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TELOMERASE IN HEMATOLOGICAL AND OTHER MALIGNANCIES: THERAPEUTIC IMPLICATIONS

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Telomerase in hematological and other malignancies: Therapeutic implications

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The Telomere is a nucleoprotein complex consisting of TTAGGG repeated sequences covered by specialized binding proteins termed shelterins masking the end of linear chromosomes and thereby protecting the end of the chromosome from genomic instability. Telomeres become progressively shortened during cellular replication and acts as a mitotic clock to confer a limited lifespan to normal cells. Telomerase reverse transcriptase (TERT) is responsible for lengthening telomeric DNA. The enzyme is silent in most normal cells due to the transcriptional repression of the *TERT* gene encoding the telomerase catalytic component. Numerous studies have demonstrated that the induction of TERT and subsequent activation of telomerase is prerequisite to malignant transformation of human cells through a telomere-lengthening mechanism. Moreover, evidence has recently shown that TERT or telomerase possesses many other biological activities contributing to tumor development and progression, therefore targeting TERT/telomerase has been suggested as a novel anti-cancer strategy. In the present project we studied telomere-lengthening-dependent and independent activities of TERT in malignant cells and explored therapeutic implications of TERT inhibition combined with other anti-cancer strategies in acute myeloid leukemia (AML) and gastric cancer.

Paper I is focused on whether TERT inhibition and telomere dysfunction is involved in the anti- tumor effect of the DNA methyltransferase inhibitor 5-azacytidine (5-AZA). We demonstrated that 5-AZA induced DNA damage and telomere dysfunction in AML cell lines coupled with diminished TERT expression, telomere attrition and cellular apoptosis. The results suggests that 5-AZA-mediated TERT inhibition and telomere dysfunction contributes to its anti-cancer activity.

Paper II was aimed to define the relationship between the oncogenic cyclooxygenase (COX2) and TERT and to evaluate the synergetic anti-cancer action of simultaneous COX2 and TERT inhibition. We found that the depletion of TERT led to elevated COX2 expression by activating p38. The COX2 inhibitor celecoxib or TERT inhibition alone was insufficient to affect cell viability, however, the combination synergistically killed cancer cells both in vitro and in vivo. Thus, the combined application of COX2 and telomerase inhibitors may be more efficient in cancer treatment.

About 30% of AML patients exhibit somatic mutations of FMS-like tyrosine kinase 3 (FLT3), the majority of which carry internal tandem duplication (ITD) in the juxtamembrane. FLT3 inhibitors have been developed for AML treatment. In paper III, we determined whether FLT3-ITD regulated TERT expression and whether TERT affected the therapeutic efficacy of FLT3 inhibitors. We found that the FLT3 inhibitor PKC412 down-regulated TERT expression in a MYC-dependent manner, while ectopic expression of TERT attenuated killing efficacy of PKC412 in AML cells.Altogether, our findings demonstrated that the interplay between TERT and FLT3ITD plays important roles in AML carcinogenesis and that FLT3 inhibitors, when combined with TERT inhibition, are more efficient in the induction of AML cell apoptosis.

In paper IV, we determined the effect of bortezomib on telomere homeostasis and its functional consequences. Bortezomib treatment inhibited TERT and telomerase expression, dysregulated shelterin proteins and shortened telomeres in AML and gastric cancer cell lines. The disrupted telomere structure triggered DNA damage response and cellular apoptosis. TERT overexpression significantly decreased DNA damage and telomere dysfunction and attenuated apoptosis mediated by bortezomib. Our findings collectively reveal a profound impact of bortezomib on telomere homeostasis/function, and down-regulation of TERT expression and telomere dysfunction induced by bortezomib thus both contributing to its cancer cell killing actions.

In summary, the present results provide novel insights of the biological functions of TERT/telomerase in malignant cells. In addition, the finding that the combination of telomerase inhibition with other anti-cancer agents induced a robust and synergistic anti-tumor effect may have future important clinical implications

LIST OF SCIENTIFIC PAPERS

Ⅰ: Xiaolu Zhang*, **BINGNAN LI***, Nick de Jonge, Magnus Björkholm, Dawei Xu (*Equal contribution)

The DNA methylation inhibitor induces telomere dysfunction and apoptosis of leukemia cells that is attenuated by telomerase over-expression

Oncotarget, 2015, 6, 4888-4900.

Ⅱ: Tiantian Liu, Xiuming Liang, **BINGNAN LI**, Magnus Björkholm, Jihui Jia, Dawei Xu

Telomerase reverse transcriptase inhibition stimulates cyclooxygenase 2 expression in cancer cells and synergizes with celecoxib to exert anti-cancer effects

British Journal of Cancer, 2013, 108, 2272-2280.

Ⅲ: Xiaolu Zhang*, **BINGNAN LI***, Jenny Dahlström, Anh Nhi Tran, Jihui Jia, Magnus Björkhom, Dawei Xu (*Equal contribution)

MYC-dependent down-regulation of telomerase reverse transcriptase (hTERT) by FLT3 inhibitors is required for their therapeutical efficacy on acute myeloid leukemia (AML)

In manuscript.

Ⅳ: Xinyu Ci, **BINGNAN LI**, Xueping Ma, Feng Kong, Chengyun Zheng, Magnus Björkholm, Jihui Jia, Dawei Xu

Bortezomib-mediated down-regulation of telomerase and disruption of telomere homeostasis contributes to apoptosis of malignant cells.

Oncotarget, 2015, advanced pulications, 2015

References to these studies will be made by the above Roman numerals

Table of Contents

ABBREVIATIONS

5-AZA	5-aza-2’-deoxycytidine
AKT	Protein kinase B
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BRCA1	the breast cancer 1, early onset
CAB	Cajal box motif
cAMP	Cyclic adenosine monophosphate
CBFB	Core-Binding Factor, Beta Subunit
CEBPA	CCAAT/Enhancer Binding Protein (C/EBP), Alpha
CDKN2A	Cyclin dependent kinase inhibitor 2A
COX	Cyclooxygenase
CP	Ciliated protozoan motif
CR	Complete remission
CSF	Colony-stimulating factor
DDR	DNA damage response
DEK	DEK Proto-Oncogene
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DNMTI	DNA methyltransferase inhibitor
DOK3	Docking Protein 3
dNTP	Deoxy-ribonucleotide triphosphate
DSB	Double strand break
EGFR	Epidermal growth factor receptor
ERK	Extracellular-signal-regulated kinases
EVI1	Ecotropic virus integration site 1 (protein homolog)
FAB	French-American-British
FBS	Fetal bovine serum
FL	FLT3 ligand
FLT3	Fms-like tyrosine receptor 3
FISH	Fluorescence in situ hybridization
HER2	Human epidermal growth factor 2

ITD	Internal tandem duplication
JM	Juxtamembrane
KL	Kit ligand
IκB	Inhibitors of NF-κB
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic Syndrome
MKL1	Megakaryoblastic Leukemia (Translocation) 1
MLH1	MutL homolog 1
MLLT3	Myeloid/Lymphoid Or Mixed-Lineage Leukemia; Translocated To, 3
MMLV	Moloney murine leukemia virus
MPN	Myeloproliferative neoplasm
MYH11	Myosin, Heavy Chain 11
NHEJ	Non-homologous end joining
NID-1	Nidogen-1
NK cell	Natural killer cell
NPM1	Nucleophosmin (Nucleolar Phosphoprotein B23, Numatrin)
NUP214	Nucleoporin 214kDa
OB-fold	Oligonucleotide/oligosaccharide-binding fold
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K/AKT	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PML	Promyelocytic leukemia
qRT-PCR	Quantitative Real-time PCR
RARA	Retinoic Acid Receptor, Alpha
RAP1	Repressor/activator protein 1
RBM15	RNA Binding Motif Protein 15
RNA	Ribonucleic acid
RPN1	Ribophorin I
RT	Reverse transcription
RTK	Receptor tyrosine kinase
RUNX1	Runt-Related Transcription Factor 1
RUNX1T1	Runt-Related Transcription Factor 1; Translocated To, 1 (Cyclin D-Related)
siRNA	Small interfering RNA

snRNA	Small nuclear RNA
SULF2	Sulfatase 2
TCAB1	Telomerase Cajal body protein 1
TEN	Telomerase essential domain
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TKI	Tyrosine kinase inhibitor
TIF	Telomere-induced foci
TIN2	TRF1-interacting nuclear protein 2
TPP1	TIN2 interacting protein 1
TRBD	TERT RNA binding domain
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
UPS	Ubiquitin dependent Proteolysis System
UTR	Untranslated region
VHL	Von Hippel-Lindau tumor suppressor
WDR79	Telomerase Cajal body protein 1
WHO	World Health Organizati

1 INTRODUCTION

1.1 Acute Myeloid Leukemia (AML)

1.1.1 Definition

Acute myeloid leukaemia (AML) is a malignant disorder characterized by abnormal hematopoietic cell growth and differentiation. The expansion of immature myeloid cells (myeloblasts) in the bone marrow and peripheral blood occurs at the expense of the normal production of their terminally differentiated counterparts such as red cells, platelets and neutrophils[1,2]. The clinical presentation of AML varies from an incidental finding on a routine blood test through to a life-threatening illness requiring immediate intervention. AML in the Western world is characterized by a slight male predominance and a median age at diagnosis of approximately 70 years. In AML excluding acute promyelocytic leukemia (APL) the age-specific incidence rates rise gradually from around 40-50 years and more steeply from around age 60-64 years.

1.1.2 Classifications

There are two main systems that are used for classification of AML. In 1976, the French-American-British (FAB) proposed the first classification system for AML based on leukemic blast morphology, lineage differentiation direction and the maturation degree[3] (Table 1). This proposal was modified in 1985 and clinically used until the World Health Organization (WHO) released a new classification system which emphasizes more on the prognostic relevance of genetic abnormalities and classifies the AML based on genetic alterations, immunophenotypic changes, biological and clinical features[4] (Table 2). The WHO classification was revised in 2008 (Table 3).

