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The role of telomerase reverse transcriptase (TERT) in human malignancies: genetic regulation and telomere lengthening-independent oncogenic activities

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my family

ABSTRACT

Telomerase is a ribonucleoprotein enzyme maintaining telomere length. Telomerase reverse transcriptase (TERT), which acts as a catalytic unit, is tightly repressed in differentiated human cells while activated in cancer cells for telomere lengthening. Beyond that, TERT has also been shown to contribute to oncogenesis via its telomere lengthening-independent functions. This thesis is designed to define the role of TERT in oncogenesis and the biological implications of the genetic alterations in the *TERT* gene/promoter.

The first paper was focused on the role of TERT in acute myeloid leukemia (AML) therapy targeting mutations of FMS-like tyrosine kinases 3 (FLT3). The internal tandem duplication (ITD) mutation in the juxtamembrane domain in the *FLT3* gene has been reported as one of the most frequent mutations in AML, and PKC412 was developed as its specific inhibitor. We observed that in FLT3ITD-harboring primary cells from AML patients and AML cell lines, PKC412 down-regulated TERT expression and telomerase activity in a MYC-dependent manner. Moreover, TERT restored the activity of FLT3 downstream effectors and alternative tyrosine kinase signaling pathways inhibited by PKC412, thereby attenuating PKC412-mediated apoptosis of leukemic cells. Taken together, FLT3ITD regulates TERT expression via a MYC-dependent manner, and TERT down-regulation is required for PKC412-mediated anti-AML efficacy.

The recurrent TERT promoter mutations have been demonstrated to stimulate TERT transcription by generating new E26 transformation-specific (ETS)-binding sites in different human malignancies. Furthermore, rs2736098 and rs2736100, the two single nucleotide polymorphisms (SNPs) in the *TERT* locus, have been reported to associate with cancer susceptibility. In paper II, we found that compared to hepatocellular carcinoma (HCC) patients with wild type TERT promoter or healthy controls, a significant difference in these two genotypes was present in patients carrying TERT promoter mutations. We observed a negative association between TERT promoter mutations and rs2736098_TT and rs2736100_CC genotypes. There was no association between TERT promoter mutations and clinico-pathological variables or *CTNNB1* mutations. In summary, the germline TERT rs2736098 and rs2736100 polymorphisms may play a role in TERT promoter mutation occurrence in HCC.

The dysregulation of DNA methyltransferases (DNMTs) and the aberrant DNA methylation is a cancer hallmark. In paper III, we showed that TERT up-regulated DNA methyltransferase 3B (DNMT3B) expression and thereby contributing to the repression of downstream tumor suppressors as well as the activation of AKT. We found a positive correlation between TERT and DNMT3B expression in both HCC cell lines and primary HCC tumors. Mechanistically, TERT promotes DNMT3B transcription by cooperating with the transcription factor (TF) Sp1. The depletion of TERT expression led to significant demethylation in the tumor suppressor PTEN promoter and a reduced global DNA methylation by down-regulating DNMT3B expression. The restoration of PTEN expression mediated by TERT depletion inhibited AKT activity. Higher levels of TERT and DNMT3B expression predicted a significantly shorter survival in HCC patients according to analysis of The Cancer Genome Atlas (TCGA) dataset. Taken together, we identified the TERT-DNMT3B-PTEN-AKT axis in HCC cells, which promotes HCC progression via aberrant DNA methylation.

In conclusion, our studies demonstrated the effect of genetic alterations on TERT/telomerase activation, and the novel telomere lengthening-independent roles of TERT in carcinogenesis, which should be implicated in cancer therapy/precision oncology.

LIST OF SCIENTIFIC PAPERS

I. Xiaolu Zhang*, Bingnan Li*, **Jingya Yu**, Jenny Dahlström, Anh Nhi Tran, Magnus Björkholm, Dawei Xu. MYC-dependent downregulation of telomerase by FLT3 inhibitors is required for their therapeutic efficacy on acute myeloid leukemia. Annals of Hematology. 2018;97(1):63-72.

II. Xiaotian Yuan*, Guanghui Cheng*, **Jingya Yu**, Shunzhen Zheng, Chao Sun, Qing Sun, Kailin Li, Zhaomin Lin, Tiantian Liu, Ping Li, Yiteng Xu, Feng Kong, Magnus Björkholm, Dawei Xu. The TERT promoter mutation incidence is modified by germline TERT rs2736098 and rs2736100 polymorphisms in hepatocellular carcinoma. Oncotarget. 2017;8(14):23120-9.

III. **Jingya Yu**, Xiaotian Yuan, Louise Sjöholm, Tiantian Liu, Feng Kong, Tomas J. Ekström, Magnus Björkholm, Dawei Xu. Telomerase reverse transcriptase is required for DNMT3B expression/ aberrant DNA methylation phenotype and AKT activation in hepatocellular carcinoma. (Submitted manuscript)

* Contributed equally

CONTENTS

LIST OF ABBREVIATIONS

IDH	isocitrate dehydrogenase
AML	acute myeloid leukemia
ASXL1	additional sex combs-like 1 transcriptional regulator
CBFB	core-binding factor subunit-β
CEBPA	CCAAT/enhancer-binding protein-α
CHIP	C terminus of HSC70-Interacting Protein
CI	confidence interval
CpG	cytosine-phosphate-guanine
CR	complete remission
ddNTP	dideoxyribonucleoside triphosphate
DKC	dyskerin
DNMT	DNA methyltransferase
DNMT3A	DNA methyltransferase 3A
DNMT3B	DNA methyltransferase 3B
dNTP	deoxy-ribonucleoside triphosphate
dsDNA	double-stranded DNA
ERK	extracellular signal-related kinase
ETS	E26 transformation-specific
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FLT3	FMS-like tyrosine kinases 3
GAR1	GAR1 ribonucleoprotein
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus

HDAC	histone deacetylase
HSCT	hematopoietic stem cell transplantation
IL	interleukin
ITD	internal tandem duplication
LSD1	lysine- specific demethylase 1
LUMA	Luminometric Methylation Assay
MKRN1	Makorin Ring Finger Protein 1
mTOR	mammalian target of rapamycin
MYH11	myosin heavy chain 11 smooth muscle
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NHP2	NHP2 ribonucleoprotein
NOP10	NOP10 ribonucleoprotein
NPM1	nucleophosmin
OR	Odds ratios
PDGFR	platelet-derived growth factor receptor
PHF6	PHD finger protein 6
PI3K	phosphatidylinositol-3 kinase
PKB	protein kinase B
PKC	protein kinase C
PML	promyelocytic leukaemia
POT1	protection of telomeres 1
qPCR	quantitive real-time polymerase chain reaction
RAP1	repressor and activator protein 1
RARA	retinoic acid receptor-α
RUNX1	Runt-related transcription factor 1

RUNX1T1	RUNX1 translocated to 1
siRNA	small interference RNA
SNP	single nucleotide polymorphism
SRSF2	serine/arginine-rich splicing factor 2
ssDNA	single-stranded DNA
STAG2	stromal antigen 2
TCGA	The Cancer Genome Atlas
TERT	telomerase reverse transcriptase
TET2	tet methylcytosine dioxygenase 2
TF	transcription factor
TIN2	TRF1-interacting nuclear protein 2
TKD	tyrosine kinase domain
TNF-α	tumor necrosis factor-α
TRF1	telomeric repeat-binding factor 1
TSG	tumor-suppressor genes
U2AF1	U2 small nuclear RNA auxiliary factor 1
WHO	World Health Organization
WT1	Wilms tumour 1

1 INTRODUCTION

1.1 Telomere and telomerase

1.1.1 Telomere

1.1.1.1 Structure

The telomere is a protective complex consisting of tandem DNA repeats at chromosome ends. The repeated sequence is TTAGGG (G-rich strand 5’ –3’) and the length varies widely among different eukaryotic species. In human the length of repeats ranges between 8 and 15kb. Telomeric DNA is double-stranded DNA (dsDNA) with a 3’ single-stranded overhang. The tail functions not only as a primer for telomerase, but also as a binding place for specific protective proteins [1]. The telomeric single-stranded DNA (ssDNA) usually inserts into the dsDNA and forms a T-loop, protecting the chromosome end [2].

1.1.1.2 Telomere-binding proteins

Telomeric DNA is capped by a protein complex known as shelterin, which includes repressor and activator protein 1 (RAP1 or TERF2IP), protection of telomeres 1 (POT1) and TPP1 (also known as ACD), telomeric repeat-binding factor 1 (TRF1 or TERF1), TRF2 (also known as TERF2), TRF1-interacting nuclear protein 2 (TIN2 or TINF2). TRF1 and TRF2 bind to double-stranded telomeric DNA while TPP1 and POT1 bind to the single-stranded tail. They connect to each other by the bridging component TIN2. RAP1 is associated with TRF2 [2].

1.1.1.3 Telomere function

The telomere serves two key functions. First, it protects the chromosome by preventing the ssDNA from being recognized as a broken end or an end-to-end fusion. Otherwise the processes including DNA end-joining, DNA recombination, and DNA repair would result in unstable chromosomes. Second, the general chromosome replication process cannot cover the ends (so-called the end-replication problem), which leads to attrition of the telomere. This problem can be resolved by telomerase, which adds TTAGGG repeats at the end of chromosomes. However, most human cells lack or only have very limited telomerase activity, and therefore the telomere is shortened along with cell division. Cells would stop dividing when the shortened telomere reaches a critical size [3]. Thus, the telomere-

shortening process works as a mitotic clock, counting the number of cell replications and thereby conferring limited lifespan to somatic cells.

