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TELOMERASE REVERSE TRANSCRIPTASE (TERT): PROMOTER MUTATION AND NOVEL FUNCTION IN HUMAN CANCER

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Telomerase reverse transcriptase (TERT): Promoter mutation and novel function in human cancer

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In memory of my father

ABSTRACT

Telomerase reverse transcriptase (TERT) is the catalytic component of telomerase, an RNA-dependent DNA polymerase responsible for telomere elongation. TERT is transcriptionally repressed in most normal human somatic cells with limited life-span, which contributes to telomerase silence. It is well established that maintenance of telomere length is essential to cellular immortalization and malignant transformation, and predominantly achieved through TERT induction and subsequent telomerase activation. Evidence has also accumulated that, apart from its canonical telomere-lengthening function, telomerase or TERT exhibits many other biological activities, thereby promoting cancer progression. Given its critical role in cancer formation and differential expression between normal and malignant cells, TERT/telomerase has been suggested as a novel cancer diagnostic marker and therapeutic target. To rationally develop these telomerase-based clinical applications, it is a demanding task to gain profound insights into the regulatory mechanism underlying cancer-specific TERT expression or telomerase activation, and to delineate various roles of TERT/telomerase in cancer development/progression. My thesis thus focuses on these important issues. Specifically, I sought to determine: (i) the biological role and clinical implication of the somatic TERT promoter mutation, and (ii) the regulatory effect of TERT on the oncogenic factor cyclooxygenase2 (COX2) in human cancer.

The somatic TERT promoter mutation, initially identified in malignant melanoma, stimulates *TERT* gene transcription and activates telomerase. To see whether this genetic event occurs in the pathogenesis of thyroid and gastric cancer, we sequenced the TERT promoter region in tumor specimens derived from patients with premalignant thyroid lesions, thyroid and gastric cancer (papers I, II and III). A moderate to high frequency of TERT promoter mutations was observed in follicular cell-derived but not in parathyroid C cell-derived thyroid cancer. The mutation was correlated with senior age, shorter telomere length, aggressive disease and shorter disease-specific survival in thyroid cancer. Importantly, we found the mutation in a fraction (up to 17%) of premalignant thyroid lesions, coupled with TERT and telomerase expression, which indicates that TERT promoter mutations are early genetic events in thyroid carcinogenesis. In contrast, none of tumors from 200 patients with gastric cancer harbored TERT promoter mutations, suggesting different mechanisms for telomerase activation in gastric cancer.

One of non-canonical TERT functions is to serve as a transcription co-factor to regulate gene expression. We thus determined whether the manipulating TERT expression affected the abundance of the oncogenic factor COX2 in cancer cells (paper IV). The inhibition of TERT in cancer cell lines using RNA interference led to a robust increase in COX2 expression at both mRNA and protein levels through a p38 MAPK signal pathway. TERT depletion alone did not affect cell survival, while the COX2 inhibitor together with TERT inhibition synergistically induced apoptosis of cancer cells. TERT depletion combined with the COX2 inhibitor administration significantly inhibited tumor growth in the mouse xenograft model of human gastric cancer. These findings reveal a novel function of TERT and have implications in telomerase-based anti-cancer therapy.

In conclusion, the TERT promoter mutation is an early genetic event activating telomerase in the thyroid carcinogenesis, and associated with the aggressive disease and serves as a useful prognostic factor in thyroid cancer. The COX2 induction by TERT inhibition likely protects cancer cells from apoptosis. Thus simultaneously targeting TERT and COX2 may have synergistic killing effects on cancer cells, thereby improving treatment efficacy in a telomerase-based anti-cancer strategy.

LIST OF SCIENTIFIC PAPERS

I. **T Liu***, N Wang*, J Cao, A Sofiadis, A Dinets, J Zedenius, C Larsson and D Xu. *The age- and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas.* Oncogene, 2013. doi: 10.1038/onc.2013.446.

II. N Wang*, **T Liu***, A Sofiadis, J Zedenius, A Höög, C Larsson and D Xu. *The TERT promoter mutation as an early genetic event activating telomerase in follicular thyroid carcinogenesis.* Cancer. (in press)

III. **T Liu**, X Liang, M Björkholm, J Jia and D Xu. *The absence of TERT promoter mutations in primary gastric cancer.* Gene, 2014. 540(2): 266-267.

IV. **T Liu**, X Liang, B Li, M Björkholm, J Jia and D Xu. *Telomerase reverse transcriptase inhibition stimulates cyclooxygenase2 expression in cancer cells and synergizes with celecoxib to exert anti-cancer effects.* Br J Cancer, 2013. 108(11): 2272-2280.

* Contributed equally.

OTHER RELATED PUBLICATIONS:

1. **T Liu***, T C. Brown*, C. C Juhlin, A Andreasson, M Bäckdahl, J Healy, M L Prasad, R Korah, T Carling, D Xu and C Larsson. *The activating TERT promoter mutation C228T is recurrent in subsets of adrenal tumors.* Endocrine-related Cancer. 2014. 21 (3):427-34.
2. K Wang*, **T Liu***, L Liu*, J Liu, C Liu, C Wang, N Ge, H Ren, K Yan, S Hu, M Björkholm, Y Fan and D Xu. *TERT Promoter Mutations in Renal Cell Carcinomas and Upper Tract Urothelial Carcinomas.* Oncotarget, 2014. 5(7):1829-1836.
3. P Li, **T Liu**, J Liu, Q Zhang, F Lou, F Kong, G Cheng, M Björkholm, C Zheng and D Xu. *Promoter Polymorphism in the Serotonin Transporter (5-HTT) Gene Is Significantly Associated with Leukocyte Telomere Length in Han Chinese.* PloS ONE, 2014. 9(4): e94442.
4. Z Liu, Q Li, K Li, L Chen, W Li, M Hou, **T Liu**, J Yang, C Lindvall, M Björkholm, J Jia and D Xu. *Telomerase reverse transcriptase promotes epithelial-mesenchymal transition and stem cell-like traits in cancer cells.* Oncogene, 2013. 32(36): 4203-13.

* Contributed equally.

CONTENTS

LIST OF ABBREVIATIONS

AFTA	Atypical follicular thyroid adenoma
AKT (PKB)	Protein Kinase B
ALT	Alternative Lengthening of Telomere
Ap1	Activator protein 1
ATC	Anaplastic thyroid carcinoma
COX1	Cyclooxygenase1
COX2	Cyclooxygenase2
CTNNB1	Beta-catenin
ddNTP	Dideoxy-ribonucleoside triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxy-ribonucleoside triphosphate
DSS	Disease specific survival
E6 AP	E6 associated protein
EBV	Epstein–Barr virus
ELISA	Enzyme linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ETS	E-twenty-six
EWS	Ewing's sarcoma
FBS	Fetal Bovine Serum
FTA	Follicular thyroid adenoma
FTC	Follicular thyroid carcinoma
HBG	Beta-globin
HIF-1α	Hypoxia-inducible factor 1-alpha

HPV	Human Papillomavirus
IL-6	Interleukin-6
MAPK	Mitogen-activated protein kinase
M-MLV	Moloney Murine Leukemia Virus
MTC	Medullary thyroid carcinoma
OS	Overall survival
PAX8	Paired box gene 8
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PPARG	Peroxisome proliferator-activated receptor gamma
Rb	Retinoblastoma protein
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
Q-FISH	Quantitative Fluorescent in situ hybridization
RBP2	Retinoblastoma binding protein 2
RIPA	Radio Immunoprecipitation Assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SMYD 3	SET and MYND domain-containing protein 3
Sp1	Specificity Protein 1
STAT3	Signal transducer and activator of transcription 3
TCF	Ternary Complex Factor

TERC	Telomerase RNA template
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TRF1	Telomeric repeat binding factor1
TSS	Transcription start site
WT1	Wilms' tumor 1

1. INTRODUCTION

1.1 Telomere

1.1.1 Telomere structure

Telomere is the structure at the end of linear chromosomes. In all eukaryotes, the chromosome ends with tandem guanine-rich repeats. The human telomere sequence is $(TTAGGG)_n$ repeats ranging from 8 to 20 kb, and is in dynamic homeostasis. [1] [2] [3] There is a 3' overhang in telomere that usually loops back and inserts into the double strand of the telomere, thereby forming the t-loop and making the chromosome more stable. [4]

1.1.2 Telomere-binding proteins

Telomeric DNA is associated with a specific protein complex, which is essential for maintaining the structure and functionality of telomere. The most important protein complex structure is shelterin, which includes TRF1 (TTAGGG-repeat binding factor 1), TRF2, TIN2, RAP2, TPP1 and POT1. TRF1 and TRF2 bind the double-stranded telomere and combine with the rest proteins to form a special structure at the end of chromosomes. Shelterin regulates 3' overhang and t-loop formation. [5]

1.1.3 Telomere shortening with cell division

In most normal human cells, the telomere length is shortened with each cell division due to lack of telomere maintenance mechanism plus the end replication problem. When telomere is shortened to a certain limit, cells will stop divide and undergo permanent growth arrest so-called replication senescence. [6, 7] Therefore, most normal human cells are incapable of dividing indefinitely.

1.1.4 Telomere function

Telomere helps to maintain the stability and integrity of chromosomes. Telomere and their binding proteins form special structure at the end of chromosomes,

thereby protecting chromosomes from being recognized as double-stranded breaks or end-to-end fusion and suppressing DNA damage response. Telomere also prevents chromosome end degradation by excessive nuclease. Importantly, progressive telomere shortening occurs as cells divide, which serves as a mitotic clock to control cell life-span and to prevent unlimited cellular proliferation.

1.2 Telomerase

Telomerase is a ribonucleoprotein enzyme that synthesizes telomeric TTAGGG repeats at the end of linear eukaryotic chromosomes. Telomerase is a RNA-dependent DNA polymerase that consists of a catalytic subunit telomerase reverse transcriptase (TERT), a RNA template TERC and many other components (Figure 1). [3, 8, 9] TERC is constitutively expressed in cells whereas TERT expression is tightly regulated in time, place and quantity, which makes TERT the rate-limiting subunit of telomerase. [10] [11] Normal cells stop divide at the hayflick-limit, but tumor cells need a telomere maintenance mechanism to evade the hayflick-limit or senescence. Telomerase activation is the major mechanism for maintaining telomere length in cancer cells.