Table 1. FAB classification of AML

FAB subtype	
M0	Undifferentiated AML

M1	AML without maturation
M2	AML with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia (AMML) subtype M4 eos: Acute myelomonocytic leukemia with over 5% eosinophils
M5	Acute monocytic Leukemia Subtype M5a: Acute monoblastic Leukemia M5b: Acute monocytic Leukemia
M6	Acute erythroblastic Leukemia
M7	Acute megakaryoblastic Leukemia

Table 2. WHO classification of AML in 2001

1 AML with genetic abnormalities
AML (M2) with t(8;21) translocation
AML (M4eos) with a translocation or inversion of chromosome 16 (AMML Eos)
AML with chromosome 11 abnormalities (secondary AML)
AML (M3) with t(15;17) or rarely t(11;17) translocation (APML)
2 AML with multilineage dysplasia
With dysplasia in at least 50% of cells in at least two bone marrow cell lines
3 Secondary AML
Related to previous chemotherapy or radiation treatment
4 AML not otherwise specified

AML that doesn't fall into one of above groups, similar to FAB classification: Undifferentiated AML(M0), AML with minimal maturation (M1), AML with maturation (M2), AMML (M4), AMoL (M5), Acute Erythroid Leukemia (M6), Aute Megakaryoblastic leukemia (M7), Acute Basophilic leukemia, Acute Panmyelosis with fibrosis, Myeloid sarcoma
5 Undifferentiated or biphenotypical acute leukemias
Both lymphoid and myeloid features

Table 3. WHO revised AML classification in 2008

1 Acute Myeloid Leukemia with recurrent genetic abnormalities
AML with t(8;21)(q22;q22), RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or with t(16;16)(p13.1;q22); CBFB-MYH11
APL with t(15;17)(q22;q12); PML-RARA
AML with t(9;11)(p22;q23); MLLT3-MLL
AML with t(6;9)(p23;q34); DEK-NUP214
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1
AML (Megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
2 Acute myeloid Leukemia with myelodysplasia-related changes
3 Therapy related myeloid neoplasms
4 Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation

AML with maturation
Acute Myelomonocytic leukemia
Acute Monoblastic/Monocytic Leukemia
Acute Erythroid Leukemia
Pure Erythroid Leukemia
Erythroleukemia, erythroid/myeloid
Acute Megakaryoblastic Leukemia
Acute Basophilic leukemia
Acute Panmyelosis with myelofibrosis
5 Myeloid Sarcoma
6 Myeloid proliferations related to Down syndrome
Transcient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
7 Blastic plasmacytoid dendritic cell neoplasm

1.1.3 Treatment

Combination chemotherapy is the cornerstone of treatment and approximately 80% of patients below 60 years at diagnosis reach a complete remission (CR) which is essential for prolonged survival of AML patients.[2] Induction therapy usually include (one or two courses) three days of an anthracycline and five to seven days of cytarabine to achieve CR. CR is defined as less than 5% blasts in the bone marrow, and normal values for absolute neutrophil count (>1.0 x 10^9/L) and platelet count (>100 x 10^9/L), and independence from red cell transfusion[5]. Postremission therapy often include consolidation therapy and allogeneic or sometimes autologous hematopoietic stem cell transplantation. AML is now cured in 35 to 40% of adult patients who are 60 years of age or younger at diagnosis and in 5 to 15% of patients who are older than 60 years of age.

AML is characterized by an enormous molecular heterogeneity and the prognostic importance of this biologic heterogeneity is well accepted. However, only recently has the translation of this new information slowly led to improved/targeted therapy.

1.1.4 FMS-Like tyrosine kinase (FLT3) mutations in AML

FLT3 is a type III Receptor tyrosine kinase (RTK) expressed in hematopoietic stem cells in the bone marrow. FLT3 facilitates cell growth, proliferation and differentiation of primitive hematopoietic progenitor cells[6]. FLT3-ITD is the somatic mutation of the FLT3 in the juxtamembrane domain and observed in approximately 20-25% of AML patients[7-11]. The juxtamembrane domain is autoinhibitory, and thus the ITD mutation disrupts this autoinhibitory function and constitutively activates the enzyme[12,13]. FLT3-ITD is considered as driver in the development of AML and a predictor for poor outcome in AML patients.[7-11,14-19]

1.1.5 FLT3 inhibitors

In recent years, small-molecule tyrosine kinase inhibitors (TKIs) have been synthesized to target FLT3 and are now under evaluation in clinical trials for induction chemotherapy and postremission therapy[20,21]. These TKIs include tandutinib[22], lestaurtinib[23], midostaurin[24], sorafenib[25], sunitinib and quizartinib. Resistance to treatment is always a major obstacle in AML therapy[26-28]. With respect to FLT3 inhibition the underlying mechanism involves the occurrence of new mutations in the FLT3 genomic region. However, these new mutations were only detected in a part of FLT3TKI resistant patients.

1.2 Telomere

1.2.1 Telomere structure and shelterin proteins

Human telomeres are composed of tandem repeats of TTAGGG DNA sequences, usually extending 10-15kb. Telomeres are double stranded repeats with a single strand 3’ G rich tail. The overhang can then invade the double strand telomere tract and forms a lasso-like structure termed telomeric loop (T-Loop)[29,30]. The single strand tail acts as the docking site for binding protective capping proteins, like POT1 (see below), to maintain genomic stability (Figure 1).

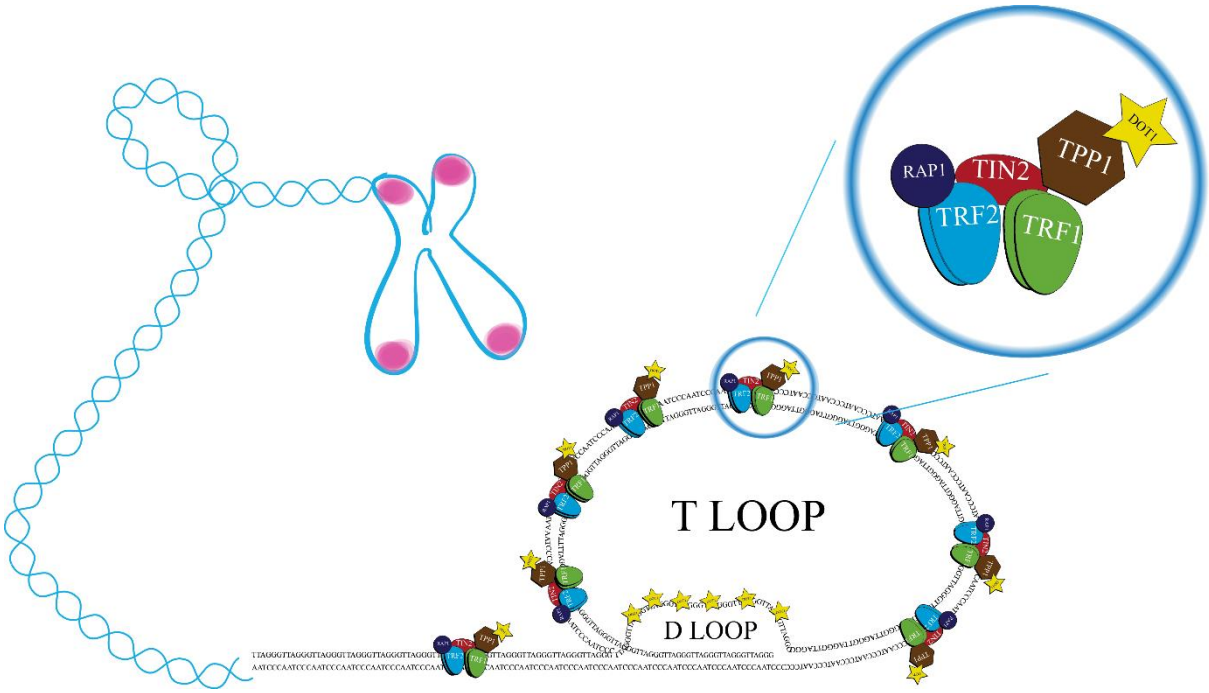

Figure 1. Telomere protects the chromosome end and shelterin proteins bind to the telomere

Human telomeres are bound by a specific group of six proteins, termed shelterin proteins: telomere repeat binding factor 1 (TRF1) and 2 (TRF2), protection of telomeres 1 (POT1), repressor/activator protein 1 (RAP1)[31], TRF1-interacting nuclear protein 2 (TIN2) and TIN2 interacting protein 1 (TPP1)[32,33]. The proper combination of shelterin proteins as well as the sufficiently long telomere protect chromosome ends from DNA damage, degradation, genomic destabilization[34] and genomic recombination or fusions[35]. The shelterin proteins form the bridge to bring together the binding proteins of ssDNA and dsDNA and protects the chromosome end from being recognized as DNA damage or breaks. TRF1 and TRF2 bind to the double strand telomere, each of them contains the TRFH domain for homodimerization[36] and Myb domain for dsDNA telomere binding[37-39].

POT1 binds the single strand telomere part with the DNA-binding domain (DBD) (Figure 2), formed by two oligonucleotide/oligosaccharide-binding fold (OB-fold) elements[40-45]. The further formation of the ternary complex with TPP1 enhances the interaction[46,47]. The crucial bridging unit TIN2 not only joins the TRF1 and TRF2 but also puts TRF1, TRF2, POT1 and TPP1 together. As TRF1 cannot heterodimerize or interact with TRF2, the linking role of TIN2 seems necessary. N-terminal of TIN2 recruits TPP1 to the telomere by binding its C-terminal[48-52].

