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The role of telomerase reverse transcriptase in human malignancies: Genetic polymorphisms and promoter mutations

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THE ROLE OF TELOMERASE REVERSE TRANSCRIPTASE IN HUMAN MALIGNANCIES: GENETIC POLYMORPHISMS AND PROMOTER MUTATIONS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Telomerase is a ribonucleoprotein with its catalytic subunit telomerase reverse transcriptase (TERT) as a key component, lengthening telomeres. In differentiated human cells, telomerase is silent due to the transcriptional repression of the *TERT* gene, but activated in oncogenesis. Telomerase activation/TERT induction is essential to unlimited proliferation of cancer cells via telomere lengthening, whereas recent evidence also suggests that TERT may be a master contributor of cancer hallmarks. It is thus important to define regulatory mechanisms underlying cancer-specific TERT expression, and to delineate oncogenic effects of TERT. This thesis is designed to address these issues with the following specific aims: (1) The association between single-nucleotide polymorphisms (SNPs) of the *TERT* gene and cancer susceptibility and (2) Biological/translational implications of cancer-specific TERT promoter mutations.

The *TERT* SNP association with cancer risk has been extensively investigated, most studies being focused on rs2736100 and rs2736098. The rs2736100_CC genotype has been shown to be associated with higher risk for a number of cancer types. Consistently, we observed that male individuals carrying the rs2736100_CC exhibited greater susceptibility to myeloproliferative neoplasms (MPNs), clonal diseases with myeloid cell origin (PAPER I). Furthermore, a comparison between Swedish and Chinese populations revealed a significantly higher fraction of rs2736100_CC in Swedes, coupled with a higher MPN incidence (compared to that in China). In addition, we made the same genotyping in upper tract urothelial carcinoma (UTUC) and hepatocellular carcinoma (HCC). The rs2736100_AC genotype was associated with reduced UTUC risk compared to the rs2736100_AA and CC carriers (PAPER II), while there were no significant differences in the rs2736100 or rs2736098 genotype distribution between HCC patients and healthy individuals (PAPER III). Collectively, male/female and ethnical groups may harbor different germline *TERT* variants, thereby contributing to different incidences and susceptibility dependent on origins of malignancies.

The recurrent TERT promoter mutations, recently identified in different human malignancies, stimulate *TERT* transcription and activate telomerase. To explore the biological and clinical implication of TERT promoter mutations, we sequenced the TERT promoter region in tumor specimens derived from patients with UTUC, bladder cancer (BC) and HCC (PAPERS III and IV), and mutations were observed in 65/220 (30%) UTUC, 41/70 (59%) BC and 57/190 (30%) of HCC patients, respectively. In UTUC, the presence of TERT promoter mutations was significantly correlated with metastases, whereas for HCC, there was a significant difference in rs2736098 and rs2736100 genotypes between wt and mutant TERT promoter-bearing tumors. The cancer risk genotype rs2736100_CC was significantly associated with a reduced incidence of TERT promoter mutations, while the rs2736098_CT genotype was significantly higher in HCCs with TERT promoter mutations. Thus, the germline *TERT* genetic background may substantially affect the incidence of TERT promoter mutations in HCCs.

As TERT promoter mutations are absent in normal cells, we evaluated the mutant TERT promoter as a urinary biomarker for non-invasive detection of UTUC and BC. The mutant TERT promoter was indeed detectable in urine from the mutation-positive UTUC and BC patients using Sanger sequencing, but the sensitivity was only 60%. To improve it, we developed a Competitive Allele-Specific TaqMan PCR (castPCR), and achieved an overall sensitivity of 89% and specificity of 96%. Thus, castPCR assays of TERT promoter mutations may be useful tools for non-invasive, urine-based diagnostics of UTUC and BC.

In summary, our findings gain new insights into the association of *TERT* SNPs with cancer risk and TERT promoter mutations. These results will hopefully contribute to the rational development of a TERT-based strategy for precision oncology.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Dahlström J*, Liu T*, **Yuan X**, Saft L, Ghaderi M, Wei YB, Lavebratt C, Li P, Zheng C, Björkholm M, Xu D. (2016) TERT rs2736100 genotypes are associated with differential risk of myeloproliferative neoplasms in Swedish and Chinese male patient populations. *Annals of Hematology*, 95(11), 1825-1832.

II. **Yuan X***, Meng Y*, Li P, Ge N, Kong F, Yang L, Björkholm M, Zhao S, Xu D. (2016) The association between the TERT rs2736100 AC genotype and reduced risk of upper tract urothelial carcinomas in a Han Chinese population. *Oncotarget*, 7(22), 31972-31979

III. **Yuan X***, Cheng G*, Yu J, Zheng S, Sun C, Sun Q, Li K, Lin Z, Liu T, Li P, Xu Y, Kong F, Björkholm M, Xu D. (2017) The TERT promoter mutation incidence is modified by germline TERT rs2736098 and rs2736100 polymorphisms in hepatocellular carcinoma. *Oncotarget*, 8(14), 23120-23129.

IV. Wang K*, Liu T*, Ge N*, Liu L, **Yuan X**, Liu J, Kong F, Wang C, Ren H, Yan K, Hu S, Xu Z, Björkholm M, Fan Y, Zhao S, Liu C, Xu D. (2014) TERT promoter mutations are associated with distant metastases in upper tract urothelial carcinomas and serve as urinary biomarkers detected by a sensitive castPCR. *Oncotarget*, 5(23), 12428-12439.

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Other related publications not included in the thesis

I. Wang K*, Liu T*, Liu C*, Meng Y*, **Yuan X**, Liu L, Ge N, Liu J, Wang C, Ren H, Yan K, Hu S, Xu Z, Fan Y, Xu D. (2015) TERT promoter mutations and TERT mRNA but not FGFR3 mutations are urinary biomarkers in Han Chinese patients with urothelial bladder cancer. *The Oncologist*, 20(3): 263-269.

II. **Yuan X***, Liu C**,** Wang K*, Liu L, Liu T, Ge N, Kong F, Yang L, Björkholm M, Fan Y, Zhao S, Xu D. (2016) The genetic difference between Western and Chinese urothelial cell carcinomas: infrequent FGFR3 mutation in Han Chinese patients. *Oncotarget*, 7(18): 25826-25835.

III. Liu T, **Yuan X**, Xu D. (2016) Cancer-specific telomerase reverse transcriptase (TERT) promoter mutations: biological and clinical implications. *Genes*, 7(7): 38.

IV. Cheng G*, **Yuan X***, Wang F, Sun Q, Xin Q, Li K, Sun C, Lin Z, Luan Y, Li P, Xu Y, Kong F, Xu D. (2017) The association between the telomerase (TERT) rs2736098_TT genotype and a lower risk of chronic hepatitis B and cirrhosis in Chinese males. *Clinical and Translational Gastroenterology*, 8(3): e79.

V. Han H, Liang X, Ekberg M, Kritikou JS, Brunnström Å, Pelcman B, Matl M, Miao X, Andersson M, **Yuan X**, Schain F, Parvin S, Melin E, Sjöberg J, Xu D, Westerberg LS, Björkholm M, Claesson HE. (2017) Human 15-lipoxygenase-1 is a regulator of dendritic-cell spreading and podosome formation. *FASEB Journal*, 31(2): 491-504.

VI. Liang X, **Yuan X**, Yu J, Wu Y, Li K, Sun C, Li S, Shen L, Kong F, Jia J, Björkholm M, Xu D. (2017) Histone chaperone ASF1A predicts poor outcomes for patients with gastrointestinal cancer and drives cancer progression by stimulating transcription of β-catenin target genes. *EBioMedicine*, 21:104-116.

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CONTENTS

LIST OF ABBREVIATIONS

AP	Activating enhancer-binding protein
BC	Bladder cancer
CSC	Cancer stem cell
CTNNB1	Beta-catenin
ddNTP	Dideoxy-ribonucleoside triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxy-ribonucleoside triphosphate
EGFR	Epidermal growth factor receptor
ET	Essential thrombocythemia
ETS	E26 transformation-specific
FISH	Fluorescence in situ hybridization
GWAS	Genome-wide association studies
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HIF	Hypoxia-inducible factor
JAK 2	Janus kinase 2
MPN	Myeloproliferative neoplasm
NF-κb	Nuclear factor - kappa B
PCR	Polymerase chain reaction
PMF	Primary myelofibrosis
POT1	Protection of telomeres 1
PV	Polycythemia vera
RAP1	Repressor/Activator protein 1
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RPC	Renal pelvic carcinoma
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1

TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TFII-I	General transcription factor IIi
TGF-β	Transforming growth factor beta
TIN2	TRF 1 interacting nuclear factor 2
TP53	Tumor protein p53
TPP1	TINT1, PTOP, PIP1 – POT1-TIN2 organizing protein
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
UC	Ureter carcinoma
UTUC	Upper tract urothelial carcinoma
β2-M	Beta 2- microglobulin

1 INTRODUCTION

1.1 Telomere

1.1.1 Telomere structure and shelterin proteins

Telomere is the special structure at the ends of chromosomes. The story dates back to the last century. In 1930s and 1940s, both Hermann J Muller and Barbara McClintock found that broken chromosomes were unstable and prone to rearrangements and fusion, which earned them the Nobel Prize in 1946 and 1983, respectively [1-3].