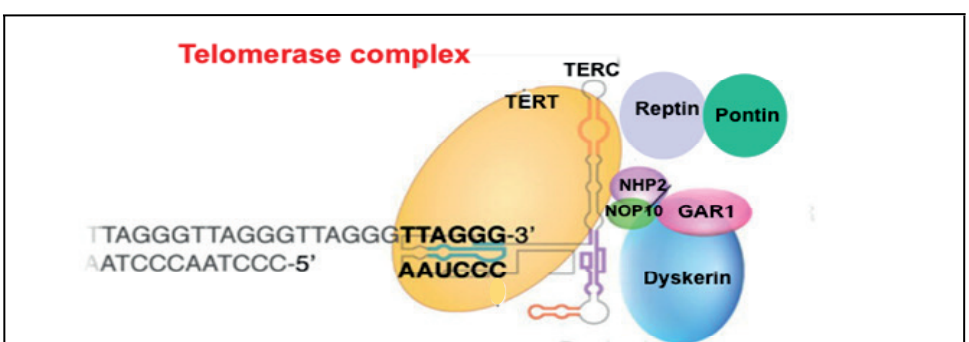

Figure1. **The telomerase complex**. It consists of a rate-limiting, catalytic subunit TERT, an RNA template TERC, and other components. TERT and TERC are the core of the telomerase complex and sufficient to constitute telomerase activity.

1.2.1 Telomerase activity and subunit expression in normal and cancer cells

Telomerase is a huge ribonucleoprotein complex with the key components of TERT and TERC (Figure 1). [12] TERT and TERC are sufficient to constitute telomerase activity *in vitro*. In most differentiated human somatic cells, telomerase activity is undetectable or very low. [13-15] However, human germline cells have telomerase activity and thus maintain stable telomere length. Stem/progenitor cells, lymphocytes, and other cells with highly proliferative potentials express various levels of telomerase activity. [15-17] TERT expression is in general associated with acquisition of telomerase activity, and introduction of TERT into telomerase-deficient cells is sufficient to activate telomerase, suggesting a key role of TERT in controlling telomerase activity. [18]

Telomerase activity is detectable in up to 90% of human malignant cells and the induction of TERT expression is a determinant step for acquisition of telomerase activity during cellular transformation as described above. [15, 17, 19] One of the key hallmarks of cancer is limitless replication potential, which is mainly achieved by TERT expression and telomerase activation. [20] Experimentally, TERT was shown to be one of essential elements for conversion of normal human cells into malignant ones. [19] Given the important role of TERT in cancer development, numerous efforts have been made to elucidate the mechanism governing TERT expression. [21, 22]

1.2.2 The regulation of TERT expression

The *TERT* gene was first cloned in 1997, [21] and since then, its regulation has been exclusively explored. TERT is regulated at multiple levels by numerous factors. [23-26]

1.2.2.1 Genetic regulation of TERT expression

Abnormal gene copy numbers. The *TERT* gene spans 40 kbs and is localized at chromosome 5p15.33. [25] Normal somatic cells from healthy individuals carry 2 copies of the *TERT* gene, and loss or gain of the copy numbers causes

abnormities. In Cri du chat syndrome, patients were found to have one *TERT* allele deletion, coupled with lower telomerase activity in their cells. *TERT* is haploinsufficient for telomere maintenance in these patients. [27, 28]

The gain of *TERT* gene copies occurs in human cancers, either from gain of 5p or gene amplification. Over 30% of human malignancies carry more than 3 *TERT* copies, [29, 30] which contributes to telomerase activation in those malignancies.

***TERT* gene mutations.** The *TERT* gene point mutation has been found to result in many degenerative diseases, such as aplastic anemia, idiopathic pulmonary fibrosis and others. Non-synonymous *TERT* mutations cause stem cell dysfunction and lead to a low telomerase activity. [31, 32] In addition, the polymorphism or mutation at the regulatory region of the *TERT* gene also affects TERT expression and telomerase activity (See the TERT promoter mutation chapter below for details).

1.2.2.2 Transcriptional regulation of TERT expression

The TERT promoter is GC rich and lacks both TATA and CAAT boxes. It contains at least five Sp1 binding motifs, two E-boxes and a single transcription start site that binds multi-functional transcription factor TFII-I for gene expression. [33, 34] Numerous studies have shown that the TERT transcription machinery is controlled by various transcription and epigenetic factors (Figure 2).

Positive transcription factors

c-Myc

The oncoprotein c-Myc is the well characterized transcription factor that activates *TERT* transcription. Myc forms a dimer with Max that binds to the 5′-CACGTG-3′ consensus core sequence (E-box) in promoters, thereby trans-activating target genes. [35] c-Myc is known to stimulate cell proliferation, block cellular differentiation, and promote tumorigenesis. Its role in trans-activation of the *TERT* gene is one of important driving-forces to transform cells. [36]

Sp1 family transcription factors

The Sp1 transcription factor belongs to the Specificity Protein/Krüppel-like Factor (SP/KLF) transcription factor family and its consensus binding sequence is GGGGCGGGG or GGGTGGG. There are at least 5 Sp1 binding GC-boxes in the TERT core promoter region. [33] Sp1 cooperates with c-Myc to activate the TERT transcription, and when all the Sp1 binding sites are disrupted, the TERT promoter activity is erased, and even over-expression of c-Myc is unable to activate the promoter any longer. [37]

Other positive regulators

Apart from the above two major factors, many other positive factors regulate TERT transcription directly or indirectly. Survivin increases the DNA binding ability of c-Myc and Sp1, thus augmenting TERT transcription. [38] HPV E6 oncoprotein forms a tertiary complex with E6AP and c-Myc, and binds to the E-box, thereby increasing TERT transcription. STAT3, c-Jun, EWS-ETS and HIF-1α have direct promoter interaction, stimulating TERT transcription. [39-43]

Negative transcription factors

Mad/Max heterodimer

Mad is expressed in non-proliferating cells, inhibits proliferation, promotes differentiation, and prevents malignant transformation. Mad and Max forms a heterodimer that binds the same E-box as Myc/Max in the TERT core promoter, and competes with Myc/Max heterodimer binding to the promoter, thus repressing TERT expression. [29] During HL60 leukemic cell differentiation, there is a switch of E-box-binding from c-Myc/Max to Mad1/Max at the TERT promoter, and this switch is concomitant with shutting down of TERT transcription and silencing of telomerase. [29, 44]

p53

p53 is a negative regulator of TERT expression, which is compatible with its tumor suppressive role. p53 protein interacts with Sp1, a known TERT activator,

inhibiting Sp1 binding to the GC-box of the TERT promoter and repressing transcription. [45, 46] Expression of wild-type p53 in cancer cells causes decrease in TERT expression and telomerase activity, while tumor cells with mutant p53 have high-levels of telomerase activity. [45, 46]

Other negative regulators

Many tumor suppressors are also negative transcription regulators of TERT expression. Wilms' tumor 1 (WT1) and menin can directly bind the promoter and repress TERT transcription. [44, 47] Rb, TGF-β/Smad and AP1 also contribute to TERT repression at the transcriptional level. [39, 44, 48, 49]

1.2.2.3 Epigenetic regulation of TERT transcription

The transcriptional regulation by above transcription factors is critical for TERT expression. However, there is an additional transcriptional controlling, the epigenetic mechanism, which also plays roles in regulating TERT expression. The epigenetic mechanism involves nucleosome/chromatin remodeling, DNA methylation and histone modifications. For example, histone acetylation/deacetylation is a common underlying feature to TERT transactivation/repression in human normal and malignant cells. [29] Histone methylation transferase SMYD3 is required for optimal stimulation of c-MYC and Sp1 on the TERT promoter, while histone demethylase RBP2, can interact with Mad1, and be recruited to the TERT promoter, thereby inhibiting TERT transcription. [50, 51]

1.2.2.4 Post-transcriptional and post-translational regulation of TERT expression

Alternative splicing of TERT mRNA is the key post-transcriptional mechanism in the regulation of TERT expression. At least five TERT mRNA variants have been identified in human cells but only the full-length transcript is capable of being translated into a catalytically active protein. [23, 52, 53] Post-translational regulation is involved in TERT protein modifications. Phosphorylated TERT protein has a longer half-life while its ubiquitination leads to a fast degradation.

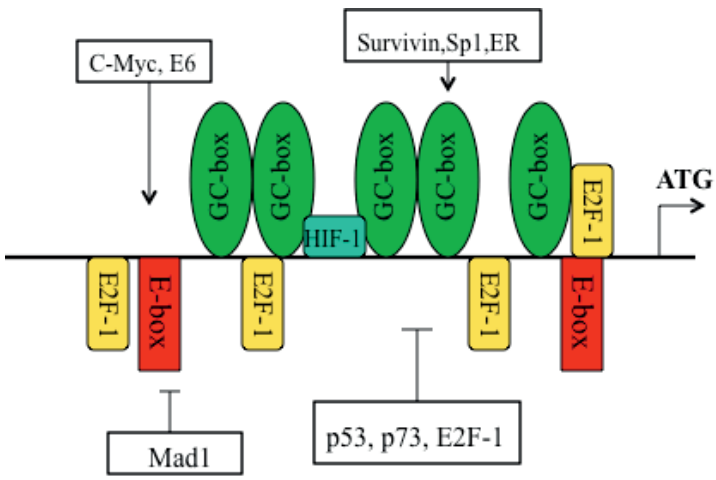

Figure 2. **Structure and regulation of the TERT promoter**. Proteins that activate TERT are located above the line; repressors are located below the line.

1.2.3 Telomerase function independent of telomere-lengthening

The canonical function of TERT is to maintain telomere length by synthesizing TTAGGG telomeric repeats at the end of chromosomes. Apart from its telomere lengthening function, TERT has many other activities in essential cellular processes, such as regulating gene expression, cell transformation, cell survival, mitochondrial function and epithelial-mesenchymal transition (EMT). [54]

TERT can function as a transcriptional modulator. It occupies the Wnt target promoters, working as a cofactor in the Wnt-β-catenin transcriptional complex and regulating Wnt target gene expression. [55] The RNA component of mitochondrial RNA-processing endoribonuclease (RMRP) is a small non-coding RNA found in mitochondrial. TERT has been shown to interact with RMRP and functions as RNA-dependent RNA polymerase that produces double-stranded RNAs. These RNAs are further processed into small-interfering RNAs and regulate gene expression in a post-transcriptional way. [56] TERT also binds to

the p65 subunit of NF-κB and promotes the expression of NF-κB target genes, including interlukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). [57] EMT is an important player in cancer metastasis. Ectopic TERT expression promotes whereas its inhibition attenuates EMT in cancer cells. [58]

1.3 TERT promoter mutations

Telomerase activation and TERT expression are important in cell immortalization and tumor progression. Although the regulation of TERT transcription has been extensively explored, the underlying mechanisms are still incompletely understood. More recently, TERT promoter mutations have been identified as important genetic events activating telomerase in different types of cancer.