1.1.2 Telomerase

Telomerase is a cellular ribonucleoprotein enzyme consisting of two core subunits, telomerase reverse transcriptase (TERT) and telomerase RNA (TERC). The TERT subunit provides the active site for catalysis whereas TERC functions as a RNA template for telomeric DNA synthesis. Studies have revealed that TPP1 plays a central role in recruiting telomerase to chromosome ends by binding to TERT. After recruiting, telomerase elongates the telomere by forming a product-template duplex and recycling of the internal template [4]. Beyond TERT and TERC, some auxiliary components are also necessary when telomerase functions *in vivo*, including dyskerin (DKC), NHP2 ribonucleoprotein (NHP2), NOP10 ribonucleoprotein (NOP10), GAR1 ribonucleoprotein homolog (yeast) (GAR1), reptin and pontin (Figure 1) [5].

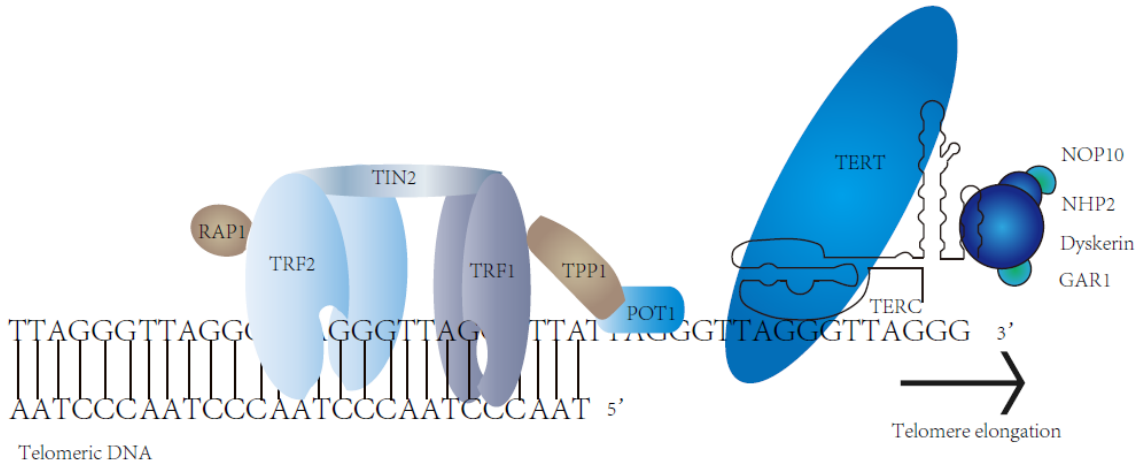

Figure 1. Shelterin complex and telomerase.

1.1.3 Telomerase reverse transcriptase (TERT)

While TERC is ubiquitously expressed in human cells, TERT expression is tightly limited to stem/progenitor cells, activated lymphocytes and other cells with high proliferation potentials, which indicates a rate-limiting role of TERT in controlling telomerase activity.

1.1.3.1 TERT regulation

TERT expression can be regulated at both the transcription and posttranslation levels. In general, the regulation of TERT transcription is crucial, and posttranslational modification is also responsible for controlling telomerase activity [6].

1.1.3.1.1 Transcription factors (TFs) and TERT expression

At the level of transcription, the TERT promoter is a region containing plenty of consensus sequences for TFs, including GC-boxes, E-boxes, ETS and E2F consensus sites, but lacking a TATA-box [6]. The most important TF in TERT trans-activation is c-Myc, which binds to E-boxes [7]. However, there are also some other E-box binding TFs (Mad1 and USF1) repressing TERT expression. Beyond that, some GC-box binding TFs including Sp1 and Sp3 are important for the initiation of TERT transcription. In addition, different negative regulatory factors might repress TERT transcription by binding to X-box, E2F, MT-box, and MZF-2 sites [8].

1.1.3.1.2 Epigenetic regulation of TERT

The regulation of TERT promoter activity occurs at multiple levels, and histone modification and cytosine-phosphate-guanine (CpG) methylation also play critical roles in TERT transcription [8].

It has been shown that the TERT promoter could be activated by hyperacetylation of core histones. Inhibition of class I and II histone deacetylases (HDACs) by trichostatin A could induce TERT expression. The increase of methylation at H3K4 resulting from over-expression of SMYD3 or inhibition of lysine-specific demethylase 1 (LSD1) leads to TERT transcription activation [9].

CpG methylation is another important part of epigenetic regulation; however, plenty of data supported a dual role of methylation in TERT promoter regulation. On the one hand, treatment of immortal fibroblast line with the demethylating agent 5-aza-2'-deoxycytidine resulted in increased TERT transcription, which indicated that promoter methylation could lead to TERT promoter repression. On the other hand, a CpG island locating across the TERT promoter and 5’ end of the *TERT* gene is usually methylated in human cancer cells, and demethylation in some cancer cell lines resulted in the decrease of TERT expression. This is probably because some binding sites of transcriptional repressors are blocked by

CpG methylation, which suggests that CpG methylation can promote TERT transcription in cancer cells [9].

1.1.3.1.3 Posttranslational regulation of TERT expression

The posttranslational modification of TERT is mainly composed of two parts: the phosphorylation of TERT by both protein kinase C (PKC) and AKT/protein kinase B (PKB) leads to telomerase activation; ubiquitination by Makorin Ring Finger Protein 1 (MKRN1), C terminus of HSC70-Interacting Protein (CHIP), and Hdm2 E3 ligases results in a telomerase activity decrease [6].

1.1.3.2 TERT promoter mutations

1.1.3.2.1 Function of TERT promoter mutations

Besides the TFs mentioned above, ETS TFs are also reported to participate in the TERT expression regulation. Interestingly, most cancer-associated TERT promoter mutations function by generating *de novo* ETS binding sites. Two major mutations are -124C/T and -146C/T, which are named C228T and C250T, respectively. These two mutations generate putative ETS-binding sites (TTCC), thereby activating TERT transcription. Many studies support the concept that TERT promoter mutations contribute to tumorigenesis by increasing TERT transcription [5].

Besides the two major mutations mentioned above, CC-to-TT tandem mutations at -124/-125bp and - 138/-139bp, and the C-to-T mutation at -57 are also found in a small proportion of cancers [5].

1.1.3.2.2 TERT promoter mutations in human cancers

TERT promoter mutations have been identified in several human cancers. Among them, bladder, renal pelvic, thyroid, HCC, glioblastoma and melanoma have shown a high mutation frequency [10,11]. However, TERT promoter mutations are rare in tumors such as prostate and breast cancer and leukemia [12].

1.1.3.3 Single nucleotide polymorphisms (SNPs) of the TERT gene and cancer susceptibility

It is known that SNPs are the most common sources of human genetic variations, which may contribute to cancer risk. Many studies suggest that SNPs in the *TERT* gene are associated with susceptibility of different cancers [13]. Among the multiple TERT SNPs,

the two most studied are rs2736100 at intron 2 and rs2736098 at exon 2. Lung cancer, basal cell carcinoma and pancreatic cancer show strong associations with the TERT rs2736100 CC genotype. Moreover, the association between rs2736098 and cancer risk has also been demonstrated in multiple types of cancer including HCC, lung cancer, breast cancer, and others [14-16].

1.1.3.4 Noncanonical functions of TERT in cancers

TERT was first recognized for its telomere-lengthening activity. However, over the past decade, accumulating data have suggested that TERT can function as a regulator in the transcription of genes and therefore contribute to cell survival and proliferation in cancers [17-19]. The two well-studied signaling pathways regulated by TERT are the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and Wnt/β-catenin pathways [20,21]. NFκB pathway is critical in cellular and developmental processes, and the key TF P65 in this pathway has been reported to stimulate expression of its target genes, most of which are involved in inflammation and cancer progression [22,23]. It has been demonstrated that P65 can activate TERT by binding to its promoter as well as to TERT protein, which promotes TERT nuclear translocation [24,25]. Intriguingly, studies showed that the interaction between TERT and P65 could also promote the complex localizing to promoters of the NFκB downstream genes, including interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α) [26,27]. Taken together, there might be a positive feedback link between TERT and the NFκB signaling pathway, which serves as the mechanism underlying chronic inflammation and the development from inflammation to various manifest cancers.

Beyond that, TERT has been demonstrated to play a role in the Wnt/β-catenin pathway. The Wnt/β-catenin signaling pathway is the key regulator in organ development in the embryo as well as cell fate decision in stem cells, and the constitutive activation of Wnt/β-catenin pathway in cells can lead to tumor development [28]. The interaction between TERT and β-catenin was first indicated in mice. The study demonstrated that TERT could promote the activity of Wnt/β-catenin by functioning as a cofactor in the β-catenin transcriptional complex and occupying promoters of Wnt/β-catenin downstream genes [29]. Moreover, TERT is a direct target of β-catenin, which can regulate TERT transcription by binding to the TERT promoter in cancer cells and stem cells [30,31]. Overall, the accumulating evidence indicates a feed-forward regulatory cycle between TERT and Wnt/β-catenin

pathway, which maintains telomerase activity and stimulates downstream oncogene transcription.

Studies further showed the noncanonical functions of TERT besides cooperating with P65 and β-catenin. TERT can promote VEGF transcription by acting as a cofactor with TF Sp1 [32]. It is reported that the interaction between TERT and AKT might also promote phosphorylation of each other [33-35].

