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PROMOTER MUTATIONS/METHYLATION IN THYROID AND UROTHELIAL CARCINOMAS: BIOLOGICAL AND CLINICAL IMPLICATIONS

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PROMOTER MUTATIONS/METHYLATION IN THYROID AND UROTHELIAL CARCINOMAS: BIOLOGICAL AND CLINICAL IMPLICATIONS.

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By

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To my family

POPULAR SCIENCE SUMMARY OF THE THESIS

Most of us are now living in a world where we become pessimistic whenever we talk about "Cancer". Indeed, cancer is one major reason for mortality and the occurrence of cancer diseases is increasing. But how does cancer begin? And what can we do to get rid of it? Therefore, the aim of cancer research is to explore why and how cancer is formed, getting worse and finally leading to death, as well as the development of new methods to prevent, detect, and eventually cure it.

The gene is the smallest leader to guide how our bodies work daily. Occasionally, the gene function may be prone to errors caused by tiny alterations of genetic information (genetic change) that could escalate into huge consequences. Besides, gene function also can be changed due to alterations of its external environment. In this case, although genetic information is not changed, the efficiency and accuracy of the gene function may change completely (epigenetic change). Throughout the years, our understanding of cancer is similarly evolving like the development of the modern society. It could be pretty much well-structured but yet highly dynamic due to the involvement of both genetic and epigenetic changes. In this thesis, we studied the function of several important genes and their genetic and/or epigenetic changes focusing on thyroid cancer (TC) and upper tract urothelial cancer (UTUC).

Every single cell of our body contains a countdown clock (telomere attrition) for limited lifespan. The *TERT* gene is capable of reversing this clock (maintenance/elongation of telomere length) but repressed in most human somatic cells so that cells may eventually get older and experience death. However, when *TERT* is reactivated abnormally, cells may become immortal, grow out of control, and finally become life threatening cancer. In fact, *TERT* reactivation is a prerequisite step for malignant transformation, which occurs in up to 90% of human cancers. One of the well-known factors to affect *TERT* expression is through the genetic mutation in its promoter region. Indeed, *TERT* promoter mutations have been found in various cancers including TC and UTUC, and commonly occur in patients with worse outcome. Therefore, it has been suggested as a sign (biomarker) for fatal tumor. However, it is still unclear how the mutant *TERT* promoter and its activators (e.g. GABPB1) work together in the process of tumor development and progression.

While the frequency of *TERT* promoter mutations in TC and UTUC is relatively low, we still need to find more useful biomarkers to facilitate clinical management. When searching for new biomarkers, the *PLEKHS1* gene came to our attention as its promoter mutations have been identified in TC and other types of cancers. In addition, we were also interested in the *GPR126* gene for the similar reason.

In general, the thesis explored how genetic and/or epigenetic changes of *TERT*, *GABPB1*, *PLEKHS1* and *GPR126* genes occur in TC and UTUC, and how these factors work together or separately to participate in the process of TC and UTUC. Our findings are important in understanding the underlying molecular secrets as well as providing new useful biomarkers in TC and UTUC.

ABSTRACT

Genetic and epigenetic aberrations are well established to drive cancer development and progression. However, the underlying mechanisms are incompletely understood, and the translational applications of aberrant alterations are limited in gene non-coding regions, such as the promoter locus. The present thesis aims to address the relevant molecular basis to contribute further insights of how genetic/epigenetic events involved in oncogenesis of thyroid cancer (TC) and upper tract urothelial carcinoma (UTUC).

In **Paper I,** we characterized Pleckstrin homology domain-containing family S member 1 (*PLEKHS1*) promoter mutation/methylation, gene expression and its role in TCs including papillary and anaplastic thyroid carcinomas (PTCs and ATCs). The *PLEKHS1* promoter mutation was rare in TC, but *PLEKHS1* was significantly over-expressed in TCs compared to adjacent thyroid tissues. ATC tumors, the most aggressive TC subtype, expressed the highest level of PLEKHS1. Mechanistically, the demethylation of the *PLEKHS1* promoter contributes to its over-expression in TC tumors. We further revealed that higher PLEKHS1 expression led to increased AKT phosphorylation and invasive phenotype of TC cells. Consistently, *PLEKHS1* over-expression was associated with TC metastasis, and predicted shorter patient survival. Taken together, it is the hypomethylation of *PLEKHS1* promoter rather than its mutation that induces *PLEKHS1* over-expression, thereby enhancing AKT activity and aggressive behavior of TC cells. Clinically, *PLEKHS1* may be a useful prognostic factor in PTCs.