1.3.1 TERT promoter mutations - overview

Gene mutations are frequently related with tumorigenesis, and the cancer-specific mutations occur mostly in the protein-coding area, while rarely in the gene regulatory area. Recently, however, somatic TERT promoter mutations have been observed, which are located within 200 base pairs (bp) upstream of the ATG translation start site, and these mutations stimulate the TERT transcription by creating *de novo* ETS binding motifs. [59-61]

TERT promoter mutations and their functional implication

The TERT promoter mutations were first reported by Huang, et al and Horn, et al in 2013. [59-62] Both groups identified recurrent somatic mutations in the TERT promoter region in malignant melanoma. Two major mutations, which caused a cytidine-to-thymidine (C >T) dipyrimidine transition, localized at -124 and -146 bp (chromosome 5 1,295,228 and 1,295,250) from the translation start site, and are named C228T and C250T, respectively (Figure 3). The two

mutations are found to be mutually exclusive. [59-61] The mutations exhibited the signature of an ultraviolet-induced DNA damage, and were found to be more frequent in sun-exposed sites in primary cutaneous melanoma. [59-61, 63]

The above mutations in the TERT promoter region have functional implications. Both C228T and C250T create a new sequence 5′-CCCCTTCCGGG-3′, which is the E-twenty-six (ETS) transcription factor binding motif (GGAA, from the reverse strand) (Figure 3). Huang, et al constructed a TERT promoter luciferase reporter, and observed that the mutant TERT promoter activity increased by two to four fold compared with the wild-type one in same cancer cells. [59-61]

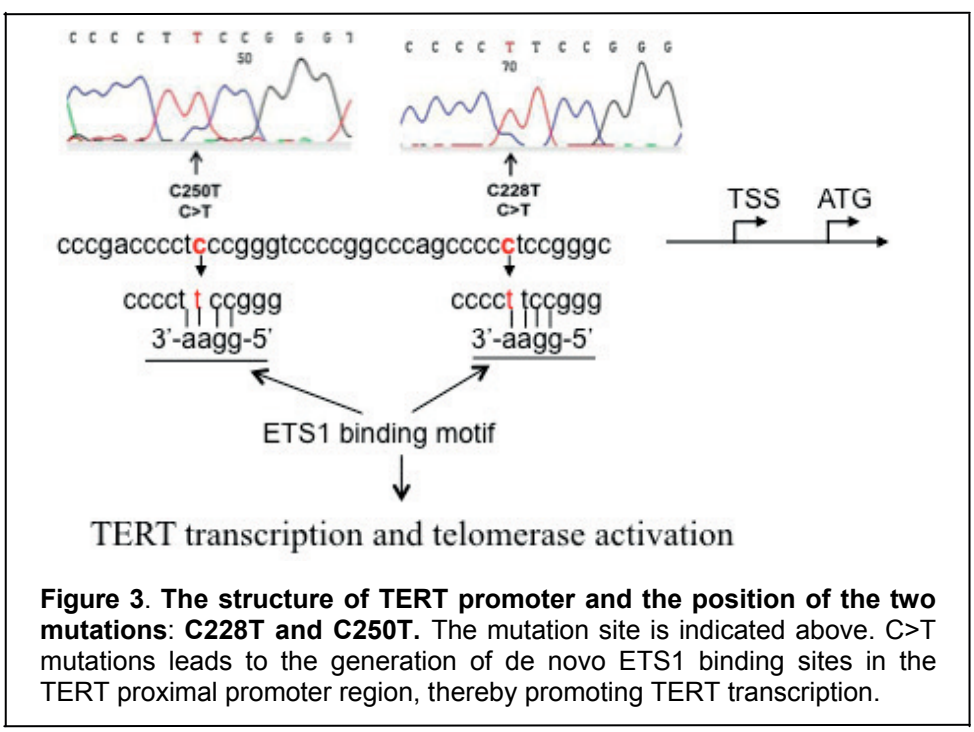

Figure 3. The structure of TERT promoter and the position of the two mutations: C228T and C250T. The mutation site is indicated above. C>T mutations leads to the generation of de novo ETS1 binding sites in the TERT proximal promoter region, thereby promoting TERT transcription.

In addition to these two major TERT promoter mutations, other mutations are also identified in that region. CC>TT tandem mutations at position -124/-125bp and -138/-139, and C>T mutations at position -57 are found in a small proportion of cancer. [62] The two tandem mutations also contributed to the generation of an extra ETS transcription factor-binding motif. Moreover, the mutation at position -57 creates a CCGGAA ternary complex factor (TCF) binding site, which also enhances TERT transcription. [62]

1.3.2 TERT promoter mutations in human cancer

TERT promoter mutations are present in a vast type of cancers with different frequency and correlated with clinical variables. They are the most frequent mutation in hepatocellular carcinoma, bladder cancer and malignant melanoma. [59-61, 64-66] By contrast, no TERT promoter mutations were found in tumors such as breast and prostate cancer, leukemia, etc. [59-61, 67, 68] Details of TERT promoter mutations in human cancer are summarized in Table 1.

Malignant melanoma was shown to express significantly higher levels of TERT mRNA in mutation-positive tumors than in negative ones. [59-61] Consistent with these observations, tumors from thyroid cancer, liver cancer and primary gliomas also exhibited higher TERT mRNA expression in the mutation-positive tumors. [66, 67, 69, 70] However, in bladder cancer, such an association was not seen. [65]

Table 1. A Summary of TERT promoter mutations in cancer

Tumor type	Mutation frequency (%)	References
Urinary system		
Bladder cancer	55.6 - 85	[61, 64, 65, 68, 71-74]
Renal cell carcinoma (RCC)	8.3	[71]

Clear cell RCC (ccRCC)	9.3	[75]
Chromophobe RCC	13	[75]
Upper Tract Urothelial Carcinomas (UTUCs)	47.3	[68]
Renal pelvic cancer	60 - 63.7	[71, 75]
Ureter cancer	11	[75]
Digestive System		
Hepatocellular carcinoma (HCC)	44 - 59	[76]
HCC borderline lesions	17	[76]
Cirrhotic preneoplastic macronodules	25	[66]
Primary gastric cancer	0 - 0.7	[77]
Esophageal adenocarcinoma	0	[78]
Esophageal squamous cell carcinomas	1.60	[79]
Laryngeal cancer	0	[80]
Gallbladder cancer	0 - 9.1	[81]
Central nervous system		
Glioma	49 - 55	[82]
Glioblastomas	54 - 84	[83-85]
Medulloblastomas (MB)	21.0	[71]
MB_{SHH}	34 - 83	[71, 86]
MB_{WNT}	31	[60, 71]
MB_{Group3}	<5	[60, 71]
MB_{Group4}	<5	[60, 71]
Meningiomas	28	[87]
Gliosarcomas	81	[71]
Solitary fibrous tumors	13 - 50	[71]
Oligodendrogliomas	78 - 74	[84]
Oligoastrocytomas	58	[84]
Diffuse astrocytomas	19	[69]
Anaplastic astrocytomas	25	[69]
Malignant peripheral nerve sheath tumors	6	[88]
Skin tumor		
Melanoma	22 - 71	[59-63, 89, 90]
Acral lentiginous melanomas	6	[91]
Non-acral cutaneous melanomas	33	[91]
Uveal melanomas	0	[92]
Conjunctival melanomas	32	[92]

Basal cell carcinomas (BCCs)	56 - 78	[93, 94]
BCCs from X-irradiated patients	27	[89]
BCCs from nonirradiated patients	51	[89]
Primary melanocytic tumors	0	[95]
Cutaneous squamous cell carcinomas	50	[93, 94]
Atypical fibroxanthomas	93	[96]
Pleomorphic dermal sarcomas	76	[96]
Endocrine system		
Thyroid tumor		
Papillary thyroid carcinoma (PTC)	7.5 - 25	[70, 97-99]
Follicular thyroid carcinoma (FTC)	13.9 - 36.4	[70, 97, 98, 100]
Poorly differentiated carcinomas (PDTC)	29	[97, 98]
Anaplastic thyroid carcinomas (ATC)	33.3 - 50	[70, 97, 98]
Medullary thyroid carcinoma (MTC)	0	[70]
Adrenal neoplasms	4.8	[61]
Reproductive system		
Ovarian clear cell carcinomas	15.9%	[101]
Other tumors		
Malignant pleural mesothelioma	15	[73]
Myxoid liposarcomas	74 - 79	[68, 88]
Synovial sarcomas	0.4	[68, 88]
Osteosarcomas	4.3	[68]
Oral cavity cancers	17	[68]
Cell lines		
Gastric cancer cell lines	0	[81]
MTC cell lines	0	[70]
ATC cell lines	75	[70]
Bladder cancer cell lines	88	[74]
Melanoma cell lines	0.74	[62]

The TERT promoter mutation tends to be related to aggressive forms of malignancies. In thyroid carcinomas, anaplastic thyroid carcinoma (ATC), the most aggressive one, has the highest mutation rate (50%), while the differentiated thyroid carcinomas have a mutation rate 22 - 27%. [70] In renal

cell carcinoma, the mutation is also correlated with an advanced clinical stage (stage III/IV) and metastatic disease. [75] In primary glioblastoma and thyroid carcinomas, the mutation predicts a shorter patient survival. [68]

The TERT promoter mutation is the most frequently observed genetic event in bladder cancer. The presence of the mutant TERT promoter in voided urine has been suggested to be a marker for bladder cancer diagnosis and recurrence. [65] However, the mutations detected in the urine could also come from other urological malignancies. In the analysis of upper tract urinary carcinoma, a renal pelvic carcinoma patient was also found to have a TERT promoter mutation in his urine sample. [75] When the mutant TERT promoter is detected in voided urine, differential diagnosis must be made between bladder cancer and other urological malignancies.

The TERT promoter mutation frequency varies substantially from cancer to cancer. Killela et al hypothesized that it occurred mainly in tissues with low self-renewal rate and was mutually exclusive with the alternative lengthening of telomere. [68] However, more and more recent studies have suggested that malignancies with a high self-renewal may also acquire TERT promoter mutations. It is currently unclear what causes such differences in the mutation frequency among different malignancies.