1.2 Epigenetics and cancer

Epigenetics refer to heritable changes of gene expression without alteration of the underlying DNA sequence. Epigenetic mechanisms play a crucial role in normal cell development and differentiation as well as metastasis and progression of cancer. Epigenetic mechanisms mainly consist of histone modification and DNA methylation [36].

1.2.1 Histone modification

The histone is a spool-like highly alkaline protein present in eukaryotic cell nuclei. DNA is twisted around the histones and further compacted to form nucleosomes. The histone includes five family proteins. Histone 1 and histone 5 are linker proteins, while histones 2, 3 and 4 are core assembly proteins. Modifications of histones have a direct impact on chromatin structure, thereby affecting gene expression. Modifications such as ubiquitination, phosphorylation, methylation, and acetylation at N-terminal tails of histones can regulate gene activities together with other epigenetic mechanisms [36,37].

1.2.2 DNA methylation

It is well established that DNA methylation plays an essential role in the regulation of gene expression [38]. In most cases, DNA methylation occurs at 5th carbon of cytosine in CpG dinucleotides. The clusters of CpG are named CpG islands, and they are widely present in the human genome, especially at 5’ regions that consist of promoter and transcription sites. Most of the CpG islands are unmethylated or methylated at a very low level in normal cells. In contrast, abnormal methylation occurs in most tumor cells. Specific promoter hypermethylation can be associated with inactivation of tumor-suppressor genes (TSGs) while global hypomethylation have been associated with genomic instability [36]. Silencing of TSGs such as *PTEN* and *RASSF1A* is achieved by hypermethylation of CpG islands in promoter sequences, thereby leading to abnormal expression of downstream genes [38,39].

An increasing number of studies indicate that the aberrant methylation of CpG islands is one of the cancer hallmarks [40].

It is reported that cellular DNA methylation patterns are established by at least three independent DNA (5-cytosine)-methyltransferases (DNMTs): DNMT1, DNMT3A and DNMT3B, which are enzymes that methylate the cytosine residue of CpG. DNMT1 expression is more abundant in somatic cells compared to DNMT3A and DNMT3B. The reason might be that DNMT1 usually works as 'maintenance' methyltransferase. DNMT1 mainly catalyzes hemi-methylation and maintains the methylation patterns in newly replicated DNA fragments. DNMT3 family enzymes are required for the *de novo* methylation of a cytosine residue. DNMT3A and DNMT3B are highly expressed during stages when new DNA methylation patterns are being established, such as the blastocyst stage and germ cell developing stage [41]. Increasing expression of DNMT1, DNMT3A and 3B has been observed in various types of tumors [42].

1.3 Hepatocellular carcinoma (HCC)

1.3.1 Epidemiology

HCC is the primary malignancy of the liver. Most patients with HCCs are diagnosed in developing countries. HCC ranks third in terms of incidence in Eastern and South-Eastern Asia, Sub-Saharan Western and Eastern Africa. An estimated number of 466,100 new cases and 422,100 deaths occurred in China in 2015, which is almost half of all cases diagnosed globally. It was also the third cause of death from cancer worldwide in 2014. HCC is more common in males with a male: female ratio of 2.4 worldwide [43].

1.3.2 Etiology

There are multiple risk factors for HCC development; there is a strong association between infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and cirrhosis and HCC development. The regions with high incidence rates of HCC overlap with the regions where HBV and HCV infections are endemic. Around 80%–90% of HCC cases occur in cirrhotic livers. Other causes including alpha1-antitrypsin deficiency, hereditary hemochromatosis, autoimmune hepatitis, and Wilson's disease also play a role in HCC development but are less common. In addition, there is an incremental effect of presence of more than one risk factor responsible for HCC. And alcohol abuse further increases this risk by synergistic interaction with hepatitis virus infections [44,45].

1.3.3 Genetic alterations

Improved knowledge of oncogenic processes has revealed the genetic alterations and signaling pathways involved in HCC tumorigenesis. The most studied ones are the RAF/MEK/ extracellular signal-related kinase (ERK) pathway, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway, insulin-like growth factor pathway, WNT/β-catenin pathway, growth factor-regulated angiogenic signaling and hepatocyte growth factor/c-MET pathway [46-49]. Moreover, drugs targeting some components in these signaling pathways have been developed as therapies for HCC [50,51].

In addition, TERT and DNA methylation was also found to be important in HCC tumorigenesis. Up to 60% of HCC patients carry TERT promoter mutations, and it is reported that TERT rs2736100 and rs2736098 are associated with an increased HCC risk [15]. Studies have shown that methyltransferases DNMT1 and DNMT3B are up-regulated in HCC compared to non-tumorous liver tissues, thereby contributing to HCC by suppressing TSGs [42,50].

1.4 Acute myeloid leukemia (AML)

1.4.1 Definition

Leukemias are clonal disorders characterized by hematopoietic insufficiency due to the abnormal proliferation of incompletely matured myeloid or lymphoid cells. Normally, the common pluripotent stem cell can differentiate into a lymphoid precursor or a myeloid precursor. Based on the origin of the cells and the usual onset and progression of the disease, leukemia is classified into four major types: acute lymphocytic leukemia, chronic lymphocytic leukemia, AML and chronic myeloid leukemia [52].

AML, just as its name implies, is myeloid precursors-derived acute disorder. It can be phenotypically and genetically heterogeneous but with a common characteristic of abnormal accumulation of blast cells in the bone marrow and peripheral blood. The manifestations of AML can be diverse and nonspecific, but most of them are attributed to the cytopenias. The leukemic expansion of the bone marrow leads to anemia (fatigue and dyspnea), neutropenia (infections) and thrombocytopenia (hemorrhages) [53].

1.4.2 Classifications

Nikolaus Friedreich defined and used the term of “acute leukemia” for the first time in 1857, but it was not classified into myeloid and lymphoid until 1877, when polychromatic staining was developed by Paul Ehrlich [54]. Afterwards, many ways to classify AML were proposed, and the most widely accepted one is the World Health Organization (WHO) classification [53,55]. WHO classification not only divides AML according to the morphology, but also reflects the genetic and clinical diversity [53].

1.4.3 Epidemiology

1.4.3.1 Incidence

AML is a relatively rare cancer which represents 1.3% of all new cancer cases in the US 2017, but it is the most common myeloid leukemia. AML is more common in older adults with a median age at diagnosis of 68. Beyond that, the number of new cases is larger among men compared to women [56]. AML is one of the most common pediatric cancer with the incidence of 7.7 per million children aged 0-14 in the United States [57]. Ethnic origin is another strong factor influencing AML incidence. Studies have shown that the incidence of AML is much lower in Asia than that in western countries [58].

1.4.3.2 Risk factors

The risk factors contributing to leukemogenesis can be various; however, the most well-known ones are high-dose ionizing radiation exposure, chronic, high-dose benzene exposure, and alkylating chemotherapeutic agents. Most of them function by producing oxidative DNA damage [58]. On the other hand, the genetic background, especially the susceptibility genes of hematological malignancies shared by first-degree relatives of patients increase their risk of similar malignancies [59].

1.4.4 Mechanisms/ pathophysiology

Along with the development of new genomic techniques, next-generation sequencing is used to define the genomic landscape of AML as well. TCGA Research Network analyzed the genomes of 200 patients with AML and identified nine functional categories of genes that are commonly mutated in AML (Table 1) [60]. Among the 23 genes that were frequently mutated, three genes were mutated even more frequently than others in AML patients: *FLT3*; *DNMT3A* and nucleophosmin (*NPM1*) [61].

Table 1. Functional Categories of Genes Commonly Affected in AML

Functional Category	Selected Gene Members
Activated Signaling	Kinases (eg, *FLT3*, *KIT*), RAS family members (eg, *KRAS*, *NRAS*)
DNA methylation-associated genes	*DNMT3A*, *TET2*, *IDH1*, *IDH2*
Myeloid TF gene fusions	*PML-RARA*, *MYH11-CBFB*, *RUNX1-RUNX1T1*
Myeloid TF gene mutations	*RUNX1*, *CEBPA*
Chromatin-modifying genes	Mutations (eg, *ASXL1*, *EZH2*) or *KMT2A* fusions
Nucleophosmin (NPM1) gene	*NPM1*
Tumor-suppressor genes	*TP53*, *WT1*, *PHF6*
Spliceosome-complex genes	*SRSF2*, *U2AF1*
Cohesin-complex genes	*STAG2*, *RAD21*

Abbreviations: AML, acute myeloid leukemia; TF, transcription factor; *ASXL1*, additional sex combs-like 1 transcriptional regulator; *CBFB*, core-binding factor subunit-β; *CEBPA*, CCAAT/enhancer-binding protein-α; *DNMT3A*, DNA methyltransferase 3A; *EZH2*, enhancer of zeste 2 polycomb repressive complex 2 subunit; *FLT3*, FMS-related tyrosine kinase 3; *IDH*, isocitrate dehydrogenase; *MYH11*, myosin heavy chain 11 smooth muscle; *NPM1*, nucleophosmin; *PHF6*, PHD finger protein 6; *PML*, promyelocytic leukemia; *RARA*, retinoic acid receptor-α; *RUNX1*, Runt-related transcription factor 1; *RUNX1T1*, RUNX1 translocated to 1; *SRSF2*, serine/arginine-rich splicing factor 2; *STAG2*, stromal antigen 2; *TET2*, tet methylcytosine dioxygenase 2; *U2AF1*, U2 small nuclear RNA auxiliary factor 1; *WT1*, Wilms tumor 1.

