the genome (Fig. 3).

Telomere loss that is not rapidly repaired causes chromosomal instability due to degradation of DNA from the end of the chromosome. Lost telomeres can be recovered via several mechanisms. First, telomere sequences can be added directly to the open ends of broken chromosomes with the help of telomerase; this process, termed chromosome healing, results in a terminal deletion. Secondly, sister-chromatid fusions after telomere loss, followed by propagation of chromosomal instability via breakage/fusion/bridge (BFB) cycles (McClintock 1941), result in terminal deletions, inverted duplications, DNA amplification, duplications, and nonreciprocal translocations; all of these types of chromosomal rearrangements related to telomere instability have been associated with human cancer. Additionally, anaphase bridges during BFB cycles can also cause losses of whole

chromosomes, leading to aneuploidy (or an abnormal number of chromosomes). Moreover, telomeres can be acquired through telomere capture, involving the translocation of the ends of other chromosomes via break-induced replication (BIR). Finally, chromosomal rearrangements that do not involve telomere restoration may arise, without compensating for telomere loss, such as rings or dicentric chromosomes, which are usually unstable in mammalian cells.

Higher levels of chromosomal instability and structural chromosomal alterations after telomere loss are often associated with functional loss of cell cycle control mechanisms, such as p53 or p16. Dicentric chromosomes that are formed by BFB cycles cause cell cycle arrest in normal cells with intact cell cycle checkpoints; however, cells lacking normal checkpoints continue to divide, and the unstable dicentric chromosomes break during cell division, leading to further genetic instability.

Applications

Telomeres and Carcinogenesis

Telomeres and telomere length are involved in the pathology of several age-related human diseases and carcinogenesis (reviewed in Shim et al. 2014) via (1) loss of cell cycle checkpoints that allow the bypass of senescence and continued cell proliferation that may eventually lead to carcinogenesis or (2) abnormal regulation of telomere length, including abnormal telomerase activity or activation of ALT pathways. Telomerase is upregulated in about 85 % of human cancers, suggesting its important role in the process of cellular immortalization and tumorigenesis. Its downregulation or inhibition would therefore target and impair telomerase-positive malignant cancers, while normal somatic cells, which do not express telomerase, are minimally affected. Clinical trials including telomerase inhibitors in cancer treatments already demonstrated that the use of these inhibitors hindered tumor growth and spread of metastases. Additionally, inhibition of telomerase may sensitize tumors to conventional

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chemotherapy agents. Therefore, telomerase inhibition may be most effective when used in combination with conventional chemotherapy and/or radiotherapy regimens, since telomerase inhibitors may require a period of treatment before chemotherapy or radiotherapy to produce telomeres that are short enough to trigger death in cancer cells.

On the other hand, telomerase deficiency in stem cells can lead to untimely telomere shortening; a variety of degenerative disorders, including dyskeratosis congenita, pulmonary fibrosis, and bone marrow failure, are all characterized by the presence of short telomeres (reviewed in Armanios et al. 2012).

Additionally, a significant subset of human tumors (10–15 %) employs the telomerase-independent ALT pathway. Whether the cancer cell uses telomerase activation or the ALT pathway appears to depend on the origin of the tumor. This pathway can coexist with telomerase activity in some tumors, rendering these tumors resistant to treatment with telomerase inhibitors (Reddel et al. 2001).

Telomerase Activity in Tissues

The appearance of various cancers (colon, skin, thyroid, breast, etc.) is accompanied by changes in the telomere status including either an increase or decrease of telomere length. There is evidence that the quantification of telomerase activity – playing a major role in the telomere maintenance – allows the characterization and analysis of cancerous tissue. To perform these analyses, the amount of human TERT (hTERT) is quantified, assuming that the level of expression is correlated with its in vitro activity (Hao et al. 2005).

Telomerase expression is studied, i.e., by immunohistochemical staining of tissue sections (Volpi et al. 2005) or real-time polymerase chain reaction (RT-PCR) and PCR-ELISA mostly performed in liquid nitrogen-frozen tissues (Ayiomamitis et al. 2014; Capezzone et al. 2011). With the latter two protocols, overall hTERT expression is analyzed, while individual cells with telomerase activity can be identified and distinguished from normal cells only in tissue

sections by colorimetric differences. Quantification of telomerase activity could help to classify histologic grades of carcinomas. This represents an important feature to evaluate the cancer status and therewith the need and type of follow-up. The expression level of hTERT can be used as a new marker for an early diagnosis for certain types of cancer (Bautista et al. 2007).

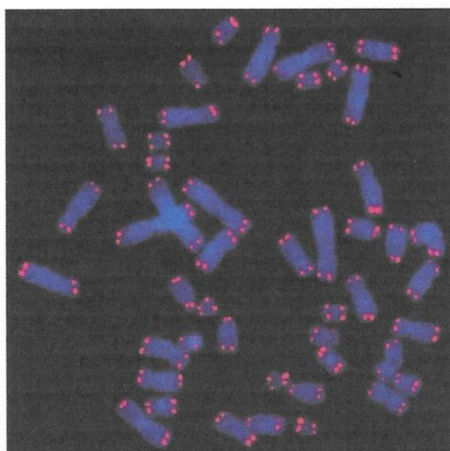
Studies of telomerase activity for a spectrum of colorectal tumors (hyperplastic polyps, adenomas, and carcinomas) – characterized in the majority of publications by short telomeres – show an increase of hTERT expression compared to adjacent healthy tissue, and stronger staining correlated proportionally with the histological grade (Simsek et al. 2010).

A clinical approach for the early detection of invasive bladder cancer was described by Soria et al. (2002). Epithelial cells have been isolated from the blood by magnetic beads coated with highly specific antibodies. Telomerase expression was detected using the telomerase-PCR-enzyme-linked immunosorbent assay test for the majority (90 %) of patients with high-grade, muscle-invasive, or metastatic bladder cancer but in none of the healthy donors. This diagnostic protocol may have great value for monitoring cancer progression and might be adjusted with regard to the type of cancer.

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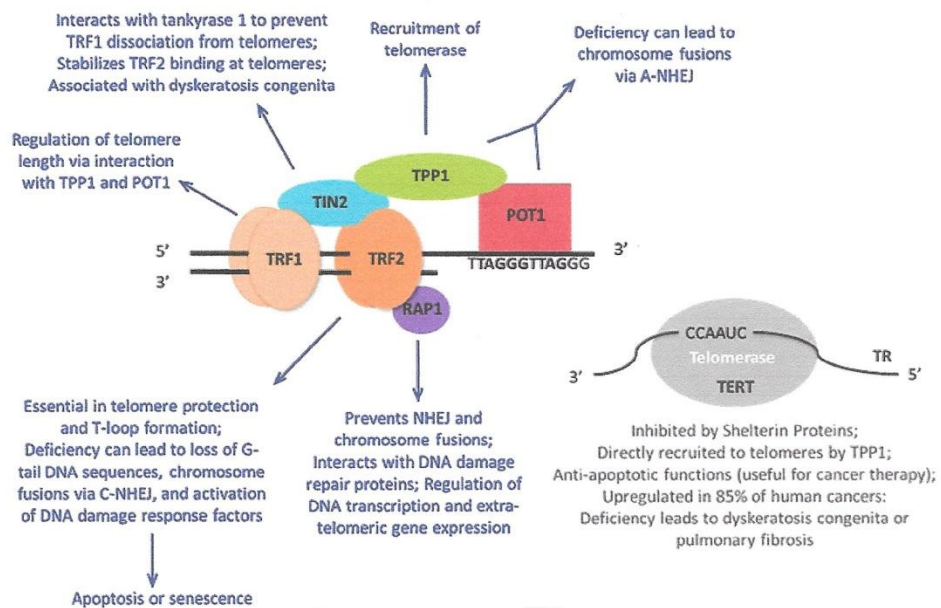
- 336 Soria, J. C., Morat, L., Durdux, C., Housset, M., Cortez, Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., 341
337 A., Blaise, R., & Sabatier, L. (2002). The molecular & Shay, J. W. (1996). Telomerase activity in human 342
338 detection of circulating tumor cells in bladder cancer germline and embryonic tissues and cells. *Develop-* 343
339 using telomerase activity. *The Journal of Urology*, mental Genetics, 18, 173–179. 344
340 167(1), 352–356.



Telomere, Fig. 1 Visualization of telomeres in metaphasic chromosomes using PNA (peptide nucleic acid)-telomere-FISH (fluorescence in situ hybridization) staining. Telomeric regions are marked quantitatively with PNA probes corresponding to the telomeric sequence that is labeled with the fluorescent dye cyanine-3 (550 nm excitation, 570 nm emission)

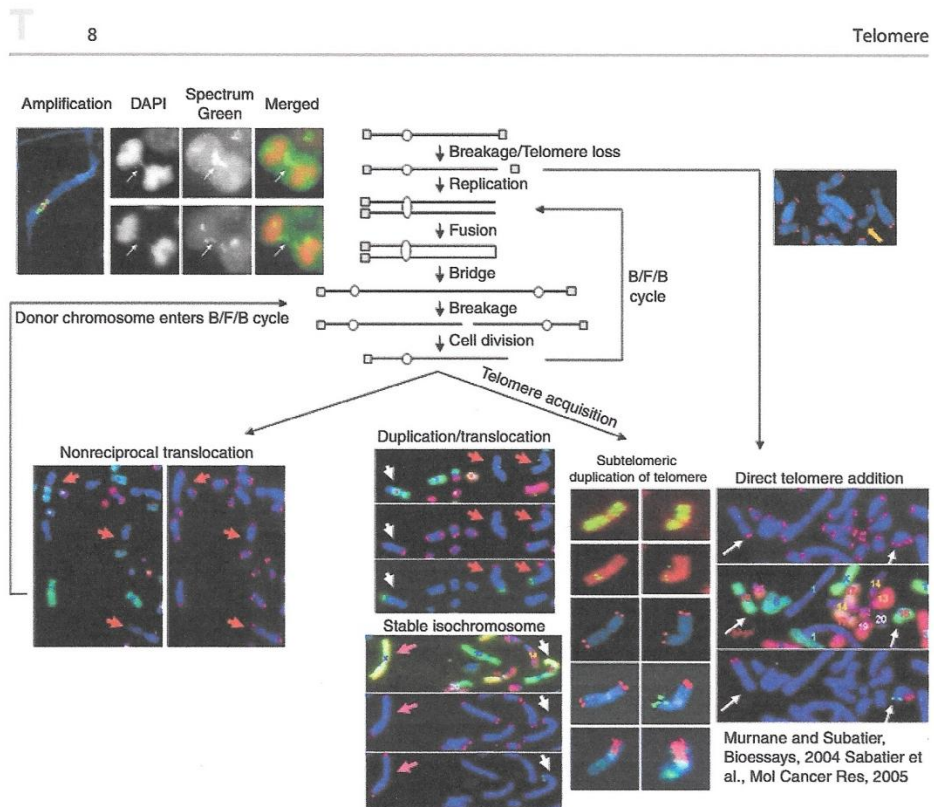
Telomere

7



Telomere, Fig. 2 Composition of the shelterin complex: TRF1 and TRF2 are associated with the double-stranded region of telomeric DNA, while POT1 is associated with

the single-stranded region and interacts with TRF1 and TRF2 with the help of TIN2 and TPP1. RAP1 increases the stability of the shelterin complex



Telomere, Fig. 3 The B/F/B cycle is initiated when sister chromatids fuse after the loss of a telomere (gray squares). Due to the presence of two centromeres (circles), the fused sister chromatids form anaphase bridges that break when the cell attempts to divide. Breakage occurs at locations other than the site of fusion, resulting in amplification of sequences in one daughter cell and deletions in the other. Lost telomeres can be restored by sister-chromatid fusion following DNA replication or

different mechanisms of telomere acquisition: duplication/translocation, formation of stable isochromosomes, subtelomeric duplication, or direct telomere addition by the telomerase. The translocations can be also nonreciprocal, leaving one uncapped, damaged chromosome. The loss of the telomere from the donating chromosome can initiate new B/F/B cycles. Therefore, a loss of a single telomere can lead to cascades of instability involving multiple chromosomes

Annex 2

**New tool for biological dosimetry : Reevaluation and automation
of the gold standard method following telomere and centromere
staining**



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New tool for biological dosimetry: Reevaluation and automation of the gold standard method following telomere and centromere staining[☆]



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ARTICLE INFO

Article history:

Received 7 July 2014

Received in revised form 5 September 2014

Accepted 16 September 2014

Available online 28 September 2014

Keywords:

Gold standard technique

Dicentric

Biological dosimetry

Telomere

Centromere staining

ABSTRACT

Purpose: The dicentric chromosome (dicentric) assay is the international gold-standard method for biological dosimetry and classification of genotoxic agents. The introduction of telomere and centromere (TC) staining offers the potential to render dicentric scoring more efficient and robust. In this study, we improved the detection of dicentrics and all unstable chromosomal aberrations (CA) leading to a significant reevaluation of the dose–effect curve and developed an automated approach following TC staining.

Material and methods: Blood samples from 16 healthy donors were exposed to ¹³⁷Cs at 8 doses from 0.1 to 6 Gy. CA were manually and automatically scored following uniform (Giemsa) or TC staining. The detection of centromeric regions and telomeric sequences using PNA probes allowed the detection of all unstable CA: dicentrics, centric and acentric rings, and all acentric fragments (with 2, 4 or no telomeres) leading to the precise quantification of estimated double strand breaks (DSB).

Results: Manual scoring following TC staining revealed a significantly higher frequency of dicentrics ($p < 10^{-3}$) (up to 30%) and estimated DSB ($p < 10^{-4}$) compared to uniform staining due to improved detection of dicentrics with centromeres juxtaposed with other centromeres or telomeres. This improvement permitted the development of the software, TCScore, that detected 95% of manually scored dicentrics compared to 50% for the best currently available software (DCScoreTM).

Conclusion: The use of TC staining has permitted a reevaluation of the dose–response curve and the highly efficient automation of the scoring process, marking a new step in the management and follow-up of populations exposed to genotoxic agents including ionizing radiation.

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1. Introduction

The analysis of induced chromosomal aberrations (CA) is a powerful approach for the assessment of the genotoxic effects of exposure to chemicals or ionizing radiation, relating such exposures to the risk of cancer development [1]. Indeed, the potential of ionizing radiation to cause DNA damage is a prime example of genotoxic exposure.

In a radiological or nuclear accident, in particular in the absence of physical dosimetry, the estimation of dose received by the

exposed population using the scoring of CA, must be performed in the days following the exposure in order to optimize the care of exposed individuals, to reassure the exposed populations, and to adapt their treatment as a function of the received radiation dose. Post-Fukushima, the recent paper of Harada et al. [2] highlights the importance of individual radiation dose estimates that will permit health risk assessment. An evaluation of personal external dose remains a challenge and a necessity as individual behavior (and thus, exposure) varies largely amongst individuals. The dicentric chromosome (dicentric) assay remains the most precise, sensitive and widely used method in biological dosimetry and is medically and legally recognized [3]. The lowest detected dose is on average 0.1 Gy. This dose is at the limit of risk assessment as epidemiological studies have failed to observe a statistically significant increase in cancer risk for doses below 100 mSv. The dicentric is considered

[☆] This paper is dedicated to Prof. Maurice Tubiana and Prof. Claude Parmentier.