In summary, the detection of the mutant TERT promoter as a diagnostic marker seems promising. This marker has also been tested to distinguish benign tumors from malignancies and to judge the tumor origins, because of its specific mutation profile in different tumor types. Patients with thyroid cancer carrying the mutation tended to have a poor outcome, thus making the TERT promoter mutation a prognostic factor. Several strategies based on telomerase inhibition have entered into clinical trials. [102] Whether the detection of the

TERT promoter mutation will contribute to a better-individualized telomerase-based therapy is worthy of exploring.

1.4 Thyroid cancer

The thyroid gland is the largest endocrine organ. It is located under the thyroid cartilage, and anterior to the larynx and trachea. The thyroid gland consists of two lobes with joint by isthmus, and a pyramidal lobe. Histologically, the basic unit of thyroid gland is the thyroid follicle, which is composed of epithelial follicular cells. Follicular cells constitute 90% of thyroid cells, and are responsible for secreting thyroid hormones. The remaining 10% of thyroid cells are called parathyroid cells or C-cells, which have a different histological origin and secret another hormone calcitonin. [103]

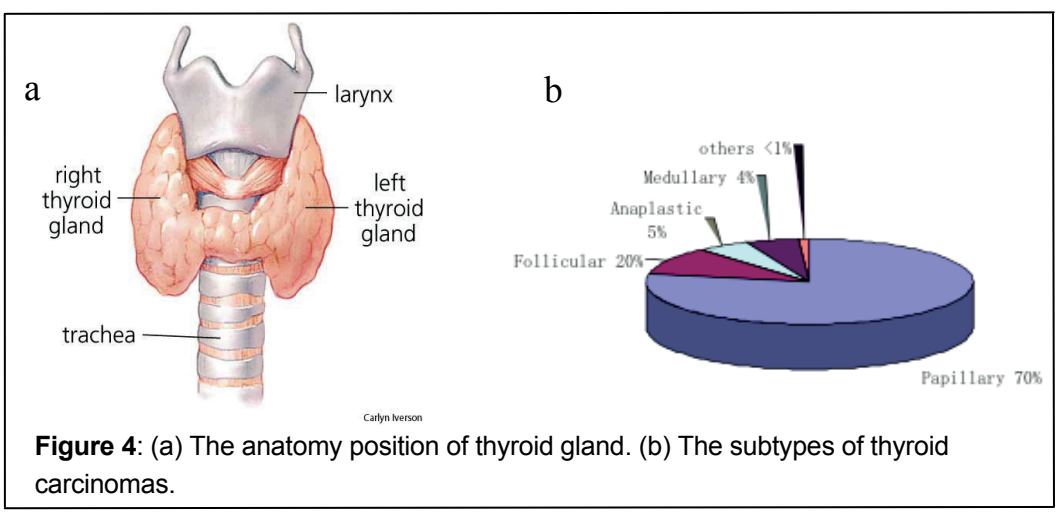

Figure 4: (a) The anatomy position of thyroid gland. (b) The subtypes of thyroid carcinomas.

The incidence of thyroid cancer is growing in the past decades although the death rate is low. [104] According to its histological constitution, thyroid cancer has two origins: the follicular cell-derived tumors, which consist of follicular thyroid adenoma (FTA), follicular thyroid carcinoma (FTC), papillary thyroid

carcinoma (PTC), anaplastic thyroid carcinomas (ATC), and the parathyroid cell-derived tumor, medullary thyroid carcinoma (MTC). [104]

1.4.1 FTA: Follicular thyroid adenoma

FTA is a benign thyroid tumor, which accounts for 3 - 4% of all thyroid tumors. FTA is surrounded by an entire thin fibrous capsule, and is distinguishable from its malignant form, FTC, based on cytology or clinical features. No invasion of capsule or vascular could be the definition of FTA. [105] AFTA is anaplastic FTA with uncertain malignancy.

Although a benign tumor, certain genetic alterations occur in FTA. Oncogenic *HRAS/NRAS* mutations are found in 20 - 25% FTA. *A*s an activator of the MAPK and PI3K–AKT pathways, the presence of *RAS* mutations indicates oncogenic alterations in those benign thyroid tumors. Moreover, a transition from FTA to FTC has been observed, and thus FTA is a pre-malignant lesion of the thyroid. [104]

1.4.2 FTC: Follicular thyroid carcinoma

FTC is a differentiated thyroid cancer, which accounts for approximately 15% of thyroid malignancies. FTC usually has thick and irregular capsule, and necrosis and mitosis occur frequently. Capsule or vascular invasion is a key feature distinguishing FTC from FTA. [105]

The paired box 8 (*PAX8*) peroxisome proliferator-activated receptor-γ (*PPARG*) fusion gene (*PAX8–PPARG*) is the predominant genetic alteration in FTC. *NRAS* mutations and *PTEN* mutations or deletions are also oncogenic drivers in FTC pathogenesis. [104]

1.4.3 PTC: Papillary thyroid carcinoma

PTC is the most common thyroid cancer and constitutes 80 - 85% of all thyroid cancers. [104] It has a good prognosis and the 10-year survival rate is over 80%. PTC is also a differentiated cancer. It has papillary architecture and special

characteristic nuclear features like enlargement, elongation, etc., which distinguish PTC from other follicular cell-derived thyroid cancer. [104]

The *BRAF* gene T1799A point mutation, which generates BRAF V600E mutant protein, is the most common genetic alteration in PTC. $BRAF^{V600E}$ is found in 30 - 70% PTCs. The mutant protein is required for tumor growth and responsible for PTC pathogenesis. *BRAF* mutations in general predict poor patient outcomes. [104]

1.4.4 ATC: Anaplastic thyroid carcinoma

ATC is undifferentiated thyroid cancer. It is the least common while the most aggressive type of thyroid cancer. It accounts for 2 - 3% of all thyroid cancers. [104] ATC patients have a poor prognosis. According to a survey in USA, the patients' median survival was 5 months, and one-year survival is only 20%. [106] ATC may come from a differentiated thyroid cancer or occur as a *de novo* disease.

ATC has multiple genetic abnormalities. They include mutations in *TP53, CTNNB1, BRAF, RAS, PTEN,* and other genes, making ATC the most malignant thyroid cancer.

1.4.5 MTC: Medullary thyroid carcinoma

MTC is the only malignancy that arises from parafollicular or C-cells and accounts for 5 - 8% of all thyroid cancers. The proto-oncogene *RET* mutation is considered to be the major genetic alteration in MTC and a negative prognostic factor. Mutations related with follicular cell-derived carcinomas, such as *BRAF* and *RAS* mutations, are not found in MTC. [107]

1.5 Gastric cancer

1.5.1 Overview

Gastric cancer is the fourth most-common cancer in the world. It's on the second place of cancer mortality, just after lung cancer. The incidence of gastric cancer has declined in the past decades in developed countries, but in most developing countries, like China, the incidence is still high, which leads to heavy social burdens. [108] In 2004, 264,000 patients were diagnosed with gastric cancer, and 206,000 patients died in China. Over 90% of gastric cancer are adenocarcinoma and mainly divided into two histologic subtypes: intestinal and diffuse. [108]

Helicobacter pylori, an infectious agent, have been recognized to be strongly associated with gastric cancer development. *H. pylori* was classified as class I carcinogen by the International Agency for Research on Cancer (IARC) in 1994. [108] Epstein-Barr Virus (EBV), an oncogenic virus, is also related to the pathogenesis of gastric cancer.

A precancerous to cancerous cascade is well defined in gastric cancer: normal→ chronic nonatrophic gastritis → atrophic gastritis → gastric cancer. Gastric cancer has poor outcomes in general.

1.5.2 The role of cyclooxygenae-2 (COX2) in cancer

Cyclooxygenase-2 is also called Prostaglandin-endoperoxide synthase 2. It is the rate-limiting enzyme for the conversion of arachidonic acid into prostaglandin H2. There are two isoforms of cyclooxygenase: COX1, constitutively expressed in most tissues, and COX2, unexpressed under normal conditions in most cells, while induced during inflammation. COX2 is also an oncogenic factor; it promotes angiogenesis, induces tumor cell growth, inhibits apoptosis and attenuates immune-surveillance function. [109-113]

2. AIMS OF THE STUDY

The overall objective of the present study is to delineate regulatory mechanisms underlying cancer-specific TERT expression, novel roles of TERT/telomerase in cancer development/progression, and their clinical relevance. Specifically, the study is aimed at

1. determining TERT promoter mutations in thyroid and gastric cancer.
2. defining the relationship between TERT promoter mutation and clinical variables in thyroid carcinomas.
3. defining novel functions of TERT in oncogenesis for rational development of telomerase-based anti-cancer strategies.

3. MATERIALS AND METHODS

3.1 Human patient samples

3.1.1 Thyroid carcinoma/ adenoma tissues (Paper I and II)

We collected tissue samples from 144 patients with thyroid cancers who were diagnosed and underwent surgery at Karolinska University Hospital Solna. The patients included 20 ATCs, 51 PTCs, 52 FTCs and 37 MTCs. We also collected 58 FTAs and 18 AFTAs tumor specimens from 76 patients at the same hospital. The specimens were collected after surgical treatment within Karolinska University Hospital and kept frozen at -70 ^{o}C until use. Tumors were diagnosed according to the criteria of the World Health Organization. All samples were collected with informed consent and approved by the local ethics committee. Detailed clinical information of patients is given in Supplementary Tables S1-S3 in paper I and paper II.

3.1.2 Gastric cancer tissues (Paper III)

Tumor tissues from two hundred patients with gastric cancer were collected from Qilu hospital, Shandong, China. Formalin-fixed paraffin embedded tumor tissues were used. The study was approved by the local ethics committee. Tumors were diagnosed according to the criteria of the World Health Organization.

3. 2 Established human cell lines

3.2.1 Thyroid cancer cell lines (Paper I)

We used 10 human thyroid cancer cell lines in the study (Table 2), and they were C643W, SW1736, U-hth7, U-hth74, U-hth112, U-hth104, ARO and KAT-4, [114] MTC-TT (ATCC) and MZ-CRC-1 (provided by Dr. BG.Robinson at University of Sydney).

3.2.2 Gastric cancer and other cell lines (Paper IV)

Human gastric cancer cell line AGS and cervical cancer cell line HeLa were from American Type Culture Collection (ATCC) (Table 2). The gastric cancer

cell line BGC-823 was bought from Beijing Cancer Institute, China in 2008. Normal human fibroblasts were from Life Technology and the Osteosarcoma cell line U2OS was a gift from professor Yusheng Cong, Hangzhou Normal University, China (Table 2).