1.4.5 Treatment

The primary goal is to achieve complete remission (CR) first and then maintain the CR by giving consolidation treatment. CR is defined as less than 5% blasts in the bone marrow, a neutrophil count greater than 1.0×10^9 /L, a platelet count more than 100×10^9 /L and independence of red cell transfusion [62].

The initial phase of the treatment to achieve CR is referred to induction therapy. The general therapeutic strategy has almost remained unchanged, which is the combination of continuous-infusion cytarabine with an anthracycline (eg, daunorubicin, idarubicin, or the anthracenedione mitoxantrone) [63,64].

If CR is achieved after induction therapy, appropriate postremission treatment is essential for maintenance. The aim of postremission treatment is to kill leukemic cells remaining in the bone marrow or blood. The strategies include intensive chemotherapy and hematopoietic stem cell transplantation (HSCT). For patients younger than 60, the standard therapy is 2 to 4 cycles of intermediate-dose cytarabine, however, the most appropriate number of cycles and dose depend on individuals. For older patients, neither more intensive consolidation chemotherapy nor less intensive consolidation chemotherapy gives a satisfying outcome, which means that the exploration of new maintenance therapies is necessary [65-69].

HSCT is known as the most successful curative treatment. Based on the different level of risk in patients, autologous HSCT or allogeneic HSCT can be the option. While autologous HSCT is not recommended in patients with high-risk cytogenetics, allogeneic HSCT can offer a relatively satisfying outcome in patients with intermediate- and high-risk AML [70,71].

1.5 FMS-like tyrosine kinase3 (FLT3) in AML

1.5.1 FLT3 mutations in AML

FLT3 is a gene located on chromosome 13q12 encoding the FLT3 tyrosine kinase receptor, which falls into Class III receptor tyrosine kinase [72,73]. The other members in this class are platelet-derived growth factor receptor (PDGFR), stem cell factor receptor (c-KIT), and macrophage colony-stimulating factor receptor (FMS). Activation of FLT3 by ligand (FL) promotes cell proliferation through downstream pathways, including PI3K, AKT, RAS, ERK and mTOR [74]. However, mutations can result in constitutive activation of the receptor even without ligand. The internal tandem duplication (ITD) mutation in the *FLT3* gene has been reported as one of the most frequent mutations in AML, which occurs in 20% -30% of AML patients and is associated with poor prognosis [75]. Single base mutation in the tyrosine kinase domain (TKD) of FLT3 is also a gain-of-function mutation, however, its prognosis significance is still unclear due to its rarity [76,77].

1.5.2 FLT3 inhibitors

Because of the association between FLT3-ITD and the higher relapse rate and poor disease-free and overall survival in AML, the effort to develop FLT3 inhibitors has been made in the recent decade. Nowadays, more than 20 small-molecule inhibitors targeting FLT3 have been reported and clinical trials to evaluate them are ongoing. The well-studied FLT3 inhibitors include sunitinib, tandutinib, sorafenib and PKC412 [78-81].

PKC412 (midostaurin), the N-benzoyl derivative of staurosporine, was named after its characterization as an inhibitor of PKC. It has activity not only against both FLT3-IDT and FLT3-TKD mutation, but also against multiple other kinases, including PDGFR and c-KIT [82]. In a phase I study including various solid tumors, the results showed inhibition of FLT3 activity with 75 mg thrice daily of PKC412. In phase II clinical trials, this dose was also well tolerated by 20 patients with FLT3-mutant AML and resulted in a significant decrease in peripheral blast counts. The phase III trials combining PKC412 with standard induction and postremission chemotherapy is ongoing, and the preliminary results show an improved overall survival in younger adult patients [77,83].

2 AIMS OF THE STUDY

The overall objective of this study is to define novel role of TERT and telomerase in cancer development, progression and cure, as well as the clinical implication of genetic alterations in TERT in cancer. More specifically, the study aims are:

1. To determine whether FLT3-ITD regulates TERT expression in AML cells and whether TERT expression affects FLT3 inhibitors' therapeutic efficacy in AML (Paper I).
2. To determine whether the rs2736100/rs2736098 variants in the *TERT* gene are associated with the incidence of TERT promoter mutations and HCC susceptibility (Paper II).
3. To define whether TERT promotes HCC development by regulating DNA methylation (Paper III).

3 METHODS

3.1 Patient samples (Papers I - III)

Patients' peripheral blood samples were collected at the Department of Hematology, Karolinska University Hospital, Stockholm, Sweden. The study was approved by the Stockholm Regional Ethics Review Committee, and written informed consent was obtained from the subjects (Paper I). Two hundred and forty-five patients with HCC as well as two hundred and fifty-seven healthy individuals were recruited from Shandong Provincial Hospital and Shandong University Second Hospital, Jinan, China. Fifty-three patients with newly diagnosed histologically confirmed HCC were recruited from Qilu Hospital, Jinan, China. Tumor specimens and/or blood samples were obtained after written informed consent was obtained from the patients. The study was approved by the Shandong University Second Hospital Ethics Committee (Papers II - III). All experiments were performed in accordance with relevant guidelines and regulations.

3.2 Cell lines and culture conditions (Papers I - III)

AML cell lines HL60, MV4:11 and MOLM-13 were used in Paper I. The specific FLT3 inhibitor PKC412 (Sigma-Aldrich, Buchs, Switzerland) was diluted in DMSO, and added into cells at different concentrations (0.01, 0.025, 0.05, and 0.1 μM) for various time periods (Paper I). HCC cell lines PLC/PRF/5 and HUH-7 were used in Paper III. The PIK3/AKT inhibitor LY2490024 was purchased from Millipore Sigma, and added into wells at 30 μM (Paper III).

3.3 Mutation analysis of FLT3-ITD (Paper I)

QIAamp Blood & Cell Culture DNA Kit (QIAGEN, Germany) was used to extract DNA from AML patients. PCR primers were fluorescently labeled with 6-FAM, HEX or NED. The length of amplified fragments with dye was detected by Applied Biosystems 3130XL and FLT3-ITD mutations were detected qualitatively by comparing it to a size standard using the GeneMapper Software.

3.4 Primary AML cell separation and culture (Paper I)

Leukemic cells were isolated from AML patients by Lymphoprep gradient centrifugation (Nycomb, Oslo, Norway) from peripheral blood and then cultured in complete medium in the absence or presence of PKC412 as described above.

3.5 RNA extraction and quantitative real-time PCR (Paper I & III)

Total cellular RNA in cells with different treatments and primary tumor tissues was extracted using Trizol, and RNA was reverse transcribed to cDNA. Quantitative real-time polymerase chain reaction (qPCR) was carried out using SYBR Green and specific primers. Relative expression of target mRNAs were calculated based on the CT values and normalized to β2m CT values. Primers used for qPCR are listed in Table 2.

Table 2. Primers and small interference RNAs (siRNAs) used in the study

Primers for qPCR	
β2m-F	5′-GAATTGCTATGTGTCTGGGT-3′
β2m-R	5′-CATCTTCAAACCTCCATGATG-3
RASSF1A –F:	5’- TCATCTGGGGCGTCGTG -3’
RASSF1A –R:	5’- CGTTCGTGTCCCGCTCC -3’
PTEN-F:	5’-CCGGCAGCATCAAATGTTTC-3’
PTEN-R:	5’-GTTCCACCCCTTCCATCTGC-3’
DNMT3b-F	5’-TGTTTCTGTGTGGAGTGC-3’
DNMT3b-R	5’-CAGCAATGGACTCCTCAC-3’

TERT-F	5’-CGGAAGAGTGTCTGGAGCAA-3’
TERT-R	5’- GGATGAAGCGGAGTCTGGA-3’
c-Myc-F	5′-TACCCTCTCAACGACAGCAGCTCGCCCAACTCCT-3′
c-Myc-R	5′-TCTTGACATTCTCCTCGGTGTCCGAGGACCT-3′
c-KIT-F	5′-TCATGGTCGGATCACAAAGA-3′
c-KIT-R	5′-AGGGGCTGCTTCCTAAAGAG-3′
DOK3-F	5′-GTCCCCATGGAGGAAAACTC-3′
DOK3-R	5′-AAGTGGTAGGGCCAGCTGTA-3′
SULF2-F	5′-CCGCCCAGCCCCGAAACC-3′
SULF2-R	5′-CTCCCGCAACAGCCACACCTT-3′
siRNAs	
DS NC1 Negative Control	5‘-CGUUAAUCGCGUAUAAUACGCGUAT-3’
siPTEN	5‘-AUGUGCAGUGUUGAAUCAUUUCUTC-3’
siTERT.1	5‘-CAUUUUUCCUGCGCGUCAUCUCUGA-3’
siTERT.2	5‘-GGUGAACUUCCCUGUAGAAGACGAG-3’
siSP1.1	5‘-GGUGCAAACCAACAGAUUAUCACAA-3’

siSP1.2	5‘-GGUGAGAUAGUAAAACACUUAUUCC-3’
Primers for Pyrosequencing	
PTEN.PCR primer F	5’-TTGTTATTATTTTTAGGGTTGGGAA -3’
PTEN.PCR primer R	5’-Biotin-CTAAACCTACTTCTCCTCAACAACC -3’
PTEN.Pyrosequencing primer	5’- GTTGGTATATTTAGGGATT -3’

3.6 Western blot (Papers I & III)

Total proteins from cells were extracted and quantified. Thirty μg of proteins were separated in Mini-PROTEAN TGX Gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk diluted in TBST, and then incubated with primary antibodies and followed by anti-mouse or rabbit secondary antibodies before imaged. Primary antibodies used were: AKT, p-AKT, FLT3 and p-FLT3, (Cell Signaling Technology, Boston, USA); β-Actin, c-MYC, Sp1 and PTEN (Santa Cruz Biotechnologies); TERT and DNMT3B (Abcam). β-Actin immunoblotting was performed in parallel as a loading control.