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<http://dx.doi.org/10.1016/j.mrfmmm.2014.09.007>

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the aberration of choice because its generation is nearly specific to DSB caused by ionizing radiation, and its natural occurrence is low [4]. It has been a major tool in the estimation of the dose received by individuals following nuclear accidents such as Chernobyl in 1986 and Fukushima in 2011 [5–7]. This technique has also shown efficacy in the estimation of the dose received in patients treated with ionizing radiation for whom physical dosimetry is difficult to apply such as the case of patients with differentiated thyroid carcinoma who were treated with ^{131}I (3.7 GBq), and can provide the most accurate quantification of the risk incurred by ^{131}I exposure [8,9]. Similarly, the scoring of unstable CA has been used in the follow-up of populations exposed to ionizing irradiation [10–12].

A major drawback of the dicentric assay is the time consuming scoring of aberrations and the high level of necessary expertise making it difficult to apply this approach to a mass casualty event or the follow-up of a large cohort exposed to genotoxic agents. The automation of dicentric scoring is a prerequisite to increasing analysis capacity. Currently it takes a group of 5 trained specialists 7 days to supply results on 100 samples. There are existing tools for the automation of dicentric scoring following the uniform staining of metaphases [13,14]. However, the existing software detects only half of the dicentrics and rejects many of the metaphases [15,16]. Recently, there has been a joint effort to harmonize the use of this software by an international network [17–19]. However, the difficulty in obtaining sufficient numbers of metaphases from irradiated samples due to the lack of sensitivity of the existing software, limits the capacity to perform sensitive and precise biological dosimetry at both lower and higher doses.

Here, we demonstrate that the introduction of simultaneous staining of telomeres and centromeres (TC) using peptide nucleic acid (PNA) probes not only makes it easier to detect dicentrics and rings [20], but also renders the scoring of radio-induced CA more reliable and robust allowing for rapid and improved detection of all CA; dicentrics, centric and acentric rings and acentric fragments (with 0, 2, or 4 telomeres). These improvements reduce the analysis time while dramatically improving precision compared to conventional techniques, which rely on uniform staining, without significantly increasing the cost. They facilitate the automated scoring of CA as it is aided by TC staining and not based solely on chromosome morphology. We have developed and implemented an automated system for fluorescent image analysis (TCScore) capable of detecting not only 95% of dicentrics and centric rings, but also different acentric fragments following TC staining with the same efficacy as manual scoring, but in a fraction of the time.

2. Materials and methods

2.1. Irradiation procedure and metaphase preparation

Peripheral blood samples were obtained from 16 healthy donors (5 females and 11 males, mean age 37.1 years (20–58 years)) and exposed to gamma irradiation using an IBL637 ^{137}Cs irradiator at 9 different doses (0, 0.1, 0.2, 0.5, 1, 2, 4 and 6 Gy) at a dose rate of 0.61 Gy/min. Blood lymphocytes were cultured for 48 h and metaphase preparations were performed using standard procedures [8]. Slides were spread and stored at -20°C until use.

2.2. Uniform staining

Uniform staining was performed using standard procedures. Fluorescence plus Giemsa (FPG) staining was performed in order to score unstable CA only in the first generation metaphase cells [3] (Fig. 1A and B).

2.3. Telomere and centromere staining

Telomeres and centromeres were stained using the Q-FISH technique with a Cy-3-labeled PNA probe specific for TTAGGG for telomeres and a FITC-labeled PNA probe specific for centromere sequences (both from Panagene, Daejeon, South Korea) as described in Pottier et al. [21]. Briefly, slides were washed with $1\times$ PBS for 5 min at room temperature. Fixation was performed with 4% formaldehyde for 2 min at room temperature, and the slides washed 3 times with PBS for 5 min. After 7 min of pepsin treatment (0.5 mg/ml) at 37°C , the slides were washed in PBS and refixed in 4% formaldehyde for 2 min. After 3 PBS washes during 5 min, slides were sequentially dehydrated with 50%, 70%, 100% ethanol and air-dried. The probe solution consisted of 50 μl of PNA probe (0.3 $\mu\text{g}/\text{ml}$ telomere and centromere). The slides with the probe solution were denatured on a hot plate at 80°C for 3 min and incubated in the dark for 1 h at room temperature. After hybridization, the slides were washed with 70% formamide/10 mM Tris pH 7.2 3 times during 15 min and then in 50 mM Tris pH 7.2/150 mM NaCl pH 7.5/0.05% Tween.20 ($3\times$ 5 min). After washing with PBS, the slides were counterstained with DAPI and mounted with PPD. Quantification image acquisition and analysis were performed using Isis software (version 3.9.1, MetaSystems, Newton, MA).

2.4. Chromosomal aberration scoring

Due to the loss of signal intensity and increase in background on the hybridized slides following uniform staining, we decided not to carry out successive Geimsa and TC staining on the same slide. Two slides were used each for uniform and TC staining. For each condition, an average of 100 metaphases was counted for each slide. Following uniform staining, CA were scored based on chromosomal morphology. Following TC staining, CA were detected based on the detection of centromeric regions and telomeric sequences. Only, metaphases with 46 centromeres were analyzed. For men, the centromere of chromosome Y had a very weak signal (Fig. 1E and F) and for this reason we used the DAPI image (strong heterochromatin) to identify this chromosome for manual and automatic scoring. We detected dicentrics, centric and acentric rings and fragments with 4 telomeres resulting from a fusion event generally accompanying the formation of a dicentric or centric ring. We also detected fragments with 2 telomeres representing terminal deletions as well as acentric fragments without telomeres representing interstitial deletions. All of the information provided by TC staining, allowed us to precisely calculate the number of DSB that generated the CA: either a dicentric or a centric ring with a fragment containing 4 telomeres are considered as 2 DSB. Excess acentric fragments are considered as resulting from one DSB for terminal deletions with only two telomeres and 2 DSB for interstitial deletions fragments with no telomeres.

2.5. Slide scanning and metaphase acquisition

Images of metaphase cells were acquired using automated acquisition module Autocapt software (MetaSystems, version 3.9.1) using a ZEISS Plan-Apochromat $63\times/1.40$ oil and CoolCube 1 Digital High Resolution CCD Camera. For manual scoring after uniform or TC staining, the analysis was carried out using Isis software (MetaSystems, version 5.5). For automatic scoring of dicentrics after uniform staining, DCscore software (version 3.8.101 MetaSystems) was used to detect dicentrics and an operator validated and excluded the false dicentrics [15].

Automatic scoring following TC staining was performed using TCScore (CEA) as described in supplementary data, Fig. 3.

2.6. Statistical analysis

A script in R[®] was developed according to the IAEA recommendations [3]. The curves generated in this study are primarily based on generalized linear models using the *glm* function of the standard *stats* package.

3. Results

3.1. Improvement of the technique: detection of chromosomal aberrations after TC staining with PNA probes

The detection of CA using the gold standard technique is based on chromosome morphology following uniform staining (FPG) (Fig. 2A) and permits the scoring of only first generation metaphases (Fig. 1A and B). The introduction of centromere staining allowed the visualization of the centromeric regions and, as a result, the identification of dicentric, centric rings and acentric fragments. Similarly, telomere staining permitted the identification of the chromosome ends improving the identification of CA contributing to an improvement in the scoring of acentric fragments (Fig. 2B), in particular in the case of overlapping chromosomes. TC staining also made it possible to eliminate the false positives which are impossible to detect with uniform staining (Fig. 2C) or centromere staining only (Fig. 2D). In addition, TC staining improved the detection of dicentric when the centromere was in close proximity to or in contact with the telomeres (Fig. 2E) and where both centromeres were in close proximity (Fig. 2G and F). It was also possible to easily score and distinguish centric and acentric rings (Fig. 2F and G). Of note, TC staining also allowed the easy identification of 1st to 2nd generation metaphases, representing another advantage of this approach (Fig. 1C and D). The use of PNA probes, characterized by their higher signal intensity and very low cost compared to DNA probes, for TC staining does not significantly increase the cost compared to uniform staining.

3.2. Increased sensitivity of the technique following TC staining

Following uniform staining the lowest detectable dose was 0.1 Gy ($p < 0.03$) after the scoring of dicentric. Taking into account all DSB, no significant difference was observed between controls and 0.1 Gy. Following TC staining, it was possible to detect a difference between controls and 0.1 Gy after dicentric scoring ($p = 0.01$). This difference increased when we calculated the DSB which gave rise to the CA ($p = 0.001$). The higher sensitivity observed when taking into account the estimated DSB is due, for the most part, to the improvement in the detection and scoring of interstitial acentric chromosomes.

3.3. Scoring of CA following TC staining does not require a high level of expertise

Two galleries of 100 metaphases following exposure to 1 and 2 Gy were scored by five different operators, 3 of which had no previous experience in cytogenetics. The variability of the ratio between the different operators was very low ($\mu = -0.0147$, $\sigma^2 = 0.0170$ with $p = 0.7449$ for 1 Gy and $\mu = 0.0170$, $\sigma^2 = 0.00367$ with $p = 0.5226$ for 2 Gy) demonstrating the independence of the scoring of CA following TC staining from the operator, the robustness of the approach, and the transferability of this technique to multiple cytogenetic laboratories.

3.4. Dose–response curve after manual scoring of chromosomal aberrations

Table 1 shows the number of analyzed cells, the number of dicentric, centric rings, acentric fragments, the excess of acentric fragments and the estimated DSB following uniform or TC staining. A linear quadratic dose–response curve was obtained for up to 6 Gy after the scoring of dicentric as well as that of DSB taking into account all scored aberrations (Fig. 3A and B).

No significant difference was observed between the dose–response curve obtained after uniform staining by the CEA and the dose–response curve published by the IAEA [3].

Furthermore, a significant difference was observed at low (Fig. 3C and D) and higher doses between the dose–response curves obtained after uniform staining and that obtained after TC staining. The improved detection of centromere regions and telomere sequences using PNA probes, led to the detection of a higher number of dicentric following TC staining compared to uniform staining, in particular at higher doses due to the high incidence and complexity of the CA. Indeed, the frequency of excess acentric fragments was significantly lower following TC staining (acentric fragments without or with two telomeres) than that following uniform staining (Table 1). Following exposure at 4 Gy, the analyses of 200 dicentric shows that 60% could be easily detected following uniform staining, whereas for 31%, the centromere region was close to the telomere and for 9%, the two centromeres were very close to each other (Fig. 2F and G). In addition, the improvement of TC staining for the scoring of dicentric and acentric fragments resulted in a better estimation of DSB leading to a higher frequency of estimated DSB following TC staining than that following uniform staining. The difference observed between the frequencies of estimated DSB following uniform versus TC staining was even greater than that based on dicentric scoring alone (Fig. 3B and D).

3.5. Development of new software to score CA after TC staining

Given the better detection of dicentric, centric rings and acentric fragments, we have developed a new software called TCScore[®]. Fig. 4A shows a simplified version of the workflow used by TCScore. The three channel image contains the DAPI information in the blue channel and the telomere and centromere information in the red and green channels respectively. The chromosomes are first segmented using the DAPI information and then the centromeres and telomeres are detected. The chromosomes are then defined by the number of telomeres and centromeres and finally classified according to the aberration class (supplementary data Fig. 1). This processing scheme allows for batch processing and report generation using a highly intuitive and interactive user interface designed for rapid review and correction by the user (Fig. 4B). The computer requirements for the use of this software are minimal allowing its use on any computer, and images can be treated regardless of the type of image capture platform used.

Using TCScore, we were able to detect 95% of dicentric scored manually. In addition, 85% of all unstable CA that were scored manually were detected by TCScore, leading to a better estimation of DSB. No significant difference was detected between the manual and automatic dose–response curves for either dicentric alone (Fig. 5A) or for the estimated DSB based on all CA (Fig. 5B). In addition, the analysis using TCScore, including operator correction, was more straightforward and less time consuming (1/10 of the time compared to manual scoring). For example, the scoring of 200 metaphases exposure to 2 Gy requires 20 min including operator validation.

Importantly, a significant difference was observed between the frequency of dicentric scored using the commercial image analysis software DCScore[™] (MetaSystems) following uniform staining

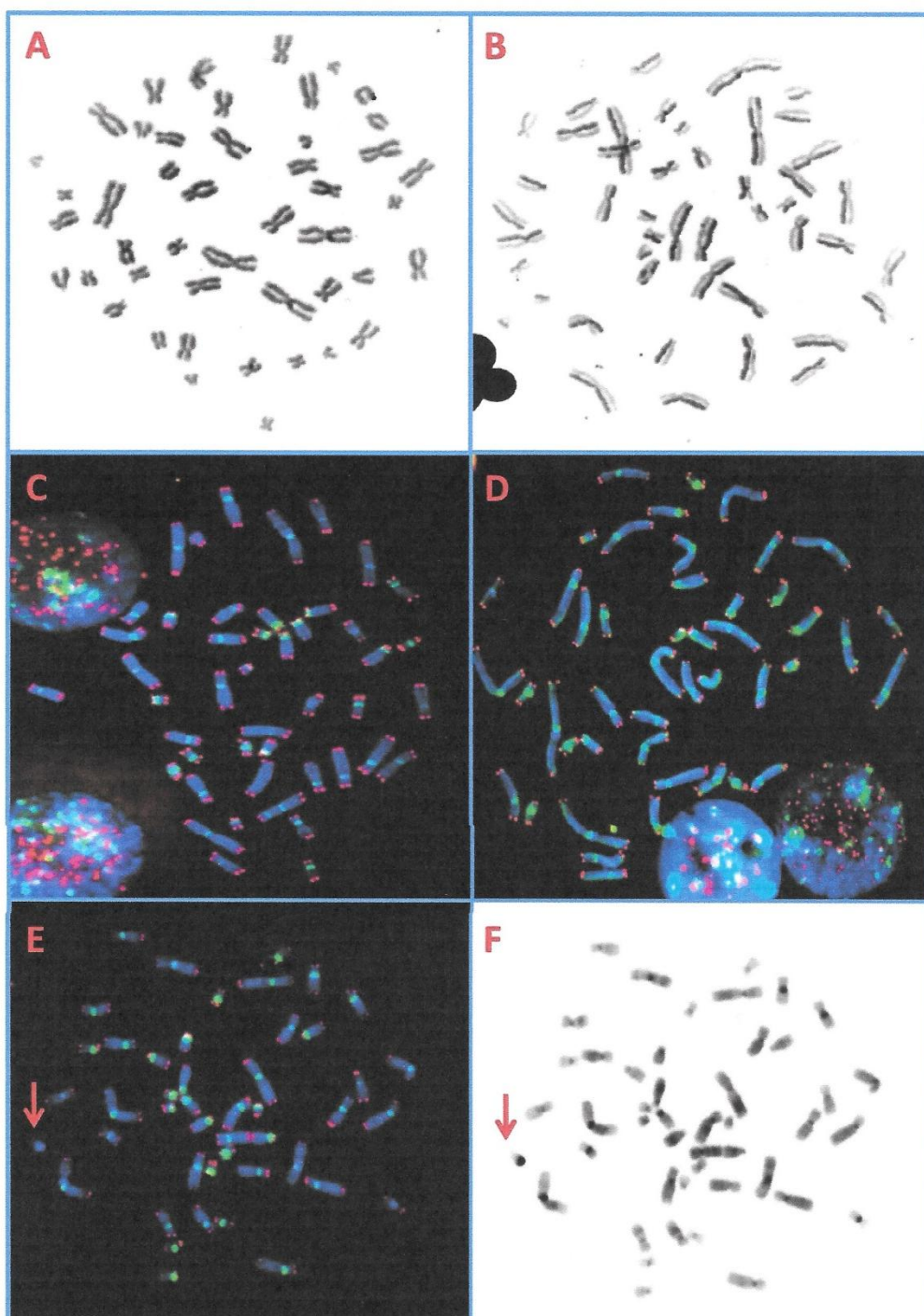


Fig. 1. In cytogenetic biological dosimetry, only the first metaphase is scored. Fluorescence plus Giemsa staining makes it possible to distinguish the 1st metaphase with homogeneous coloration (A) and the second metaphase after fluorescence plus Giemsa staining (FPG), due to the incorporation of BrdU in the second chromatin strand (B). This step adds approximately 2 h to the protocol. PNA probe staining distinguishes the 1st metaphase (C) from the 2nd due to the cross hybridization of the FITC signal in the 2nd generation (D). For men, the centromere of chromosome Y had a very weak signal (E) and for this reason we used the Dapi image (strong heterochromatin) or inverse DAPI to identify this chromosome (F).