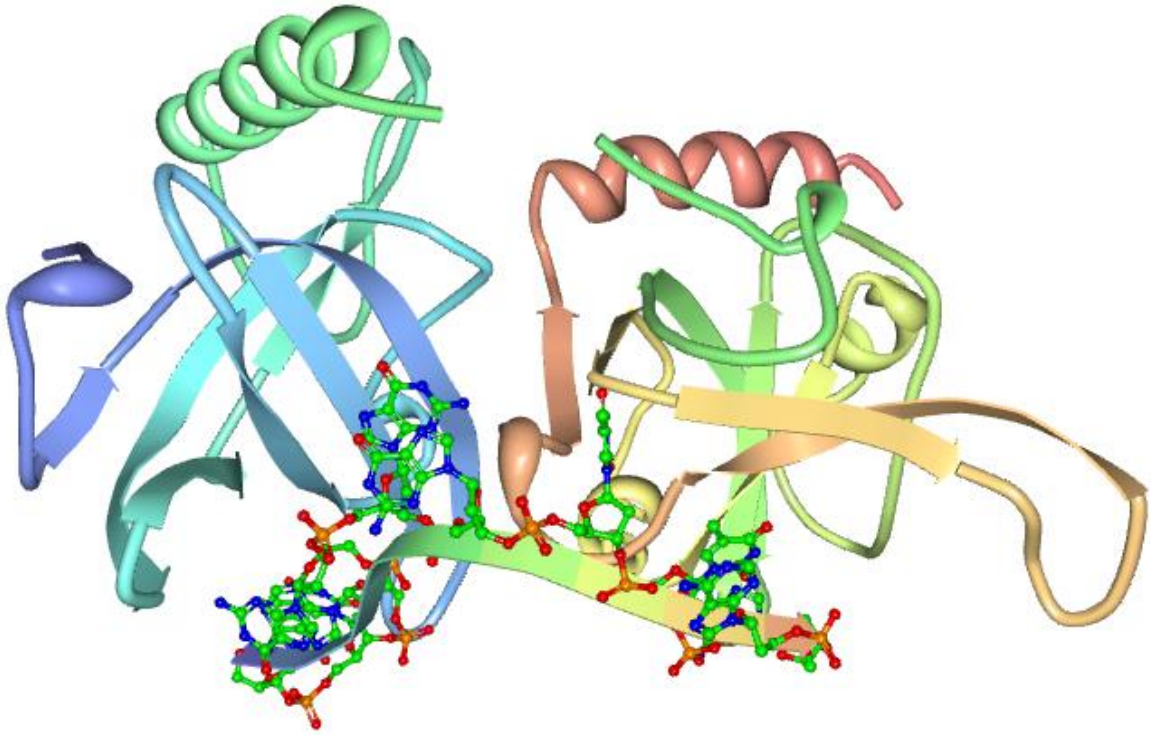

Figure 2. POT1 binds to single strand DNA of telomere[53]

1.2.2 Hayflick limit and end replication problem

Fifty years ago, Hayflick and Moorhead discovered that the proliferative capacity of somatic cells like normal human fibroblast cells were intrinsically limited[54]. After about 60-80 doubling cycles, cultured fibroblast cells stop dividing and adopt a senescent phenotype. This proliferative limit, known as hayflick limit or replicative senescence[55-57], is mainly caused by over-shortened telomeres. On the other hand, the DNA polymerase can only synthesize DNA from 5' to 3', incapable of filling the gap left behind by the 5' end, which leads to chromosome end replication problem[58].

1.2.3 Telomere function, genomic instability and cancer

Telomere conceals the linear chromosome from being detected as DNA Double Strand Break (DSB). Critical telomere shortening and disruption in telomere structure are important causes of genomic instability. When telomere length reaches below the minimal functional length, or

the telomere become uncapped, it will be recognized as the DSB, and thereby DNA damage response (DDR) is triggered[59]. The uncapped telomere will be repaired by the homologous recombination or NHEJ (non-homologous end joining) and leads to end-to-end fusion, which could be achieved, via either classic non-homologous end joining (C-NHEJ) or alternative non-homologous end joining (A-NHEJ) or other alternative pathways[60-62]. The end-to-end fusion between sister or other different chromatins leads to breakage-fusion-bridge cycle. The breakage-fusion-bridge cycle can result in deletions, amplifications and rearrangements of chromosomes that are often observed in cancer cells. This cycle will continue until the telomere healing occurs.

1.2.4 Telomere dysfunction

Studies have demonstrated that the shortest telomere length rather than traditionally thought the average telomere length determines the cell response. In human fibroblasts, the threshold of senescence is about five repeats short telomeres. Critically short telomeres would be dysfunctional and elicit the DNA damage repair activation. Phenotypically, co-localization of the telomere with the DNA damage repair proteins like 53BP1 or rH2AX would be expected.

1.3 Telomerase

1.3.1 Telomerase and its components

Telomerase is a specialized DNA polymerase responsible for synthesizing TTAGGG DNA repeats onto the chromosome ends. Telomerase holo-enzyme consists of two conserved components: the core catalytic protein TERT[63,64] and the RNA template TERC. TERT and TERC are assembled in the Cajal body to form the functional ribonucleoprotein enzyme complex[65] (Figure 3). By synthesizing telomeric DNA (tandem repeated TTAGGG sequence), telomerase compensates for the gradually lost telomere and provides solutions to the chromosome end replication problem. Telomerase expression is low in most somatic cells[66], while high in germline and stem cells[67].

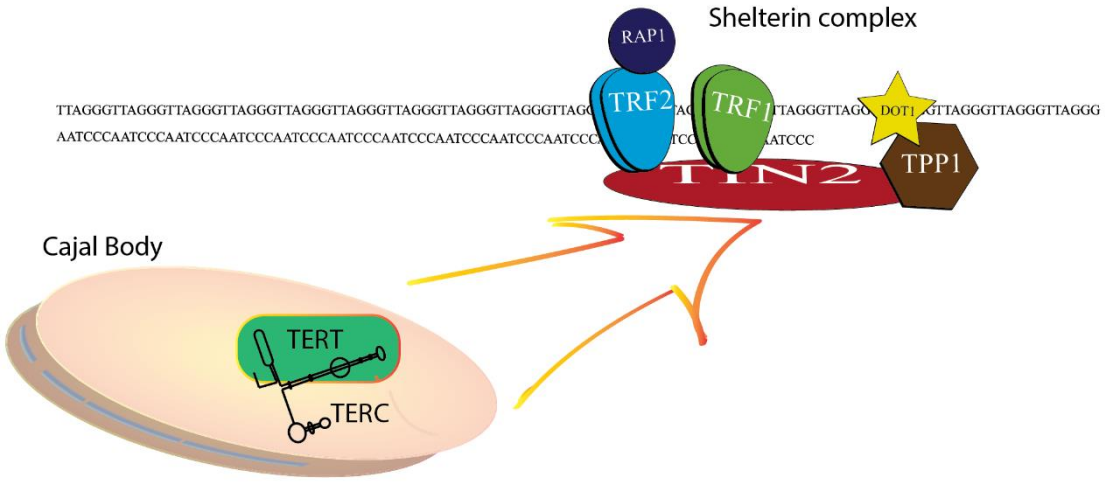

Figure 3. Telomerase is assembled in Cajal body and delivered to the telomere

1.3.2 TERT

The reverse transcriptase subunit TERT contains about 1000 amino acids[68] and contains 3 major domains: the telomerase essential domain (TEN), TERT RNA binding domain (TRBD), and the reverse transcriptase domain with finger, palm and thumb[63]. The fingers and palm recognize the RNA arm and fit the template into catalytic center. The thumb recognizes and stabilizes the DNA primer. The T and CP pockets bind the template boundary element to ensure repeat addition according to the RNA template only[69]. The TEN domain anchors on the telomeric DNA and uses the inside DAT motif for telomerase recruitment[70,71].

1.3.3 TERC

The human TERC templates for telomere synthesis is 451 nucleotide long[72]. TERC has a template boundary element that limits reverse transcriptional range[73-75]. In the site of the catalytic center, the triple helix and pseudoknot orientate the primer-template and plays a certain catalytic role[76-78]. TERC also functions by loading the template into active center and aid template relocation during multi rounds of repeat addition[79,80]. TERC binds to telomerase by the template-pseudoknot domain and CR4-CR5 domain[81-83]. The distinct RNA structure and elements of TERC provide possibility of telomerase accessory protein binding. H/ACA box on 3' end of TERC could bind dyskerin, NOP10, NHP2 and GAR1[84,85]. Another important motif on the 3' end is Cajal box motif (CAB), which binds telomerase Cajal body protein 1

(TCAB1/WDR79) and localizes the TERC into the Cajal body for assemble of telomerase package.

1.3.4 Telomerase inhibition in cancer

Telomerase plays very important roles in carcinogenesis and thus, inhibition of telomerase should be considered as one anti-cancer approach. Several telomerase inhibitors have been developed such as BIBR1532, GRN163 and GRN163L. Recently, GRN163L (imetelstat) has been shown to deliver fast and durable hematologic effects in essential thrombocythemia patients[86].

1.4 DNA methylation

1.4.1 Chemical nature of DNA methylation

DNA methylation occurs at 5' carbon of the cytosine ring by covalent addition of a methyl group, which occurs at the major group of the genomic DNA strand and blocks the transcription[87].

DNA methylation commonly takes place in cytosine in CpG dinucleotides. These CpG dinucleotide clusters in gene promoter regions, where transcription is initiated, silence the genes or non-coding genomic regions[88].

In normal cells, heterochromatin like pericentromere regions are heavily methylated and transcriptionally silenced. In females, the inactivated X chromosome is also heavily methylated. Besides, some imprinted genes that only express maternal or paternal alleles also rely on DNA methylation[88,89].

1.4.2 DNA methyltransferase (DNMT)

DNA methylation relies on DNMTs, with three major isforms: DNMT1, DNMT3A and DNMT3B. DNMT1 is mainly involved in the maintenance of existing methylation pattern, while DNMT3A and DNMT3B target unmethylated nucleotides including CpG sites, which is enriched in CpG islands, and large repetitive sequences like centromeres and retrotransposon elements.

1.4.3 DNA methylation in cancer

Cancer epigenome is characterized by altered DNA methylation patterns. Cancer cells manifest global hypomethylation and local hypermethylation. DNA hypomethylation in cancer occurs in repetitive sequences, low density CpG regions[90-97], retrotransposons, lamin associated domains[98-101], introns, etc. This hypomethylation will lead to genomic instability through chromosome rearrangement and translocation to other genomic regions. Hypomethylation also leads to activation of oncogenes and losses of genomic imprinting.