3.7 Assessment of telomerase activity (Paper I)

A protocol based on real-time telomeric repeat amplification was used to determine telomerase activity. Total cellular proteins were extracted using CHAPS lysis buffer. For each assay, 0.5 μg of protein was used for the telomerase-primer elongation with 30-min incubation at 30℃, which was then terminated by 5-min incubation at 95℃. The products containing telomeric repeats were further amplified using SYBR Green kit and detected by ABI7700 sequence detector. Total cellular protein from HCC cell line HepG2 was used as a positive control and the serial dilutions of positive control were used as standard curve [84].

3.8 Promoter activity assay (Papers I & III)

The TERT promoter reporter plasmid $p181^{wt}$ harboring the core promoter sequence of the hTERT 5′-flanking region and its mutant variant ($p181^{MYC-}$) with c-MYC motifs (E-boxes) deletion were described previously [85,86]. These two plasmids were transfected into MV4,11 and MOLM-13 cells with Lipofectamine2000 (Life Technology) according to the manufacturer's protocol in the absence or presence of PKC412. The cells were lysed and the luciferase activity was determined using a dual-luciferase reporter assay system (Promega, WI) 24 h after the transfection (Paper I).

The DNMT3B reporter construct contains the human DNMT3B promoter sequence (-469 - +260) from Shanghai Integrated Biotech Solutions (Shanghai, China). DNMT3B promoter plasmids were transfected into cells together with the control or TERT and/or Sp1 expression vectors using Lipofectamine2000, and the luciferase activity was determined using a dual luciferase reporter assay system (Promega, Madison, WI) 48 h post-transfection (Paper III).

3.9 Puro. Cre-TERT promoter-driven GFP plasmid and Lenti-III-HA-GFP-TERT vectors (Paper I)

The h3.4k-GFP plasmid was obtained from Dr. Pei-Rong Huang (National Taiwan University), which contained 3.4-kb TERT promoter (+ 1 to − 3405, ATG as + 1) located just upstream of *GFP* gene. To construct pLenti-III-HA-GFP-TERT vector, a 4.5-kb GFP-TERT fragment was cut from pBabe-hygro-GFP-TERT (addgene) and inserted into pLenti-III-HA (Applied Biological Materials Inc, BC, Canadia). A control plasmid (pLenti-BMN-GFP) was kindly provided by Rudbeck Laboratory, Uppsala University. The vectors were packaged into viral particles used for infecting AML cells to make cells with TERT promoter-driven GFP or with TERT over expression.

3.10 Cell cycle and apoptosis analyses (Paper I)

Stable TERT-over-expressed MOLM-13 cells and their control counterparts with pBMN were treated with 0.1 μM PKC412 for 24 h. The cells were fixed with ice cold 70% ethanol at +4 °C overnight, and stained with propidium iodide (50 μg/ml) in the presence of RNAse A (0.5 μg). Apoptotic cells and cell cycle distribution were determined using flow cytometry.

3.11 cDNA array (Paper III)

Stable TERT-over-expressed MOLM-13 cells and their counterparts with control pBMN were treated with DMSO or 0.1 μM PKC412 for 12 h. Total RNA was extracted for affymetrix Human Gene 1.0 ST Array. The differential gene expression between DMSO- and PKC412-treated cells with or without ectopic TERT expression was then analyzed.

3.12 DNA extraction and Sanger sequencing of the TERT promoter and *CTNNB1* gene (Paper II)

Genomic DNA was extracted using QIAGEN DNA extraction kits. DNA extracted from HCC tumors was used for Sanger sequencing to analyze *CTNNB1* gene mutations and TERT promoter mutations.

Sanger sequencing uses ssDNA as template and determines the sequence according to different length and end of amplified fragments [87]. Both deoxy-ribonucleoside triphosphate (dNTP) and dideoxyribonucleoside triphosphate (ddNTP) are added in the PCR system. The amplification will continue with a dNTP binding to the template, while the amplification will come to an end with ddNTP, which is incapable of binding to next nucleotide [88]. The binding of dNTP or ddNTP is random and therefore the amplification ends at different positions. Since four ddNTPs are labeled with different fluorescence dyes, integrating the termination signals can give the sequence of the template [89].

The mutation occurring at positions 124 and 146 bp upstream of the ATG site in the TERT core promoter were named as C228T and C250T. The primer pair for the TERT promoter sequencing was previously described: 5′-CAC CCG TCC TGC CCC TTC ACC TT-3′ (forward) and 5′-GGC TTC CCA CGT GCG CAG CAG GA-3′ (reverse). The *CTNNB1* gene hotspot mutations occur in exon 3 and the primers used to sequence this region are listed as follows: 5′-GGG TAT TTG AAG TAT ACC ATA C-3′ (forward) and 5′-TGG TCC TCG TCA TTT AGC AG-3′ (reverse). All the mutations were verified by sequencing from both directions [10,90-92].

3.13 The *TERT* rs2736100 (AC) AND rs2736098 (TC) genotyping (Paper II)

The *TERT* rs2736098 (TC) and rs2736100 (AC) were genotyped using pre-designed TaqMan SNP genotyping assay kits on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems), as described [93,94]. Both positive and negative controls were included in all assays and the running condition was as follows: 95 ℃ for 10 min, followed by 40 cycles of 92 ℃ for 15 sec and 60 ℃ for 1 min.

3.14 Small interference RNA (siRNA) transfection (Paper III)

siRNAs targeting TERT, Sp1, and PTEN were purchased from (Thermo Fisher Scientific) and Integrated DNA Technologies (IDT, Leuven, Belgium). Cells were transfected with siRNAs using Lipofactamine2000 (Thermo Fisher Scientific) according to the protocol provided. Cells were harvested for analyses 72 h post-transfection. siRNAs used in this study are listed in Table 2.

3.15 Plasmid transfection (Paper III)

TERT and Sp1 expression plasmids were kindly provided by Professor. R. Weinberg (Massachusetts Institute of Technology) and Doctor R. Tjian (Howard Hughes Medical Institute, Ashburn), respectively. The DNMT3B expression plasmid was purchased from AdeGene. Lipofactamine2000 (Thermo Fisher Scientific) was used for transfection according to the protocol provided.

3.16 Colony formation assay (Paper III)

HCC cells under different treatment conditions were seeded into six well-plates (1 000 cells/well) and incubated for 10 – 14 days. Plates were stained with Giemsa and the number of colonies with more than 50 cells was counted. The PIK3/AKT inhibitor LY2490024 was purchased from Millipore Sigma, and added into wells at 30 μM when TERT over-expressed cells were seeded.

3.17 DNA methylation assessments (Paper III)

Luminometric Methylation Assay (LUMA) and bisulfite pyrosequencing were used to determine global and PTEN promoter methylation, respectively. The LUMA assay has previously been described and exploits methylation sensitive and insensitive restriction enzymes followed by detection with Pyrosequencing [95,96]. Briefly, each DNA sample was subjected to two separate digestions, with HpaII + EcoRI and MspI + EcoRI. The digested DNA was then subjected to polymerase extension assays using Pyrosequencing. The level of cytosine methylation was finally determined by comparing the ratio of HpaII to MspI cleavages in the various samples. Pyrosequencing was previously described [97,98]. Briefly, genomic DNA was bisulfite-converted, and PCR amplification performed using PTEN promoter specific primers [99]. A biotin-labeled primer (reverse primer) was used to purify the PCR product by use of streptavidin-coated Sepharose beads (GE Healthcare, UK). PCR products were bound to Sepharose beads, purified, washed, denatured, and washed again. Then sequencing primer was annealed to the purified single-stranded PCR product and the Pyrosequencing was performed in a PyroMark Q96 (Qiagen) according to the manufacturer's instructions. The primers for PCR and sequencing are listed in Table 2.

3.18 The Cancer Genome Atlas (TCGA) dataset (Paper III)

The TCGA Research Network is available at http://cancergenome.nih.gov/. The datasets for HCC cases within the TCGA database were downloaded via Memorial Sloan Kettering Cancer Center cBioPortal for cancer genomics in January 2018 [100,101].

3.19 Statistical analyses (Papers I - III)

Mann-Whitney U-test was performed to examine the difference in DNA methylation as determined using LUMA and Pyrosequencing (Paper III). All other comparisons between control and treated cells were analyzed using Student's T-test or one-way ANOVA followed by LSD test (Paper I & III). Correlation between TERT and DNMT3B expression in HCC tumors was assessed using Pearson test. Differences in survival were compared with the log-rank test. Overall survival was visualized with Kaplan-Meier plots (Paper III). Chi-square ($\chi 2$) test was used to compare sex distribution between HCC patients and healthy controls. $\chi 2$ test or Student's T-test was used to analyze the differences in clinico-pathological variables between HCC patients with wild type or mutated TERT promoter, respectively. $\chi 2$ test was

used to evaluate the distribution differences of selected variables and alleles of the TERT rs2736098 and rs2736100 between patients and healthy controls. Hardy–Weinberg equilibrium of the genotype distribution among the controls was tested by a goodness-of-fit $\chi 2$ test. Odds ratios (OR) and their 95% confidence interval (CI) were estimated using unconditional univariate and multivariate logistic regression analyses for risk of HCC or tumors with and without TERT promoter mutation (Paper II). All the tests were two-tailed. P values <0.05 were considered significant.