In human, telomere is TTAGGG repeats up to 20 kb long. Telomere structure consists of double stranded DNA sequences with 3' G rich tails and protein complexes [4-6]. The 3'overhangs usually invade and insert into the double stranded telomere repeats to form a T-loop, which makes the chromosome stable. The protein complexes include those directly binding to telomeric DNA and their interacting factors. The most important members of binding proteins are collectively named shelterins, consisting of TTAGGG repeat binding protein factor 1 (TRF1), TRF2, POT1, TIN2, TPP1 and RAP1. TRF1 and TRF2 bind to the double stranded telomeric DNA, while POT1 binds the single strand overhang [7]. They interact with RAP1, TIN2 and TPP1 to regulate T-loop formation and maintain chromosome structure stability [8].

1.1.2 Telomere function

Telomere and its binding proteins form a complex structure at the end of the chromosome, protecting chromosomes from end-to-end fusions, double-strand breaks and degradation [9]. Importantly, most normal human cells exhibit progressive telomere shortening with cellular division due to "the end replication problem", and when telomere length becomes too short to maintain its function and structure, cells stop dividing and enter into a permanent growth arrest or senescent stage [10]. Therefore, telomere shortening serves as a mitotic clock, counting/controlling the number of cell population doublings. By doing so, telomere shortening prevents unlimited cell proliferation and thus form a strong barrier to immortalization and malignant transformation [11].

1.2 Telomerase

1.2.1 Discovery of telomerase and its structure/function

Telomerase is a RNA-dependent DNA polymerase that extends TTAGGG repeats at the end of chromosomes. In 1987, Carol Greider and Elizabeth Blackburn discovered the enzyme to be a ribonucleoprotein complex critically dependent on both the protein and RNA component and named it as telomerase. They received the Nobel Prize in 2009 [12].

Telomerase consists of an RNA template (TERC), telomerase reverse transcriptase (TERT) and other components [13]. TERT is the catalytic, rate-limiting subunit of the enzyme. TERC is constitutively expressed in normal human cells, while TERT is absent in most differentiated human cells. In general, TERT expression is highly correlated with telomerase activity and lack of TERT expression leads to telomerase silence in normal human cells where progressive telomere erosion occurs, as described above. On the other hand, different levels of TERT/telomerase expression are detectable in stem/progenitor cells, activated lymphocytes and other cells with high proliferative potentials and required for their sustained proliferation by compensating for telomere loss [14].

1.2.2 Biological role of telomerase in oncogenesis

In most human somatic cells, telomerase is silent and TERT expression is repressed, and these cells have finite lifespan and telomeres shorten with cell division [15-17]. However, telomerase activation occurs widely in tumor cells to maintain telomere length, thereby overcoming proliferation limitation and senescence. Experimentally, ectopic TERT expression and telomerase activation is absolutely required for oncogene-mediated transformation of normal fibroblasts [18]. Furthermore, telomerase or TERT inhibition leads to the loss of tumorigenic potential of cancer cells. Consistent with these data, numerous clinical studies showed TERT/telomerase activity to be detectable in up to 90% of malignancies [19-20]. Therefore, telomerase activation is an essential step in malignant transformation. TERT, as a rate-limiting unit for telomerase activity, is equally critical to cancer development.

Telomere elongation is an established function of telomerase or TERT, however, accumulated evidence suggests novel properties of TERT without involvement of telomere maintenance [21]. Ectopic expression of TERT promotes carcinogenesis independently of telomere lengthening, or stimulates cell proliferation by up-regulating growth factor expression [22]. TERT was recently shown to interact with NF-κb p65, activating NF-κb target genes [23]. TERT was associated with β-catenin, and synergized with β-catenin to induce epithelial-mesenchymal transition, thereby facilitating cancer metastasis [24]. Importantly, our recent findings further revealed a key role of TERT in self-renewal and expansion of prostate CSCs [25]. In addition, TERT enhances the chromatin-remodeling factor Brg1 recruited to β-catenin targets, promoting normal stem cell proliferation [26]. Thus, TERT plays parts far beyond its telomere-lengthening activity in cancer biology, significantly contributing to multiple cancer hallmarks.

1.3 Regulation of TERT expression

Given an important role of TERT/telomerase in oncogenesis, its regulatory mechanism has long been a central issue addressed in cancer research. TERT expression is controlled at multiple levels by many factors [27-30], however, it is predominantly

regulated at the transcriptional level. The TERT promoter is a region with GC-rich content rather than with a TATA box, which contains at least five upstream Sp1 binding motifs, two E-boxes and a single transcription start site that binds multi-functional transcription factor TFII-I [31-32].

1.3.1 Positive regulators of TERT transcription

The c-Myc oncogene, promoting cell growth and proliferation in a variety of human cancer, is a key trans-activator for the *TERT* gene. The TERT proximal promoter harbors two E-boxes with the sequence of 5'-CACGTG-3', which are bound by c-Myc [33-35]. The c-Myc-induced TERT over-expression is one of the important mechanisms underlying its oncogenic potential [34].

Another key molecule in TERT regulation is Sp1 family transcription factors. Sp1 is a Zinc-finger transcriptional factor binding to GC boxes in promoters [31]. It directly stimulates or co-operates with c-Myc to activate TERT transcription [36].

Besides c-Myc and Sp1, many other positive transcriptional factors regulate TERT transcription directly or indirectly. For example, the E26 transformation-specific (ETS) family proteins regulate TERT transcription by interacting with their binding motifs in the proximal promoter region [37-38]. Survivin could enhance TERT transcription by increase DNA binding ability [39]. Hypoxia-inducible factor-1α (HIF-1α) was observed to stimulate TERT transcription by binding to the TERT promoter in cancer cells [40]. Activating enhancer-binding protein-2 (AP-2) is capable of facilitating TERT transcription in lung cancer cells [41].

1.3.2 Negative regulators of TERT transcription

The *TERT* gene is stringently repressed at the transcriptional level in normal differentiated human cells, however, the underlying mechanism remains incompletely understood. The tumor suppressors Mad1, TGF-β and Menin were identified as key negative factors repressing TERT transcription in normal fibroblasts [42]. Consistently, Mad/Max/c-Myc network proteins were also shown to be the master regulator of the TERT transcription in human cancer cells [34]. Mad is expressed in non-proliferating cells, and has the role to promote cell differentiation and prevent malignant transformation. Mad/Max heterodimers competitively binds to E-boxes on the TERT promoter to repress TERT transcription [43]. In HL60 leukemic cells, TERT mRNA is highly expressed because c-Myc binds to the E-boxes on the TERT promoter. Once cells are induced to undergo terminal differentiation, c-Myc expression is diminished whereas Mad1 levels increase and subsequently replace c-MYC on the TERT promoter, thereby silencing TERT transcription [34, 44]. Tollefsbol's group determined the *TERT* gene trans-activation by endogenous c-Myc during the conversion from normal to transformed human fibroblasts, and they found that the induction of c-Myc expression

led to a switch from Mad1/Max to c-Myc/Max binding to sequences containing the TERT promoter distal and proximal E-boxes, coupled with telomerase activation [45].

P53, as a tumor suppressor, regulates cell proliferation, differentiation, apoptosis and senescence. It interacts with Sp1, a TERT activator binding to the TERT promoter, thereby attenuating the role of Sp1 in TERT transcription [46-47]. Thus, wild-type P53 leads to decreased TERT expression and telomerase activity in cancer cells.

In addition, many other transcriptional factors also negatively regulate TERT transcription. For instance, Wilms' tumor 1 (WT1) [48-49], Rb, Ap1 and TGF-β/SMAD all down-regulate TERT transcription [50-52].

1.4 *TERT* gene polymorphisms and its promoter mutations

According to recent genome-wide association studies (GWAS), single nucleotide polymorphisms (SNPs) are associated with susceptibility of human malignancies [53-56].

The genetic variation in the *TERT* gene and cancer-specific expression of TERT or telomerase activity play an important role in malignant transformation and cancer progression. More recently, TERT promoter mutations have been identified as important genetic events that trigger telomerase activation in different types of cancer [57-65].

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

The association between *TERT* rs2736100 and rs2736098 variants and cancer

The presence of multiple SNPs in the *TERT* gene has been documented, among which rs2736100 (located at intron 2) and rs2736098 (at exon 2) are most studied. We and others previously analyzed the rs2736100 association with lung cancer risk, and observed a significantly elevated risk in C variant-carriers [66]. Recently, the rs2736100_C allele was further identified to be more intimately associated with female, non-smoking, EGFR-mutation-positive lung adenocarcinoma [67]. In addition, the rs2736100_C has also been shown to be a risk allele for malignant glioma, colorectal carcinoma, cervical, pancreatic, bladder, and ovarian cancer, acute myeloid and lymphoblastic leukemia, and other malignancies [68]. rs2736098 variants and association with cancer risk have also been demonstrated in multiple types of cancer including hepatocellular carcinoma (HCC), lung cancer, breast cancer, and more others [69-72]. However, it is unclear whether the TERT variants are associated with upper tract urothelial carcinoma (UTUC) risk or disease progression.