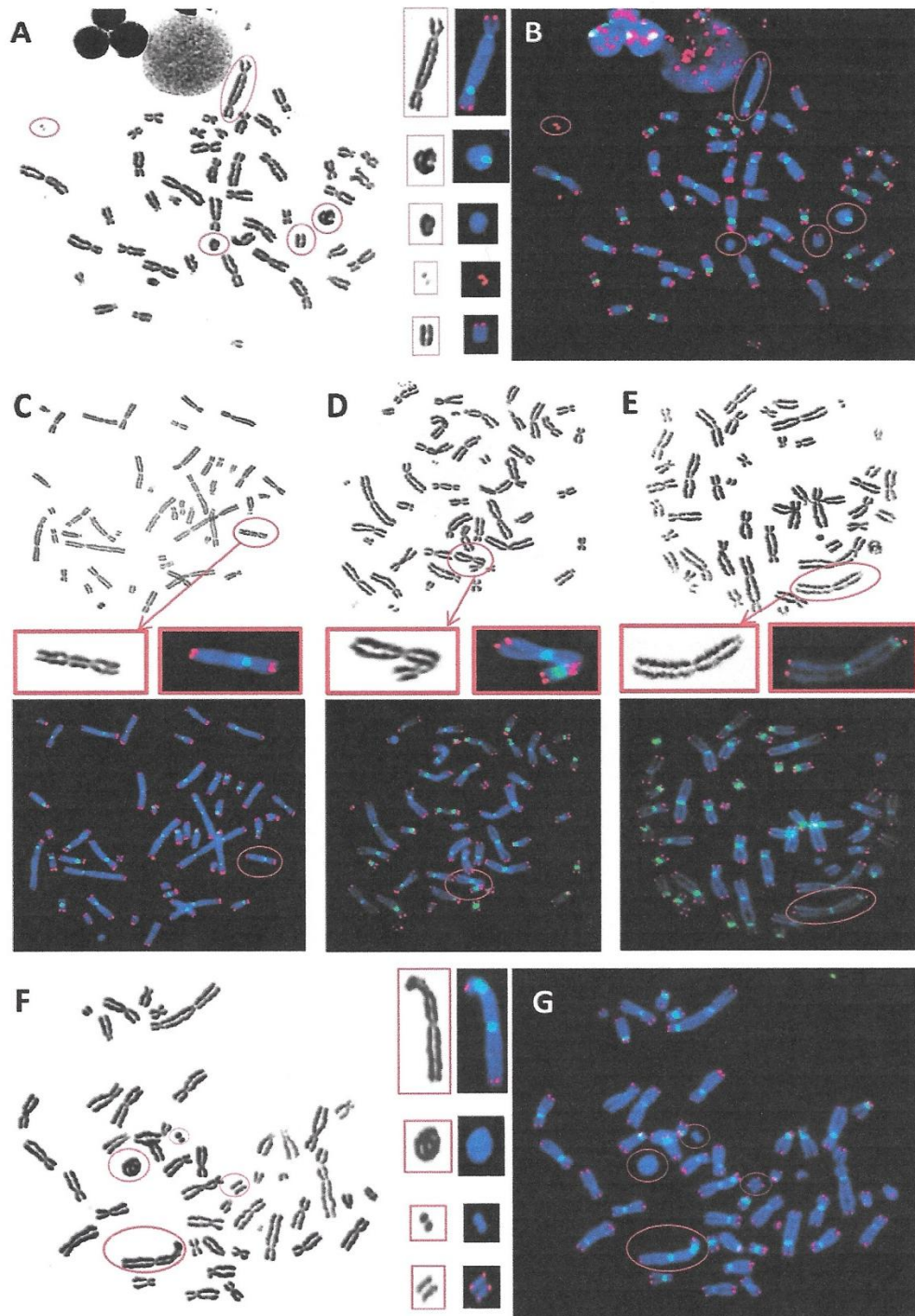


Fig. 2. Detection of unstable CA of irradiated circulating lymphocytes of healthy donors either following: (A) Uniform staining based on chromosome morphology, (B) telomere (red) and centromere (green) staining according to the following criteria: (1) dicentric – two green centromere signals with 4 red telomere signals (top inset) (2) ring chromosomes – one green signal without a red signal (2nd inset from the top) (3) acentric ring – no green signal with no red signal (middle inset), acentric fragments with 2 or 4 red signals (bottom two insets). (C–E) Three examples of potentially ambiguous scoring of Uniform stained chromosomes resolved by telomere/centromere PNA staining consisting of a false dicentric detected by uniform (C), overlapping chromosomes and the importance of telomere staining to define the chromosome ends without ambiguity (D), and a dicentric with the implication of an acentric chromosome in its formation, difficult to detect by uniform staining (E). (F and G) Example of the ability of telomere/centromere PNA staining (G) to permit the unambiguous detection a tricenric chromosome (top inset), an acentric ring (2nd panel from the top) and acentric fragments with no telomeres (2nd panel from the bottom) or with 4 telomeres (bottom) panel, which would not be possible with uniform staining (F).

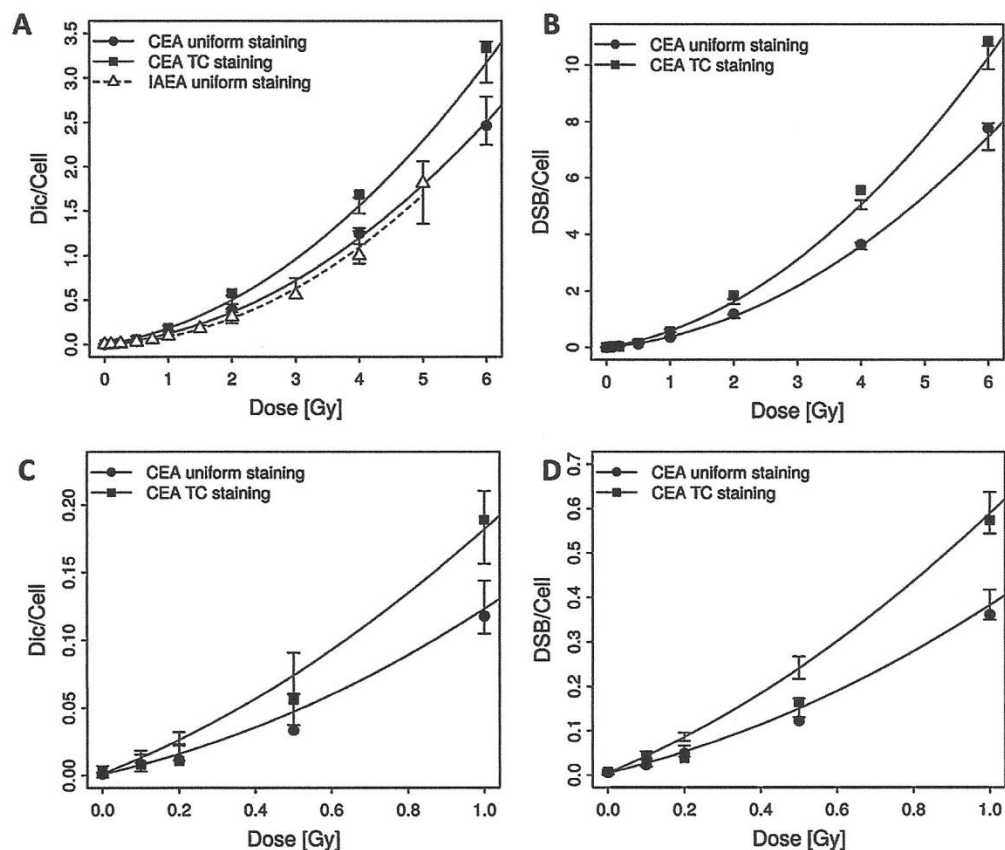


Fig. 3. Dose-response curves following uniform or TC staining. The 95% confidence interval is indicated in all cases. (A) Dose-response curves obtained following uniform staining and TC staining after scoring of dicentrics and compared with the IAEA dose-response curve. (B) Dose-response curves for the number of estimated DSB based on dicentrics, rings and acentric fragments. (C) Zoom below 1 Gy of the dose-response curves for dicentrics and (D) estimated DSB.

and TCScore (CEA) software following TC staining ($p < 10^{-3}$) (Fig. 5). This difference between the two softwares is essentially due to the improvement in the detection of the CA after TC staining, leading to a more than threefold improvement for dicentric detection and a fivefold improvement for the estimation of DSB based on all CA as it is not possible to calculate the DSB following scoring by DCScore based on CA other than dicentrics.

4. Discussion

The introduction of PNA probes, to shorten hybridization times and increase signal intensity, to radiation cytogenetics has not only lead to the better detection of unstable CA, but has also made it possible to develop an appropriate automated system for the scoring of dicentrics as well as centric rings and acentric fragments that was hitherto only possible with uniform staining.

Table 1

Frequency of chromosomal aberrations in circulating lymphocytes after in vitro exposure at different doses detected after telomere and centromere staining or uniform staining.

Telomere and centromere staining									Uniform staining					
Dose (Gy)	Scored cells	Dicentrics	Centric rings	Ac with 4 telo	Ac with 2 telo	Ac with 0 telo	Acentric ring	DSB	Scored cells	Dicentrics	Rings	Ac	Excess	DSB
0	3244	6	0	6	3	1	0	17	4993	5	1	31	25	26
0.1	1787	14	6	19	8	13	1	76	1045	9	1	14	4	24
0.2	1874	20	0	18	6	8	5	72	1364	16	2	49	31	67
0.5	1031	58	10	70	8	9	4	170	1110	37	6	93	50	136
1	1031	195	30	243	36	37	16	592	1240	146	23	279	110	448
2	975	563	100	634	113	114	65	1797	1327	527	81	974	366	1582
4	792	1334	249	1590	319	351	110	4407	930	1162	195	1357	675	3389
6	248	828	149	929	184	192	83	2688	133	328	75	403	230	1034

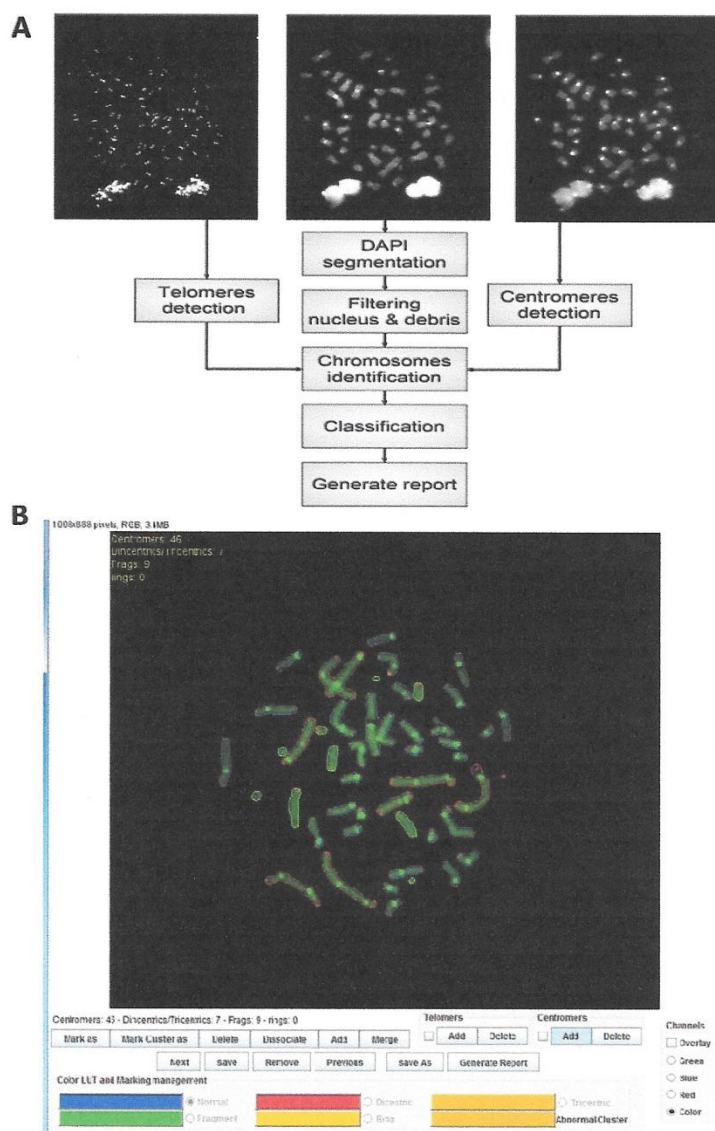


Fig. 4. Automation of CA scoring following TC staining. (A) Workflow of the TCScore image analysis software. Chromosomes are segmented following DAPI staining and the telomeres and centromeres are detected. The chromosomes are then defined by assigning for each object its corresponding attributes and finally classified to the aberration class, and a report is generated. (B) Screen shot from TCScore showing a metaphase from cultured lymphocytes following 6 Gy of irradiation. The software detected 7 dicentric chromosomes (circled in red) and 9 acentric fragments (circled in green).