4 RESULTS & DISCUSSION

4.1 MYC-dependent down-regulation of telomerase by FLT3 inhibitors is required for their therapeutic efficacy on AML (Paper I)

4.1.1 PKC412 down-regulated TERT expression and TERT promoter activity in FLT3ITD-carrying AML cells

PKC412, a specific FLT3ITD inhibitor, was used in this study. In AML cell lines MV4, 11 and MOLM-13, which carry FLT3ITD mutations, PKC412 inhibited FLT3 phosphorylation and activity in both time- and dose-dependent manners. TERT mRNA expression was down-regulated in MV4, 11, MOLM-13 and primary FLT3ITD-positive AML cells after PKC412 treatment. The qPCR results showed that PKC412 inhibited TERT mRNA expression also in a dose-dependent manner. To confirm the role of FLT3ITD mutations in PKC412-mediated down-regulation of TERT expression, two wild-type FLT3-carrying cell lines HL60 and HeLa were treated with PKC412, however, there were no detectable changes in TERT mRNA level, which suggested that PKC412 inhibited TERT expression via FLT3ITD. A significantly diminished telomerase activity was detected in MV4, 11 and MOLM-13 as well 24h after treated with PKC412.

4.1.2 PKC412 inhibited TERT transcription in a MYC-dependent way

To determine the underlying mechanism, a GFP expression vector driven by a 3.4-kb-long TERT promoter was constructed and transfected into MV4, 11 and MOLM-13 cells and the cells were then treated with PKC412. PKC412 led to reduced GFP+ cells compared to DMSO. A core TERT promoter reporter construct (p181) was transfected into the same cells followed by DMSO or PKC412 treatment. The luciferase activity driven by p181 was significantly inhibited in these cells after PKC412 treatment.

PKC412 treatment led to diminished c-MYC mRNA and protein expression in MV4, 11 and MOLM-13 cells. Because c-MYC is a well-established TF regulating TERT expression [102], our hypothesis is that FLT3ITD regulates TERT transcription via c-MYC. Wild-type p181 vector and its counterpart with MYC binding motif-deletion were transfected into the

same cell lines, respectively. When these cells were treated with PKC412, wild-type TERT promoter activity was down-regulated compared to that in DMSO-treated control cells. In contrast, no difference between DMSO and PKC412 treatment was observed when cells were transfected with the MYC binding site-deleted p181 vector.

4.1.3 The attenuation of PKC412-mediated AML cell apoptosis by ectopic TERT expression

To explore whether TERT expression was associated with the PKC412 effect in AML cell killing, a variant subline of MOLM-13 with ectopic TERT expression (MOLM-13-hTERT) and the control subline with empty vectors (MOLM-13-pBMN) were made. IC_{50} was 17.2 and 34.1 μM for MOLM-13-pBMN and MOLM-13-TERT, respectively. The viability was assessed by incubating these two sublines with or without PKC412 (0.0125 μM) for different time periods. The viability in MOLM-13-TERT cells was significantly higher than that in MOLM-13-pBMN at all the time points ($P = 0.009$). Almost all MOLM-13-pBMN cells were dead by 120 h. The results from flow cytometry revealed that the ectopic TERT expression significantly attenuated apoptosis mediated by PKC412, which was consistent with the cell viability results.

4.1.4 The enhanced activity of alternative tyrosine kinase signaling pathways and AKT mediated by TERT in the presence of PKC412

Comparing the gene expression profiles between MOLM-13-pBMN and MOLM-13-hTERT cells treated with DMSO or PKC412, we found that ectopic TERT expression contributed to the activation of alternative tyrosine kinase pathways in the presence of PKC412. c-KIT, another tyrosine kinase receptor, and SULF2, an activator in the PDGF signaling pathway, were up-regulated; DOC3, a negative regulator of the RAS signaling pathway was repressed (Figure 2).

Moreover, in PKC412-treated cells, ectopic TERT expression also increased the phosphorylation and activation of AKT, a well-studied downstream effector in tyrosine kinase signaling pathways which promotes cell survival [103].

In summary, this study demonstrated that FLT3ITD mutations play an important role in constitutive TERT expression, and the FLT3 specific inhibitor PKC412 contributes to AML

cells killing by suppressing TERT expression. PKC412 inhibited both FLT3 activation and TERT expression, and ectopic TERT expression rescued the PKC412-mediated apoptosis of MOLM-13 cells. Furthermore, we demonstrated that the induction of TERT transcription by FLT3ITD was MYC-dependent. It has been reported that TERT can contribute to cell survival by up-regulating growth factors and pro-survival factors through its telomere lengthening-independent function [104-106]. In this study we found ectopic TERT expression enhanced alternative tyrosine kinase signaling pathways as well as the downstream effector AKT. Taken together, TERT may play an important role in the resistance to AML-targeted therapy, and the positive feedback between FLT3ITD and telomerase may shed light on AML pathogenesis and therapy.

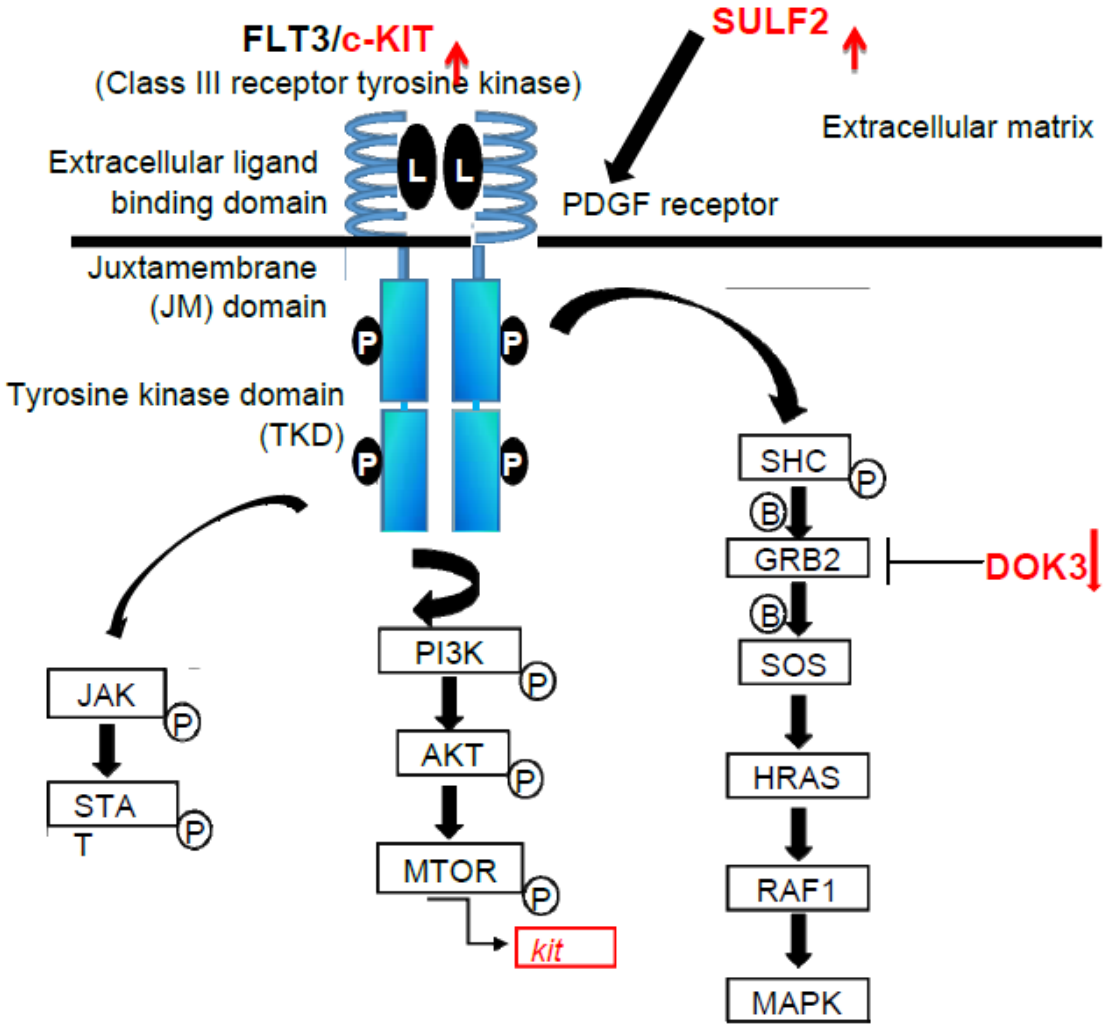

Figure 2. TERT stimulates the FLT3 downstream effectors and alternative tyrosine kinase pathways in the presence of PKC412, and the schematic of c-KIT, DOC3, and SULF2 as regulators of the FLT3 and other tyrosine kinase signaling pathways.