The mechanism underlying the association between *TERT* variants and cancer susceptibility remains poorly defined. The rs2736100_CC genotype was shown to promote TERT transcription and to maintain telomere length much more strongly than

its AA and AC variants [67], which provides a potential explanation for linking rs2736100_CC genotype with cancer risk. It is not established whether there exist other mechanisms, and further investigations are required to elucidate this issue.

1.4.2 TERT promoter mutations in human cancer

In 2013, the hotspot TERT promoter mutation was first reported in human melanoma. Huang et al and Horn et al identified somatic mutations in the TERT promoter region in malignant melanoma [56, 73]. Two major point mutations are named C228T and C250T, which are cytidine-to- thymidine change occurring at -124 and -146 from the translation start site, respectively. These mutations create a new ETS binding motif, and thereby activate TERT transcription [58, 74] (Figure 1). Since then, TERT promoter mutations have been identified in many types of cancer. TERT promoter mutations occur most frequently in bladder, renal pelvic, thyroid cancer, HCC, malignant glioblastoma and melanoma [75], while they are rarely present in hematological malignancies, prostate, gastrointestinal, breast and lung cancer [57, 76]. Many studies demonstrate that tumors bearing the mutant TERT promoter in general express higher levels of TERT mRNA than those with a wild type promoter [77-78].

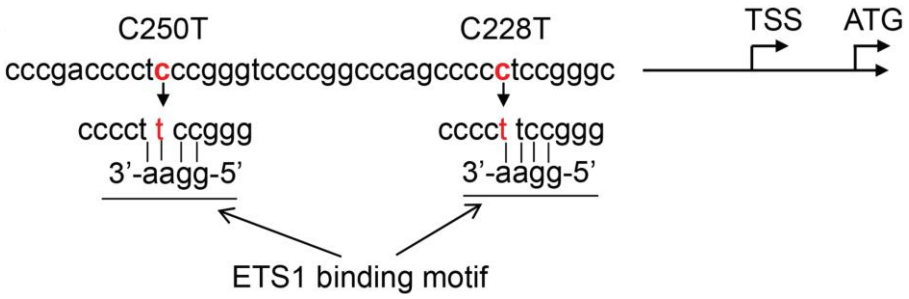

Figure 1. The structure of TERT promoter and positions of C228T and C250T. C>T mutations lead to new ETS1 binding sites, therefore promoting TERT transcription.

In addition to C228T and C250T mutations, other mutations with a low frequency are also identified in the TERT promoter region. For example, CC-to-TT tandem mutations occur at -124/-125 and -138/-139, and the C-to-T mutation at -57, are found in a small proportion of cancer [56]. All these mutations contribute to enhanced TERT transcription by creating new transcriptional factor binding sites.

1.4.3 Overview of myeloproliferative neoplasms (MPNs)

MPNs are a group of clonal disorders within the myeloid lineages in the bone marrow [79]. MPNs have the character of hyperproliferation, resulting in excessive number of terminally differentiated cells from one or more of myeloid lineages. MPNs are sub-grouped into polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) [80].

A number of important genetic alterations have been identified in MPNs and the most common one is the $JAK2^{V617F}$ mutation, which is observed in 95% of PV, 50% of ET and PMF [81-82]. Other genetic aberrations include calreticulin (CALR), MPL mutations, etc. [83]. All these mutations are believed to act as drivers for MPN development.

1.4.4 Telomere biology in MPNs and efficacy of telomerase inhibition

A number of studies have reported that telomere length in MPN patients is shorter than that in the healthy population, and shorter telomere length in bone marrow cells indicated MPN progression [84-86]. Moreover, we observed a widespread dysregulation of shelterin factor expression [87-88], and this together with shortened telomere length significantly contributed to telomere dysfunction and genomic instability occurring in MPNs. Thus, aberrant telomere length and shelterin protein expression play an important part in the pathogenesis of MPNs. In addition, the variant at SNP rs2736100 has recently been reported to be associated with MPN susceptibility [89-90].

The above findings promoted testing of the telomerase inhibitor GRN163L (Imetelstat®) in MPN treatment. GRN163L inhibits telomerase activity by interfering with TERC function. In PMF, approximately one-fifth of patients (7/33) treated with Imetelstat® either had a complete remission (CR) (defined as normalization of hepatosplenomegaly, blood counts including white blood cell differential together with reversal of bone marrow fibrosis) or a partial remission (defined with the same criteria as for complete remission apart from reversal of the bone marrow fibrosis) [91]. In addition, GRN163L could significantly inhibit megakaryocyte maturation in MPNs, thereby achieving its efficacy in ETs [92].

1.5 Upper tract urothelial carcinomas (UTUCs)

1.5.1 Overview

Urothelial carcinomas are malignant tumors that arise from the urothelial epithelium and may involve the lower urinary tract (bladder and urethra) [93] or the upper urinary tract (renal pelvis and ureter) (Figure 2).

UTUCs, like bladder cancer (BC), belong to transitional cell carcinomas and consist predominantly of renal pelvic carcinomas (RPCs) and ureter carcinomas (UCs). UTUCs account for 5% to 10% of all primary urothelial cancers [94], and their recurrence and progression rates are high due to difficulties in early diagnosis [95]. Therefore a better understanding of UTUC pathogenesis might lead to early identification, and hence improved therapeutic possibilities.

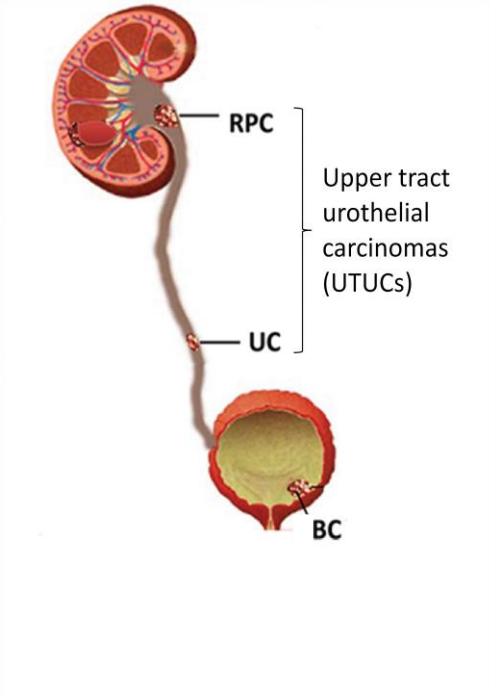

Figure 2.The diagram shows the location of UTUCs and BCs.
RPC, Renal pelvic carcinoma; UC, Ureter carcinoma; BC, Bladder cancer.

1.5.2 Treatment

Urothelial carcinoma is the ninth most common cancer globally and the eighth most lethal neoplasm in men in the United States [96-97]. It is the most costly cancer in the US health care system on a per-patient basis, because these patients are prone to frequent relapses and need life-long surveillance.

Surgery is the mainstay of UTUC therapy, but the prognosis remains poor. Recently, there is increasing enthusiasm for combined-modality approaches in both the adjuvant and neoadjuvant settings. Nephron-sparing surgical strategies, including partial ureterectomy and purely endoscopic tumor resection, are also increasingly used [98].

1.6 Hepatocellular carcinoma (HCC)

1.6.1 Overview

HCC is derived from primary liver cells and represents 70-85% of primary liver cancer [99]. It is the fifth most common cancer in men and the seventh in women, being diagnosed in more than half a million individuals worldwide every year [100]. The main risk factor for HCC include hepatitis B (HBV) and hepatitis C (HCV) infection [101-104], and liver diseases caused by excessive alcohol consumption, aflatoxin exposure, and non-alcoholic fatty liver disease [100, 105-107].

1.6.2 The role of TERT in HCC

Several studies have reported the correlation between TERT and HCC [108-111]. A recent study showed that TERT promoter mutations were identified as the most frequent genetic alterations in HCC with an overall frequency around 60% [112]. Moreover,

SNPs rs2736098 on the *TERT* gene and rs2853669 on TERT promoter region were found to be associated with increased risk and poor prognoses of HCC [113-115].

2 AIMS OF THE STUDY

The overall aim of this PhD project is to define the association between the *TERT* SNPs and cancer susceptibility, relation to TERT promoter mutations, new roles of TERT and telomerase in cancer development/progression, and their clinical relevance. Specifically, the study is aimed at:

1) Determining whether there exist disparities in the rs2736100 distribution between Chinese and European populations and its relation to MPN susceptibility.

2) Determining whether the rs2736100/rs2736098 variants in the *TERT* gene are associated with UTUC and HCC susceptibility.

3) Defining whether the germline variants in the *TERT* gene affect the incidence of TERT promoter mutations in UTUC and HCC.