Table 2. Cell lines used in this thesis.

Name	Origin	Subtype	Paper
C643W	Thyroid cancer	ATC	I
SW1736	Thyroid cancer	ATC	I
U-hth7	Thyroid cancer	ATC	I
U-hth74	Thyroid cancer	ATC	I
U-hth12	Thyroid cancer	ATC	I
U-hth104	Thyroid cancer	ATC	I
ARO	Thyroid cancer	ATC	I
KAT-4	Thyroid cancer	ATC	I
MZ-CRC-1	Thyroid cancer	MTC	I
MTC-TT	Thyroid cancer	MTC	I
AGS	Gastric cancer		IV
BGC-823	Gastric cancer		IV
HeLa	Cervical cancer		IV
U2OS	Osteosarcoma		IV
Normal human skin fibroblasts	Normal cells		IV

3.3 DNA extraction and Sanger sequencing (Paper I, II and III)

In the present study, genomic DNA was extracted from frozen tumor tissue samples using standard methods and from paraffin tissues using a DNA FFPE

Tissue Kit (QIAGEN). The genomic DNA was then applied with the primers spanning the hTERT promoter, *BRAF*, *HRAS, KRAS* and *NRAS* genes using a QIAGEN Hotstar DNA polymerase. PCR was performed based on the manufacture's instruction and primers sequences are listed in Table 3.

The TERT promoter region is GC-rich and is difficult to amplify using conventional PCR conditions. We solved this problem by adding betaine in the reaction mixture. Betaine is a chemical compound that increases PCR amplification efficiency by reducing the formation of secondary structure in the GC-rich DNA region. [115]

Sanger sequencing was developed in 1975 by Sanger as the first generation sequencing technology based on four-color fluorescence and automated capillary electrophoresis (CE) systems. [116] It is the conventional method to analyze DNA sequences. Both deoxy-ribonucleoside triphosphate (dNTPs) and dideoxy-ribonucleoside triphosphate (ddNTps) are added into PCR reaction systems. The ddNTPs, which may be radioactively or fluorescently labeled for detection, lack the 3'-OH and cannot be amplified. dNTPs and ddNTPs bind to the template randomly. The PCR reaction will cease when ddNTPs bind to the template, which generate different sized PCR fragments. These fragments can be separated by size using gel electrophoresis and different termination signals can be detected and analyzed to get the template sequence.

After PCR reaction, products were purified and precipitated with ethanol and EDTA. The Sanger sequencing was conducted with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in an ABI 3730 DNA analyzer machine. We used CodonCode Aligner software to analyze the sequencing results and the mutation samples were confirmed from both directions.

Table 3. Primers used for PCR and Sanger sequencing in this thesis.

Purpose / Target	Description	Sequence
Sequencing of TERT		
TERT promoter	Forward	5' - CACCCGTCCTGCCCCTTCACCTT - 3'
TERT promoter	Reverse	5' - GGCTTCCCACGTGCGCAGCAGGA - 3'
Sequencing of HRAS		
HRAS exon 1 codon 12/13	Forward	5' - ATGACGGAATATAAGCTGGT - 3'
HRAS exon 1 codon 12/13	Reverse	5' - CTCTATAGTGGGGTCGTATT - 3'
HRAS exon 2 codon 61	Forward	5' - AGGTGGTCATTGATGGGGAG - 3'
HRAS exon 2 codon 61	Reverse	5' - AGGAAGCCCTCCCCGGTGCG - 3'
Sequencing of KRAS		
KRAS exon 1 codon 12/13	Forward	5' - GGCCTGCTGAAAATGACTGAA - 3'
KRAS exon 1 codon 12/13	Reverse	5' - GGTCCTGCACCAGTAATATGC - 3'
KRAS exon 2 codon 61	Forward	5' - CAGGATTCCTACAGGAAGCAAGTAG - 3'
KRAS exon 2 codon 61	Reverse	5' - CACAAAGAAAGCCCTCCCCA - 3'
Sequencing of NRAS		
NRAS exon 1 codon 12/13	Forward	5' - ATGACTGAGTACAAACTGGT - 3'
NRAS exon 1 codon 12/13	Reverse	5' - CTCTATGGTGGGATCATATT - 3'
NRAS exon 2 codon 61	Forward	5' - TCTTACAGAAAACAAGTGGT - 3'
NRAS exon 2 codon 61	Reverse	5' - GTAGAGGTTAATATCCGCAA - 3'

3.4 Telomere length assessment (Paper I, II and IV)

3.4.1 Quantitative PCR (qPCR)

We used two methods to measure telomere length. In paper I and II, a well-established qPCR method was used to determine telomere length from frozen tissues. [117] TEL primers were specially designed to amplify telomere (T) repeats and beta-globin (HBG) was used as a standard (S) reference gene.

Relative telomere length was determined by calculating T/S values using the formula, $T/S=2^{-\Delta Ct}$, where ΔCt=average $Ct_{telomere}$ −average $Ct_{\beta\text{-globin}}$.

3.4.2 Q-FISH

Quantitative Fluorescent in situ Hybridization (Q-FISH) is a more informative method to measure telomere length. It provides visible and individual telomere length assessment with the resolution to 200 bps. [118] Cells are fixed with 4% paraformamide and incubated with fluorescence-labeled PNA (CCCTAA) 3' probe. The signal intensity in 50 cells was determined using NIS software (Nikon, Stockholm, Sweden) and expressed in arbitrary units.

3.5 RNA extraction, reverse transcription and qPCR

To detect gene mRNA expression in different cells or tissues, we used qPCR. Total RNA was extracted using Trizol method (Invitrogen). RNA was then used as the template to synthesize cDNA with random primers (N6, Amersham, Buckinghamshire, UK) and M-MLV reverse transcriptase (Invitrogen). qPCR was carried out in an ABI7900HT fast real-time system (Applied Biosystems) using SYBR green master mix kit (Applied Biosystems). The primers used in the study are listed in Table 4. We used β-2 microglobulin (β-2M) as the internal control and $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression. [119]

Table 4. Primers used for qPCR determination of gene expression

Target	Description	Sequence
TERT	Forward	5'-CGGAAGAGTGTCTGGAGCAA-3'
	Reverse	5'-GGATGAAGCGGAGTCTGGA-3'
COX2	Forward	5'-GCCCAGCACTTCACGCATCAG-3'
	Reverse	5'AGACCAGGCACCAGACCAAAGACC-3'
COX1	Forward	5'-CGGCTGCAGCCCTTCAATGAGT-3'
	Reverse	5'-CTCTCCCCAAAGATAGAGTTTGGA-3'
β2-M	Forward	5'-GAATTGCTATGTGTCT GGGT-3'
	Reverse	5'-CATCTTCAAACCTCCATGATG-3'

3.6 Telomerase activity measurement (Paper II and IV)

We used a commercial Telomerase PCR ELISA kit (Roche Diagnostics Scandinavia AB, Stockholm, Sweden) to detect telomerase activity. 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.

3.7 COX2 promoter activity assay (Paper IV)

The COX2 reporter plasmid contains the core promoter sequence (-327/+59) inserted into a luciferase-encoding DNA fragment and its luciferase activity indicates the transcriptional level of the *COX2* gene. [120] The plasmid was transfected into the treated or control BGC cells, and the COX2 promoter-driven luciferase activity was then determined using a dual luciferase reporter assay system (Promega, Madison, WI, USA) 48 hrs post transfection, and the target promoter-driven firefly luciferase activity was normalized to the renilla activity included in the kit.

3.8 Western blot (Paper IV)

Western blot or protein immunoblot is widely used to detect proteins from cells/tissues. Total cellular proteins were extracted using RIPA lysis buffer, and the concentration of proteins was measured using a BCA method. For the assessment of phosphorylated p38 MAPK, sodium orthovanadate was added to lysis and washing buffers to inhibit phosphatases. Proteins were then denatured at 100 ^{o}C for 5 minutes. Twenty microgram of proteins were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was then incubated with 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), and finally developed with the chemiluminescence method (ECL, Amersham, Little Chalfont, UK).

3.9 Flow cytometry (Paper IV)

Flow cytometry (FCM) is a laser based, biophysical technology employed, and multi-parameter biotechnology. FCM is widely used in cell counting, apoptosis and cell cycle analysis, cell sorting and biomarker detection, etc.. The amount of chromatins varies in different cell phases. S-phase cells have double amounts of chromatin comparing to G0/1-phase cells, and apoptotic cells have chromatin breakages. Propidium iodide (PI) is a fluorescent molecule that can bind to nucleic acid. Cells were fixed with ethanol and stained with PI, and the fluorescent signal shows the different DNA contents in each cell phase, which helps to define cell cycle distribution and to differentiate necrotic and apoptotic cells from living ones.

In our experiments, cells are fixed with 70% ethanol at 4 ^{o}C overnight and stained with RNAse A (0.5 mg)-containing PI (50 μg/ml). Cell cycle distribution and apoptosis were determined using flow cytometry with ModFit (BD Biosciences, Franklin Lakes, NJ, USA).

3.10 Cell culture and *TERT* gene knock down (Paper IV)

Human gastric cancer cell lines and cervical cancer cell line were cultured in RPMI-1640 medium with 10% Fetal Bovine Serum (FBS) and L-glutamine in a 37^{o}C, 5% CO_2 incubator.

3.10.1 Small-interfering RNA (siRNA)

siRNA is a class of 20-25 base pairs in length, double-stranded RNA molecules. Each siRNA is unwound into two single-stranded (ss) ssRNAs, the passenger strand and guide strand. The passenger strand is degraded whereas the guide strand is incorporated into the RNA-induced silencing complex (RISC), which causes the transcriptional silence of complementary message RNA (mRNA). siRNA is a widely used tool in transcriptional silencing. In our study, we bought chemical modified Stealth control (5'-CCUACAUCCCGAUCGAUGAUGUUGA-3') and TERT (5'-AGGCACUGUUCAGCGUGCUCAACUA-3') siRNAs from Invitrogen. The

siRNAs were transfected into cells using lipofectamine 2000 (Invitrogen) according to the manufacture's instruction.

3.10.2 Lenti-viral vector construction

The gene knock down by siRNAs is transient. In order to stably inhibit TERT expression, we infected cells with a lenti-viral vector encoding TERT siRNA (Invitrogen).