Another apparent epigenetic change in cancer is the hypermethylation in CpG island promoter regions[102-104] which silences the tumor-suppressor genes, such as the breast cancer 1, early onset (BRCA1), CDKN2A/p16INK4A (cyclin dependent kinase inhibitor 2A), MLH1 and VHL, and CpG island shores[105-107]. Hypermethylation occurs in hematopoietic malignances like myelodysplastic syndromes (MDS) and contributes to leukemic transformation[108-112].

1.4.4 DNA methylation inhibitors

The DNA methylation inhibitors include 5-azacitidine (5-AZA)[113], decitabine, zebularine[114], NPEOC-DAC[115], CP-4200[116,117], and RX-3117[118-120]. They induce the recovery of methylation silenced genes in cancer and lead to apoptosis and growth arrest in cancer cells. Both 5-AZA and decitabine have shown positive clinical effects in subsets of AML and MDS patients[121].

1.5 The ubiquitin dependent proteolysis system (UPS) and proteasome inhibition in cancer

1.5.1 UPS function

The UPS is the main player in regulated protein degradation in prokaryotic cells and functions critically in regulation of variety of pathways, including apoptosis[122], proliferation[123], DNA repair[124,125], transcription[126], etc. UPS initializes an enzymatic cascade via minimal of Ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3 activities. Ubiquitin-activating enzyme E1 forms a highly energetic thioester bond between glycine residue of ubiquitin and cysteine residue within E1 itself, utilizing energy from the ATP

hydrolysis. This energized ubiquitin is then transferred to the cysteine of the ubiquitin conjugating enzyme E2. Finally, the E2 cooperates with E3, the ubiquitin ligase, to transfer this high energy ubiquitin to the lysine of target protein. This initial ubiquitin acts as the acceptor for further ubiquitin modification and forms a polyubiquitin chain onto the target protein. The ubiquited proteins are then targeted towards the proteasome. This proteasome is a large complex responsible for degradation of large variety of intracellular proteins.

1.5.2 Proteasome inhibition in cancer

The proteasome pathway takes part in cell proliferation and anti-apoptosis, especially involving the pro-oncogenic NF-κB pathway, which plays vigorous roles in many cancer types. IκBs retains the NF-κB within cytoplasm and keeps it inactive. When polyubiquitylated, polyubiquitinated IκBs will be degraded by the proteasome and thus unleash the NF-κB. The proteasome inhibitor bortezomib (Velcade/PS-341)[127-132] targets the active sites of 20S proteasome reversibly and prevent the degradation of IκB, thus holding NF-κB in leash[133]. The inhibition of NF-κB reduces the pro-inflammation gene expression and results in elevated expression of the cyclin-dependent kinase inhibitors $P21^{Cip1}$ and $P27^{Kip1}$, leading to apoptosis of tumor cells. Aside from this, evidence shows that bortezomib also induces endoplasmic-reticulum stress thereby increasing cell death.

1.6 Cyclooxygenase (COX), COX2 inhibition and cancer

1.6.1 Cyclooxygenase

Cyclooxygenase (COX) is a key enzyme responsible for inflammation, converting arachidonic acid to prostaglandin and thromboxane[134]. COX has at least two isoforms, COX-1[135] and COX-2[136]. While COX-1 is constitutively expressed in most tissues for maintaining physiologic homeostasis, COX-2 is induced by inflammatory stimuli but not normally present except in CNS[137] and kidneys[138] including cytokines and growth factors. Studies have shown that COX-2 contributes to cancer development and progression in various types of malignancy.

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1.6.2 COX2 inhibitors and cancer

COX2 plays an important role in suppressing apoptosis and promoting cell survival via PI3K/AKT[139,140], ERK[141,142], cAMP[142], EGFR[143,144], RAS-MAPK signaling[145], COX2/PGE_2/EP4 axis[146] and promote tumor survival. Thus, COX2 specific inhibitors[147-149] have been utilized in cancer therapy and some in clinical trials, such as celecoxib[150,151], rofecoxib[152-155], nimesulide, etc.

2 AIMS OF THE STUDY

The overall objective of the present study is to define the role of telomerase in oncogenesis and to identify potential novel therapeutic strategies for human malignancies. Specifically, the study is aimed at

- probing the effect of the DNA methylation inhibitor 5-AZA on telomere function, and exploring mechanisms of DNMT inhibitors' action
- defining the effect of TERT inhibition on COX2 expression and exploring the synergistic anti-tumor efficacy of COX2 and TERT inhibition;
- probing the functional interplay between FLT3-ITD and telomerase in AML;
- exploring the mechanism underlying the anti-cancer effect of bortezomib and its effect on telomere homeostasis and function.

3 MATERIALS AND METHODS

3.1 Cell culture

Cells grew in 10% fetal calf serum-containing RPMI-1640 (Life Technologies, Paisley, Scotland, UK) with addition of 2 mM L-glutamine and antibiotics (50 mg/mL penicillin, and 50 mg/mL streptomycin) in a humid atmosphere at 37°C/5% CO_2.

In PAPER I, AML cell lines KG1A and HEL were used.

Human gastric cancer cell lines AGS (ATCC, 2007) and BGC-823 (Beijing Cancer Institute, China, 2008), the cervical cancer cell line HeLa (ATCC, 2001), normal human skin fibroblasts and osteosarcoma cell line U2OS were used in PAPER Ⅱ.

In PAPER Ⅲ,FLT3-ITD-harboring AML cell lines MV4,11 and MOLM-13, acute promyelocytic leukemia cell line HL60 and cervical cancer cell line were used.

In PAPER Ⅳ, the leukemia cell line HEL and the gastric cancer cell line BGC-823 were cultured.

3.2 Primary AML cell separation and culture

Primary AML cells were derived from five AML patients. Peripheral blood was drawn into heparinized glass tubes and leukemic cells were isolated by Lymphoprep gradient centrifugation (Nycomed, Oslo, Norway), and subsequently incubated in complete medium in the absence or presence of 5-AZA as described above.

3.3 Small interfering RNA transfection

Chemically modified Stealth control and TERT siRNAs were bought from Invitrogen.

Cells were incubated in 6-well plates, with density 1.0×10^5 cells per well, overnight and then transfected with siRNA (sequence in table) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

Table 4. SiRNA sequence

	Sequence
Control	50 -CCUACAUCCCGAUCGAUGAU- GUUGA-30
TERT	50 -AGGCACUGUUCAGCGUGCU- CAACUA-30

3.4 Lentivirus infection

To knock down TERT expression, we infected cells with a lenti-viral vector encoding TERT siRNA (Invitrogen) and the cells were maintained in puromycin-containing medium (2μg/ml) 48h post infection

A lenti-III-HA-GFP-TERT vector was constructed and a Lenti-BMN-GFP vector (a gift from Rudbeck Laboratory, Department of Immunology, Genetics and Pathology of Uppsala University) was used as control. The lentiviral vector was packaged in 293FT cells and supernatant collected to infect HEL cells. The cells were selected using puromycin (2 μg/ml).

3.5 Drug treatment

To inhibit COX2, COX1 and p38 mitogen-activated protein kinase (MAPK) activity, BGC-823 cells were treated with the specific COX2 inhibitor celecoxib (50 μM), aspirin (0.4 mM) and p38 MAPK inhibitor SB203580 (10 or 20 mM) for different time periods (all from Sigma-Aldrich, St Louis, MO, USA), respectively.

The DNA methylation inhibitor 5-AZA was bought from Sigma-Aldrich (St. Louis, USA) and exponentially growing cells were cultured in the presence and absence of 5-AZA (0, 0.5, 1, 2 and 5 μM) for up to 144 hours or 6 days. Culture medium was replaced with freshly prepared 5-AZA-containing medium every second day.

The specific FLT3 inhibitor PKC412 (Sigma-Aldrich, Buchs, Switzerland) was diluted in DMSO and cells were incubated with different concentrations of PKC412 for various time periods.

Bortezomib was bought from Selleck Chemicals (Houston, TX, USA) and exponentially growing cells were incubated with bortezomib at different concentrations for various time periods.

3.6 RNA extraction, reverse transcription and quantitative real-time PCR (qPCR)

Total cellular RNA from cells with different treatments was extracted using Trizol (Life Technology, Paisley, Scotland, UK).

cDNA was synthesized using random primers (N6; Amersham, Buckinghamshire, UK), and M-MLV reverse transcriptase.

qPCR was carried out in an ABI7700 sequence detector (Applied Biosystems, Foster City, CA, USA) using SYBR Green kit (Applied Biosystems) and the following primers:

TERT, β2-M in PAPER I;

TERT, COX2, COX1, β2-M in PAPER Ⅱ;

TERT, c-MYC, c-KIT, DOK3, NID-1 and SULF2 in PAPER Ⅲ;

TERT, TERC, TRF1, TRF2, TPP1, POT1, RAP1 and TIN2 in PAPER Ⅳ.

Levels of TERT, COX2, COX1, TERC, TRF1, TRF2, TPP1, POT1, RAP1, TIN2, c-MYC, c-KIT, DOK3, NID-1 and SULF2 mRNA were calculated based on the cycle threshold (CT) values and normalization of human β2-M expression.