4) Evaluating the detection of mutant TERT promoter sequences as urinary biomarkers for non-invasive UTUC diagnosis and disease surveillance.

3 METHODS

3.1 Patients and healthy controls (PAPERS I-IV)

3.1.1 MPN patients and healthy controls (PAPER I)

One hundred and one Chinese MPN patients and 101 age- and gender- matched healthy adults were recruited from Shandong University Hospitals, China. One hundred and twenty-six patients, diagnosed with MPN at Karolinska University Hospital were included in the study. Age-and-gender matched Swedish healthy adults (N=756) were used as controls for the Swedish MPN patients. Peripheral blood was collected from both MPN patients and controls and myeloid cells then isolated.

3.1.2 UTUC patients and healthy controls (PAPERS II & IV)

In PAPER II, 212 recently diagnosed UTUC patients were recruited from the Shandong University Qilu Hospital and the Second Hospital, and 289 age- and gender- matched healthy adults were used as control populations. Both patients and controls have Han Chinese ethnic background. Tumors and their adjacent normal tissues were collected from patients. Peripheral blood was collected from healthy controls and mononuclear cells then isolated.

In PAPER IV, 98 patients with RPC, 122 with UC and 70 with BC were recruited from Shandong University Qilu Hospital and the Second Hospital, China. Spontaneously voided urine was collected from 16 RPC, 20 UC and 70 BC patients prior to surgical treatment. In 13 of 36 RPC and UC patients, urine was also obtained one week after surgery.

3.1.3 HCC patients and healthy controls (PAPER III)

Two hundred and forty-five newly diagnosed HCC patients were recruited from Shandong University Second Hospital and Shandong Provincial Hospital. Sex-matched healthy adults served as controls. Age (mean $\pm$ SD) for patients and controls was 45 ± 16 years and 54 ± 10 years, respectively. Both patients and controls have Han Chinese ethnic background. Tumors and their adjacent normal tissues and blood were collected from HCC patients. Peripheral blood was collected from healthy controls and mononuclear cells then isolated.

3.2 Telomere length analysis using flow-FISH (PAPER I)

The average telomere length was measured with flow-FISH following the protocol by Baerlocher et al. [116], with minor modifications. Calf thymocytes were kindly donated from Ö-slakt AB (Värmdö, Stockholm). Stained cells were captured with Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and the analysis was done using the Kaluza software (Beckman Counter, Brea, CA, USA). Fluorescent MESF-FITC beads

(Bangs Laboratories, Fisher, IN, USA) were used and the fluorescent signal was quantified using the QuickCal v.2.3 data analysis program (Bangs Laboratories, Fishers, IN, USA).

3.3 RNA extraction and quantitative real-time PCR (PAPER I)

Total RNA from MPN patients was isolated using TRIzol reagent (Life technologies, Carlsbad, CA, USA). Two μg of RNA was used for reverse transcription using M-MLV (Life technologies, Carlsbad, CA, USA) according to the recommended protocol. Real-time amplification was performed in triplicate using SYBR Green PCR Master Mix (Life technologies, Carlsbad, CA, USA) with QuanStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). The primers used in this study are listed in Table 1. β2 microglobulin (β2-M) was used as the internal control and $2^{-\Delta\Delta Ct}$ method was used to calculate relative mRNA expression [117].

Table 1. Primers used for quantitative real-time PCRamplification of gene expression

TERT	Forward	5'-CGGAAGAGTGTCTGGAGCAA-3'
	Reverse	5'-GGATGAAGCGGAGTCTGGA-3'
β2-M	Forward	5'-GAATTGCTATGTGTCTGGGT-3'
	Reverse	5'-CATCTTCAAACCTCCATGATG-3'

3.4 DNA extraction (PAPERS I-IV)

In PAPER I, DNA was isolated using QIAmp DNA blood kit (QIAGEN, Hilden, Germany) from both Swedish and Chinese MPN patients and Chinese healthy controls. For Swedish healthy controls, DNA was extracted from saliva using Oragene saliva collection kit (DNA Genotek Inc., Ottava, Canada) [118].

In other PAPERS, DNA was extracted using QIAGEN DNA extraction kits (QIAGEN) and the concentration was measured by NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

3.5 Genotyping of *TERT* rs2736098 and rs2736100 (PAPERS I-III)

The TERT rs2736098 (T/C) and rs2736100 (A/C) genotyping was performed using pre-designed TaqMan SNP genotyping assay kits on a QuanStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) [119]. Both positive and negative controls were included in all assays and the running condition was as followed: 95℃ for 10 min followed by 40 cycles of 92℃ for 15s and 60℃ for 1 min.

3.6 Sanger sequencing of the TERT promoter region and *CTNNB1* gene (PAPERS II-IV)

Sanger sequencing is based on single-strand DNA template, DNA primers and DNA polymerase [120]. Both deoxy-ribonucleoside triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs) lacking 3'-OH group were added into the PCR system [121]. Since ddNTPs cannot form phosphodiester bonds with the nucleotide next to it, the DNA amplification will be stopped when ddNTPs bind to the template. The ddNTPs were labeled by four different fluorescence dyes, so the different sized PCR fragments with different termination signal could be separated and detected by capillary electrophoresis and sequence analysis [122].

In PAPER II-IV, point mutations of the TERT promoter and *CTNNB1* gene were detected using Sanger sequencing. Since the TERT promoter is a GC-rich region, bataine was added into the PCR system to increase PCR amplification efficiency [123-124]. After PCR reaction, products were purified with ExtraStar and then precipitated with EDTA and ethanol. Sanger sequencing was performed with Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) in ABI 3730 DNA analyzer machine. Primers used for Sanger sequencing are listed in Table 2.

The results were analyzed with Codon Code Aligner software and the mutations were confirmed in both forward and reverse directions.

Table 2. Primers used for PCR and Sanger sequencing

TERT promoter	Forward	5'-CACCCGTCCTGCCCCTTCACCTT-3'
	Reverse	5'-GGCTTCCCACGTGCGCAGCAGGA-3'
CTNNB1	Forward	5'-GGGTATTTGAAGTATACCATA-3'
	Reverse	5'-TGGTCCTCGTCATTTAGCAG-3'

3.7 Competitive allele-specific TaqMan™ PCR (castPCR) (PAPER IV)

castPCR analysis is highly specific and sensitive technology to detect rare amount of mutated DNA in a sample containing large amount of wild-type DNA [125-127]. In this study, it was performed by using ABI 7900 Real-time PCR system. The method requires genomic DNA template, mutant/wild type assay and 2×Taqman genotyping master mixture. The reaction system included 5 μl Taqman genotyping master mixture, 1 μl mutant/wild type assay, 2 μl DDW and 20 ng DNA template (diluted into 2 μl). The PCR conditions were: 95℃ for 10 mins, then (92℃ for 15s and 58℃ for 1 min) ×5 cycles followed by 45 cycles of 92℃ for 15s and 60℃ for 1 min. The PCR result was

analyzed using SDS 2.4 software program and Mutation Detector Software 2.0 (Life technologies).

For the sensitivity and specificity evaluation, the results from castPCR were compared with the results from Sanger sequencing, and the obtained results were also compared with the TERT promoter mutation status in tumors.

3.8 Statistical analyses (PAPERS I-IV)

The comparison of telomere length and mRNA expression was made using 2-tailed Student's t-test or Mann-Whitney U test. For genotype distributions of *TERT* rs2736098 and rs2736100, Fisher's exact test was used to generate odd ratio (OR), 95% confidence interval (CI) and *P*-value (PAPER I). Sex and age were compared between patients and healthy controls using Chi-square test or Fisher's exact test (PAPERS II-IV). Two-tailed student's t-test was used to analyze differences in tumor sizes between the TERT promoter mutation-positive and negative patient groups. Sensitivity and specificity difference between castPCR and Sanger sequencing were evaluated using McNemar's test (PAPER IV). All the tests were made using SigmaStat 3.1 software (Systat Software, Inc., Richmond, CA), and *P*-values<0.05 were considered as statistically significant.

4 RESULTS AND DISCUSSION

4.1 *TERT* gene variation in cancer and clinical implication (PAPERS I-III)

4.1.1 Distribution of *TERT* rs2736100 alleles in healthy Swedish and Chinese populations (PAPER I)

The characteristics of the MPN patients and healthy controls are showed in table 3. Genotyping of rs2736100 was performed in Swedish and Chinese patients and healthy controls. The A-allele is more frequent in the Chinese cohort. Moreover we collected the published genotyping data from other cohorts from Shandong and Guangzhou areas, and both studies showed the rs2736100 genotype distribution to be similar to our data (Table 4). The rs2736100 genotype distribution in healthy populations from Sweden and other European countries was also compared to that in China, and all of the published studies displayed similar rs2736100 variant frequency: lower A allele and higher C allele (48.0% vs 57.4% and 52.0% vs 42.6% for A and C, respectively, $P < 0.001$) (Table 4).