Lentivirus belongs to the retrovirus family. It delivers viral RNA into host cells and infects both dividing and non-dividing cells. We used the second-generation packaging system, which include the packaging plasmid psPAX2 and envelop plasmid pMD2. G (Addgene). The TERT siRNA psPAX2 and pMD2.G were transfected into 293FT virus package cells. Supernatant was collected 24 hrs and 48 hrs post-transfection. The virus-containing medium was then centrifuged and concentrated. Cells are selected with 2 μg/ml puromycin 48 hrs after infection.

3.11 Mouse model (Paper IV)

The mouse model maximally mimics the *in vivo* biological progress without the added risk of harming actual human. In our study, we used a mouse model to investigate the synergistic anti-cancer efficacy by targeting both TERT and COX2.

BGC-823 cells infected with control and siTERT lenti-viral vectors were harvested, and 2×10^6 cells were suspended in 100 μl PBS. The cells were then subcutaneously injected into nude mice (Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China) at the right back. The experiments were carried out with the university ethics committee approval and met the standards required by the UKCCCR guidelines. [121] Mice were killed after 3 weeks and tumors were collected for the measurement of weight, size and other analyses.

4. RESULTS

4.1 TERT promoter mutations in cancer and their clinical implication

4.1.1 The frequency of TERT promoter mutations in cancer

In this thesis, we sequenced the TERT promoter in different tumors, including thyroid carcinoma, benign thyroid tumor and gastric cancer. The frequency of TERT promoter mutation was different in these tumors and cancer subtypes (Table 5).

Table 5. Summary of *TERT* promoter mutations detected in thyroid and gastric tumors

	Number	Mutated	Wild-type
Tumor type	**Investigated**	**Number**	**Number**
Thyroid tissue samples			
Anaplastic thyroid carcinoma (ATC)	20	10 / 20 (50%)	10 / 20
Papillary thyroid carcinoma (PTC)	51	13 / 51 (27%)	38 / 51
Follicular thyroid carcinoma (FTC)	36	8 / 36 (22%)	28 / 36
Medullary thyroid carcinoma (MTC)	37	0 / 37 (-)	37 / 37
Follicular thyroid adenoma (FTA)	58	1/58 (2%)	57/58
Anaplastic follicular thyroid adenoma (AFTA)	18	3/18 (17%)	15/18
Radioactive related thyroid carcinoma	51	0/51 (-)	51/51
Primary gastric cancer	200	0/200 (-)	200/200
Established cell lines			
ATC cell lines	8	6 / 8 (75%)	2 / 8
MTC cell lines	2	0 / 2 (-)	2 / 2

As controls, we analyzed 20 normal thyroid samples and none of them carried TERT promoter mutations. In follicular cell-derived thyroid cancers, TERT promoter mutation C228T or C250T was detected. Particularly, mutations were also detected in benign thyroid adenoma (FTA) and the pre-malignant tumor AFTA, indicating the TERT promoter mutations being present in pre-malignant

lesions of the thyroid, too. The two mutations are mutually exclusive. C228T is the predominant form of mutations in the cancer types examined in the present study, consistent with other observations.

However, none of 37 medullary thyroid carcinomas (MTCs) carried either C228T or C250T mutation, which indicates that the TERT promoter mutation occurs only in follicular cell-derived thyroid cancers (Table 5).

We also got access to a cohort of 51 young PTC patients (age range 19–39 years; median 31 years) surgically treated at Kyiv City Teaching Endocrinological Center, Ukraine, and all these patients had been exposed to radioactivity from the accident at the Chernobyl nuclear power station in Ukraine in 1986. [122] Unexpectedly, none of the 51 patients carried TERT promoter mutations. Thus, this genetic event is not related to radiation in thyroid cancer.

In 200 patients with primary gastric cancer, none of their tumors were found to carry TERT promoter mutations.

In summary, the frequency of TERT promoter mutations differs among cancers, even in different subtypes of the same cancer. Exposure to radioactivity did not induce the mutation in thyroid cancer.

4.2 TERT expression and telomerase activity in FTA and AFTA

Since the TERT promoter mutation increases TERT transcription, [59] we analyzed *in vivo* TERT mRNA expression and telomerase activity in FTA and AFTA samples. All four TERT promoter-carrying samples were observed to express TERT mRNA and telomerase activity. For comparison, we further analyzed TERT mRNA expression in 7 FTAs and 11AFTAs without TERT promoter mutations. Only 1 of these 7 FTAs and 2 of 11 AFTAs expressed TERT mRNA. Altogether, there was a highly significant difference in TERT expression between C228T-positive and negative tumors.

4.3 TERT promoter mutations and telomere length

Very short telomere is a strong trigger for telomerase activation during cellular immortalization. We thus sought to determine whether there was an association between TERT promoter mutations and telomere length. In PTC patients, telomere length was significantly shorter in patients with TERT promoter mutations (0.873 ± 0.45) compared to patients without mutations (1.569 ± 0.627) (P < 0.0001) (Figure 5). Because mutations occurred in elderly PTC patients, and telomere shortened with age, we further determined the relationship between telomere length and age in these patients. The Pearson correlation analysis demonstrated that telomere length in PTC cancer cells was independent of patients' age.

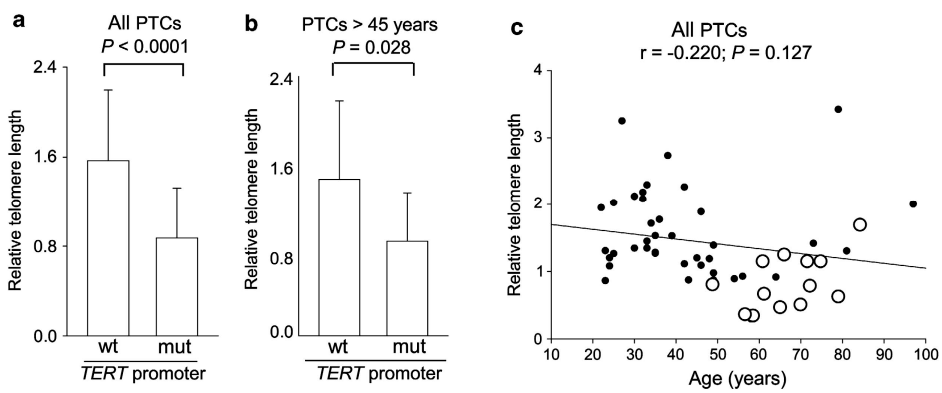

Figure 5. **Association of TERT promoter mutations with age and shorter telomere in PTC patients**. (a) Significantly shorter telomere in PTC patients with the TERT promoter mutation (mut) compared with the wild-type (wt) carriers. Bars: s.d. (b) Significantly shorter telomere in PTC tumors with the TERT promoter mutation stratified by age >45 years. Bars: s.d. (c) Age-independent telomere length in PTC tumors. Open circles represent telomere length in 13 TERT promoter mut-carrying tumors and filled circles represent wt cases.

We also determined the mean telomere length in all FTA and AFTA specimens and the results showed that telomere length varied substantially. The C228T mutation-positive tumors tended to have shorter telomere compared to the negative ones, but the difference was not statistically significant. In addition, there was no correlation between patients' age and telomere length in these tumor tissues, either.

4.4 TERT promoter mutations and other gene mutations

4.4.1 TERT promoter mutations are not associated with BRAF mutations in PTC

BRAF mutations are the most common genetic event in PTC with a frequency of 30 – 70%. [104] To explore whether there is a relationship between these two events, we screened the presence of $BRAF^{V600E}$ mutations in PTC patients. We identified 32 $BRAF^{V600E}$ positive tumors in 51 PTC patients, but no association between these $BRAF^{V600E}$ and TERT promoter mutations were observed.

4.4.2 TERT promoter and *NRAS* gene mutations in AFTA

Activating *RAS* gene mutations are known to occur in both FTA and AFTA. We further screened all 18 AFTAs and the C228T-positive FTA for mutations at codons 12, 13 and 61 of the *HRAS*, *KRAS* and *NRAS* genes. All 19 tumors carried wild type *HRAS* and *KRAS* genes. In contrast, five of them were shown to have an NRAS missense mutation at codon 61. Three of the NRAS mutated cases (1 FTA and 2 AFTA) were positive for C228T. There was a significant difference in the presence of the C228T mutation between tumors with and without NRAS mutations ($P = 0.016$).

4.5 TERT promoter mutations and patients' age

TERT promoter mutations were more common in older age. In the 51 PTCs, all the 13 mutation-carrying patients were older than 49 years, and no mutations were found in patients <45 years old. The mutation frequency was 52% (13/25) in PTC patients >45 years. Thus, the age difference was highly significant between patients with and without the TERT promoter mutation ($P < 0.0001$).

In FTC patients, one of eight TERT promoter mutation-positive FTC patients was 31 years old and the remaining were >45 years. The age range was 31 – 83

years (median 69 years) and 17 – 77 years (median 54 years) for FTC patients with and without TERT promoter mutations, respectively, and this difference reached a statistically significant level ($P = 0.05$).

In the 58 FTA and 18 AFTA patients, all 4 patients with a TERT promoter mutation were ≥45 years old at diagnosis. In contrast, none of 28 patients with age <45 carried the mutation. However, a statistical analysis did not show a significant difference (<45 vs. ≥45, Fisher exact test, $P = 0.29$), likely due to the small number of patients with TERT promoter mutations.

Furthermore, we sequenced the cohort of 51 young PTC patients (age range 19 – 39 years old; median 31 years) surgically treated at Kyiv City Teaching Endocrinological Center, Ukraine, and all these patients had been exposed to radioactivity from the accident at the Chernobyl nuclear power station in Ukraine in 1986. Consistently, none of these 51 young patients carried the TERT promoter mutation in their tumors, which further supported an association between senior age and TERT promoter mutations in PTC patients.

4.6 TERT promoter mutations and clinical characteristics

4.6.1 TERT promoter mutations in aggressive thyroid cancer

The results from both thyroid cell lines and primary tumors, showed that the most aggressive carcinomas had the highest prevalence of TERT promoter mutations in all tumor subtypes (the mutation rates for ATC, PTC, FTC, AFTA, and FTA were 50%, 27%, 22%, 17%, and 3%, respectively), indicating a link between mutations and progressive diseases.

In FTAs, the only C228T-carrying patient had a recurrence at the scar site three years after primary surgery. This patient subsequently developed metastasis and died from the disease. A final diagnosis of FTC was confirmed at autopsy. In contrast, none of 57 TERT mutation-negative FTA cases progressed into FTC.

Taken together, the TERT promoter mutation predominantly occurs in more aggressive thyroid cancer and its presence in pre-malignant lesions of the thyroid may be a risk factor for malignant transformation.