4.2 The TERT promoter mutation incidence is modified by germline TERT rs2736098 and rs2736100 polymorphisms in HCC (Paper II)

4.2.1 TERT promoter mutations and their relation to CTNNB1 mutations and clinico-pathological variables in HCCs

Tumor DNA from 200 HCC patients was extracted and sequenced to identify TERT promoter mutations, among which 190 were evaluable (Figure 3). The results showed 57 of 190 (30%) tumors carrying TERT promoter mutations, and the frequency of C228T was higher (50 out of 57; 88%) than that of C250T (7 out of 57; 12%). It is reported that the *CTNNB1* gene mutation occurs frequently and is associated with TERT promoter mutations in HCC [107,108]. DNA from 81 HCC tumors was used for Sanger sequencing to assess the hotspot mutations in *CTNNB1* exon 3. The sequencing was successful in 70 of them, among which 17 (24%) tumors carried mutations. No association between *CTNNB1* mutations and TERT promoter mutations was observed in these HCC tumors.

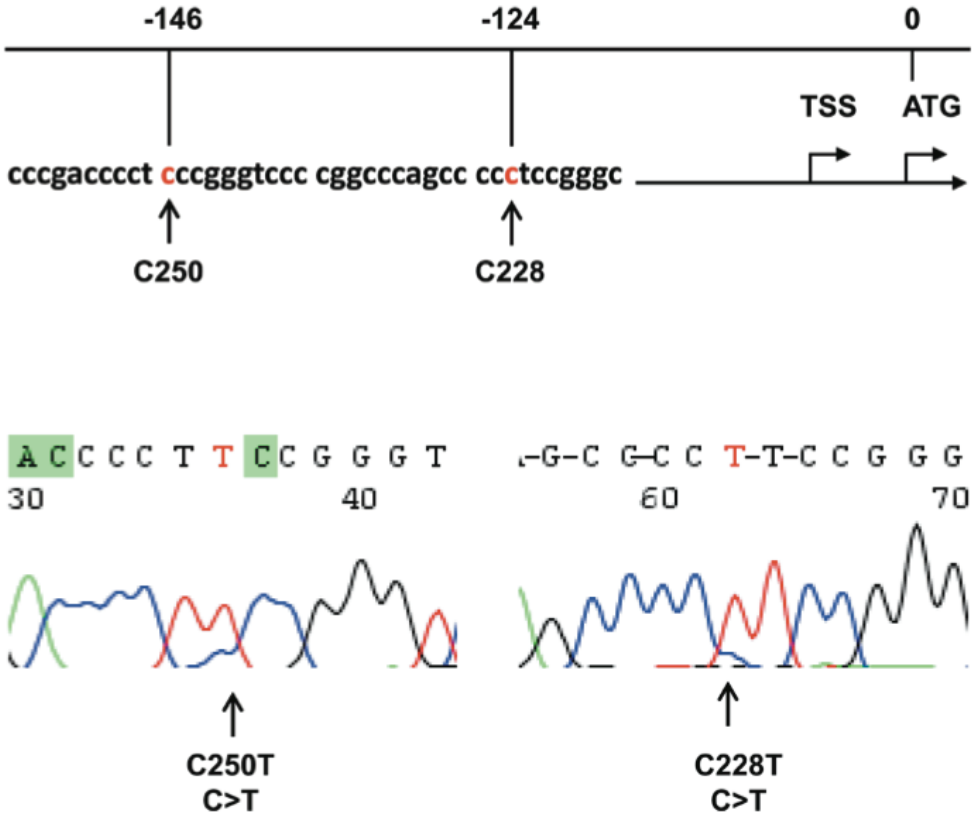

Figure 3. Identification of TERT promoter mutations (C250T and C228T) in HCC

We further analyzed the relationship between TERT promoter mutations and clinico-pathological variables, and no significant differences were observed in sex, age, α-fetoprotein levels, liver cirrhosis, HBV infection, tumor sizes, differentiation status and metastasis.

4.2.2 The association between *TERT* genetic variants and TERT promoter mutations in HCC

Accumulating studies showed that *TERT* rs2736098 and rs2736100 variants are associated with cancer risk. We thus carried out *TERT* rs2736098 (TC) and rs2736100 (AC) genotyping. For rs2736098, data were available in 231 HCC patients and 240 healthy controls; for rs2736100, data were available in 201 HCC patients and 237 healthy controls. When comparing genotype and allele frequencies of the two variants between patients and healthy controls, we found that HCC patients exhibited a significantly lower frequency of rs2736100_CC genotype.

Since it is reported that both rs2736098 and rs2736100 variants are capable of regulating TERT expression and telomerase activity [109-111], next we sought to define if there is a link between these two *TERT* genetic variants and TERT promoter mutations. When comparing HCC patients bearing TERT promoter mutations and healthy controls, we found that the frequencies of rs2736098_TT and rs2736100_CC were significantly lower in the group with mutations (Table 3). When comparing HCC patients with wild-type and mutant TERT promoters, similar distributions were observed: the group with mutations exhibited significantly lower frequencies of both rs2736098_TT and rs2736100_CC (Table 4).

According to the result of linkage disequilibrium analysis, the association between rs2736098 and rs2736100 is non-significant in the Han Chinese population.

In summary, we observed a negative association between TERT promoter mutations and rs2736098_TT/ rs2736100_CC. It is known that both C228T and C250T TERT promoter mutations generate new ETS binding sites, thereby activating TERT transcription, and tumors with shorter telomere are prone to undergo TERT promoter mutations [10,112-115]. However, studies have shown that rs2736100_CC and rs2736098_TT have opposite effects on TERT transcription. rs2736098_TT tends to repress TERT transcription and telomere elongation, while rs2736100_CC has been shown to be associated with higher TERT

expression and longer telomeres. The lower frequency of rs2736100_CC in patients with HCC tumors with TERT promoter mutations can be explained by genetic stress, since the higher TERT expression resulting from rs2736100_CC gives HCC patients less stress for telomerase activation and therefore TERT promoter mutations. In contrast, the lower frequency of rs2736098_TT in HCC patients with TERT promoter mutations cannot be explained by this genetic stress.

We failed to find an association between TERT promoter mutations and clinico-pathological variables including sex, age, α-fetoprotein levels, liver cirrhosis, HBV infection, tumor sizes, differentiation status and metastasis as reported previously [107,108,116-122]. We found that TERT promoter mutations and CTNNB1 mutations were independent of each other, contrasting to previously published results [107,108].

Table 3. TERT promoter mutations and association with rs2736100 and rs2736098 in HCC patients

Genotype Odds	Cases	Healthy	ratio (95% CI)	*P*
rs2736098				
wt TERT promoter vs controls	128 (100%)	240 (100%)		
TT	13 (10.1)	31 (12.9)	1.0 (Ref.)	
CT	61 (47.7)	115 (47.9)	1.265 (0.617–2.594)	0.643
CC	54 (42.2)	94 (39.2)	1.370 (0.661–2.840)	0.504
CT + CC	115 (89.9)	209 (87.1)	1.310 (0.660–2.607)	0.543
mt TERT promoter vs controls	55 (100%)	240 (100%)		
TT	2 (3.6)	31 (12.9)	1.0 (Ref.)	
CT	40 (72.7)	115 (47.9)	5.391 (1.234–23.553)	**0.025**
CC	13 (23.7)	94 (39.2)	2.144 (0.458–10.030)	0.505
CT + CC	53 (96.4)	209 (87.1)	3.931 (0.912–16.948)	0.083
rs2736100				
wt TERT promoter vs controls	114 (100%)	237 (100%)		
AA	38 (33.3)	69 (29.1)	1.0 (Ref.)	
CA	52 (45.6)	108 (45.6)	1.141 (0.683–1.916)	0.705
CC	24 (21.1)	60 (25.3)	0.726 (0.392–1.346)	0.389
AA + AC	90 (78.9)	177 (74.7)	1.0 (Ref.)	
CC	24 (21.1)	60 (25.3)	0.787 (0.460–1.346)	0.457
mt TERT promoter vs controls	52 (100%)	237 (100%)		
AA	15 (28.8)	69 (29.1)	1.0 (Ref.)	
CA	34 (65.4)	108 (45.6)	1.448 (0.735–2.854)	0.389
CC	3 (5.8)	60 (25.3)	0.230 (0.0635–0.833)	**0.032**
AA + AC	49 (94.2)	177 (74.7)	1.0 (Ref.)	
CC	3 (5.8)	60 (25.3)	0.181 (0.0543–0.601)	**0.004**

HCC, Hepatocellular carcinoma; CI, confidence interval.

Table 4. rs2736098 and rs2736100 genotype frequency in HCC patients bearing wt and mutant TERT promoter in tumors

	wt	mutant	*P* value
rs2736098	128 (100%)	55 (100%)	
TT	13 (10.1)	2 (3.6)	
CT	61 (47.7)	40 (72.7)	
CC	54 (42.2)	13 (23.7)	**0.007**
rs2736100	114 (100%)	52 (100%)	
AA	38 (33.3)	15 (28.8)	
CA	52 (45.6)	34 (65.4)	
CC	24 (21.1)	3 (5.8)	**0.018**

HCC, hepatocellular carcinoma.

4.3 TERT is required for DNMT3B expression/ aberrant DNA methylation phenotype and AKT activation in HCC (Paper III)

4.3.1 TERT regulates DNMT3B expression and the downstream tumor suppressors in HCC cells

TERT is known to be important in tumorigenesis by both canonical telomere-lengthening function and its activities independent of telomere stabilization. The aberrant DNA methylation is another key factor contributing to human malignancies [123,124]. In this study, we wanted to determine the potential link between TERT and cancer-specific DNA methylation. DNMT3B is a member of DNMTs and has been reported to promote HCC progression [125]. Our results showed that in HCC cell lines PLC/PRF/5 and HUH-7, TERT depletion down-regulated DNMT3B expression while TERT over-expression up-regulated DNMT3B expression at both mRNA and protein levels. Consistent with the results in HCC cell lines, a positive correlation was observed between TERT and DNMT3B mRNA expression in 53 primary HCC tumors.