Table 3. Characteristics of healthy controls and patients with MPN

	Sweden		China	
	Controls	MPN	Controls	MPN
Number	756	126	101	101
Age (years)				
Mean ± SD	64±5	64±14	58±15	58±15
Median (range)	64(54-74)	65(25-106)	60(17-82)	60(17-82)
Sex (% female)	53	53	50	50
MPN subtype, n (%)				
PV		41(32.5)		16(15.8)
ET		40(31.7)		38(37.6)
PMF		28(22.3)		15(14.9)
MPN-NOS		17(13.5)		32(31.7)
JAK2-station, n (%)				
JAK2 V617F^{+}		60(47.6)		38(37.6)
JAK2 V617F^{-}		66(52.4)		56(55.4)
Unknown		0		7(7.0)
CALR mutation		45		Unknown

Table 4. Published rs2736100 genotype distributions of healthy populations in China and Europe

Author	Number	AA (%)	AC (%)	CC (%)	A (%)	C (%)	Area	Reference
China								
Dahlström et al	101	33(32.7)	50(49.5)	18(17.8)	116(57.4)	86(42.6)	North[a]	This study
Yuan et al	289	86(29.8)	144(49.8)	59(20.4)	316(54.7)	262(45.3)	North[a]	[128]
Wei et al	2520	814(32.3)	1269(50.4)	437(17.3)	2897(57.5)	2143(42.5)	South[b]	[67]
Total	2910	933(32.1)*	1463(50.3)*	514(17.6)*	3329(57.2)*	2491(42.8)*		
Europe								
Dahlström et al	756	167(22.1)	377(49.9)	212(28.0)	711(47.0)	801(53.0)	Sweden	This study
Jäger et al	202	47(23.3)	88(43.6)	67(33.2)	182(45.0)	222(55.0)	Italy	[129]
Krahling et al	400	111(27.8)	188(47.0)	101(25.2)	410(51.3)	390(48.7)	Hungary	[130]
Total	1358	325(23.9)**	653(48.0)**	380(28.1)**	1303(48.0)**	1413(52.0)**		
		AA vs AC+CC	AC vs AA+CC	CC vs AA+AC		C va A		
p value (* vs **)		＜0.001	0.106	＜0.001		＜0.001		

OR and p values were generated using chi-squared test

[a]From Shandong area; [b]From Shanghai and Guangzhou areas

4.1.2 TERT SNP rs2736100_C is a risk factor for MPNs in males (PAPER I)

Since rs2736100 allele has different distributions in control groups between China and Sweden, we decided to analyze MPN patients from these two countries separately. According to our analyses, both Chinese and Swedish MPN patients had a higher frequency of the rs2736100_C allele compared to their corresponding healthy controls (both *P*=0.004, Table 5). Compared to the AA variant carriers, both Swedish and Chinese patients bearing the CC genotype showed significantly increased risk of MPNs (OR = 2.47; 95% CI: 1.33 - 4.57, *P* = 0.003, for Swedish, and OR = 3.45; 95% CI: 1.52 - 7.85, *P* = 0.005, for Chinese patients) (Table 5). Further analyses showed that the CC genotype and C allele had a higher frequency only in male MPN patients.

Interestingly, we notice that the MPN incidence is much higher in Sweden (5.8/100,000) than in China (2/100,000) [131], which is correlated with their rs2736100_C allele frequencies. Racial or ethnic disparities in cancer incidence and pathogenesis due to different genetic backgrounds have been well characterized, and a difference in TERT rs2736100 variants between Swedish and Chinese populations may partially explain their differential MPN incidences.

Table 5. Comparison of TERT rs2736100 genotypes in MPN patients and healthy controls

rs2736100 genotype	Sweden				China			
	Controls n (%)	MPN n (%)	OR (95%CI) *	p value	Controls n (%)	MPN n (%)	OR (95%CI) *	p value
All	756 (100)	123 (100)			101 (100)	101 (100)		
Alleles								
A	711 (47.0)	94 (37.3)	1.0 (ref)		116 (57.4)	86 (42.6)	1.0 (ref)	
C	801 (53.0)	158 (62.7)	1.49 (1.13-1.96)	**0.004**	86 (42.6)	116 (57.4)	1.82 (1.23-2.70)	**0.004**
Genotypes								
AA	167 (22.1)	15 (11.9)	1.0 (ref)		33 (32.7)	17 (16.8)	1.0 (ref)	
AC	377 (49.9)	64 (50.8)	1.89 (1.05-3.41)	**0.034**	50 (49.5)	52 (51.5)	2.02 (1.00-4.08)	0.057
CC	212 (28.0)	47 (37.3)	2.47 (1.33-4.57)	**0.003**	18 (17.8)	32 (31.7)	3.45 (1.52-7.85)	**0.005**
AA+AC	544 (72.0)	79 (62.7)	1.0 (ref)		83 (82.2)	69 (68.3)	1.0 (ref)	
CC	212 (28.0)	47 (37.3)	1.53 (1.03-2.27)	**0.044**	18 (17.8)	32 (31.7)	2.14 (1.11-4.14)	**0.033**
AC+CC	589 (77.9)	111 (88.1)	1.0 (ref)		68 (67.3)	84 (83.2)	1.0 (ref)	
AA	167 (22.1)	15 (11.9)	0.48 (0.27-0.84）	**0.009**	33 (32.7)	17 (16.8)	0.42 (0.21-0.81)	**0.014**

*OR, Odds ratio; CI, Confidence interval

OR and p values generated using Fishers' exact test Significant p values are shown in bold.

4.1.3 TERT mRNA expression and telomere length in patients with MPN carrying different *TERT* rs2736100 genotypes (PAPER I)

MPN patients with the *TERT* rs2736100_CC genotype display the highest TERT mRNA expression in their myeloid cells compared with the AA and AC carrying patients (P = 0.024) (Figure 3). The difference in TERT expression between the AC and AA variants was not significant. The rs2736100_CC-carrying patients tended to have longer telomere than did those with AA and AC genotypes; however, the difference did not reach statistical significance.

The present finding is consistent with the result by Wei et al. [67], who observed that the rs2736100_CC genotype promoted *TERT* gene transcription and up-regulated telomerase activity more strongly than did AA or AC variants. Conceivably, higher TERT expression and telomerase activity facilitates the pathogenesis of MPNs via both telomere lengthening-dependent and independent mechanisms.

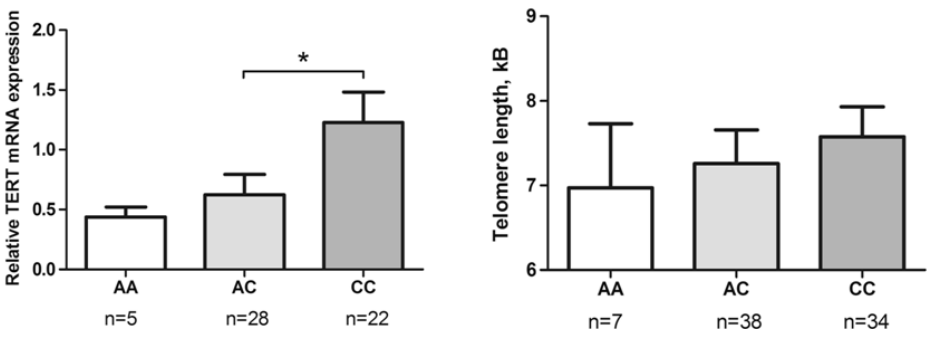

Figure 3. TERT mRNA expression and telomere length in Swedish MPN patients with different *TERT* rs2736100 genotypes. * p<0.05.

4.1.4 *TERT* gene variation and UTUC risk (PAPER II)

In this study, 212 UTUC patients were included, and the clinical characteristics are summarized in Table 6.

Table 6. Clinical characteristics of patients with UTUC

	RPC*	UC*	Total
informative cases (n=)	92	120	212
Age at diagnosis (n=212)			
Mean ± SD	63±11	66±11	64±11
Median (range) years	64(36-85)	67(32-87)	66(32-87)
Gender (n=212)			
Female	37	45	82
Male	55	75	130
Metastases or capsular invasion (n=189)			
Yes	6	11	17
No	77	95	172
Stage (n=189)			
Pa+Ⅰ	16	24	40
PⅡ+Ⅲ+Ⅳ	67	82	149
grades (n=189)			
G1	13	12	25
G2	9	13	22
G3	61	81	142

*RPC: Renal pelvic carcinoma; UC: Ureter carcinoma

TERT rs2736100 AC and rs2736098 GT genotype were analyzed in the age- and sex-matched healthy population and UTUC patients. The genotype distributions are listed in Table 7. In UTUC patients, the prevalence of the rs2736100 heterozygous AC genotype was significantly lower than that in healthy controls, which indicates a reduced risk for UTUCs (Odds ratio = 0.583; 95% CI: 0.388 - 0.875; *P* = 0.012). When we combined the AA and CC genotypes together and compared with the AC variant, a significant difference remained (OR = 0.613, 95% CI: 0.428 - 0.879, *P* = 0.010) (Table 7).