The first step for the improvement of the conventional gold standard technique was to obtain a better visualization of CA. In this study, we demonstrate not only the improvement of the scoring of dicentrics and centric rings following TC staining, but also the improvement of the scoring of different types of acentric fragments leading to the precise estimation of the DSB which gave rise to the CA.

The resulting improvement of unstable CA detection has led to an increase in the sensitivity of CA detection following low dose exposure. By uniform staining, it is possible to detect an exposure of 0.1 Gy after scoring dicentrics [3]. Following TC staining, the ability to detect the presence of CA at 0.1 Gy increased significantly when taking into account the telomeric status of scored acentric

fragments and the detection of the interstitial fragments without telomeres. The ability to detect doses below 0.1 Gy can now be tested in future work. The increased sensitivity following TC staining makes it possible to apply this technique to the field of low doses research, as low dose exposure due to radiodiagnostic procedures (CT scan) is an ongoing health concern [22].

Furthermore, the use of TC staining has rendered the analyses independent of the level of expertise of the operator as demonstrated by the high reproducibility of scoring (low μ as σ^2 values). This improvement allows the transfer of this technique, currently used only in a few laboratories, to, for example, prenatal diagnostic cytogenetic laboratories. This is an important point as the scoring of uniform stained slides requires a high level of expertise and

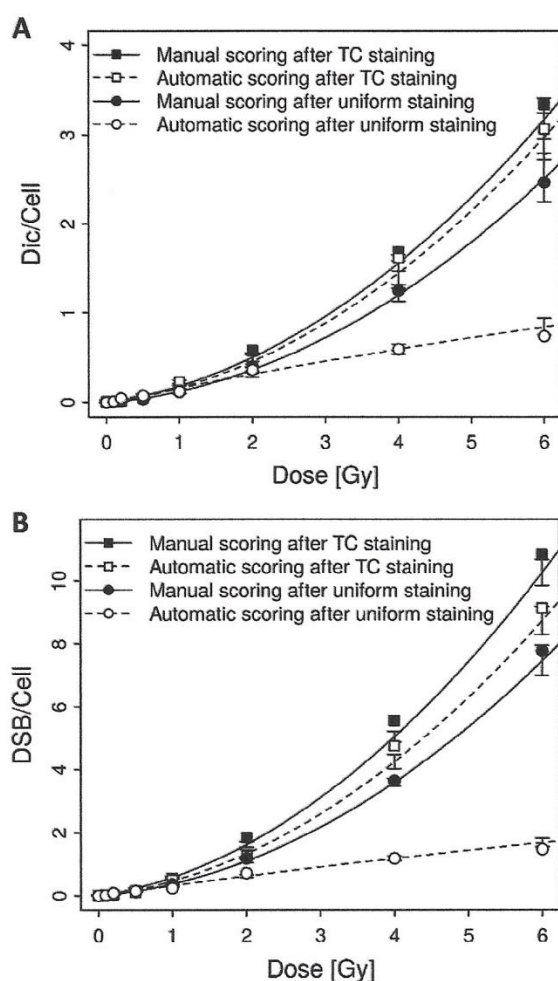


Fig. 5. Dose–response curves following manual and automated scoring of aberrations. (A) Comparison of the dose–response curves obtained after manual or automated scoring of dicentric following uniform using DCScore (Metasystem) or TC staining using TCScore (CEA). (B) The performance of TCScore was superior to that of DCScore based on the estimated DSB. The 95% confidence interval is indicated.

is quite operator dependent making inter-laboratory comparisons and the transfer of this technique quite difficult. The introduction of TC staining to biological dosimetry offers the possibility to have a unique dose–response curve for each type of irradiation and dose rate between all laboratories.

These improvements in the detection of CA can explain the significant difference observed between the dose–response curve obtained following uniform staining versus that following TC staining, especially at higher doses. In this study the dose–response curve obtained following uniform staining was in concordance with the dose–response curve of the IAEA [3] and that published previously [23]. The detection of certain configurations of dicentric, especially when both centromeres are very close to each other or when the centromere is very near the telomere sequences, is very difficult following uniform staining and can explain the difference in the dose–response curves following TC versus uniform staining. Such differences could be detectable after COBRA or M-FISH analysis, but as the dicentric in excess are centromere–centromere or

centromere–near telomere, they can be easily mistaken for translocations as centromeric regions are not labeled by M-FISH probes [24]. In addition, the lower excess of acentric fragments observed following TC staining confirms this amelioration in the detection of dicentric and centric rings. Interestingly, the dose–response curve obtained after the scoring of estimated DSB, following TC staining, was significantly higher than that observed following uniform staining. This work demonstrates the ability to establish a sensitive and precise dose–response curve using the estimated DSB for all CA detected following TC staining, which is a prerequisite for the detection of very low doses [25]. The dose–response curves obtained after the scoring of dicentric as well as the estimated DSB following TC staining are the first to be published.

Using this improvement for the detection of CA, we have successfully automated the scoring of CA that is as efficient as manual scoring. This software is operational and allows for batch processing and report generation using a highly intuitive user interface designed for fast review and correction. The application of TCScore software is independent of the image analysis system and can be applied to all types of images.

In this study, we used the commercial software DCScore by MetaSystems [26] to automatically score the frequency of DC following uniform staining. Using DCScore, half of the dicentric were detected compared to manual scoring and the lowest detectable dose was 0.5 Gy [15]. These data are comparable to the best published results using DCScore [15,23]. The efficiency of this software depended highly on the quality of the cytogenetic preparation and the major problem was the detection of dicentric with corresponding acrocentric chromosomes and the rejection of metaphases with overlapping chromosomes.

A significant difference was observed between the frequency of dicentric scored using DCScore (MetaSystems) and TCScore (CEA) software ($p < 10^{-3}$). The difference between the performance of the two softwares is essentially due to the improvement in the detection of CA following TC staining, leading to a threefold improvement for dicentric detection and a fivefold improvement for estimated DSB based on all CA.

The need to improve the automatic detection of dicentric has been driven by the need to transfer this technique, which is used in only a few laboratories, to all cytogenetics laboratories to achieve high sample throughput for the processing of large cohorts of exposed populations and their follow-up. Furthermore, it would make it possible for such laboratories to refine the determination of complex CA detected in those patients where such knowledge is necessary such as the presence of dicentric [27]. Cytogenetics laboratories confront radiation-induced chromosomal abnormalities without adequate tools for their detection and analysis. It is desirable that this technique be widely disseminated for the scoring of radiation-induced CA as well as for other applications. This automation would also allow the follow-up of patients exposed to chemical agents (chemotherapy, etc.) and ionizing radiation (radiation therapy, radiodiagnostics, etc.).

5. Conclusion

The improvement in the detection of chromosomal aberrations, the reevaluation of dose–response curves and the automation of the gold standard technique following TC staining provides the most precise cytogenetic biological dosimetry currently available. This improved approach will open up new horizons for the assessment of genotoxic risk and for biological dosimetry, in particular for low doses. It will mark a new step in the management of populations exposed to ionizing radiation and/or chemical agents be it accidental, professional or medical, as it associates telomere quantification with the scoring of CA. This automated approach permits long-term

follow-up of exposed populations and would make possible the construction of a database in order to respect the radioprotection norms and to define the risk associated with the use of chemical agents and ionizing radiation.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from the European Community's Seventh Framework Program (EURATOM) contracts Fission-2011-249689 (DoReMi), Fission-2011-295513 (RENEB) and NRBC-C2. We are indebted to Aude Lenain and Wala Najjar for their valuable technical assistance.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mrfmmm.2014.09.007>.

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Annex 3

**Telomere shortening : a new prognostic
factor for cardiovascular disease post-radiation exposure**

TELOMERE SHORTENING: A NEW PROGNOSTIC FACTOR FOR CARDIOVASCULAR DISEASE POST-RADIATION EXPOSURE

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Telomere length has been proposed as a marker of mitotic cell age and as a general index of human organism aging. Telomere shortening in peripheral blood lymphocytes has been linked to cardiovascular-related morbidity and mortality. The authors investigated the potential correlation of conventional risk factors, radiation dose and telomere shortening with the development of coronary artery disease (CAD) following radiation therapy in a large cohort of Hodgkin lymphoma (HL) patients. Multivariate analysis demonstrated that hypertension and telomere length were the only independent risk factors. This is the first study in a large cohort of patients that demonstrates significant telomere shortening in patients treated by radiation therapy who developed cardiovascular disease. Telomere length appears to be an independent prognostic factor that could help determine patients at high risk of developing CAD after exposure in order to implement early detection and prevention.

INTRODUCTION

Recent epidemiological studies have documented vascular and cardiovascular effects following exposure to radiation at doses much lower than those normally associated with cardiac injury when the irradiation is localised to the mediastinal field^(1, 2). According to the International Commission on Radiological Protection⁽³⁾, excess cardiovascular risk has been proven with a threshold acute dose of ~0.5 Gy for both cardiovascular and cerebrovascular diseases⁽⁴⁾. In addition, several cardiovascular risk factors that could potentially account for most of the risk for coronary heart disease in a population⁽⁵⁾ were proposed. At the individual level, there is wide variation in both the occurrence of coronary heart disease and the age of manifestation, even in individuals with the same classical risk factors. The reasons for this wide inter-individual variation in susceptibility are poorly understood. The hypothesis has emerged that inter-individual variation for the risk of coronary heart disease might result from variations in the rate of biological ageing^(6, 7).

Telomeres are found at the very ends of chromosomal DNA and are involved in the maintenance of genome stability^(8, 9). Mean telomere length is considered to be a marker for biological age with shorter telomeres indicative of greater biological age⁽⁹⁾. Telomere shortening

in peripheral blood lymphocytes has been linked to cardiovascular-related morbidity and mortality in the general population^(10, 11). However, a link between telomere shortening and the vascular effects of ionising radiation is still lacking⁽¹²⁾.

The aim of the present study was to investigate conventional risk factors as well as radiation dose and telomere shortening in a large cohort of HL patients who developed coronary artery disease (CAD) post-treatment as biological age could affect susceptibility to coronary heart disease.

Hodgkin lymphoma is a malignant haematologic disease for which current treatment provides a very high cure rate. Because of this success, the first amongst the best examples in oncology, and because this disease affects young patients, it is the object of intense interest amongst oncologists and radiobiologists, insofar as lessons learned from the study of this disease and its treatment can serve as a basis for the therapeutics and the follow-up of other cancers. Nevertheless, the cohort study of Hodgkin's disease patients shows that the survival of these patients who are essentially cured is much lower than that which would be expected⁽¹³⁾. The risk of these former patients of dying from a complication of treatment,

given in the distant past, is greater than the risk of dying from a recurrence of the disease itself. The extent and nature of these morbidities and late mortalities has been the subject of numerous and detailed studies. For these reasons, the authors decided to investigate conventional and non-conventional prognostic factors, such as telomere length, for the occurrence of CADs in HL patients.

The authors demonstrate, in this study, that following multivariate analysis, hypertension and telomere length were the only significant prognostic factors for the occurrence of secondary cardiovascular diseases.

STUDY DESIGN

Patients

A prospective cohort of 179 HL patients treated and followed up at the Gustave Roussy Institute from 2007 to 2012 without a prior history of CAD was entered into the study (Table 1). Treatment was standard: all patients underwent mediastinal radiation therapy, and chemotherapy was given to 173 of 179 HL patients. All radiation treatment charts or computerised records were reviewed, and the radiation doses were estimated according to isodose curves encompassing the area of the coronary artery origins (CAOs)⁽¹⁴⁾.

Blood samples were obtained before Coronary CT angiography (CCTA). The study was approved by the local ethics committee (approval no. 97-56). All subjects signed an informed consent form.

Table 1. Clinical characteristics, modalities of treatment and CCTA findings of HL patients.

Characteristics	Patients (N = 179)
Age at treatment (median and range)	29 (9–75)
Age at CCTA (median and range)	42 (19–79)
Male/female ratio	82/97 (0.85)
Stage	
Early stage	151 (84 %)
Advanced stage	28 (16 %)
Treatment	
Chemotherapy	173 (97 %)
Radiation therapy	179 (100 %)
Total radiation dose (Gy)	36 (35.4–36.8)
Radiation dose to the origin of coronary arteries (Gy)	33.4 (32.4–34.6)
Patients with abnormal CCTA	46 (26 %)
Treatments	
Surgery	10 (22 %)
Angioplasty with stent placement	8
Bypass grafting	2
Medical treatment	24 (52 %)
Follow-up alone	10 (22 %)
Outcome	
Alive	43
Deceased	3

Coronary artery status

Coronary CT angiography was performed on patients using a dual-source CT scanner (Somatom Definition Flash, Siemens AG, Forchheim, Germany). The mean radiation dose delivered during the CCTA procedure was 4.5 mSv with a standard deviation of ± 2 . All imaging data were reviewed by two experienced radiologists.

Telomere quantification

Blood lymphocytes were cultured for 48 h, and metaphase preparations were performed using standard procedures⁽¹⁵⁾. Slides were spread and stored at -20°C until use. Telomeres were stained using the Q-FISH technique with a Cy-3-labelled PNA probe specific for TTAGGG for telomere sequences (from Panagene, Daejeon, South Korea). Quantification image acquisition and analysis were performed using Metacyte software (version 3.9.1, MetaSystems, Newton, MA, USA).

Statistical analysis

All the parameters including radiation dose and telomere length were first tested for their association with CCTA abnormalities by univariate analysis. Cox Proportional Hazard regression analyses assessed the association of all parameters and CCTA abnormalities. Multivariate analysis included all the parameters whose *p*-values were <0.10 in the univariate analysis (all tests were two-sided; *p*-values of <0.05 were considered significant).

RESULTS AND DISCUSSION

The incidence of CCTA abnormalities is given in Table 1. In the total cohort of 178 patients, 3 patients died due to myocardial infarction and 46 patients had abnormalities of their CCTA. Univariate analysis of risk factors was performed and showed the presence of significant conventional risk factors for cardiovascular disease such as hypertension ($p < 2 \times 10^{-5}$), hypercholesterolaemia ($p = 0.001$), age at treatment ($p < 0.04$) and at CCTA ($p = 0.004$). The radiation dose to CAO was also a significant risk factor ($p = 0.015$). A less conventional risk factor, telomere length, was highly significant ($p = 0.006$). Multivariate analysis of conventional risk factors demonstrates that radiation dose to CAO is the most significant factor ($p = 0.005$). Age at treatment ($p < 0.02$), hypertension ($p = 0.006$) and hypercholesterolaemia ($p = 0.01$) were independent prognostic factors. The second multivariate analysis was performed with all risk factors including telomere length. Only hypertension ($p = 0.007$) and telomere length ($p = 0.03$) were found to be significant prognostic factors.