DNMTs contribute to tumorigenesis by repressing downstream tumor suppressors, and therefore we further assessed the expression of PTEN and RASSF1A, two well-studied TSGs repressed by DNMT3B-mediated promoter hypermethylation [39,40,126,127]. TERT depletion increased, whereas ectopic TERT inhibited PTEN expression at both mRNA and protein levels. Moreover, the over-expression of DNMT3B rescued the de-repression of PTEN resulting from TERT depletion at both mRNA and protein levels. Similar changes of RASSF1A transcripts were observed in the experiments above.

4.3.2 TERT inhibition leads to the PTEN promoter demethylation and global decline in DNA methylation in HCC cells

To see whether TERT regulates DNMT3B downstream tumor suppressors by affecting their promoter methylation, we first performed Pyrosequencing to examine the methylation status of the PTEN promoter region in PLC/PRF/5. TERT depletion led to a significant demethylation at 3 of 9 CpG positions sequenced successfully in the PTEN promoter. Beyond that, we further performed LUMA to assess global methylation in PLC/PRF/5 after TERT knock-down. The results showed a significant decline in global methylation compared to the control counterparts. Taken together, TERT plays an important role in the aberrant methylation in HCC cells.

4.3.3 TERT cooperates with Sp1 to stimulate DNMT3B promoter activity

It is reported that the TF Sp1 can bind to the DNMT3B promoter and stimulate its transcription [128]. Studies have also shown that TERT can interact with Sp1 as a co-factor to facilitate Sp1 target transcription [32]. We hypothesize that this may be the case in TERT-mediated DNMT3B expression. As expected, the ectopic expression of Sp1 increased abundance of DNMT3B while the depletion of Sp1 decreased DNMT3B expression in both PLC/PRF/5 and HUH-7. Moreover, both the gain and loss-function assays of Sp1 revealed that PTEN expression was altered in the opposite manner to DNMT3B. Over-expression of Sp1 rescued the decreased DNMT3B expression resulting from TERT depletion. We further constructed the reporter plasmid containing DNMT3B promoter sequence. When co-transfected cells with TERT or Sp1 expression vector alone, only a slight increase in DNMT3B promoter activity was observed, whereas DNMT3B promoter activity was significantly increased when co-transfected with both TERT and Sp1 expression vectors. The results suggested that TERT regulates DNMT3B transcription by cooperating with Sp1.

4.3.4 TERT-DNMT3B-PTEN-AKT axis in HCC

According to the results presented in Paper I, TERT plays a positive role in AKT activation in leukemic cells. We thus examined whether it was the same in HCC cells. PTEN is a tumor suppressor, and negatively regulates the PI3K/AKT signaling pathway [129]. Consistently, TERT over-expression enhanced while its depletion diminished pAKT abundance. Furthermore, the down-regulation of pAKT mediated by TERT knock-down was rescued

when blocking PTEN expression using PTEN specific siRNA. Taken together, the results reveal a TERT-DNMT3B-PTEN-AKT axis in HCC cells.

We further evaluated the functional effect of this axis by assessing clonogenic formation of HCC cells. Consistent with previous studies reporting that TERT promotes cancer cell survival and proliferation, TERT depletion led to a significant decline in the number of colonies. The over-expression of either DMT3B or its TF Sp1 restored the decline. Moreover, a blockade of AKT activity by its inhibitor LY2940024 abolished the enhancement of clonogenic potential mediated by TERT over-expression.

We also sought to determine the clinical relevance of TERT-DNMT3B-PTEN-AKT axis. The analysis in 380 HCC patients from the TCGA database revealed that higher levels of TERT and DNMT3B expression both predict shorter survival, which suggests a promoting effect of this axis on HCC disease progression.

In summary, we demonstrated a novel telomere lengthening-independent role of TERT by showing a link between TERT and aberrant DNA methylation in HCC. Mechanistically, TERT promotes DNMT3B transcription by cooperating with Sp1, thereby contributing to the maintenance of global DNA hypermethylation as well as gene-specific hypermethylation in HCC cells. Our results reveal that PTEN, one of the well-studied DNMT3B downstream tumor suppressors, is regulated by TERT via promoter DNA methylation. In addition, we found the TERT-mediated PTEN repression enhances AKT activity, which in turn promotes cell survival and proliferation. While previous studies mainly focused on the role of AKT in TERT phosphorylation and stabilization, our results indicate a positive feedback loop between TERT and AKT. Taken together, the TERT-DNMT3B-PTEN-AKT axis may greatly drive HCC pathogenesis and be the hub for three oncogenic signaling cascades and targets for cancer therapy (Figure 4).

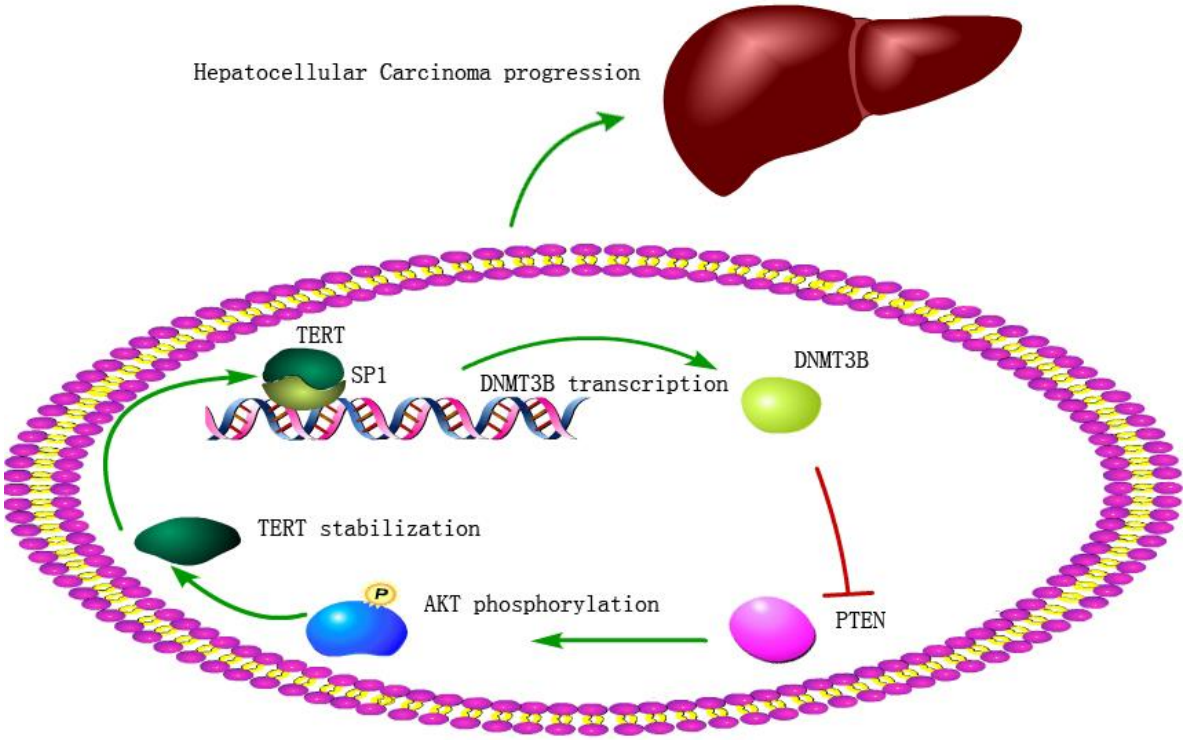

Figure 4. TERT-DNMT3B-PTEN-AKT axis in HCC progression.

5 SUMMARY& CONCLUSIONS

1. The FLT3 inhibitor PKC412 represses TERT expression and telomerase activity in AML cells carrying FLT3ITD mutations, and this effect is MYC-dependent. On the other hand, ectopic TERT expression attenuates the PKC412-mediated apoptosis of AML cells, and activates alternative tyrosine kinase signaling pathways as well as downstream effector AKT. The findings indicate a link between FLT3ITD mutations and telomerase. Moreover, our study also demonstrated that TERT is required for therapeutic efficacy of PKC412 in AML and TERT confers resistance to the PKC412 targeted AML therapy (Paper I).
2. HCC patients bearing tumors with mutant TERT promoter showed lower frequencies of rs2736098_TT and rs2736100_CC compared to wild type TERT promoter-carrying HCC patients or healthy controls. This indicates a negative association between TERT promoter mutations and these two genotypes, and also reveals the effect of germline TERT genetic background on TERT promoter mutations occurrence (Paper II).
3. TERT promotes DNMT3B transcription by cooperating with the TF Sp1, thereby contributing to the maintenance of hypermethylation and the repression of downstream tumor suppressors PTEN and RASSF1A. TERT depletion led to significant demethylation in the PTEN promoter as well as a reduced global DNA methylation. The restoration of PTEN expression mediated by TERT depletion inhibited AKT activity, which forms a positive feedback between AKT and TERT. Higher levels of TERT and DNMT3B expression predicted significantly shorter survival in HCC patients. Taken together, we identified the TERT-DNMT3B-PTEN-AKT axis in HCC cells which promotes HCC progression via aberrant DNA methylation (Paper III).

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