Table 7. TERT rs2736100 genotypes in healthy controls and patients with UTUC

Genotype	Control	RPC*			UC*			Total UTUC		
	N (%)	N (%)	Odds ratio (95% CI*)	*P*	N (%)	Odds ratio (95% CI*)	*P*	N (%)	Odds ratio (95% CI*)	*P*
rs2736100 (N/%)	289	92			120			212		
AA	86 (29.8)	34 (37.0)	1.0 (ref.)		49 (40.8)	1.0 (ref.)		83 (39.2)	1.0 (ref.)	
AC	144 (49.8)	32 (34.8)	0.562 (0.324 - 0.976)	0.055	49 (40.8)	0.597 (0.370 - 0.963)	**0.045**	81 (38.2)	0.583 (0.388 - 0.875)	**0.012**
CC	59 (20.4)	26 (28.2)	1.115 (0.606 - 2.049)	0.846	22 (18.4)	0.714 (0.396 - 1.288)	0.330	48 (22.6)	0.878 (0.542 - 1.423)	0.685
AA + CC	145 (50)	60 (65)	1.0 (ref.)		71 (59.2)	1.0 (ref.)		133(62.1)	1.0 (ref.)	
AC	144 (49.8)	32 (35)	1.862 (1.144 - 3.031)	**0.016**	49 (40.8)	0.695 (0.452 - 1.069)	0.121	81 (37.9)	0.613 (0.428 - 0.879)	**0.010**

*RPC: Renal pelvic carcinoma; UC: Ureter carcinoma; CI: Confidence interval. Significant p values are shown in bold.

To see if the rs2736100_C allele showed any association with clinical variables, we analyzed the rs2736100 variants according to the disease stage and grade. A significantly negative association was found between the heterozygous rs2736100 AC genotype and early stage (pTa+T1) and low grades of UTUCs (OR = 0.358, 95% CI: 0.167 - 0.769, *P* = 0.012) (Table 8).

The present study is the first report showing the association between *TERT* SNPs and UTUC susceptibility. Moreover, we observed that the rs2736100 AC variant, a protective genotype, was significantly associated with a reduced risk for wt TERT promoter-carrying UTUCs. In addition, there exists an association between the rs2736100 AC genotype and reduced Ta and T1 stages of UTUCs. Thus, the germline *TERT* variants affect both UTUC susceptibility and disease progression.

Table 8. Association of TERT rs2736100 variants with disease characteristics variables and TERT promoter mutations in patients with UTUC

Genotype	**Cases**	**Healthy controls**	**Odds ratio (95% CI*)**	***P*-value**
Stages pTa - I vs controls				
AA	20 (48.8%)	86 (29.8%)	1.0 (ref.)	
AC	12 (29.2%)	144 (49.8%)	0.358 (0.167 - 0.769)	**0.012**
CC	9 (22.0%)	59 (20.4%)	0.656 (0.279 - 1.540)	0.455
Stages pII + III + IV vs controls				
AA	50 (33.8%)	86 (29.8%)	1.0 (ref.)	
AC	64 (43.2%)	144 (49.8%)	0.764 (0.484 - 1.206)	0.229
CC	34 (23.0%)	59 (20.4%)	0.991 (0.573 - 1.713)	0.914
Grade G1 vs controls				
AA	10 (41.7%)	86 (29.8%)	1.0 (ref.)	
AC	8 (33.3%)	144 (49.8%)	0.478 (0.182 - 1.257)	0.203
CC	6 (25.0%)	59 (20.4%)	0.875 (0.301 - 2.537	0.983
Grade G2 + G3 vs controls				
AA	60 (36.4%)	86 (29.8%)	1.0 (ref.)	
AC	68 (41.2%)	144 (49.8%)	0.675 (0.437 - 1.049)	0.101
CC	37 (22.4%)	59 (20.4%)	0.899 (0.531 - 1.522)	0.793
wt TERT promoter vs controls				
AA	50 (37.5%)	86 (29.8%)	1.0 (ref.)	
AC	50 (37.5%)	144 (49.8%)	0.597 (0.372 - 0.960)	**0.044**
CC	33 (25.0%)	59 (20.4%)	0.962 (0.555 - 1.668)	0.988
mt TERT promoter vs controls				
AA	20 (35.7%)	86 (29.8%)	1.0 (ref.)	
AC	26 (46.4%)	144 (49.8%)	0.776 (0.409 - 1.474)	0.543
CC	10 (17.9%)	59 (20.4%)	0.729 (0.318 - 1.668)	0.586

*CI: Confidence interval. Significant p values are shown in bold.

4.1.5 *TERT* rs2736098 and rs2736100 polymorphisms in HCC (PAPER III)

The genotyping data were obtained from 240 healthy controls and 231 HCC patients for rs2736098 and 237 healthy controls and 201 HCC patients for rs2736100, respectively. The clinical characteristics are shown in Table 9.

Table 9. TERT promoter mutations and clinical characteristics of HCC patients

Variable	**TERT promoter mutated**	**TERT promoter wild-type**	***P* value**
informative cases (n = 190)	**(n = 57)**	**(n =133)**	
Age at diagnosis (n =)	56	128	0.191
Mean years	54.71	52.63	
Median (range) years	55.5 (32 - 75)	51 (25 - 76)	
Gender (n =)	56	128	0.898
Female	8	19	
Male	48	109	
*HBV infection** (n =)	55	129	0.105
Yes	50	103	
No	5	26	
Cirrhosis (n =)	57	130	0.394
Yes	30	58	
No	27	72	
α-fetoprotein (ng/ml) (n=)	54	120	0.927
<200	38	82	
≥ 200	16	38	
Tumor size (n =)	56	121	0.328
< 5 cm	32	58	
> 5 cm	24	63	
Differentiation (n =)	55	123	0.609
Well or moderate	37	89	
Poor	18	34	
CTNNB1 (n =) or TERT (n =)	19	49	0.535
mutated	6	11	
wt	13	38	
Metastases (n =)	56	129	0.670
Yes	1	5	
No	55	124	

*HBV: Hepatitis B virus

Table 10 shows the summary of genotyping results. For the rs2736098 genotype, there was no significant difference between HCC patients and healthy controls. The rs2736100_CC genotype was significantly lower in HCC patients compared to the healthy controls (OR = 0.544, 95% CI: 0.320 - 0.925, *P* = 0.034) (Table 10). However, the difference was no longer significant after Bonferroni correction. Taken together,

neither rs2736100 nor rs2736098 variants are associated with HCC susceptibility. Because there were only 200 patients in the present HCC cohort, our result is unlikely conclusive and further studies recruiting more patients with HCCs are required to validate our findings.

Table 10. TERT rs2736098 and 2736100 genotyping in healthy adults and HCC patients

Genotype	HA*	HCC	Odds ratio (95% CI*)	*P* value
rs2736098 (N)	240 (100%)	231 (100%)		
TT	31 (12.9)	19 (8.2)	1.0 (ref.)	
CT	115 (47.9)	127 (55.0)	1.802 (0.965 - 3.364)	0.088
CC	94 (39.2)	85 (36.8)	1.475 (0.7763 - 2.804)	0.303
CT + CC	209 (87.1)	212 (91.8)	1.655 (0.906 - 3.023)	0.133
CC	94 (39.2)	85 (36.8)	1.0 (ref.)	
TT + CT	146 (60.8)	146 (63.2)	1.106 (0.762 - 1.605)	0.664
rs2736100 (N)	237 (100%)	201 (100%)		
AA	69 (29.1)	74 (36.8)	1.0 (ref.)	
AC	108 (45.6)	92 (45.8)	0.794 (0.517 - 1.221)	0.347
CC	60 (25.3)	35 (17.4)	0.544 (0.320 - 0.925)	**0.033**
AC + CC	168 (74.7)	127 (63.2)	0.705 (0.472 - 1.053)	0.107
CC	60 (25.3)	35 (17.4)	1.0 (ref.)	
AA + AC	177 (73.7)	166 (82.6)	1.608 (1.007 - 2.566)	0.06

*HA: Healthy adults; CI: Confidence interval. Significant p value is shown in bold.

4.2 TERT promoter mutations in human cancer

4.2.1 TERT promoter mutations in HCC (PAPER III)

For the HCC cohort, TERT promoter mutations were also analyzed and we identified the presence of the mutations in 30% (57/190) HCC patients (Table 9). In addition, the *CTNNB1* gene, encoding β-Catenin, is frequently mutated in HCC [59, 132], and therefore we sequenced this gene for mutation detection in HCC tumors, too. The *CTNNB1* mutation was identified in 17/83 (24.3%) of HCC tumors and was not associated with the TERT promoter mutation. Clinical characteristics were compared between patients with and without TERT promoter mutations in their tumors, and no significant difference was found regarding age, sex, HBV infection, liver cirrhosis, α-fetoprotein levels, tumor size, differentiation status or presence of metastasis.

Analyzing the relationship between the rs2736098/rs2736100 genotype and TERT promoter mutations, we observed that HCC patients bearing a mutant TERT promoter had remarkably lower frequencies of rs2736098_TT and rs2736100_CC genotypes compared with those of healthy controls (mutant cases vs controls: 3.6% vs 12.9% and 5.8% vs 25.3% for rs2736098_TT and rs2736100_CC, respectively) (Table 11).