The multivariate analysis of conventional risk factors corroborated the significance of well-known

TELOMERE SHORTENING AND CARDIOVASCULAR DISEASES

prognostic factors. The details for the conventional risk factors are given in Girinsky *et al.*⁽¹⁶⁾ Figure 1 shows the mean and the range of telomere lengths of circulating lymphocytes from HL patients with CCTA abnormalities compared with patients with no evident disease. The mean age and the number of patients are represented. There was a significant association between telomere length and age in HL patients.

In this study, the authors compared only cardiovascular disease versus no cardiovascular disease. Some patients exhibit drastic telomere shortening (<5 kb). The principle finding of this investigation is the prognostic factor of telomere length for cardiovascular disease in HL patients, post-treatment. This study is the first to demonstrate the major role of telomeres in the occurrence of cardiovascular disease in a population exposed to ionising radiation for medical purposes. It is very well documented that individuals with short telomeres of their peripheral blood lymphocytes carry a higher risk for dying of cardiovascular disease in the general population⁽⁶⁾. Cardiovascular complications are the second most frequent fatal post-radiation therapy complication, and epidemiological studies have linked this to exposure to ionising radiation. However, the link between telomere shortening and the higher risk of cardiovascular disease post-exposure was lack.

These findings demonstrate, using multivariate analysis, on the one hand, that the dose to CAO plays a major role in the incidence of CCTA abnormalities, whereas on the other hand, the implication of telomere shortening in the occurrence of these abnormalities.

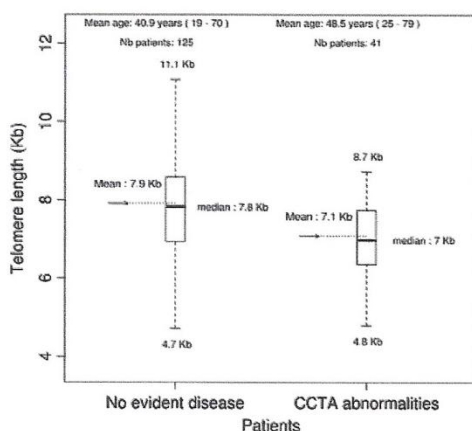


Figure 1. Telomere length in peripheral blood lymphocytes of HL patients with CCTA abnormalities compared with those with no evident cardiovascular disease. Higher heterogeneity in the mean telomere length was observed in patients with no evident cardiovascular disease, which can be explained by the short follow-up of some patients as well as the possible occurrence of another complication such as secondary cancer.

The link between telomeres and the DNA damage response has been strengthened by several studies, both at the cellular and organismal levels, demonstrating that telomere shortening is a determinant of radiation sensitivity^(15, 17-19). Interestingly, in a previous study, the authors have demonstrated that a subset of newly diagnosed, untreated patients with HL demonstrated pre-treatment telomere shortening associated with increased *in vitro* radiation sensitivity and higher frequency of chromosomal aberrations⁽¹⁵⁾. These findings suggest that HL patients exhibiting short telomeres prior to treatment who received mediastinal radiation might be at higher risk of developing CCTA abnormalities. This plausible hypothesis that telomere shortening may represent an important mediator between radiation exposure and vascular damage could be used to define new radiation protection strategies and supports the quantification of telomere as a prognostic factor of cardiovascular risk of population exposed to ionising radiation.

Two possible hypotheses, oxidative stress and viral infections, could be investigated to elucidate the mechanisms of this telomere shortening. Hodgkin lymphoma patients offer a specific profile from the double point of view of oxidative stress (immunosuppression) and viral infection (role of EBV)⁽²⁰⁾. These hypotheses can be used in the monitoring of telomere length in populations before they are exposed to genotoxic agents in order to define new radiation protection strategies on an individual basis (Figure 2).

This study is the first to link telomere length in peripheral blood lymphocytes to cardiovascular disease in a population medically exposed to ionising radiation and provides additional evidence that telomere

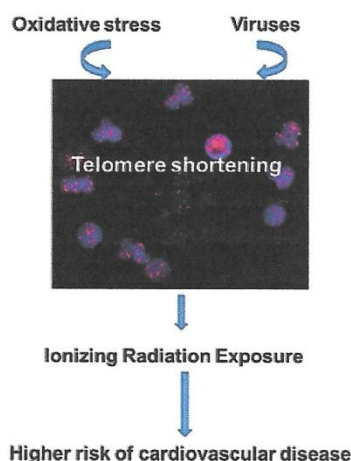


Figure 2. Schematic representation of telomere shortening and higher risk of cardiovascular disease hypothesis in HL patients.

length may be a proxy for underlying inter-individual sensitivity.

These findings need to be confirmed in larger exposed cohorts (accidentally exposed populations, professionally exposed personnel, breast cancer patients, etc.). The introduction of telomere length as a prognostic factor for cardiovascular diseases will require the development of a reliable, easy and automated method to quantify telomere length.

ACKNOWLEDGMENTS

The authors are grateful to the nurses, technologists and secretaries in the department of Medicine and Radiation Oncology of the Institut Gustave Roussy (Villejuif) and Marie Lannelongue (Chatenay-Malabry).

FUNDING

This work was supported by a grant from the European Community's Seventh Framework Program (EURATOM) contracts Fission-2011-249689 (DoReMi), Fission-2011-295513 (RENEB).

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Annex 4

Lead exposure induces telomere instability in Human cells

Lead Exposure Induces Telomere Instability in Human Cells

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Abstract

Lead (Pb) is an important environmental contaminant due to its widespread use over many centuries. While it affects primarily every organ system of the body, the most pernicious effects of Pb are on the central nervous system leading to cognitive and behavioral modification. Despite decades of research, the mechanisms responsible for Pb toxicity remain poorly understood. Recent work has suggested that Pb exposure may have consequences on chromosomal integrity as it was shown that Pb exposure leads to the generation of γ H2Ax foci, a well-established biomarker for DNA double stranded break (DSB formation). As the chromosomal localization of γ H2Ax foci plays an important role in determining the molecular mechanism responsible for their formation, we examined the localization of Pb-induced foci with respect to telomeres. Indeed, short or dysfunctional telomeres (uncapped or damaged telomeres) may be recognized as DSB by the DNA repair machinery, leading to "telomere-Induced Foci" (TIFs). In the current study, we show that while Pb exposure did not increase intra-chromosomal foci, it significantly induced TIFs, leading in some cases, to chromosomal abnormalities including telomere loss. The evidence suggests that these chromosomal abnormalities are likely due to perturbation of telomere replication, in particular on the lagging DNA strand. We propose a mechanism by which Pb exposure leads to the loss of telomere maintenance. As numerous studies have demonstrated a role for telomere maintenance in brain development and tissue homeostasis, our results suggest a possible mechanism for lead-induced neurotoxicity.

Citation: Pottier G, Viau M, Ricoul M, Shim G, Bellamy M, et al. (2013) Lead Exposure Induces Telomere Instability in Human Cells. PLoS ONE 8(6): e67501. doi:10.1371/journal.pone.0067501

Editor: Arthur J. Lustig, Tulane University Health Sciences Center, United States of America

Received: November 27, 2012; **Accepted:** May 20, 2013; **Published:** June 26, 2013

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Funding: M.V. is supported by the European 7th framework grant BOOSTER: grant agreement no. 242361 (http://ec.europa.eu/research/fp7/index_en.cfm). W.H. is supported by the European 7th framework grant DoReMi: grant agreement no. 249689 (http://ec.europa.eu/research/fp7/index_en.cfm). This work was supported by the grants from the Agence Nationale de la Recherche (ANR) (<http://www.agence-nationale-recherche.fr/>) and the Institute National Du Cancer (INCa) (<http://www.e-cancer.fr/recherche>), Hemi-BREAKS and HEMIBREAKS-T. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Lead (Pb) is a naturally occurring metal which has been used for over 8000 years in a wide range of applications [1]. As Pb is non-biodegradable, it has historically been an important environmental pollutant in air and water, and while environmental Pb levels have decreased in western countries, due to strict regulatory policies, it remains a problem in developing countries, and inner city neighborhoods.

The first accurate description of symptoms from Pb poisoning was probably provided by Nicander, a little known poet of the 2nd century BC [2]. Since then, numerous studies have demonstrated that while Pb affects essentially every organ system in the body, including the hematopoietic, cardiovascular, renal and skeletal systems, it is the central nervous system which is particularly sensitive to the effects of Pb [3]. Alterations in cognitive function and behavior due to the neurotoxic effects following Pb poisoning have long been recognized [4]. Animal studies have shown that chronic lead exposure indeed affects neuronal development and adult neurogenesis [5]. Importantly, a lower threshold for the effects of Pb on the nervous system has not been determined [6]. Persistent low-level environmental contamination of this heavy metal has ensured that Pb contamination remains a significant

public health issue. Lead has also been identified as a probable carcinogen [7].

Despite the fact that Pb exposure has been the subject of intense research over many decades, the mechanisms responsible for its far reaching and persistent neurotoxic effects are still poorly understood. In humans, as in animal models, Pb contamination is associated with generalized oxidative stress observed through biomarkers such as GSH status, lipid peroxidation, and an increase in the activity of glutathione reductase and peroxidase, catalase, superoxide dismutase... (reviewed in [8]). Lead serves no biological function in humans but its high electronegativity favors interactions with proteins, notably those with metal binding sites (rich in oxygen and sulfur).

More recently, the genotoxic effects of lead exposure have also been the subject of a number of studies. Using a variety of different experimental models, conditions, and endpoints, these studies have generated largely contradictory findings [9]. Nonetheless, a recently published study has reported the emergence of γ H2Ax foci, commonly correlated with the formation of double-stranded DNA breaks (DSB), some hours after cell exposure to Pb [10]. Furthermore, the same study provides evidence suggesting close association between Pb molecules and DNA in the cells, suggesting that Pb directly affects DNA/chromosomal integrity.

The apparition of γ H2Ax foci may arise from different mechanisms depending on the chromosomal localization. γ H2Ax foci in intra-chromosomal regions are generally due to the presence of DSBs [11], whereas those at or near telomeres may also be due to telomere shortening, which has been shown to lead to γ H2Ax foci formation in the absence of bona fide DSBs [12]. Telomeres are DNA-protein complexes containing long 6 base pair repeats added onto the ends of chromosomes by the enzyme telomerase. Telomeres serve multiple functions including protecting the ends of chromosomes and preventing chromosome fusion. Telomeres are maintained in germline cells, but shorten with each progressive cell division in somatic cells. Telomere shortening in somatic cells is a signal of replicative cell senescence which results from the inability of the telomere to form a cap that protects the end of the chromosome. When this occurs, the cells cease to divide and often undergo apoptosis. However, if the checkpoint pathways are deficient, cells continue to divide until they reach the stage of telomeric crisis which often leads to cell death or significant genomic instability [reviewed in [13]. When several chromosomes contain very short telomeres, their extremities tend to undergo end-to-end fusion leading to the generation of dicentric chromosomes. A correlation between reduced telomere length and dicentric chromosome frequency has been demonstrated in human senescent fibroblasts [14]. In the segregation phase of mitosis, dicentric chromosomes often form an anaphase bridge that results in chromosome breakage, increased chromosomal rearrangements and chromosomal abnormalities [15].

This association between Pb exposure and the generation of γ H2Ax foci led us to study the cytogenetic consequences related to telomere maintenance. Indeed, short or dysfunctional telomeres (uncapped or damaged telomeres) may be recognized as DSB by the DNA repair machinery, leading to "telomere-Induced Foci" (TIFs). In the current study, we show that while Pb exposure did not increase intra-chromosomal foci, it significantly induced TIFs leading in some cases to chromosomal abnormalities including telomere loss. The evidence suggests that these chromosomal abnormalities are likely due to perturbation of telomere replication, in particular on the lagging DNA strand.

Materials and Methods

Cell Culture

Clone B3 was isolated from the human EJ30 bladder cell carcinoma cell line (obtained from Dr. William Dewey, University of California, San Francisco). The origin, derivation and use of this cell line are described in [15,43–49]. Cells were grown in alpha-MEM medium (Gibco®), supplemented with 10% fetal calf serum (Eurobio) and antibiotic antimycotic (Gibco®). For the detection of γ H2Ax foci in interphase nuclei, cells were seeded at 6×10^4 /slide, cultured for 24 h in the absence of Pb and a further 24 h in the presence of the indicated concentrations of $\text{Pb}(\text{NO}_3)_2$ before processing for immunofluorescence. For the detection of γ H2Ax foci and/or telomeres in metaphase spreads, cells were seeded at 5×10^5 cells/T25 flask, cultured for 24 h in the absence of Pb and a further 24 h in the presence of the indicated concentrations of $\text{Pb}(\text{NO}_3)_2$. Colchicine (6 $\mu\text{g}/\text{mL}$) was included during the last 5 hours of culture to accumulate mitotic cells before processing for FISH or IF FISH.

Preparation of Pb Solution

Lead nitrate (Sigma Chemical) solution was prepared by dissolving 3.31 g Pb nitrate in 100 ml of distilled water (i.e. 100 mM solution).

Chromosome Analysis

Preparation of metaphase chromosome for *in situ* hybridization was performed as previously described [50]. Cytogenetic analyses were performed in two steps, consisting of hybridization with a protein nucleic acid (PNA) telomere probe followed by M-FISH for all chromosome painting. Telomere analysis was performed as previously described [51] using telomere specific PNA probes labelled with CY3 (Perspective Biosystems). M-FISH was performed using multi-FISH probes (Metasystems GmbH, Althusscim, Germany), according to the manufacturer's recommendations. Images of hybridized metaphases were captured with a charge-coupled device camera (Zeiss, Thornwood, NY) coupled to a Zeiss Axioplan microscope and processed using ISIS software (Metasystems). In all cases, 50 metaphases were analysed per experiment.

Co-FISH

Chromosome Orientation FISH (CO-FISH) consists of a FISH technique that uses single-stranded DNA probes to produce strand-specific hybridization and was performed as previously described [18]. The technique relies on labelling by 5'-bromodeoxyuridine (BrdU) (Sigma) incorporation on one strand of DNA during S-phase. Metaphase chromosomes were prepared 24 h (1 cell cycle) after the addition of 1×10^{-5} M BrdU. After rinsing the slides with SSC (Saline Sodium Citrate), they were incubated for 15 min in 5 $\mu\text{g}/\text{mL}$ Hoechst 33258 (Sigma) to allow intercalation of the dye into the newly synthesized DNA. The slides were then subjected to 45 min of UV irradiation, in a bath of $2 \times \text{SSC}$, to introduce nicks at the sites of BrdU incorporation. The nicks were enlarged by incubating the slides in 10 U/ μL ExoIII (Promega) for 15 min at 37°C , leaving the parental strand as a single-stranded template for the hybridization procedure. After dehydration in successive baths of 50, 70, and 100% ethanol for 5 min each, on ice, the slides were incubated for 1 h30 with the PNA probe T_2AG_3 (FITC, Fluorescein IsoThioCyanate) and then with the probe C_3TA_2 (Cy3) for another 1 h30. After hybridization, the slides were counterstained with DAPI (1 $\mu\text{g}/\text{mL}$).