Table 5. Q-PCR primer sequences and Primer sequences for telomere length analyses

Gene	Forward	Reverse
TRF1	5’-GCTGTTTGTATGGAAAATGGC-3’	5’-CCGCTGCCTTCATTAGAAAG-3
TRF2	5’- GACCTTCCAGCAGAAGATGCT-3’	5’-GTTGGAGGATTCCGTAGCTG-3’
TPP1	5’- CCCGCAGAGTTCTATCTCCA-3’	5’-GGACAGTGATAGGCCTGCAT-3’
TIN2	5’- GGAGTTTCTGCGATCTCTGC-3’	5’-GATCCCGCACTATAGGTCCA-3
POT1	5’-TGGGTATTGTACCCCTCCAA-3’	5’-GATGAAGCATTCCAACCACGG-3’
RAP1	5’-CGGGGAACCACAGAATAAGA-3’	5’-CTCAGGTGTGGGTGGATCAT-3’
TERC	5’-TCTAACCCTAACTGAGAAGGGCGTAG-3’	5’-GTTTGCTCTAGAATGAACGGTGGAAG-3’
TERT	5’-CGGAAGAGTGTCTGGAGCAA-3’	5’-GGATGAAGCGGAGTCTGGA-3

β2-M	5'-GAATTGCTATGTGTCTGGGT-3’	5’-CATCTTCAAACCTCCATGATG-3’
COX2	5’ -GCCCAGCACTTCACGCATCAG-3’	5’ -AGACCAGGCACCAGACCAAAGACC-3’
COX1	5’-CGGCTGCAGCCCTTCAATGAGT-3’	5’ -CTCTCCCCAAAGATAGAGTTTGGA-3’
c-MYC	5'-TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT-3'	5'-TCTTGACATTCTCCTCGGTGTCCGAGGACCT-3'
c-KIT	5’-TCATGGTCGGATCACAAAGA-3’	5’-AGGGGCTGCTTCCTAAAGAG-3’
DOK3	5’-GTCCCCATGGAGGAAAACTC-3'	5’-AAGTGGTAGGGCCAGCTGTA-3’
NID-1	5’-AGGAGCTCTTTCCCTTCGGC-3’	5’-CGGGGGTTCACTCGTAGCAA-3’
SULF2	5’-CCGCCCAGCCCCGAAACC-3’	5’-CTCCCGCAACAGCCACACCTT-3’
Primer sequences for telomere length analyses		
Tel1b	5′ -CGGTTTGTTTGGGTTTGGGT-TTGGGTTTGGGTTTGGGTT-3′	
Tel2b	5′ -GGCTTGCCTTACCCTTACCCTTACCC-TTACCCTTACCCT-3′	
HBG3	5′ -TGTGCTGGCCCATCACTTTG-3′	
HBG4	5′ -ACCAGCCA-CCACTTTCTGATAGG-3′	

3.7 Telomerase activity assay

Telomerase activity was assessed using a commercial Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) as recommended by the manufacturer. For each assay, 1 μg of protein was used, and 26 PCR cycles were performed after the telomerase-primer elongation reaction. The PCR products were detected using ELISA color reaction and the level of telomerase activity was expressed as absorbance in arbitrary units.

3.8 Cell cycle and apoptosis detection with flow cytometry

In PAPER Ⅰ AML cells were treated with different concentrations of 5-AZA for three days, and then harvested and fixed in ethanol followed by PI staining. The PI fluorescence was measured with a FlowCytometer (Beckman Coulter). For each sample 1×10^6 cells were measured. Data analysis was performed with Kaluza® Flow Analysis Software. The control gate was set based on the negative control.

In PAPER Ⅱ BGC-823 cells transfected with control and TERT siRNA were incubated with and without celecoxib and harvested after 4 days. The cells were fixed with 70% ethanol at

+4℃ overnight and stained with RNase A (0.5 mg)-containing propidiumiodide (50 mg/ml) Cell cycle distribution was determined using flow cytometry with ModFit (BD Biosciences, Franklin Lakes, NJ, USA).

In PAPERⅢ Stable MOLM-13 cells were transfected with control pBMN or TERT-expressing lentiviral vectors, treated with 0.1 μM PKC412 and harvested after 24 hrs then fixed with 70% ethanol at + 4℃ overnight and stained with RNAse A (0.5 μg) containing PI, 50 μg/ml. Cell cycle distribution was determined using flow cytometry with ModFit.

In PAPER Ⅳ cells were treated with bortezomib, and then harvested for apoptosis assay. The cells were stained with a kit from BD Biosciences and analysed using a FlowCytometer (BD). For each sample 1×10^6 cells were measured. The control gate was set based on the negative control.

3.9 Western blot

Total cellular proteins were extracted with RIPA lysis buffer.

In PAPERⅡ for the assessment of phosphorylated p38 MAPK, cells were washed once with sodium orthovanadate-containing buffers to inhibit phosphatases before RIPA buffer lysis. 20μg proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were probed with the specific antibodies against COX2 (Cayman chemical, Ann Arbor, MI, USA), Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK (Cell Signaling Technology, Billerica, MA, USA) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with secondary anti-mouse or rabbit horseradish peroxidase-conjugated IgG (Bio-Rad, Hercules, CA, USA)

In PAPERⅢ proteins were probed with the specific antibody against FLT3, p- FLT3, Akt and p-Akt (Cell Signalling Technology, Boston, USA) or c-MYC (Santa Cruz Biotechnologies) followed by anti-mouse or rabbit horseradish peroxidase-conjugated IgG.

In PAPER Ⅳ proteins were probed with the specific antibodies against TRF1 (Sigma-Aldrich, St. Louis, MO, USA, T1948), TFR2 (Novus, Littleton, CO, USA, #NB110-57130), POT1 (Novus, NB500-176), Caspase3 (Cell Signaling Technology, Danvers, MA, USA, #9665), or Bcl-2 (ProteinTech, Wuhan, China, #12789-1-AP) followed by anti-mouse or rabbit horseradish peroxidase-conjugated IgG.

β-actin immunoblotting was performed in parallel as a loading control.

The membrance were then developed by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific).

3.10 Promoter activity assay

In PAPERⅡ, to determine the effect of TERT knock-down on the COX2 promoter reporter activity, we transfected control and TERT-depleted BGC-823 cells with the COX2 reporter construct, in which the COX2 core promoter sequences (-327/ +59) were inserted up-stream of a luciferase-encoding DNA fragment (Katsukawa et al, 2010; Inoue and Nakata, 2011; kindly provided by Dr Hiroyasu Inoue, Nara Women's University, Japan). The COX2 promoter-driven luciferase activity was determined using a dual luciferase reporter assay system (Promega, Madison, WI, USA) 48h post transfection, and the target promoter-driven firefly luciferase activity was normalised to the renilla activity included in the kit.

In PAPERⅢ The TERT promoter reporter plasmid $p181^{wt}$ harboring the core promoter sequence of the TERT 5'-flanking region and its mutant variant ($p181^{MYC-}$) lacking the functional c-MYC motifs (E-boxes) were described previously. Cells cultured in 24-well plates at 0.5×10^6 cells /well were transfected with $p181^{wt}$ and $p181^{MYC-}$ plasmids using Lipofectamine2000 (Life Technology) according to the manufacturer's protocol, followed by treatment with PKC412. Luciferase activity in the cell lysates was determined by using a dual luciferase reporter assay system (Promega, WI) 24 hours post-transfection of the promoter reporter.

3.11 Puro. Cre-TERT promoter-driven-GFP plasmid and lenti-III-HA-GFP-TERT vectors

The h3.4k-GFP plasmid containing 3.4kb TERT promoter (+1 to -3405, ATG as +1) was obtained from Dr. Pei-Rong Huang (National Taiwan University), and the 3.4kb TERT promoter fragment was inserted into a pPuro. Cre empty vector (Addgene) upstream of GFP gene. For pLenti-III-HA-GFP-TERT vector construction, pLenti-III-HA empty vector was

bought from Applied Biological Materials Inc. (BC, Canada), a 4.5kb GFP-TERT fragment was cut from pBabe-hygro-GFP-TERT (Addgene) and inserted into pLenti-III-HA. A control plasmid, pLenti-BMN-GFP was a gift from Rudbeck Laboratory, Uppsala University. The vectors were then packaged and viral particles collected to infect AML cells to make TERT promoter-driven GFP cells and TERT-over-expressed cells.

3.12 Quantitative Fluorescence In situ Hybridization (Q-FISH)

BGC-823 cells were transfected with control and TERT siRNA and the metaphase cells harvested at 72h. Telomere length was determined using quantitative FISH (Q-FISH) with PNA (CCCTAA)3 probe. The signal intensity in 50 cells was determined using NIS software (Nikon, Stockholm, Sweden) and expressed in arbitrary units.

3.13 Immuno Fluorescence In Situ Hybridizaiton (Immuno-FISH)

Briefly, cells were harvested and cytospined onto Superfrost plus slides (Thermo Scientific), fixed with 4% paraformaldehyde, permeabilized with Triton PBS for 20 mins and blocked with serum free Block (DAKO, Glostrup, Denmark). The slides were then incubated with 53BP1 antibody (Bethyl Inc., Montgomery, Texas, USA) followed by incubation with Alexa 594 secondary antibody (Jackson Labs Technologies Inc., Los Gatos, CA, USA). The slides were treated with frozen and thawed cycle in liquid nitrogen, and incubated in 0.1N HCL for 10 min. The PNA-telomere probe (PANAGENE Inc., Daejeon, Korea) was added. The slides were then subjected to 85℃ denaturation for 10 min and 30℃ hybridization for 2h and a following washing procedure. High-resolution images were collected using Leica Confocal TCS SP5 with 488 nm and 594 nm sequential laser scan. Z stack images was acquired with slide interval recommended optimization and between-stack mode. The co-localization of 53BP1 and telomere signals was examined and analyzed on each layer by two separate independent persons in a double-blind manner.

3.14 Flow FISH for telomere length assay

Flow FISH was performed according to previous protocols by Baerlocher *et al.* with minor modifications. Cells from calf thymus were kindly donated from the butchery Ö-slakt AB (Värmdö, Stockholm). Stained cells were captured with Gallios flow cytometer (Beckman Coulter) and analyzed using the Kaluza software (Beckman Coulter, Caguas, PR, USA). For normalization, fluorescent MESF-FITC beads (Bangs Laboratories, Fishers, IN, USA) were used and the fluorescent signal was quantified using the QuickCal v.2.3 data analysis program (Bangs Laboratories).

3.15 Subtelomeric DNA methylation at chromosome 4p

Genomic DNA, extracted from control and TERT- over-expressed HEL cells with or without 5-AZA, was subject to bisulfite conversion using an EZ DNA Methylation-Gold Kit (ZYMO RESEARCH, Irvine, CA, USA). PCR primers specific to the subtelomere region of chromosome 4p was used to amplify the target region. The obtained PCR products were then sequenced from both directions. Two independent experiments were performed.