4.6.2 TERT promoter mutations and patients' prognosis

Since there is a link between TERT promoter mutations and aggressive tumors, we asked whether these genetic events predicted outcomes in thyroid cancer.

In ATC patients, there was no difference in tumor size and metastasis between the TERT promoter mutation-positive and -negative tumors. These patients all died of the disease except one. The patients with the mutation tended to have a shorter overall survival (OS) than those with a wild-type TERT promoter.

In PTC patients, the presence of the TERT promoter mutation was strongly associated with metastasis (7/11 in mutated vs. 3/25 in wild type, Fisher exact test, $P = 0.028$). Univariate analyses showed that TERT promoter mutations and age >45 years, were significantly associated with shorter overall survival (OS) (Figure 6). On multivariate analysis, age >45 years remained significantly associated with shorter OS. However, an opposite result was obtained for disease-specific survival (DSS). Both the presence of the TERT promoter mutation and age >45 years were significantly correlated with a shorter DSS in PTC patients using univariate analysis ($P < 0.0001$ and $= 0.015$ for mutated TERT promoter and age >45, respectively), whereas the former, but not age>45 years independently predicted a shorter DSS, in multivariate analysis. ($P = 0.021$ and 0.882 for the TERT mutated promoter and age >45 years, respectively) (Figure 6).

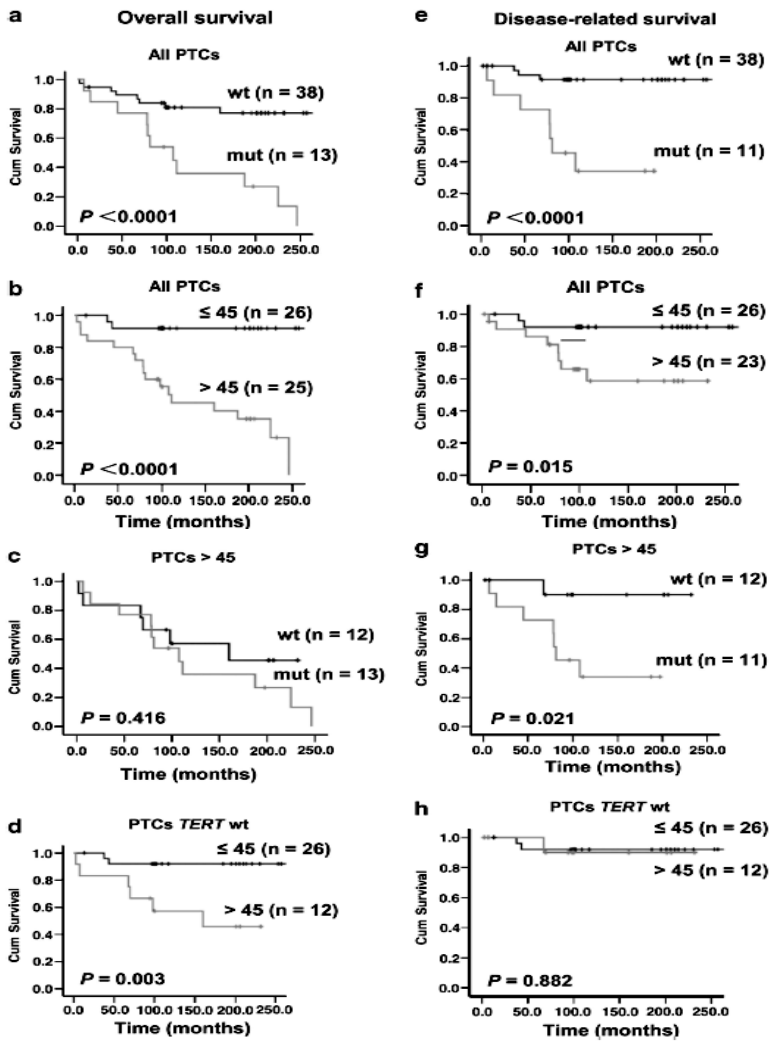

Figure 6. **The relationship between patients' survival and TERT promoter mutation/age in papillary thyroid carcinoma (PTC)**. Kaplan–Meier plots showing overall survival (left) and disease-related survival (right)

In FTC patients, the TERT promoter mutation and age >45 years were both associated with inferior OS (P = 0.038 and 0.003, respectively). Borderline significantly worse DSS was associated with the TERT promoter mutation (P = 0.058) and age >45 years was significantly associated with a shorter DSS in univariate analysis (P = 0.019). However, both of them were unable to predict DSS independently, as revealed by the multivariate analysis.

In primary gastric cancer, there were no TERT promoter mutations found in 200 patients, and these genetic alterations were not involved in telomerase activation occurring in gastric cancer.

4.7 TERT and oncogenic factor COX2 expression in cancer

4.7.1 TERT depletion leads to up-regulation of COX2 expression in cancer cells

COX2 was found to activate TERT transcription in our previous study. [123] To see whether TERT affects COX2 expression, we knocked down TERT expression in gastric and cervical cancer cells using siRNA oligos specifically targeting TERT mRNA.

Following TERT depletion, the expression of COX2 was up-regulated at both mRNA and protein levels, as determined using qPCR and western blot, respectively. TERT depletion-mediated COX2 induction occurred already at 24 hrs, and lasted for the whole observation period (96 hrs), suggesting a constitutive rather than transient up-regulation of COX2 expression (Figure 7).

To determine whether TERT knocking down specifically up-regulates COX2 expression, we determined COX1 expression, the isoform of COX2 in the same sets of cDNA, and no significant change in COX1 mRNA level was observed. When we transfected control and TERT siRNA into normal human fibroblasts and the osteosarcoma cell line U2OS lacking TERT expression and

telomerase activity, COX2 expression did not increase, which indicates a specific effect of TERT depletion on COX2 induction.

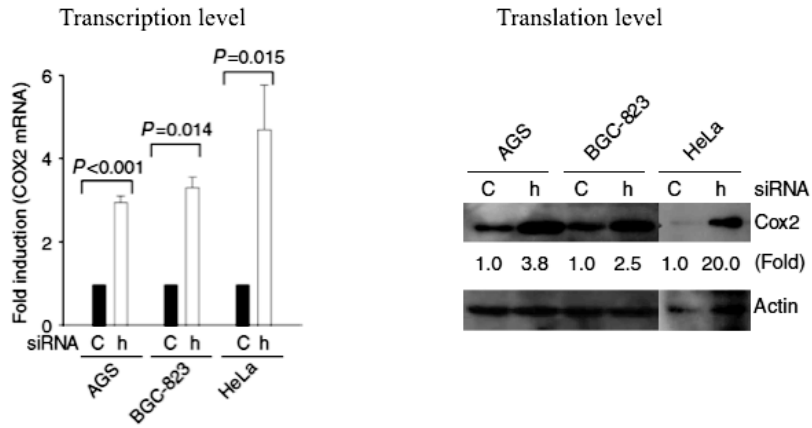

Figure 7. **TERT knocking down-mediated COX2 up-regulation**. qPCR and immunoblotting analyses were performed to assess COX2 expression at both mRNA and protein levels in cell lines 72 h post-transfection of TERT siRNA.

4.7.2 The COX2 promoter is activated in TERT-depleted cells

To probe the mechanism behind the COX2 induction mediated by TERT knocking down, we transfected BGC-823 cells with a reporter construct harboring a core COX2 promoter sequence (−327 - +59). The COX2 promoter activity was significantly higher in TERT-depleted BGC-823 cells compared to that in the cells with the control siRNA. So the induction of COX2 expression by TERT depletion occurs at the transcriptional level.

4.7.3 p38 MAPK is involved in COX2 induction by TERT depletion

The p38 MAPK signaling pathway plays an important role in the regulation of COX2 transcription [124-128], while telomerase inhibition was previously shown to activate p38 MAPK in leukemic cells. [129] Thus we wanted to probe if this regulatory loop was present in cancer cells. We compared the phosphorylated p38 MAPK at Thr180/Tyr182 (p-p38) between control and TERT siRNA-treated BGC-823 and HeLa cells, and the immunoblotting

results showed a substantial increase in p-p38 levels in TERT knocked-down cells (Figure 8).

We further tested whether inhibiting the p38 MAPK activity was capable of blocking the up-regulation of COX2 expression following TERT knocking down. Indeed, the specific p38 MAPK inhibitor SB203580 abolished the stimulatory effect of TERT depletion on COX2 expression in both BGC-823 and HeLa cells (Figure 8).

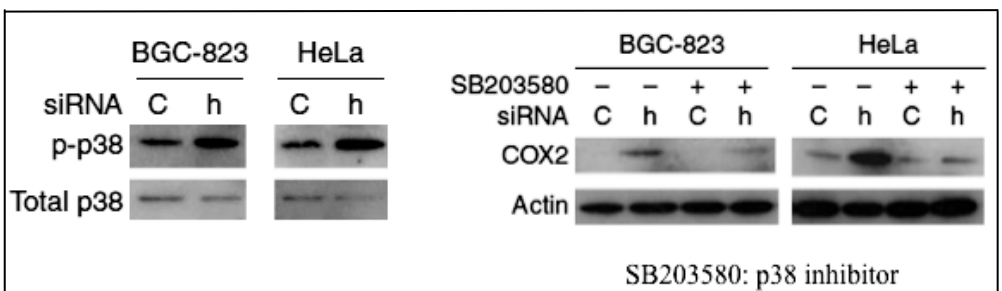

Figure 8. p-p38 expression increased after TERT depletion in cell lines and the TERT depletion-mediated COX2 up-regulation was abolished by inhibiting p38 MAPK activity.

4.7.4 The TERT depletion-mediated COX2 up-regulation is independent of telomere length

TERT is the catalytic component of the telomerase complex and its canonical function is telomere elongation. We thus asked whether TERT inhibition led to telomere shortening, thereby triggering p38 phosphorylation and COX2 up-regulation. No detectable changes in telomere length were observed in control and TERT siRNA treated BGC-823 cells, as determined using Q-FISH. The results suggest a telomere lengthening-independent effect of TERT on the stimulation of p38 MAPK activation and COX2 expression.

4.7.5 Synergistic apoptosis induction by inhibiting both TERT and COX2

In vitro studies

As COX2 promotes cancer cell survival and/or proliferation, we wanted to know whether COX2 inhibitors facilitated the anti-cancer effect of telomerase/TERT inhibition. BGC-823 cells were transfected with TERT siRNA followed by immediate treatment with celecoxib, a specific COX2 inhibitor. Compared with control cells, TERT depletion or celecoxib treatment at 50 μM alone did not lead to significant changes in the cell number, however, when combined together, there was a robust decrease of viable cells (Figure 9). Flow cytometry analysis of DNA stained with PI showed the constant result: significant apoptotic death of TERT depleted cells in the presence of celecoxib (Figure 9).