Compared to rs2736098_TT cases, HCC patients with rs2736098_CT genotype exhibited increased frequency of TERT promoter-mutation (OR = 0.181, 95% CI: 0.054 - 0.601, *P* = 0.004) (Table 11).

Table 11. TERT promoter mutations and association with rs2736100 and rs2736098 in HCC patients

Genotype	Cases	Healthy controls	Odds ratio (95% CI*)	*P* value
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
TT	13 (10.1)	31 (12.9)	1.0 (Ref.)	
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
TT	2 (3.6)	31 (12.9)	1.0 (Ref.)	
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**

*CI: Confidence interval

Significant p values are shown in bold.

It is currently unclear why the TERT promoter mutation differs substantially from cancer to cancer, or from patient to patient with the same type of cancer. Here we observed that the germline *TERT* gene variants rs2736100_CC and rs2736098_TT were negatively associated with the mutation frequency, which might provide one potential explanation. As documented in PAPER I, the rs2736100_CC stimulates TERT expression and up-regulates telomerase activity more strongly than does the AA or AC genotype. Likely, patients with the AA/AC variants may face strong pressure of

telomere shortening in tumor cells, and are thus prone to undergo TERT promoter mutations, because very short telomeres have been identified to be a trigger for TERT promoter mutations [78]. Consistently, the rs2736100_AC frequency was significantly lower in UTUC patients with a wt TERT promoter (PAPER II).

4.2.2 TERT promoter mutations in UTUCs and BCs (PAPER IV)

Tumor DNA from 220 patients with UTUC was analyzed for the TERT promoter status. A total of 29% (65 cases) were identified to harbor TERT promoter mutations in their tumors. The clinical characteristics in relation to TERT promoter mutations are shown in table 12 and table 13. In addition, 41/70 (59%) BC patients were positive for TERT promoter mutations.

Table 12. Clinical and disease characteristics in relation to TERT promoter mutations in patients with RPC

	TERT promoter mutated	TERT promoter wild-type	p value
informative cases (n=)	42	56	
Age at diagnosis (n=98)			
Mean ±SD	61.90 ±10.031	63.96 ±11.207	0.349 (n.s.)
Median (range)	64 (36-82)	65.5 (40-85)	
Sex (n=98)			0.646 (n.s.)
Female	14	30	
Male	28	26	
TNM stage (n=98)			0.216 (n.s.)
pTa+pT1	6	14	
≥pT2	36	42	
Pathology stage (n=98)			0.239 (n.s.)
G2	13	11	
G3	29	45	
Tumor size (n=93)			0.825 (n.s.)
<3cm	13	16	
≥3cm	27	37	
Distant metastases (n=98)			**0.013**
Yes	5	0	
No	37	56	
Lymph node infiltration (n=98)			0.069 (n.s.)
Yes	0	5	
No	42	51	

n.s.: not statistically significant

Significant p value is indicated in bold.

Table 13. Clinical and disease characteristics in relation to TERT promoter mutations in patients with UC

	TERT promoter mutated	TERT promoter wild-type	p value
informative cases (n=)	23	99	
Age at diagnosis (n=122)			
Mean±SD	72.39±8.994	65.05±9.612	**0.001**
Median (range)	75 (55-87)	67 (32-87)	
Sex (n=122)			0.632 (n.s.)
Female	7	38	
Male	16	61	
TNM stage (n=122)			0.589 (n.s.)
pTa+pT1	4	25	
≥pT2	19	74	
Pathology stage (n=122)			1.000 (n.s.)
G2	6	24	
G3	17	75	
Tumor size (n=101			0.623 (n.s.)
<3cm	10	46	
≥3cm	10	35	
Distant metastases (n=122)			**0.046**
Yes	3	2	
No	20	97	
Lymph node infiltration (n=122)			0.686 (n.s.)
Yes	1	9	
No	22	90	

n.s.: not statistically significant

Significant p values are indicated in bold.

We found that frequency of mutations increased with age in patients with UCs but not in RPCs (P = 0.001 and 0.349, respectively) (Table 12 and Table 13). The distribution of sex, tumor size, clinical stage and local lymph node infiltration did not differ significantly between RPC or UC patients with and without TERT promoter mutations. However, distant metastasis was closely associated with the presence of TERT promoter mutations (P = 0.013, Fisher exact test) (Table 12).

4.2.3 Detection of TERT promoter mutations in urine from UTUC patients by Sanger sequencing (PAPER IV)

Using Sanger sequencing, 4 of 8 mutations-carriers could be detected in urine from RPC patients. For UC patients, 2 of 20 patients harbored TERT promoter mutations in their tumors and the same mutations were detected in urine from these two patients, too (Table 14). The Sanger sequencing results revealed the presence of a wt TERT promoter in the urine samples from the remaining 18 UC patients with a wt TERT promoter. Moreover, we also collected urine samples from 4 RPC and 9 UC patients one week

after surgery and all three patients with mutation-positive urine samples prior to operation became negative following surgical resection of tumors (Table 14).

Taken together, Sanger sequencing showed a detection sensitivity of 60% with 96% specificity for urinary examination in UTUC patients. The mutant TERT promoter disappeared fast after surgical removal of tumors. Clearly, the assay sensitivity using Sanger sequencing is presently insufficient for clinical application, and a more sensitive, reliable approach has to be established.

4.3 TERT promoter mutations as urinary biomarkers for UTUC diagnosis and disease monitoring (PAPER IV)

Since TERT promoter mutations are absent in normal cells, the detection of the mutant TERT promoter as a diagnostic marker seems promising. For this purpose, we first performed analyses on the mixture of proportional mutant and wild type DNA using Sanger sequencing. We found that the detection threshold limit of Sanger sequencing was about 10% of mutant alleles present in mixed DNA samples. To improve the sensitivity of the assay, we developed a competitive Allele-specific TaqMan PCR (castPCR). By using this method, the threshold limit for measurement decreased from 10% to 2.5% of mutant alleles present in mixed DNA samples (Figure 4A, 4C and 4D), which indicates at least a four-fold higher detection sensitivity by castPCR in comparison to Sanger sequencing (Figure. 4A and 4D). Consistently, the detection of the mutant TERT promoter in urine from UTUC and BC patients using castPCR showed significantly improved sensitivity (89%) without compromising specificity (96%, comparable to that by Sanger sequencing), which should have great potential for clinical implications.

Table 14. TERT promoter mutations detected on both tumor and urine samples from RPC and UC patients

case number	Sex M/F	Age at diagnosis (year)	Tumor size (cm)	TNM stage	TERT promoter mutation				
					Tissue	Preoperative urine		Postoperative urine	
					Sanger	Sanger	castPCR	Sanger	castPCR
RPC-1	F	68	3.5	T1N0M0	wt	wt	wt	wt	wt
RPC-2	F	66	3	T1N0M0	wt	wt	wt	NA	NA
RPC-3	F	61	10	T1N0M0	wt	wt	wt	NA	NA
RPC-4	M	71	8	T3N0M0	C228T	C228T	C228T	NA	NA
RPC-5	M	43	5.5	T3N0M0	C228T	wt	C228T	NA	NA
RPC-6	M	65	1.8	T1N0M0	wt	wt	wt	wt	wt
RPC-7	F	82	6	T2N0M0	C228T	C228T	C228T	wt	wt
RPC-8	M	79	1	T1N0M0	wt	wt	C228T	NA	NA
RPC-9	M	71	3.5	T3N0M0	C228T	wt	C228T	NA	NA
RPC-10	M	63	5	T4N0M0	C228T	C228T	C228T	wt	wt
RPC-11	M	64	2.5	T3N0M0	C228T	C228T	C228T	NA	NA
RPC-12	M	64	3	T2N0M0	C228T	wt	wt	NA	NA
RPC-13	F	67	3.2	T1N0M0	C228T	wt	C228T	NA	NA
RPC-14	M	80	4.8	T3N0M0	wt	wt	wt	NA	NA
RPC-15	F	73	4.5	T2N0M0	wt	wt	wt	NA	NA
RPC-16	M	57	2	T3N0M0	wt	C228T	C228T	NA	NA
UC-1	M	67	2.5	T2N0M0	wt	wt	wt	NA	NA
UC-2	F	72	0.3	T1N0M0	wt	wt	wt	NA	NA
UC-3	F	68	1.7	T3N0M0	wt	wt	wt	NA	NA
UC-4	M	71	1.2	TaN0M0	wt	wt	wt	NA	NA
UC-5	M	67	4	T3N0M0	wt	wt	wt	NA	NA
UC-6	M	61	2	T1N0M0	wt	wt	C228T	NA	NA
UC-7	F	68	4.5	T1N0M0	wt	wt	wt	NA	NA
UC-8	M	78	4.5	TaN0M0	C228T	C228T	C228T	NA	NA
UC-9	F	67	1.6	T3N0M0	wt	wt	wt	NA	NA
UC-10	F	54	4	T3N2M1	wt	wt	wt	wt	wt
UC-11	M	65	3.5	T3N0M0	wt	wt	wt	NA	NA
UC-12	M	50	2.2	T2N0M0	wt	wt	wt	wt	wt
UC-13	F	58	1.9	T3N0M0	wt	wt	wt	wt	wt
UC-14	M	49	3	T1N0M0	wt	wt	wt	wt	wt
UC-15	M	70	4	T2N0M1	wt	wt	wt	wt	wt
UC-16	F	65	1.7	T2N0M0	wt	wt	wt	wt	wt
UC-17	F	61	1.6	T1N0M0	wt	wt	wt	wt	wt
UC-18	F	61	5	T2N0M0	wt	wt	wt	wt	wt
UC-19	M	57	1.5	T1N0M1	C228T	C228T	C228T	wt	wt
UC-20	M	69	3.7	T3N0M0	wt	wt	wt	NA	NA