3.16 cDNA array

MOLM-13 cells infected with control pBMN or TERT-expressing lentiviral vectors were treated with 0.1 μM PKC412 for 12 hrs and total RNA extracted. The affymetrix Human Gene 1.0 ST Array was performed. The fold change in gene expression between DMSO- and PKC412-treated cells was then calculated.

3.17 Mouse xenograft model

BGC-823 cells were infected with control and siTERT lenti-viral vectors were collected, 2.0 x 10^6 cells were suspended in 100 ml PBS. The cells were then subcutaneously injected into nude mice (Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China) at the right back. The mice harbouring control and TERT siRNA BGC-823 cells were divided into two groups each, and

one of them was fed with celocoxib containing drinking water (1.5 mg/ml), based on studies of prostate cancer tumour model. Therefore, there were a total of four groups (10 mice per group): (1) control siRNA group; (2) control siRNA plus celecoxib group; (3) TERT siRNA group; (4) TERT siRNA plus celecoxib group. Mice were euthanized after 3 weeks and tumours were isolated for the measurement of weight, size and other analyses.

3.18 Statistical analyses

In PAPER Ⅰ, Student's T-test was used to compare cell numbers, apoptotic cells, TERT mRNA levels and telomere length between control and 5-AZA-treated AML cells.

In PAPER Ⅱ, Mann–Whitney U-test or Student's T-test was used for analyses of differences between experiment groups.

In PAPERⅢ, The comparison of mRNA expression, promoter activity, telomerase activity and cell cycle analysis between control and experimental groups was made using a Student's T-test or One- way ANOVA followed by LSD test. P value less than 0.05 is considered significant.

In PAPER Ⅳ, Student's T-test or Mann-Whitney U test was used to compare cell numbers, apoptotic cells, TERT, TERC and shelterin protein mRNA levels and telomere length between control and bortezomib-treated cells.

All the tests were two-tailed and computed using SPSS or SigmaStat 3.1 software (Systat Software, Inc., Richmond, CA, USA) software. P values less than 0.05 were considered statistically significant.

3.19 Ethics

Study Ⅰ was approved by the regional ethical review board in Stockholm, Sweden.

Study Ⅱ was approved by Chinese Ethical review board in Jinan, China.

4 RESULTS AND DISCUSSION

4.1 Paper I: The DNMT inhibitor induced telomere dysfunction and the overexpression of telomerase could attenuate its anti-cancer efficacy

4.1.1 The DNMT inhibitor 5-AZA leads to apoptosis and induces DNA damage and telomere dysfunction

HEL cells seem to be more sensitive to 5-AZA treatment than KG1A cells with almost complete growth arrest at a concentration of 0.5µM. The trypan blue staining showed a progressive decline in HEL cell viability with increased concentrations of 5-AZA and longer incubation time. PI staining using flow cytometric analysis showed that the declined viability was due to increased cellular apoptosis.

Previous publications revealed DNA damage response in 5-AZA treated cancer cells[156,157]. We wanted to investigate this phenomena in AML cell lines by determining the focal formation of 53BP1 protein foci, a very well established DNA damage response marker. 53BP1 signals were detected in 5-AZA treated cell lines KG1A and HEL and were absent in corresponding untreated control cells.

Our next question was whether 5-AZA treatment could induce telomere dysfunction. Here we utilized the co-localization of the DNA damage marker protein, 53BP1 and the telomere signals to demonstrate the dysfunctional telomere induced foci (TIF) using immune-fluorescent in situ hybridization (Immuno-FISH). Telomeres were shown as green signals. In both AML cell lines (KG1A and HEL) treated with 5-AZA, the DNA damage signal 53BP1, red dots, was detected while absent in control untreated AML cell lines. In addition, the merged image showed the co-localization of 53BP1 foci on the telomere signal in 5-AZA exposed cells and this co-localization was rarely found in untreated cells (Figure 4).

In order to find the reason for telomere dysfunction, we measured telomere length. The two AML cell lines (KG1A and HEL) were exposed to 2.0 µM and 5.0 µM 5-AZA for 72h and then analyzed with Flow-FISH. Both cell lines exposed to 2.0 µM 5-AZA exhibited slightly shorter

telomeres compare to untreated cells while apparent attrition of telomeres became apparent at 5.0 µM exposure 5-AZA.

Previous reports indicated the contribution of subtelomeric regions to the telomere stability, as the methylation status of these regions locally affects the chromatin structure. Methylation specific PCR together with Sanger sequencing were performed to examine the subtelomere region methylation configuration of chromosome 4p. Out of a total of 31 CpGs in the amplicon, 25 were methylated in untreated HEL cells, 24 of which remained methylated after 5-AZA exposure. These results illustrate the resistance of subtelomeric regions to DNMT inhibitors.

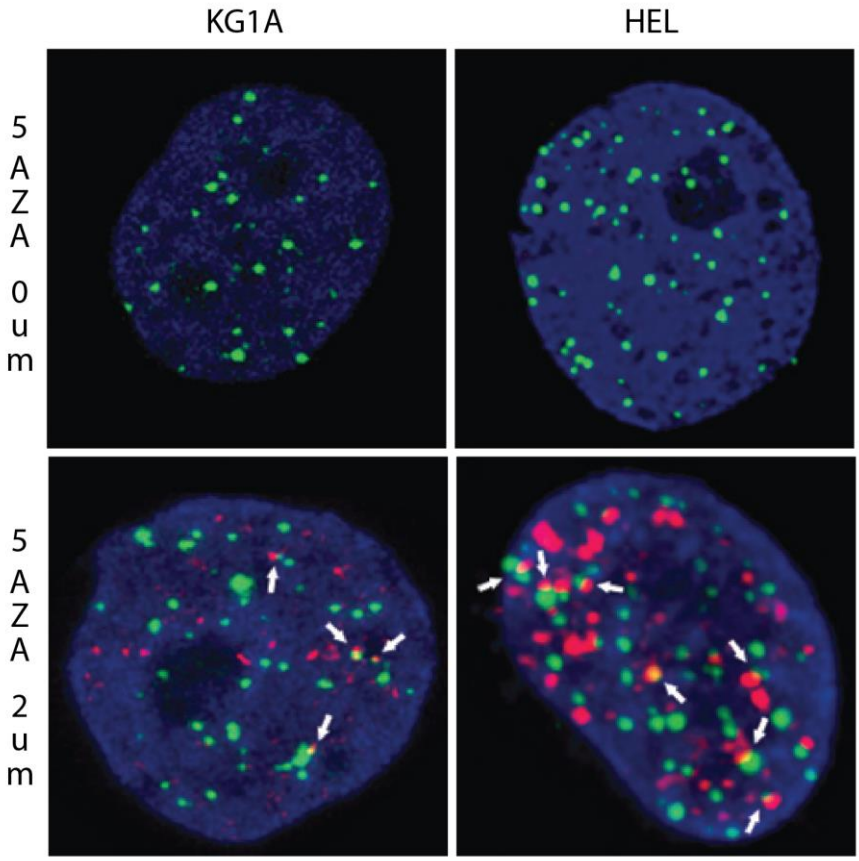


Figure 4. 5-AZA leads to apoptosis and induces DNA damage and telomere dysfunction

4.1.2 5-AZA suppresses TERT expression in AML cell lines

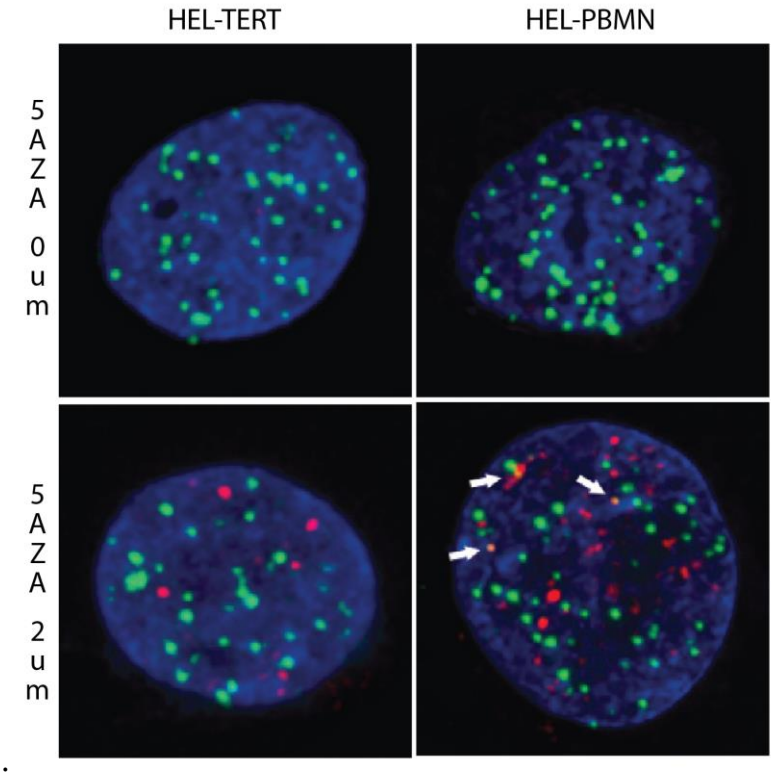
To further explore the mechanism of telomere shortening, we analysed telomerase expression and telomerease activity in 5-AZA treated KG1A and HEL cell lines. After 5-AZA exposure in KG1A cells, TERT mRNA expression was significantly downregulated in a dose dependent manner. The same results were observed in the 5-AZA treated HEL cell line.

In addition, the telomerase activity was also diminished but to a lesser extent probably due to a longer half-life of telomerase.

To further investigate whether the results achieved in AML cell lines could be duplicated in AML primary cells we tested the 5-AZA effect on primary cells obtained from newly diagnosed AML patients. Similar to the cell line experiments telomerase expression was downregulated after 5-AZA exposure in of all the five patients.