Immunofluorescence

Cells were fixed with methanol/acetone (1 V/1 V) for 10 min and permeabilized in 20 mM HEPES pH 7.9, 50 mM NaCl, 3 mM MgCl_2 , 300 mM sucrose 0.5% TRITON X-100 (Sigma) for 30 min. Cells were then incubated 1 h at 37°C in PBS-TRITON 0.5% with anti- γ H2Ax^{ser139} antibody (1/800; Upstate). After washing in PBS, cells were incubated with an anti-mouse CY3 secondary antibody (1/300) at 37°C for 45 min. Cells were mounted in p-phenylene diamine after counterstaining with 4,6-diamidino-2-phenylindole (Sigma).

IF-FISH

Immunofluorescent-FISH (IF-FISH) was performed using a protocol similar to one described previously [17]. Cells were cytospun at $112 \times \text{g}$ after 5 h of colchicine (0.09 $\mu\text{g}/\text{mL}$) treatment at 37°C in a humidified atmosphere of 5% CO_2 . The pellet was washed in $1 \times \text{PBS}$ at 37°C and re-centrifuged. The cells were subjected to hypertonic shock by resuspension in 34 mM citrate at 37°C to obtain a suspension of cells at a concentration of 6×10^5 cells/mL and incubated for 1 h at 37°C . 200 μL of the suspension was subsequently applied to polylysine slides by centrifugation. Following fixation (PFA 3%, sucrose 2%), cells were immunostained as already described. Prior to telomere hybridization with the PNA probe $(\text{CCCTAA})_3$ -FITC, cells were successively fixed

(PFA 4%, 2 min), washed in PBS and dehydrated (50/70/100 ethanol).

Statistical Analysis

Statistical analyses of the results were performed using the student t-test for two unpaired independent sample sets (treated and untreated cells), and the standard error was calculated for chromosomal alteration frequencies between the two sample sets. The formula used to compare the two averages is shown below where s = the common variance to both sample sets, N = the number of samples.

$$t = \frac{m_1 - m_2}{\sqrt{\frac{s^2}{N_1} + \frac{s^2}{N_2}}}$$

Results

Lead Induces γ H2Ax foci that are Mainly Localized in Telomeric Regions

The induction of DNA damage following Pb nitrate exposure has been previously described, based on the quantification of nuclear foci formed following H2Ax phosphorylation by immunofluorescence (IF) [10]. They deduced that Pb induces DSB that they called HEMI-breaks for Heavy Metal Induced breaks that reached a maximum level after 24 hours of treatment. Indeed, the number of nuclear foci has been previously shown to directly correlate in a one to one ratio with the number of DSB following irradiation [16]. As γ H2Ax foci formation reached a maximum level after 24 hours of Pb exposure in the paper published by Gestaldo et al, we chose to focus on the same time point for the current study. Our results confirm the generation of γ H2Ax foci in human B3 cells following exposure to increasing concentrations of Pb nitrate after 24 h of treatment (**Figure 1A**). A dose dependent increase of the mean number of γ H2Ax foci was observed that achieved significance starting with exposure of the cells to 100 μ M Pb compared to non-exposed cells ($p < 0.05$). Interestingly, there was a heterogeneous distribution of γ H2Ax foci amongst the nuclei of B3 cells (**Figure 1B**). In untreated cells, more than 50% of nuclei contained 0–9 foci, whereas only a very small percentage contained over 30 foci. Following lead exposure, however, there was a significant increase in the percentage of nuclei containing over 30 foci starting from 100 μ M ($p = 0.004$). Based on these results, subsequent experiments were performed with the lowest effective Pb nitrate concentration (100 μ M) in order to study the mechanism and the downstream consequences of γ H2Ax foci formation.

In order to position the induced γ H2Ax foci on the chromosomes, we adapted the Immunofluorescence (IF)-FISH method [17] allowing simultaneous telomere hybridization and immunofluorescent staining of γ H2Ax on the same metaphase chromosomes (**Figure 2A**). Using this approach it is possible to co-localize telomeres and chromosomal γ H2Ax foci, distinguish internal from telomeric γ H2Ax foci, and observe telomere loss. Several examples of the chromosomal localisation of the γ H2Ax foci (telomeric or intra-chromosomal) after 24 h exposure with Pb nitrate are shown in **Figure 2A**, and the comprehensive data is represented in **Figure 2B**. Several staining patterns were observed including intra-chromosomal foci or telomeric foci on one or both chromosomal arms with or without telomere staining (see next section). Surprisingly, Pb nitrate treatment (100 μ M) did not impact the percentage of intra-chromosomal foci, while the percentage of telomeric γ H2Ax foci (co-localized γ H2Ax and

telomere staining) was increased. It is important to note that the co-localisation of telomere staining and γ H2Ax foci indicate that γ H2Ax formation has occurred in the absence of DSBs, as if this was the case, the telomere would most likely be lost. These results strongly suggest that Pb nitrate preferentially impacts the telomeric region, leading to telomere dysfunction signalled initially as a DSB in the intact telomere.

Lead Induces Telomere Instability

The results obtained from the above study suggest, that in some cases, Pb exposure of B3 cells leads to telomere loss. Indeed, the preferential localization of γ H2Ax foci on telomeres may indicate the presence of extremely short or dysfunctional telomeres (uncapped or damaged telomeres) that are recognized as DSB by the DNA repair machinery [12], leading to “telomere-Induced Foci” (TIFs). As Pb is cytotoxic, it was necessary to first calculate the mitotic index of cells in the presence of the different concentrations of Pb (**Figure 3A**). Pb nitrate decreased the mitotic index of treated cells. The effect was dose-dependent and statistically significant starting with exposure of the cells to 100 μ M compared to non-exposed cells ($p < 0.05$). These data were thereafter taken into consideration and the subsequent experiments were normalized for the decrease of the mitotic index by dividing the percentage of chromosomes exhibiting telomere loss by the mitotic index for each concentration of Pb. The telomeric status of the cells was assessed after 24 h exposure with the same concentrations of Pb nitrate. The rates of telomere loss are presented in **Figure 3B–C**. Lead exposure induced a dose-dependent increase of telomere loss which was significant from 100 μ M for one chromatid. Based on these analyses, it is not possible to determine if the loss of signal for two telomeres is due to a break in the subtelomeric region or intra-chromosomally. There was also an increase in telomere doublet formation which was maximal at 100 μ M but not dose dependent (see **Figure S1**). In preliminary studies, Pb induced telomere loss and doublet formation were also observed in fresh human lymphocytes (see **Figure S2**) demonstrating that Pb exposure also leads to telomere dysfunction in primary cells. Interestingly, the level of spontaneous telomere loss in, and doublet formation and the subsequent effects of Pb exposure on human lymphocytes were highly dependent on the specific donor. The mechanism behind the donor-specific variability for the effects of Pb on telomere stability is currently under investigation.

In order to determine if the observed losses were limited to the telomeric region we performed sub-telomere staining (**Figure 3D**, middle panels) on the same slides that were used for the previous study (**Figure 3D**, top panels). Unlike the telomere probe, the sub-telomere probes are chromosome-specific, necessitating chromosome identification using the multi-FISH method prior to sub-telomere staining (**Figure 3D**, lower panels). To aid in the analysis, we decided to focus on chromosomes which demonstrated an elevated frequency of telomere loss. In addition, sub-telomere probes were not available for both arms of all chromosomes, further limiting the choice of chromosomes for analysis. Based on these constraints, we selected chromosomes 3, 5, 8 and X. Representative images in **Figure 3D** show that sub-telomere staining was preserved for chromosome 5 (left panels) and chromosome 8 (right panels). These results demonstrate that the observed loss of chromosome ends following Pb nitrate treatment is strictly limited to the telomeric region as subtelomeres are the most distal regions of unique DNA sequence found on chromosomes.

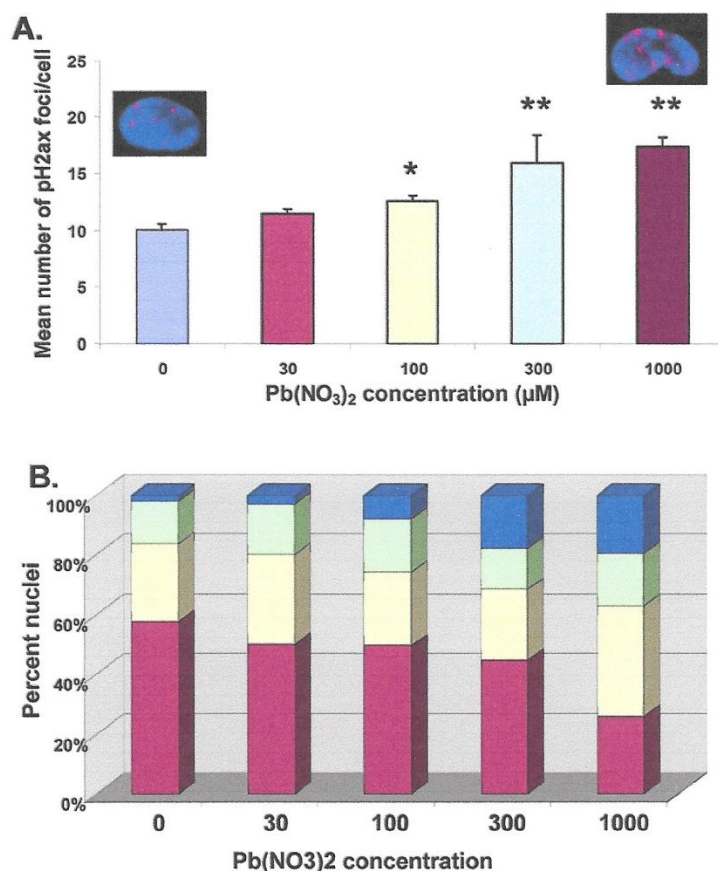


Figure 1. Lead induces γH2Ax foci in B3 cells. Results obtained with B3 cells following 24 h exposure with the indicated Pb(NO₃)₂ concentrations. **A.** Number of γH2Ax foci per cell. Inserts show representative γH2Ax signals observed for 0 and 1 mM Pb(NO₃)₂. **B.** Distribution of the number of γH2Ax foci per nucleus as a function of Pb concentration. Violet, 0–9 foci/nucleus; Yellow, 10–19 foci/nucleus; Green, 20–29 foci/nucleus; Blue, >30 foci/nucleus.
doi:10.1371/journal.pone.0067501.g001

Lead Preferentially Targets the Lagging Strand

As the leading and lagging strands of telomeres have dissimilar structures (reviewed in [13]), it seemed plausible that Pb may preferentially target one strand over the other. The telomeric probe used in the experiments described up to this point consisted of protein nucleic acid (PNA) bearing the specific sequence of one telomere strand. Chromosome orientation-FISH (CO-FISH) was used to differentiate the chromosomal strands [18] (lagging and leading) by the detection of their specific sequence (T₂AG₃ and C₅TA₂ respectively) with the complementary sequence (red and green respectively in **Figure 4A**). We analyzed the involvement of the leading and lagging strands in the loss of telomeres and the formation of doublets (**Figure 4B**). The presence of Pb nitrate in the cell culture medium induced a slight increase in all of the telomere aberrations analyzed. However, the increase was only statistically significant for the loss of the lagging strand ($p < 0.05$). It is worth noting that FITC (green probe) is less efficient than Cy3 (red probe), and thus the loss of staining of the leading strand may be partially due to poor telomere staining, whereas the loss of staining of the lagging strand is more significant as it is likely to be a true measure of telomere loss. Interestingly, in the case of

telomere doublet formation, neither strand seemed to be preferentially affected suggesting that the mechanism of doublet formation is strand-independent. These results show that exposure to Pb nitrate perturbs telomere stability and the lagging strand seems to be the preferential target of Pb nitrate induced telomere loss.

Discussion

Despite being the subject of vigorous research, the mechanism of Pb toxicity and/or carcinogenicity remains unknown. A recent study demonstrated the appearance of γH2Ax foci, a known biomarker for DSB formation, reached a maximum 24 hours after Pb contamination [10]. In the present study we have applied cytogenetic approaches to investigate the consequences of Pb-induced γH2Ax foci formation on human B3 cells.

The mechanism behind the formation of γH2Ax foci may be quite different depending on their chromosomal localization. Intra-chromosomal foci are primarily due to DSB whereas those found at telomeres may be due to short or dysfunctional telomeres, such as those which are uncapped [19] or damaged [20]. Our

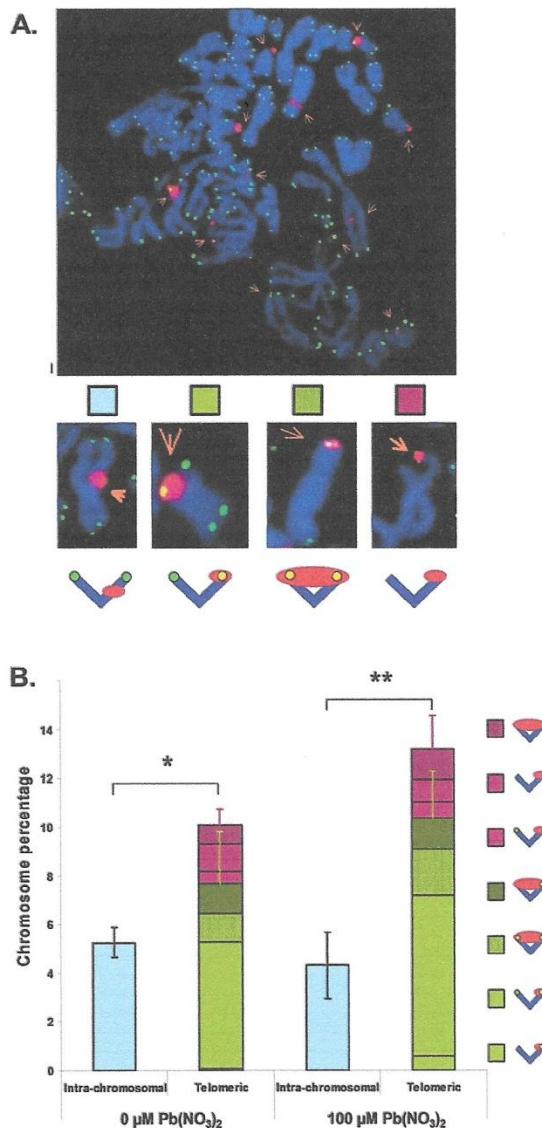


Figure 2. Lead induces Telomere-Induced Foci (TIFs). Results obtained with B3 cells following 24 h exposure with the indicated Pb(NO₃)₂ concentrations. **A.** Representative images obtained following IF-FISH. TIFs (yellow) represent co-localisation of γH2Ax (red) and telomere (green) signals. The upper panel shows a representative image of a single metaphase. The lower panel shows representative images of each type of staining pattern obtained from several metaphases. **B.** Localisation of γH2Ax and telomere staining using IF-FISH. * $p < 0.05$, ** $p < 0.01$. The different combinations of staining are shown in the legend on the right.
doi:10.1371/journal.pone.0067501.g002

results indicate that the Pb-induced γH2Ax foci form mostly at the end of chromosomes at or near the telomeres suggesting that Pb exposure may induce telomere instability most likely due to a defect in telomere maintenance resulting in the emergence of

doublets and telomere loss. Sub-telomere staining confirmed that the loss is strictly limited to telomeric sequences and the Co-FISH method allowed the identification of the lagging strand as the preferential target of these losses.