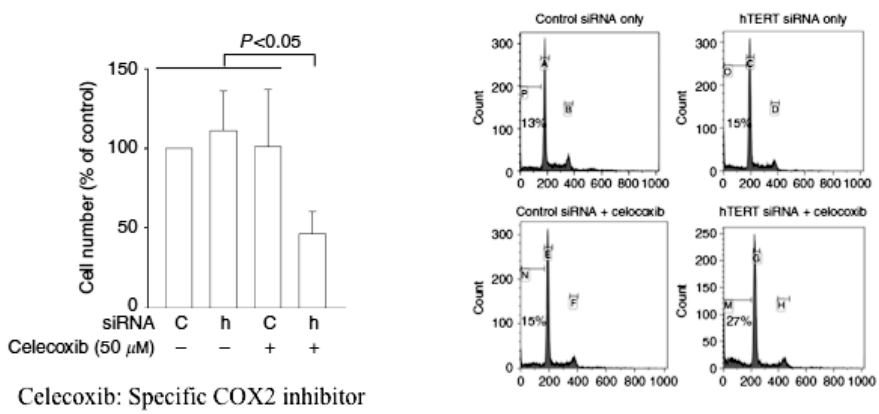

Figure 9. **TERT knocking down and celecoxib exposure synergistically induce apoptosis of BGC-823 cells in culture**. Left: Significant decrease of viable cells in TERT knocked-down cells treated with celecoxib. Right: Synergistic induction of apoptosis in BGC-823 cells treated with the combination of TERT siRNA and celecoxib. Abbreviations: C=Control; h=hTERT.

In vivo observations

To determine whether the above results obtained from *in vitro* studies could be reproduced in an *in vivo* setting, we further performed the experiments with the xenograft model of human gastric cancer in nude mice. BGC-823 cells were infected with either an empty lenti-viral vector or one generating TERT siRNA

to stably knockdown TERT expression, and cells were then subcutaneously injected into the back of nude mice following 1 week selection with puromycin. Both BGC-823 control and TERT siRNA cells formed tumors with comparable size and weight in nude mice. Tumor size and weight were significantly diminished in BGC-823 TERT siRNA cell bearing mice fed with celecoxib (Figure 10). Furthermore, compared with other three groups, cellular constituents in tumors were much less dense in the group mice bearing TERT-depleted cells and fed with celecoxib.

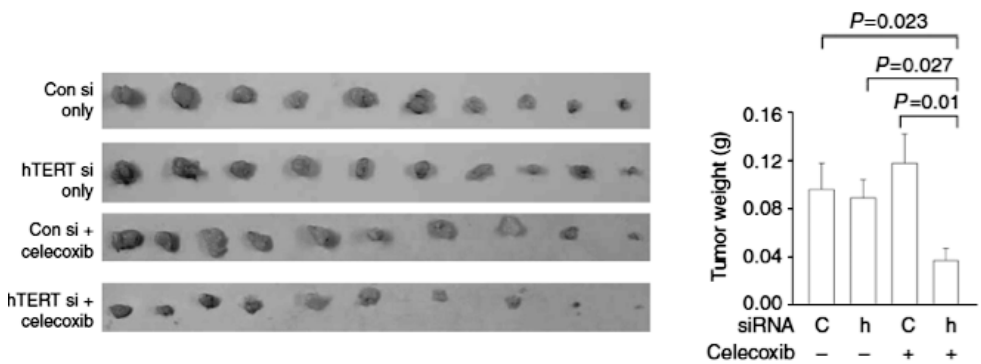

Figure 10. **TERT-depletion and celecoxib treatment synergistically inhibit *in vivo* tumor growth**. Control and TERT-depleted BGC-823 gastric cancer cells were subcutaneously injected into the back of nude mice and mice then fed with and without celecoxib. Tumor size (left panel) and weight (right panel) in mice with different treatments were measured after 3 weeks. Abbreviations: C=Control; h=hTERT.

5 DISCUSSION

Recent studies have revealed somatic TERT promoter mutations in different types of human malignancies. In our study, we determined TERT promoter mutations in thyroid cancer-derived cell lines and primary thyroid cancer specimens, pre-malignant thyroid tumors and primary gastric cancer. We observed different mutation frequencies in different types of thyroid cancer. The highest mutation rate occurred in anaplastic thyroid carcinoma (ATC) cell lines, which was 75%. In the primary tumors, ATC exhibited the most frequent mutations (50%), while differentiated follicular cell derived tumors, PTCs and FTCs, have a mutation rate of above 20%. However, in C cell-derived MTC tumors or cell lines, no mutations were found, which indicated a tumor type-specific TERT promoter mutation.

Age is an important factor in the mutation event. In both PTC and FTC patients, older age is associated with a higher mutation rate. In the most aggressive ATC, which had the highest mutation rate, all patients were above 50 at diagnosis. In contrast, 51 PTC patients from the Ukraine Chernobyl disaster were all younger than 40 years old, and no mutations were found in this cohort of patients, although PTC has a mutation rate over 20% in the Swedish patient group.

We found that in PTC, the TERT promoter mutation-positive patients had shorter telomere. Since the mutation occurred in older patients and telomere length shortened with age, we compared patients' age with their telomere length in tumors. We didn't find any association between age and telomere length. Taken together, TERT promoter mutations are associated with shorter telomere length in cancer cells.

We provide the following model to explain why TERT promoter mutations occur in old patients with thyroid cancer. Thyroid epithelial cells from later age

possess relative shorter telomeres due to their progressive erosion, and when oncogenic events drive active proliferation of these thyroid cells with diminished telomerase activity, excessive loss of telomeres leads to telomere dysfunction or even telomere crisis. Too short telomeres or telomere crisis triggers genomic instability and force cells to undergo telomerase activation, via TERT promoter mutations. In contrast, young individuals' thyroid epithelial cells have longer telomeres coupled with telomerase proficiency, and in such cases, their telomeres are likely long enough to avoid telomere crisis and telomerase may be up-regulated via other pathways rather than the TERT promoter mutation. However, TERT promoter mutations are not always present in older patients with other tumors. Medulloblastoma has a TERT promoter mutation frequency over 20%, whereas it is the most common malignant brain tumor of childhood. [68]

When analyzing an association between the mutation status and prognosis, we found that ATC patients with TERT promoter mutations tended to survive shorter compared with those without the mutation. In PTC patients, the presence of TERT promoter mutations was highly correlated with metastasis, and was a strong and independent factor to predict DSS. Age >45 years were correlated with worse OS, but did not predict DSS independently. We also found that FTC patients carrying the TERT promoter mutation exhibited substantially shorter OS and DSS. Collectively, the presence of the TERT promoter mutation may define a fraction of thyroid cancer patients with progressive disease and unfavorable outcomes.

Although TERT expression and telomerase activation is present in up to 90% of human malignancies, it is currently unclear how early this occurs in carcinogenesis. We have found one TERT promoter mutation in 58 FTA patients and 3 in 18 AFTA patients, respectively. FTA is a benign thyroid tumor and AFTA has an uncertain malignant potential. Both belong to pre-malignant lesions of the thyroid. None of the 57 mutation negative patients had progressed into FTC, whereas one of the mutation-positive patient who initially presented without features of AFTA or FTC, developed a scar recurrence with

histopathological features of AFTA and suggestive of FTC followed by recurrent metastatic FTC. This finding indicates that TERT promoter mutation may be a risk factor for FTA transforming into FTC. TERT promoter mutations were also found in cirrhotic preneoplastic macronodules and hepatocellular adenomas with malignant transformation. [66] Collectively, TERT promoter mutations occur early in the pre-malignant stage of oncogenesis, and the presence of TERT promoter mutations may predicate a future development of a full malignancy.

In the 200 primary gastric tumors that we analyzed, no TERT promoter mutations were found. There was also the absence of the mutation in breast cancer, prostate cancer or acute myeloid leukemia. [68] Despite so, there is still telomerase activity in these malignancies, and thus alternative mechanisms must account for their telomerase activation.

Telomerase has multiple activities in addition to its telomere-lengthening function. [54, 55, 58, 68] When TERT is inhibited, the effect and expression of proliferation- and survival-related factors are in general attenuated. However, our study unexpectedly showed that TERT knocking down led to a significant increase in COX2 expression. Since COX2 acts as an important oncogenic factor by promoting cell proliferation and/or survival, it's thus of importance to define the functional significance of COX2 up-regulation in TERT-depleted cancer cells. We evaluated both the *in vitro* and *in vivo* effects of TERT depletion and COX2 inhibition alone or in combination on BGC-823 cells. Neither TERT knocking down nor celecoxib treatment alone induced cell apoptosis in culture, whereas the combined treatment led to a dramatic decrease in cell numbers. This was also the case in the mouse xenograft model of human gastric cancer, which indicates their *in vivo* synergistic effect, too.

A number of previous studies have shown that telomerase or TERT inhibition induces apoptosis of certain cancer cells that occurs rapidly before telomeres become shorter. [130, 131] However, TERT depletion seemed not to inhibit proliferation and survival of BGC-823 cells at least within a 96-h period

examined. Telomerase inhibition leads to telomere shortening in cancer, which takes a period for cellular telomere become dysfunctional. This time lag might significantly affect the efficacy of telomerase inhibition and limits its clinical application. [19, 132-134] Our findings should help overcome these disadvantages by combining telomerase and COX2 inhibitors together and may have therapeutic implications in a telomerase-based anticancer strategy.

6 SUMMARY & CONCLUSIONS

1. TERT promoter mutations occur in pre-malignant thyroid lesions and are strongly associated with increased TERT mRNA expression and telomerase activity. This indicates that the mutation is an early genetic event in carcinogenesis and that telomerase can be activated in the initial stage of cancer formation.

2. TERT promoter mutations are identified in follicular thyroid cell-derived carcinomas and associated with aggressive disease, and poor outcomes.

3. TERT promoter mutations are absent in C cell-derived medullary thyroid cancer and gastric cancer, suggesting that these genetic events are not responsible for telomerase activation in both malignancies.

4. TERT inhibition-mediated COX2 induction may protect cancer cells from apoptosis. Thus simultaneously targeting TERT and COX2 has synergistic killing effects on cancer cells, which may have implications in rational development of a telomerase-based anti-cancer strategy.

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