4.1.3 Ectopic expression of TERT attenuates telomere dysfunction

Given the above observation, we would like to rule out whether ectopic expression of TERT could antagonize the 5-AZA effect. We constructed the TERT overexpression HEL cell line with lentiviral TERT expression construct, which lead to two-fold increase in telomerase activity and abolished the 5-AZA effect on telomerase expression. After ectopic expression of TERT, 5-AZA induced apoptosis was decreased and the number of TIFs declined as compared to control cells (Figure 5).



Fiugre 5. Ectopic expression of TERT attenuates telomere dysfunction

In summary, we demonstrate that 5-AZA could depress TERT expression in AML cells, shorten telomeres which led to telomere dysfunction which subsequently induced apoptosis.

Meanwhile, ectopic expression of TERT could antagonize apoptosis and telomere dysfunction induced by 5-AZA. Thus, telomere-induced dysfunction may contribute to the anti-tumor effect of DNMT inhibitors.

4.2 PAPER Ⅱ: TERT inhibition stimulates COX2 expression and synergizes with the COX2 inhibitor to conduct anti-cancer effects

4.2.1 TERT inhibition activates COX2 promoter and stimulates COX2 expression via P38 MAPK signaling

Our first question was whether TERT inhibition will affect the COX2 expression level, and whether COX2 forms a positive feedback loop with TERT. Specific TERT siRNA was transfected into gastric and cervical cancer cell lines and followed by qPCR and western blot to determine the mRNA and protein expression of COX2. Unexpectedly, we observed elevated COX2 expression. This elevated COX2 expression was observed at 24h and lasted for the whole observation period, which suggests a constitutive rather than transient elevation (Figure 6).

Our next question was what could be the reason behind this COX2 elevation. We transfected a gastric cell line BGC-823 with the reporter construct harboring the COX2 promoter. The COX2 promoter activity was higher in TERT depleted BGC-823 cells compared with that in control cells.

Previous studies showed that the P38 MAPK signaling played very important roles in COX2 regulation[158-163]. We asked whether this was the case here. We detected increased levels of p-P38 (Phospho P38 at Thr180/Tyr182) in TERT siRNA treated BGC-823 and HeLa cells compared with controls. To establish the causal link, we tested the P38MAPK specific inhibitor on cells transfected with TERT and control SiRNA. The P38MAPK inhibitor abolished the TERT depletion induced COX2 elevation.

We also measured the telomere length and the results suggested that the TERT depletion induced overexpression of COX2 is telomere length independent.

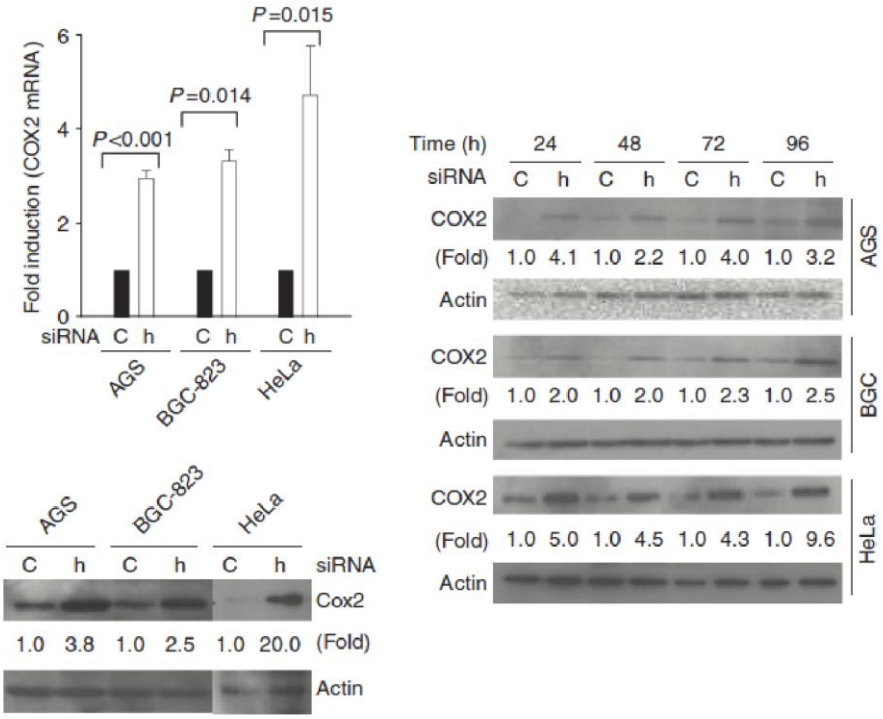


Figure 6. Western blot which shows TERT inhibition to stimulate COX2 expression

4.2.2 TERT depletion synergizes with COX2 inhibition to exert anti-tumor effect in gastric cancer cells and mouse xenograft models

In previous reports, it has been shown that COX2 promotes cancer cell survival and proliferation[164-168]. Thus, it is important to address whether the elevated COX2 expression attenuates, whereas COX2 inhibition promotes, the anticancer effect of TERT depletion. We transfected TERT specific siRNA into BGC-823 cells followed by their exposure to celecoxib, a specific COX2 inhibitor. Flow cytometry analysis showed apoptotic cell death in celecoxib and TERT siRNA exposed cells, while neither celecoxib exposure nor TERT siRNA transfection alone induced cell death. TERT depletion increased the sensitivity of cancer cells to celecoxib induced apoptosis.

To further investigate the in vivo recapitulation of the in vitro results obtained above wee injected nude mice with the TERT depleted and control BGC-823 cells. When analyzing the

tumors we could observe that both the control and the TERT depleted cells could generate comparable sized tumors in nude mice.

Then these mice were fed with celecoxib. The celecoxib could not reduce the tumor size from mice harboring BGC-823 cells with control shRNA. However, the tumors generated from the TERT depleted cells were smaller, suggesting the synergistic anti-tumor effect of TERT depletion and COX2 inhibition.

In summary, our study demonstrated that TERT depletion leads to activated phosph-p38 MAPK signaling, increases COX2 promoter activity and elevates the COX2 expression. We evaluated the anti-cancer effect of TERT depletion and COX2 inhibition separately and in combination both in vitro and in vivo. Knocking down TERT or COX2 inhibition alone could not lead to apoptosis in cultured gastric cancer cells while knocking down TERT in combination with celecoxib exposure retarded tumor growth in the mouse xenograft model, thus highlighting the clinical implications of TERT inhibitor in combination with COX2 inhibition in anti-tumor therapy.

4.3 PAPER Ⅲ: The FLT3 tyrosine kinase inhibitor PKC412 inhibits TERT expression in a MYC-dependent manner in AML cells and ectopic expression of TERT attenuates the TKI anti-tumor efficacy

4.3.1 The FLT3 TKI inhibits TERT promoter activity, decreases telomerase activity and attenuates TERT expression in a MYC-dependent manner.

PKC412 is a specific inhibitor of FLT3 tyrosine kinase that inhibits FLT3 phosphorylation and activation. As TERT plays important roles in carcinogenesis, our first question was what is the effect of FLT3 TKI on TERT expression. PKC412 exposure reduced the TERT mRNA expression at 0.1μM and almost complete abolishment of TERT mRNA was observed at 24h in Mv4,11 cells with FLT3-ITD+ phenotype. Similar results were observed in MOLM13 cells, another FLT3-ITD+ AML cell line. In contrast, the wild type FLT3.harboring AML cell line HL60 and the cervical cancer cell line HeLa manifest no alterations in TERT mRNA expression

levels following PKC412 exposure. In addition, exposure of PKC412 led to declined telomerase activity in Mv4,11 and MOLM13 cells.

Furthermore, we wanted to understand the mechanism behind the decline in TERT mRNA expression. A GFP expression vector driven by a TERT promoter was transfected into FLT-ITD cells, after PKC412 exposure the GFP signal faded to about 50% of the DMSO control, which indicates that the PKC412 may inhibit the TERT transcription and lower TERT mRNA expression.

Previous studies have demonstrated a key regulatory role of MYC on TERT expression and we further tested the involvement of MYC using the inhibitory effect of PKC412 on TERT expression. PKC412 exposure led to a robust decline in MYC mRNA and protein expression in FLT-ITD-carrying AML cell lines. To establish the causal link, we transfected the Mv4,11 cell line with the TERT core promoter harboring vector and the corresponding vector harboring TERT core promoter with a deletion in two MYC binding sites. These cells were then subjected to PKC412 treatment. PKC412 exposure led to a robust decline in TERT promoter activity, while this decline was abolished with the disruption of MYC binding sites. Thus, the above observations demonstrate the MYC dependency of TERT inhibition by TKI.

4.3.2 Ectopic expression of TERT activates alternative tyrosine kinase pathway, and abates PKC412 elicited apoptosis in a FLT-ITD+ AML cell line

Given the above observation, we would like to elucidate whether the ectopic expression of TERT could antagonize the anti-cancer effect of TKI. MOLM13 cells were infected with a lentiviral TERT expression vector and acquired a constitutive robust expression of TERT. After exposure to PKC412, MOLM13-TERT expressing cells exhibited a higher viability than control cells. In addition, flow cytometry analysis revealed attenuated apoptosis in MOLM13-TERT cells. Taken together, these results indicate that the ectopic expression of TERT abates the PKC12 elicited apoptosis in FLT-ITD+ AML cells.

We further explored the possible mechanism of ectopic TERT expression causing the decline in PKC412 induced apoptosis. Ectopic TERT expressing cells and control cells were exposed to PKC412 and subjected to microarray analysis. We found that the ectopic expression of TERT effectively influenced the FLT3 downstream effectors PI3K-AKT and RAS-MAPK, thereby antagonizing the PKC412 induced apoptosis (Figure 7).

In summary, we discovered that tyrosine kinase inhibitor (TKI) reduced TERT expression and telomerase activity in a MYC-dependent manner. Ectopic expression of TERT could activate

an alternative tyrosine kinase pathway and circumvent the PKC412 elicited apoptotic anti-cancer effect.