RPC: Renal pelvic carcinoma; UC: Ureter carcinoma; M: Male; F: Female; castPCR: Competitive Allele-specific TaqMan PCR; NA: Not Available; wt: wild-type; Sanger: Sanger sequencing

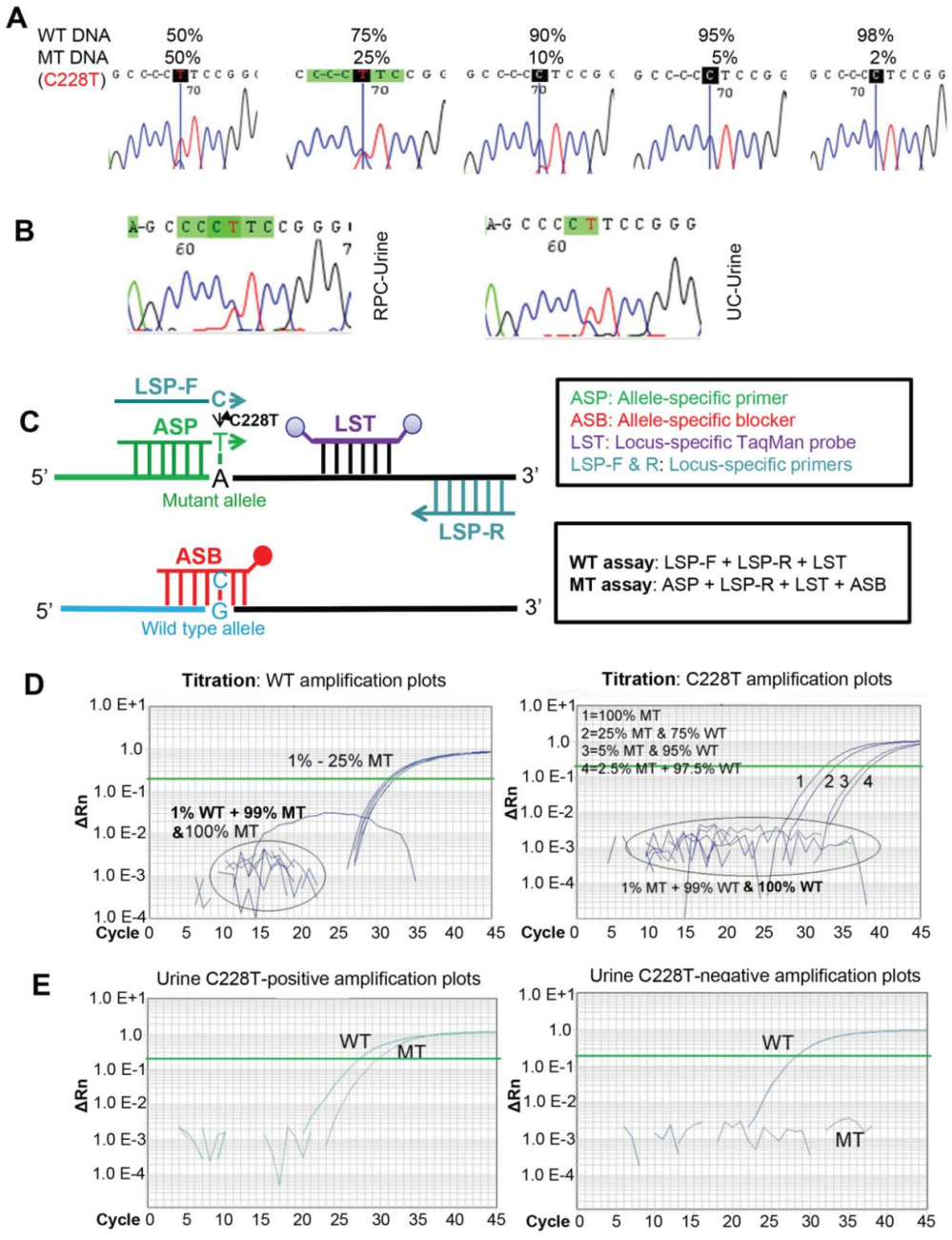
A
WT DNA
MT DNA
(C228T)
50%
50%
75%
25%
90%
10%
95%
5%
98%
2%
B
RPC-Urine
UC-Urine
C
LSP-F
ASP
C228T
LST
5'
3'
A
Mutant allele
LSP-R
ASB
C
G
Wild type allele
ASP: Allele-specific primer
ASB: Allele-specific blocker
LST: Locus-specific TaqMan probe
LSP-F & R: Locus-specific primers
WT assay: LSP-F + LSP-R + LST
MT assay: ASP + LSP-R + LST + ASB
D
Titration: WT amplification plots
1% - 25% MT
1% WT + 99% MT
&100% MT
Titration: C228T amplification plots
1=100% MT
2=25% MT & 75% WT
3=5% MT & 95% WT
4=2.5% MT + 97.5% WT
1% MT + 99% WT & 100% WT
ΔRn
Cycle
E
Urine C228T-positive amplification plots
Urine C228T-negative amplification plots
WT
MT

Figure 4. The increased sensitivity for the castPCR detection of C228T mutations in urine derived from patients with renal pelvic and ureter carcinomas (RPCs and UCs). (A) The detection sensitivity of TERT promoter mutations as determined by Sanger sequencing. DNA derived from thyroid cancer cells with (homozygous) C228T mutation and with a wt TERT promoter was mixed as indicated and the promoter region then sequenced using Sanger sequencing. The detectable load of mutant DNA by Sanger sequencing was minimally 10%. (B) Sequencing chromatographs of the TERT promoter locus in urineDNA from onerenal pelvic carcinoma (RPC) and one ureter carcinoma (UC) patient, as determined by Sanger sequencing. C228T mutation was shown. (C) Schematic illustration of the castPCR detection of C228T mutation. The C228T and wt (reference) allele assays are performed with the allele-specific primer (ASP), locus-specific primer, allele-specific blocker (ASB) and locus-specific Taqman probe (LST). In mutant assays, ASB prevents wt TERT promoter sequences from PCR amplification. (D) Representative amplification plots for the assay of different proportions of C228T mutant allele as determined by castPCR. Mixed DNA as above in (A) was analyzed for the presence of the C228T mutation using castPCR. Left panel: The amplification plots for the wt (reference) TERT promoter. 99%, 97.5%, 95% and 75% wt DNA-containing mixtures were amplified using **LSP** primers and wild type plots were generated. Of note, pure (100%) and 99% mutant DNA only yielded background signals. Right panel: The amplification plots for the mutant TERT promoter. The same DNA mixtures as described above were amplified using **ASP** primers and mutant amplification plots were generated. The CT value was inversely correlated with % of the mutant alleles. Of note, pure (100%) and 99% wt DNA-containing mixtures only gave rise to background signals. (E) C228T mutation-positive (Left) and Negative (Right) urine as revealed by castPCR. Shown are representative castPCR results obtained from two RPC patient urine samples.

5 SUMMARY AND CONCLUSIONS

1. The frequency of the *TERT* gene rs2736100_C allele is associated with an increased risk for MPNs in both Swedish and Chinese males. The rs2736100_C allele frequency is much higher in the Swedish than in the Chinese population, which are coupled with a higher and lower incidences of MPNs in Sweden and China, respectively. Such difference in *TERT* rs2736100_C allele between Swedish and Chinese populations may thus partially explain their differential MPN incidences (PAPER I).

2. The *TERT* rs2736100 AC genotype is associated with reduced risk of UTUCs in a Chinese Hans population (PAPER II).

3. The cancer risk genotype rs2736100_CC was significantly associated with a lower incidence of TERT promoter mutations, while the rs2736098_CT genotype was significantly associated with the TERT promoter mutation-positive tumors in patients with HCC. This indicates that the germline TERT genetic background may substantially affect the frequency of TERT promoter mutations (PAPER III).

4. TERT promoter mutations were detectable in urine from BC and UTUC patients, and this proof-of-concept study demonstrates that the mutant TERT promoter may serve as a useful urinary marker for non-invasive diagnosis and relapse monitoring of patients with BC and UTUC. We further developed the castPCR assay showing high sensitivity and specificity, which should have great potential for future clinical application (PAPER IV).

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