Indeed, in the previous study demonstrating the appearance of γH2Ax foci following Pb administration [10], it was never conclusively proven that Pb exposure directly led to the formation of DSB despite a concerted effort to do so. The authors concluded that DSB formation was not a direct consequence of lead exposure, but only occurred at a relatively late time point due to oxidative stress and the accumulation of single stranded breaks. Indeed, the relatively late appearance of γH2Ax foci would argue against the direct formation of DSBs following lead exposure, as intra-chromosomal DSB formation normally occurs relatively rapidly following, for example exposure of cells to ionizing radiation, and are almost completely repaired at 24 hours. In light of the results of the current study, it is likely that the γH2Ax foci observed by Gastaldo et al. were actually due to the presence of dysfunctional telomeres, in the absence of DSB, following Pb exposure.

In somatic cells, telomeres shorten at each cell division representing a mitotic clock of the senescence process. Telomeres are highly sensitive to oxidative stress due to less effective DNA repair than for intra-chromosomal sequences [21] and Pb is known to perturb the oxidative status of cells (reviewed in [8]) notably through its interaction with proteins [22]. The specific targeting of telomeres could thus be indirectly attributed to Pb-induced oxidative stress. The specific alteration of this chromosomal region may have drastic consequences on the formation and the long term transmission of chromosomal rearrangements via their interplay with the natural aging of the cells [21].

The preferential targeting of Pb to the lagging strand is intriguing. The particularity of this strand is the repetition of a well conserved G-rich sequence (d[T₁₋₃-(T/A)-G₃₋₄]_n) which has been shown to form G-quadruplexes *in vitro* under physiological conditions. G quadruplexes are 4 stranded nucleic acid structures which can be formed by G rich sequences and can readily form *in vitro* and have also been demonstrated *in vivo*, notably associated with telomeres. Once formed, G-quadruplexes are highly stable and have to be processed by enzymes to unwind the DNA, notably to allow telomere replication. Several enzymes have been shown to have this property: BLM, WRN, DHX36, GQN1, MRE11 and FANCIJ ... (reviewed in [23]; [24]). Interestingly, G-quadruplex-interacting compounds, which stabilize the G-quadruplex structure, have been shown to induce the loss of telomere maintenance leading to the induction of apoptosis in normal or tumoral cells [25–27].

The presence of monovalent cations is an imperative requirement for G-quadruplex formation ($K^+ > Na^+ > Cs^+ > Li^+$). G-quadruplexes can also be stabilized by divalent ions, sometimes more strongly than by monovalent ions because of the increase in charge. G-quadruplex structures obtained with Pb²⁺ are more compact, the hydrogen bonds are more stable than with K⁺ [28] and far fewer Pb²⁺ ions are needed to stabilize a G-rich sequence into a G-quadruplex structure.

Based on the results of the present study, it is tempting to speculate that Pb may displace the physiological ions in already formed G-quadruplexes and possibly induces the formation of additional G-quadruplex structures. In the case of DNAs, the replacement of K⁺ by Pb²⁺ may inhibit the enzymatic activity because of the induced conformation changes [29]. The high selectivity of many G-quadruplex-unwinding enzymes coupled with their high affinity for these structures could lead to their

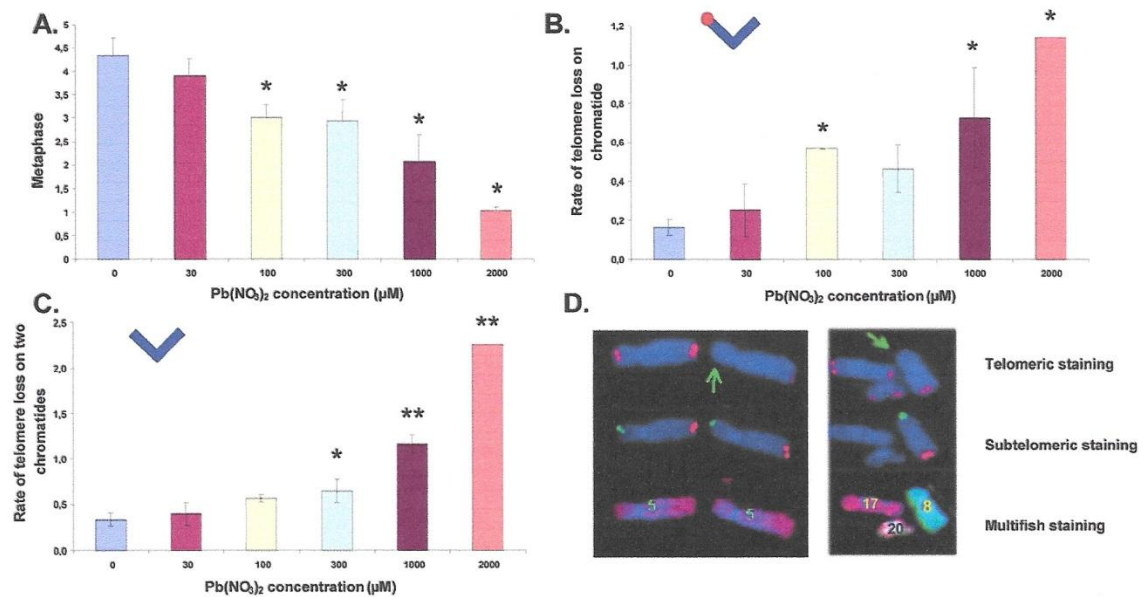


Figure 3. Lead induces telomere instability. A–C. Mean telomere loss observed in B3 cells after 24 h exposure with the indicated Pb(NO₃)₂ concentrations and normalized to the correspondent mitotic index. **A.** Mitotic index. **B.** Loss of one telomere on one chromatid. **C.** Loss of two telomeres on two chromatids. **D.** Representative images obtained with B3 cells following the indicated fluorescent *in situ* hybridization procedure. Subtelomere markers are: p-arm (FITC), q-arm (Texas Red). doi:10.1371/journal.pone.0067501.g003

instability or a reduced ability to recognize and/or cleave the Pb-compacted G-quadruplexes.

Several studies have shown that cells carrying defective G-quadruplex-unwinding enzymes demonstrate telomere instability. For example, cells deficient for the Werner Syndrome RecQ Helicase (WRN), known to be critical in the processing of G

quadruplexes, are characterized by the preferential loss of telomeres from the lagging strand [30]. The telomere instability observed in the present study could be correlated with the persistence of the G-quadruplexes during replication, underlying a defect in telomere maintenance during replication.

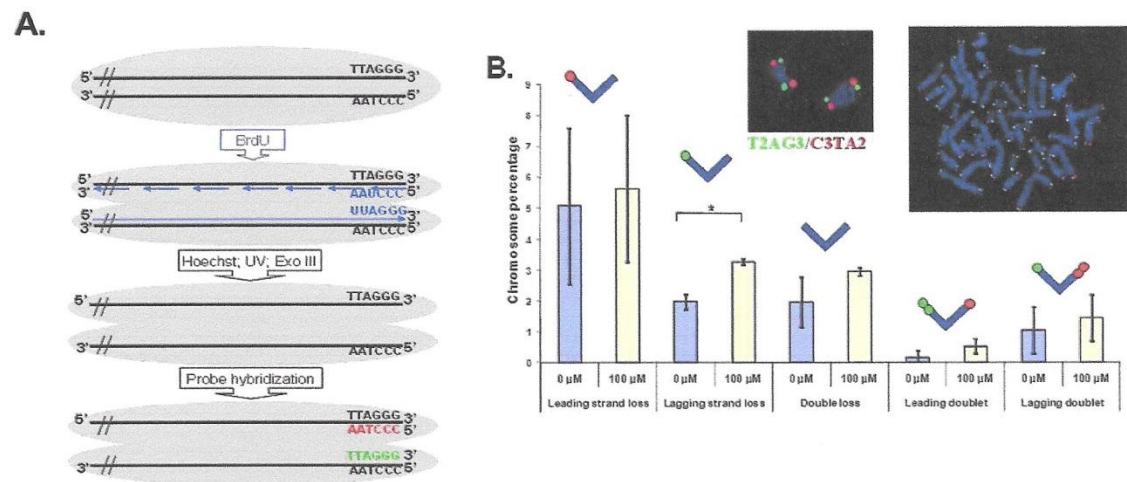


Figure 4. Lead induces mainly lagging strand instability. A. Principle of telomere hybridization by CO-FISH. B. Mean telomere instability observed with B3 cells after 24 h exposure with indicated Pb(NO₃)₂ concentrations. **p*<0.05. Representative images obtained with B3 cells are shown in the inset. doi:10.1371/journal.pone.0067501.g004

The effects of lead exposure on telomere maintenance are also intriguing in light of numerous recent studies which demonstrate a role for telomere maintenance in brain development and tissue homeostasis suggesting a possible mechanism for lead-induced neurotoxicity. Studies in mice in which either the telomerase mRNA component (mTR) [31] or telomerase reverse transcriptase (mTERT) [32] have been deleted demonstrate progressively shorter telomeres leading to numerous problems in organs with high cellular turnover after 4–6 generations [33,34]. In addition, starting from the 4th generation, developing embryos exhibit a failure in neural tube closure [35]. Furthermore, telomere shortening in neural stem cells has been shown to disrupt neurogenesis [36]. Interestingly, the effects of TERT deletion can be reversed in aging mice by inducible expression of TERT, leading to an increase in telomere length, and the reversal of tissue degeneration including in the central nervous system [37]. More recently, a strong link between levels of TERT activity in the hippocampus and depression has been demonstrated in a chronic mild stress mouse model [38].

In humans, perceived chronic stress [39] and high phobic anxiety [40] have been shown to be associated with telomere shortening of blood lymphocytes suggesting a link between environmental stressors, disruption of telomere maintenance, and psychological state. Recently, an age-independent association was also demonstrated between telomere length and cognitive function in a cohort of elderly subjects [41]. It is noteworthy that similar psychological and cognitive effects have been linked to childhood Pb exposure.

Finally, a recently published study has demonstrated that occupational exposure is associated with telomere shortening in the peripheral white blood cells of Chinese battery manufacturing plant workers [42] that was directly proportional to the body lead burden, suggesting a link between occupational lead exposure and the loss of telomere maintenance. While this study only demonstrates an association between lead exposure and telomere disruption, these results take on new significance in light of the results presented here which strongly suggest a causal link between lead exposure at the cellular level and the loss of telomere maintenance.

A model for telomere instability following Pb exposure which could explain Pb-induced neurotoxicity can be proposed. The greater affinity of Pb for G-rich sequences may induce the displacement of the physiological ions (K^+ or Na^+) to form tighter G-quadruplex complexes [29]. The complexes need to be processed to permit telomere replication but the highly specific unwinding enzymes [23] may not function properly due to the more highly compacted form of the complex. Alternative mechanisms are perhaps then recruited to obtain linear DNA, notably with the intervention of nucleases that perturb telomere integrity leading to their recognition as DSBs (γ H2Ax staining). We propose that the first anomaly is the emergence of telomere doublets subsequent to processing by the alternative unwinding

enzymes. The loss of telomere integrity then leads to the loss of the telomeric sequence on one or sometimes two chromatids. This hypothesis is consistent with the results observed where the rate of doublet formation is not dose-dependent and with the γ H2Ax staining at telomeres while telomere sequences are still detectable. The subsequent impaired telomere maintenance induces genomic and chromosomal instability that may lead to a perturbation in neurogenesis resulting in Pb-induced neurotoxicity. The loss of telomere maintenance may also be directly neurotoxic, but this remains to be demonstrated.

In light of our current knowledge of telomeres and the critical role that they play in cellular aging as well as in the development and maintenance of the central nervous system, and the downstream repercussions for cognitive and psychological status, the results presented from the current study suggest a promising novel avenue of inquiry to better understand the link between Pb exposure, telomere maintenance, and the biological responses leading to the permanent nature of the resulting neurotoxic effects.

Supporting Information

Figure S1 Lead induces the appearance of telomere doublets. Appearance of telomere doublets observed in B3 cells after 24 h exposure with the indicated $Pb(NO_3)_2$ concentrations and normalized to the corresponding mitotic index. (TIF)

Figure S2 Lead induces telomere instability in primary human blood lymphocytes. To measure inter-individual variability, the spontaneous level of telomere loss and doublet formation were measured in the blood lymphocytes of 20 healthy donors (Panels A and B). The values represent the mean of the independent analyses of 3 different evaluators. The effect of Pb on telomere loss and doublet formation were measured in human blood lymphocytes of 7 donors after 24 h exposure with the indicated $Pb(NO_3)_2$ concentrations (Panels C and D). * $p < 0.05$, ** $p < 0.01$. In all cases, 50 metaphase spreads were analyzed. (TIF)

Acknowledgments

We thank Dr. John P. Murnane for providing the B3 cell line which was derived from the EJ30 bladder cancer cell line. We are indebted to Radhia M'Kacher who kindly allowed us use her bank of human lymphocyte slides. We are grateful to Emmanuel Paul and Carolina Calderon for their help in the analysis of the human lymphocytes. We thank Nicolas Foray for stimulating discussions.

Author Contributions

Conceived and designed the experiments: LS MR. Performed the experiments: GP MR GS MB CC. Analyzed the data: GP MV MR WMH LS GS MB CC. Wrote the paper: GP MV MR WMH LS.

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Dysfonctionnement des télomères et instabilité chromosomique dans le lymphome de Hodgkin

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Le lymphome de Hodgkin est caractérisé, d'un point de vue histologique, par la présence de rares cellules tumorales nommées cellules de Reed et Sternberg, au sein d'un infiltrat cellulaire polymorphe, inflammatoire et réactionnel. Ces dernières résultent de la transformation tumorale de cellules lymphocytaires B qui acquièrent des propriétés d'échappement au système immunitaire, de prolifération, de résistance à l'apoptose et également une instabilité chromosomique.