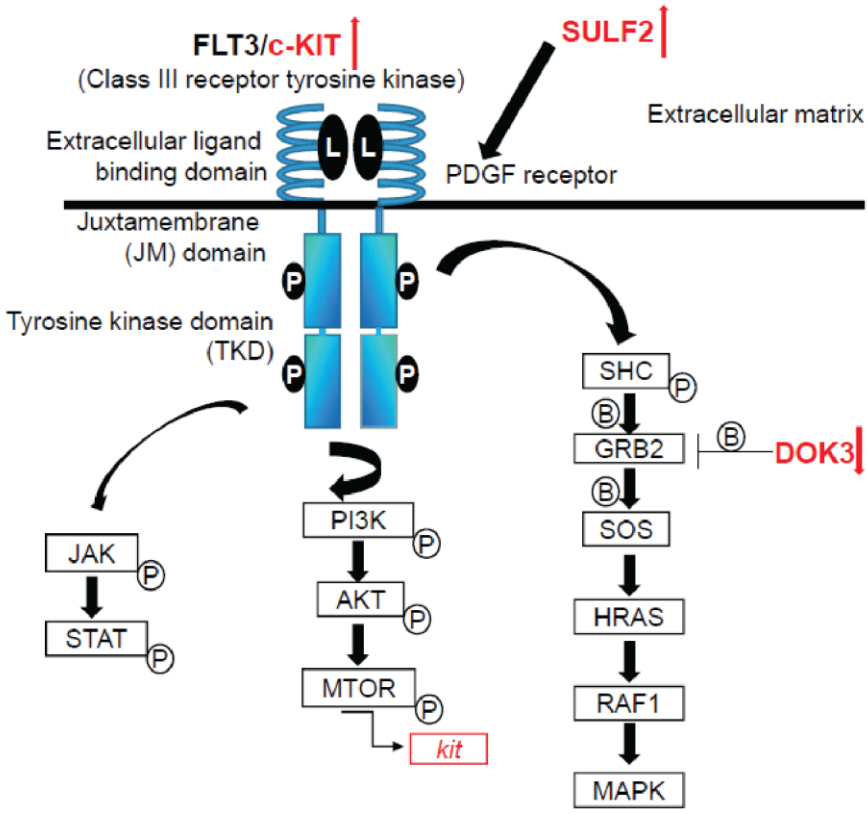


Figure 7. Ectopic expression of TERT activates alternative tyrosine kinase pathways and circumvent the TKI blocking effect

4.4 PAPER Ⅳ: Bortezomib-induced downregulation of TERT and disruption of telomere homeostasis contribute to apoptosis of malignant cells

4.4.1 Bortezomib treatment leads to decline in TERT and TERC expression, disruption of shelterin protein expression, telomere shortening and telomere dysfunction

TERT and TERC constitute the core of telomerase holo-enzyme and previous studies have shown that bortezomib leads to decline in TERT and TERC expression in subset of myeloma cell lines[169]. We raised the question whether this was also observed in other malignant cell lines.

We treated the gastric cancer cell line BGC-823 and leukemia HEL cell line with bortezomib and a sharp decline in TERT mRNA expression was observed at 48 hours. Telomerase activity also declined after bortezomib exposure.

As the shelterin protein complex functions essentially in telomere homeostasis,we explored the bortezomib effects on shelterin proteins. In HEL cells, exposure to bortezomib caused reduction in TRF1, TRF2, POT1, TPP1, RAP1 and TIN2 mRNA expression. The situation was slightly different in BGC-823 cells, where bortezomib exposure led to decrease in TRF1, TRF2, TPP1, POT1 mRNA expression but to enhancement of RAP1 and TIN2 expression.

We then analyzed the effect of bortezomib exposure on telomere length and telomere function. Flow FISH analysis revealed a significant decline in telomere length in bortezomib exposed AML and gastric cancer cells compared to DMSO controls. To illustrate the telomere dysfunction, we used co-localization of a very well established DNA damage marker and telomere in situ hybridization signal protein 53BP1. In summary, bortezomib treatment increased the frequency of telomere dysfunctional foci significantly.

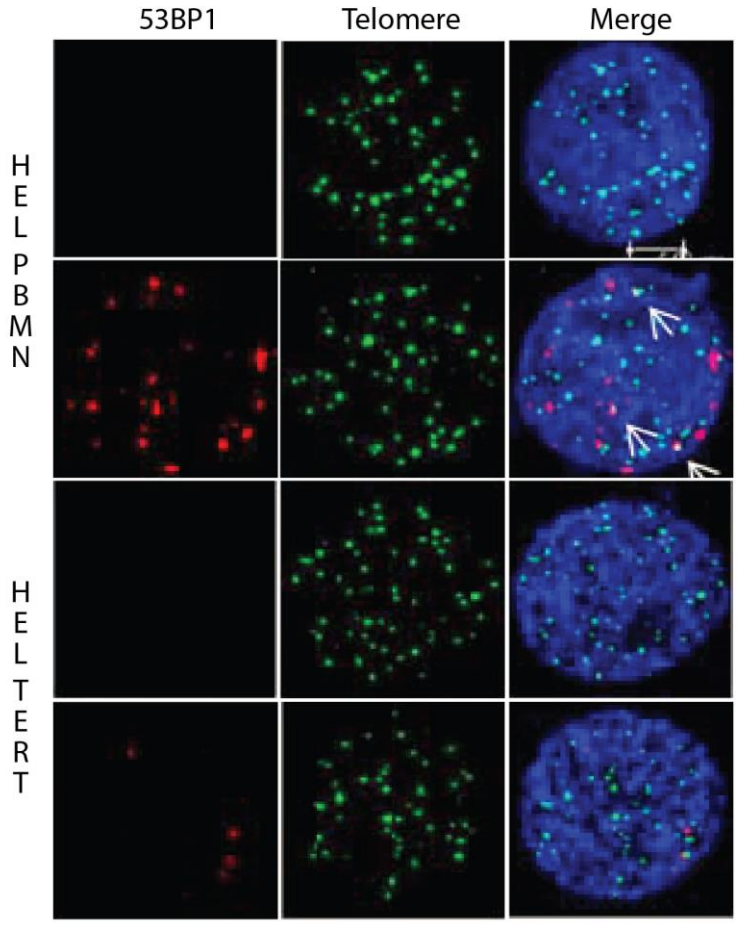


Figure 8. Ectopic expression of TERT overcomes the bortezomib induced telomere dysfunction

4.4.2 Ectopic expression of TERT attenuates bortezomib-induced downregulation of TRF1, TRF2, TPP1 and RAP1, overcomes telomere dysfunction, reduces DNA damage and rescues cancer cells from apoptosis

Given the above described observations, we further explored whether ectopic expression of TERT is capable of preventing bortezomib induced telomere dysfunction. Lenti-viral TERT infected HEL and BGC-823 cells stably expressed a two-fold increase of TERT. After exposure to bortezomib, TERT expressing cells exhibited much less telomere dysfunction foci compared to control cells. The 53BP1 DNA damage foci also declined in TERT high expressing cells compared to control cells (Figure 8).

We further explored whether ectopic expression of TERT could antagonize the bortezomib elicited disruption of shelterin protein expression. In TERT high expressing cell lines, the TRF1, TRF2, RAP1 and TPP1 expression level remained essentially the same in both bortezomib treated and untreated cells, while POT1 expression still declined and TIN2 transcripts increased.

We further investigated whether ectopic expression of TERT is able to attenuate bortezomib induced apoptosis. Both TERT high expressing and control cells were subjected to bortezomib exposure, both cell lines showed a decline in viability in a dose-dependent manner. However, more TERT high expressing cells survived compared to control cells. Flow cytometry analysis also revealed that higher percentages of control cells underwent apoptosis.

In summary, our study demonstrates that bortezomib treatment leads to a decline in TERT and TERC expression, disruption of shelterin protein expression, telomere shortening and telomere dysfunction while ectopic expression of TERT attenuates bortezomib induced down regulation of TRF1, TRF2, TPP1 and RAP1, overcomes telomere dysfunction, reduces DNA damage and rescues cancer cells from apoptosis.

5 CONCLUSION AND PERSPECTIVE

DNMTIs have been utilized clinically for therapy of hematological malignancies including AML but the mechanism of their anti-tumor activity remains to be explored. Bortezomib inhibits the ubiquitin/proteasome pathway and thereby exerts its anti-cancer effects, but the exact mechanisms remain to be clarified. In addition, the underlying mechanism of the clinically utilized TKIs could also be worth to look deeper into. Our study shows a significant impact of bortezomib and DNMTI on telomere homeostasis and function, which may contribute to bortezomib and DNMTI-mediated anti-tumor efficacy. In addition, here we demonstrated that DNMTI, bortezomib and TKI are capable of suppressing TERT expression and shortening telomeres thereby promoting apoptotic cell death of AML cells.

Recent studies revealed multiple biological activities of telomerase or TERT aside from the telomere lengthening function. For instance, TERT could facilitate the recruitment of DNA repair factors onto DNA double strand break sites, and depletion of TERT results in strengthened cell radiation-sensitivity, and declined efficacy for DNA repair. In accordance with these reports, we observed that ectopic expression of TERT attenuated DNA damage in bortezomib/DNMTI-treated malignant cells. DNA damage is required for bortezomib and DNMTI anti-cancer activity. Most likely apoptosis was attenuated in TERT over-expressed cells, when exposed to bortezomib or DNMTI. This attenuation may be due to the decreased DNA damage mediated by TERT

More importantly, our study revealed the interfering effect of TERT ectopic expression on clinically utilized anti-cancer drugs like DNMTI, TKI and bortezomib. High TERT expression could rescue cancer cells exposed to the above inhibitors, which also highlights the necessity of exploring TERT inhibitors in combination with these agents in clinical studies.

A deeper view of the molecular alterations and rewired regulatory signals induced by chemotherapeutic and other anti-cancer agents could be helpful in the future development of personalized/targeted anti-cancer treatment. . Uncovering the underlying shared mechanisms of multidrug resistance and development of the corresponding inhibitors would greatly contribute to novel anti-cancer strategies in the future.

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