En raison du succès enregistré dans le traitement de cette pathologie, qui est le premier mis en place et l'un des plus exemplaires de la cancérologie, et parce qu'elle touche le sujet jeune (Kuppers R. 2009 ; Bleyer A 2009). le Lymphome de Hodgkin fait l'objet de toutes les attentions des cancérologues et des hématologues, dans la mesure où les leçons tirées de son étude pourront servir de base à la thérapeutique des autres cancers. Or l'étude de cohortes de patients atteints du lymphome de Hodgkin montre une survie très inférieure à celle que l'on est en droit d'escompter (risque de second cancer). Le risque pour ces anciens patients de mourir d'une complication du traitement -lointaine donc ignorée au moment du traitement- devient actuellement plus grand que le risque de mourir d'une récurrence de la maladie de Hodgkin.

L'ampleur et la nature de ces morbidités et mortalités tardives fait depuis quelques années l'objet d'études. Elles concernent le rôle des médicaments et des radiations dans la survenue des effets secondaires, en particulier les leucémies et tumeurs solides secondaires ainsi que la dynamique de ces complications. En revanche, l'étude des mécanismes pouvant influencer la survenue de ces morbidités et mortalités tardives est beaucoup moins bien documentée.

La cellule tumorale du lymphome de Hodgkin est caractérisée par des aberrations chromosomiques de nombre et de structure induisant une instabilité génomique. Néanmoins, la rareté des cellules de Hodgkin et de Reed et Sternberg, impliquant des problèmes techniques mais aussi de caractérisation des événements primaires dans l'initiation de cette instabilité chromosomique, a été bien débattue dans la littérature. Mais, les mécanismes impliqués dans l'instabilité génomique dans le lymphome de Hodgkin demeurent obscurs. Très peu d'investigations cytogénétiques ont été entreprises sur ce type de lymphome et 90% de ces études ont utilisées les lignées cellulaires de lymphome de Hodgkin établies à partir des patients en rechute ou réfractaires.

L'objectif principal de cette thèse est d'élucider les mécanismes impliqués dans l'instabilité génomique dans le lymphome de Hodgkin en se servant non seulement de 7 lignées de lymphome de Hodgkin et des ganglions hodgkiniens mais aussi de plusieurs approches techniques. Une corrélation entre ces mécanismes impliqués dans l'instabilité génomique et la radiosensibilité des lignées d'une part et la survie ainsi que la survenue de complications tardives d'autre part a été établie. Ce travail nous permis de caractérisé aussi la cellule clonogénique responsable de la maintenance de la cellule de Reed-Sternberg

Partie I

La première partie de cette thèse a été dédiée à une revue de la littérature sur l'instabilité génomique dans le lymphome de Hodgkin. Nous avons revu les importantes avancées dans la compréhension de l'origine de la cellule tumorale avec les travaux du Kuppers (Kuppers et al 1994). Mais nous avons aussi décrit les derniers travaux montrant la présence d'une autre cellule de type B responsable de la génération et l'entretien des cellules de Hodgkin et de Reed Sternberg. Ces cellules ont été détectées dans les lignées hodgkiniennes, dans les ganglions tumoraux ainsi que dans les lymphocytes circulants (Newcom, Jones, Younes). Ces travaux soulèvent le rôle majeur de ces cellules tumorales dites souches dans l'établissement et le maintien des cellules « tumorales » mais aussi dans la définition d'une nouvelle stratégie thérapeutique comme la thérapie cellulaire (Gabriel Ghiaur et al 2014).

Nous avons décrit 5 voies différentes de signalisation et de transduction impliquées non seulement dans le lymphogénèse des cellules de Reed Sternberg mais aussi à la réponse aux traitements : la voie NF-KB, la voie Jack2/STAT, la voie P53, la voie ATM/ ATR ainsi que la voie FOX. Mais toutes ces voies ne sont pas spécifiques aux ni lymphomes de Hodgkin ni à l'instabilité génomiques détectées dans cette pathologie.

Néanmoins, les mécanismes impliqués dans l'instabilité génomique demeurent obscures. L'instabilité génomique via la présence des anomalies du système de réparation et des erreurs de réplication de l'ADN (système MMR, pour mismatch repair) a été vite écartée.

Seulement une centaine de cellules actuellement ont été caryotypées avec plusieurs dérivées chromosomiques non classés. Des gènes de fusion ont été décrits comme possibles évènements primaires. Mais aucune signature cytogénétique ni moléculaires n'a été mise en évidence. L'infection virale joue un rôle important dans cette pathologie. Il était bien décrit le rôle de virus Epstein-Bar (EBV) dans le lymphome de Hodgkin. Le genome virale de l'EBV est présent dans 40% des cas et pourrait atteindre 100% dans le lymphome de Hodgkin à cellularité mixte. Néanmoins, l'implication de l'EBV dans l'instabilité génomique du lymphome de

Hodgkin n'a pas été établie. Des autres candidats ont été étudiés. L'implication du HHV6 et JC virus dans le lymphogénèse du lymphome de Hodgkin a été proposée.

Cette première partie de la thèse, nous a permis de montrer l'existence des études cytogénétiques dans le lymphome de Hodgkin à l'exception de quelques études. Il est de notre grand intérêt d'élucider les mécanismes impliqués dans l'instabilité génomique dans les lymphomes de Hodgkin.

Partie II

La deuxième partie de la thèse a été consacrée à l'étude des mécanismes impliqués dans l'instabilité génomique de lymphome de Hodgkin via l'instabilité des microsatellites et l'instabilité chromosomique en utilisant 7 lignées de lymphome de Hodgkin en utilisant des approches moléculaires et cellulaires. Après la détermination du statut (fonctionnalité et mutation) de la P53 dans les 7 lignées du lymphome de Hodgkin, nous avons montré pour la première fois l'implication des microsatellites dans l'instabilité génomique des lymphomes de Hodgkin [MSI-H (microsatellite instability-high)] dans 3/7 lignées.

Pour l'instabilité chromosomique, nous avons montré que deux mécanismes favorisent l'émergence d'une instabilité chromosomique : le premier implique une instabilité télomérique qui est présente essentiellement dans les petites cellules tumorales induisant la formation des chromosomes dicentriques, des amplifications des gènes (JAK2 comme exemple) et des arrangements chromosomiques complexes. Le deuxième mécanisme est lié essentiellement à un défaut de réparation des cassures double-brin avec l'apparition des chromosomes dicentriques sporadiques et une fréquence importante des micronoyaux avec la formation des ponts anaphasiques sans séquence télomérique ni centromérique.

Nous avons trouvé que l'instabilité des télomères est liée essentiellement au scléro-nodulaire sous-groupe du lymphome de Hodgkin. Par ailleurs, les deux lignées à cellularité mixte présentent essentiellement un défaut de réparation des cassures doubles brins majeur.

L'introduction des nouvelles approches cytogénétiques nous a permis de classer tous les chromosomes et établir un caryotype précis ainsi que établir des arbres d'évolution de la cellule tumorale à partir d'une cellule diploïde.

Partie III

Au cours de cette troisième partie, nous avons montré l'établissement d'un modèle animale de xenogreffe sur des souris NOD-SCID-gammac^{-/-} (NSG) à partir de la ligne L428. Le pouvoir clonogénique des sept lignées étudiées précédemment a été évalué.

La clonogénicité de la lignée L428 est plus importante. Ce qui nous a permis d'établir des nouvelles lignées à partir des clones sélectionnées de la méthylcelluloses avec un pouvoir clonogénique plus important que la lignée parentale. Les doses de cellules injectées ainsi que la survie des souris ont été étudiées. Une meilleure reproductibilité de la prise de ce modèle a été montrée.

Ce modèle animal nous a permis une caractérisation des phénotypique des cellules clonogénique chez la souris. Ces cellules n'ont aucun marquage de lymphome de Hodgkin (CD30 et CD15 négative). Ces marqueurs de lymphome de Hodgkin apparaissent tardivement dans le développement progressif de la maladie.

Ce modèle, nous a permis d'établir des nouvelles lignées cellulaires à partir des tumeurs hépatiques. La caractérisation cytogénétique a mis en évidence des aberrations chromosomique commune entre la lignée parentale, le clone injecté et les cellules dérivant de la souris. L'efficacité de ce modèle été étudié en testant un nouveau alkylant agent inhibiteur de la déacétylase histone (HDACi).

Partie IV

Après l'étude des mécanismes impliqués dans l'instabilité génomique du lymphome de Hodgkin et l'établissement de ce modèle animal, il est important d'étudier les mécanismes de maintenance des télomères dans les ganglions tumoraux du lymphome de Hodgkin (38 patients) comme dans les lignées tumorales de lymphome de Hodgkin. Nous avons montré qu'il existe une cohabitation entre les deux

mécanismes importants de maintenance des télomères, l'activation de la télomérase d'une part et le mécanisme ALT (alternative lengthening of telomeres) d'autre part.

Nous avons identifié la présence de petites cellules dans les ganglions hodgkiniens comme dans les lignées tumorales avec une forte activité de la télomérase par contre la cellule de Reed Sternberg est caractérisée par une surexpression de la protéine PML avec une faible ou aucune activité de la télomérase. La fréquence des cellules télomérase ou ALT varie d'un ganglion à un autre et d'une lignée à une autre. Nous avons étudié la présence des cellules avec un profil ALT dans les ganglions et aussi dans les lignées tumorales du lymphome de Hodgkin. Il s'agit dans la majorité des cas des petites cellules exprimant plusieurs colocalisations du PML avec TRF2.

Cette cohabitation entre les deux mécanismes de maintenance des télomères dans le lymphome de Hodgkin montre que les mécanismes de maintenance des télomères dans le lymphome de Hodgkin n'est pas statique ni unique. L'hypermutableté des cellules tumorales du lymphome de Hodgkin pourrait être à l'origine de l'activation de ces deux mécanismes afin de permettre à ces cellules une viabilité.

Un drastique raccourcissement télomérique a été observé dans les cellules lignées exprimant la télomérase. Pour les cellules ALT, une grande hétérogénéité de la taille des télomères ainsi que la présence des chromosomes dicentriques sporadiques ont été détectées.

Le suivi des patients à long terme pendant plus que 10 ans, nous a permis d'établir une corrélation entre la fréquence des cellules avec plusieurs colocalisation entre PML et TRF2 (profil ALT) et la survenue de mortalités et de morbidités a été montré. L'étude multiparamétrique a montré que les patients EBV positifs avec une fréquence importante de PML/TRF colocalisation ainsi que des cellules macrophagiques (CD68) ont une survie nettement moins importante que les autres patients ($p=0.002$). Ces patients montrent aussi une morbidité importante par rapport aux autres patients ($p=0.0003$). De plus, l'étude de la radiosensibilité des lignées tumorales du lymphome de Hodgkin a montré que les lignées avec une

prédominance ALT sont plus résistantes que les lignées à prédominance télomérase.

L'originalité de ce travail consiste de montrer l'adaptation du système de maintenance des télomères de la cellule tumorale au cours de sa progression dans le lymphome de Hodgkin sans oublier la valeur pronostiques de la présence des cellules ALT avec un microenvironnement spécifique (EBV positif et présence des macrophages) dans la suivi des patients.

Partie V

Cette dernière partie est la validation de tous les concepts montrés précédemment sur l'instabilité génomique, le pouvoir clonogénique des cellules négatives CD30 mais aussi la maintenance des télomères. Nous montrons dans cette partie que les cellules CD30- ont un pouvoir clonogénique important d'une part et une capacité de rétablir la lignée d'origine d'autre part. Également ces cellules ont un pouvoir important de greffe chez la souris. Nous avons obtenu une efficacité importante de xenogreffe à partir de 1000 cellules injectées. La caractérisation des cellules sortant de la souris montre une présence des cellules diploïdes à l'origine de progression tumorale sans aucun marqueur de lymphome de Hodgkin.

Cette cellule clonogénique diploïde est caractérisée par des longs télomères par rapport à la cellule de Reed-Sternberg mais par des anomalies télomériques importantes (perte, délétions et dédoublement des télomères). Cette cellule exprime fortement la télomérase et en fonction de sa progression et sa différenciation elle met en route une autre voie de maintenance des télomères qui est la voie ALT en plus de la télomérase.

Une augmentation de la fréquence des dicentriques au cours de cette transformation a été montrée sans avoir une augmentation des fragments acentriques. Ce qui nous laisse penser que ces dicentriques sont issues de fusion entre deux chromosomes suite à une perte de télomères.

L'utilisation des sondes subtélomériques, nous a montré la présence des séquences subtélomériques dans le point de cassure de ces dicentriques. Cette instabilité

génomique détectée précocement dans ces cellules pourrait être un évènement initial de la transformation de la cellule clonogénique en une cellule de Reed-Sternberg.

La complexité des mécanismes de maintenance des télomères dans les cellules en voie de transformation à partir de la cellule clonogénique est due certainement à une hypermutabilité de ces cellules.

Ce travail a le mérite de faire avancer nos connaissances sur les lymphomes de Hodgkin sur plusieurs plans :

1. Les mécanismes impliqués dans l'instabilité génomique du lymphome de Hodgkin : nous montrons le rôle important du dysfonctionnement des télomères dans l'initiation et la progression de cette instabilité génomique.
2. Le déficit de système de réparations des cassures double brin joue également un rôle dans cette instabilité
3. Les mécanismes de maintenances des télomères impliqués dans le lymphome de Hodgkin sont multiples. Nous avons étudié les deux plus connus : télomérase et ALT (alternative lengthening of telomere). Nous avons montré que les cellules clonogéniques au cours de leurs différenciations et de leurs progressions activent ces deux mécanismes au même. Cette constatation est unique actuellement dans le domaine de maintenance des télomères.
4. La caractérisation phénotypique et cytogénétique des cellules clonogéniques montrent que ces cellules ne présentent pas les marqueurs du lymphome de Hodgkin. ces données pourraient nous permettre de comparer ces cellules avec les cellules souches cancéreuses trouvées dans d'autres types de lymphome (lymphome de manteau) ou d'autres types de pathologies.
5. La caractérisation de cette cellule clonogénique en vue d'une identification dans les ganglions tumoraux sera d'une grande importance non seulement dans le traitement des patients en rechute mais aussi dans la surveillance de ces patients

6. La présence d'une activité de la télomérase dans les lymphocytes circulant de patients Hodgkin pourrait être un marqueur de la présence de ces cellules dans les lymphocytes circulant.

Certainement, ce travail a le mérite d'être continué afin de mieux caractériser ces cellules clonogéniques en utilisant d'autres techniques plus spécifiques comme le CGH-array avec une grande résolution en travaillant davantage sur la caractérisation des télomères dans cette pathologie.

Une étude sur les polymorphismes des télomères dans la forme familiale du lymphome de Hodgkin sera d'une grande importance pour éclaircir le rôle des télomères dans cette pathologie.

Il est aussi important de tester des inhibiteurs d'ALT via la voie ATR afin de valider l'implication de cette voie dans la résistance au traitement.

Ces données auront une retombée clinique importante non seulement dans la compréhension et le traitement des lymphomes de Hodgkin mais aussi dans d'autres pathologies malignes.