In **Paper II,** the role for *GABPB1* in TC pathogenesis was investigated. It has been characterized that GABPB1 and its partner GABPA stimulates the mutated telomerase reverse transcriptase (*TERT*) promoter for telomerase activation in TCs or other cancers. Targeting GABPB1 is thus suggested as a strategy for telomerase-based cancer therapy. However, GABPB1 depletion promoted TC cell invasion, although *TERT* expression was reduced in those cells. We further observed that *GABPB1* expression was significantly lower in aggressive TCs, potentially attributed to its promoter hypermethylation. Collectively, *GABPB1* may function as a tumor suppressor to impede TC aggressiveness, whereas the *GABPB1* silencing via the aberrant DNA methylation is required to facilitate TC progression. Therefore, targeting GABPB1 for TC therapy might promote disease dissemination rather than cure patients.

In **Paper III**, we analyzed *TERT* and *PLEKHS1* promoter mutations in urothelial carcinomas [UTUC and urothelial bladder carcinoma (UBC)] and evaluate whether they could serve as urinary biomarkers for disease diagnosis and monitoring. In addition, the mutation of the G protein-coupled receptor 126 (*GPR126*) intron 6 was recently observed in UBC, and we included this marker in the study. By using Sanger sequencing, we identified that the frequencies of *TERT*, *PLEKHS1* and *GPR126* gene mutations occurred in UTUCs but with lower frequencies compared to UBCs. The mutant DNA sequences were readily detected in patients' urine and disappeared in most patients after surgical treatment. Thus, the mutations in the *TERT*, *PLEKHS1* and *GPR126* regulatory regions occur in UTUCs, and they may serve as urinary biomarkers for UTUC diagnostics and surveillance.

In summary, the present thesis demonstrated how mutations or aberrant DNA methylation of *TERT*, *GABPB1*, *PLEKHS1* and *GPR126* genes occur in TC and UTUC, and how these events interact together or function independently to participate in TC and UTUC pathogenesis. Our findings are of potential importance in improving the management of these patients.

LIST OF SCIENTIFIC PAPERS

I. **Xiangling Xing**, Ninni Mu, Xiaotian Yuan, Na Wang, C. Christofer Juhlin, Klas Stråât, Catharina Larsson, Dawei Xu.
PLEKHS1 over-expression is associated with metastases and poor outcomes in Papillary Thyroid Carcinoma.
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II. Xiaotian Yuan, Ninni Mu, **Xiangling Xing**, Na Wang, C. Christofer Juhlin, Klas Stråât, Catharina Larsson, Dawei Xu.
Downregulation and hypermethylation of GABPB1 is associated with aggressive thyroid cancer features.
Manuscript

III. **Xiangling Xing**, Xiaotian Yuan, Tiantian Liu, Mingkai Dai, Yidong Fan, Cheng Liu, Klas Stråât, Magnus Björkholm, Dawei Xu.
Regulatory region mutations of TERT, PLEKHS1 and GPR126 genes as urinary biomarkers in upper tract urothelial carcinomas.
Journal of Cancer. 2021; 12(13): 3853-3861.

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LIST OF ABBREVIATIONS

5-AZA	5-azacitidine
AA	Aristolochic acid
ACC	TCGA-Adrenocortical Carcinoma
ADGRG6	Adhesion G protein-coupled receptor G6
AJCC	American Joint Commission on Cancer
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ATC	Anaplastic thyroid carcinoma
CML	Chronic myeloid leukemia
CpG	Cytosine-phosphate-Guanine
CTCL	Cutaneous T-cell lymphoma
DAC	5-aza-2’ deoxycytidine
DFS	Disease-free survival
DNMTs	DNA methyltransferases
DTC	Differentiated thyroid cancer
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ETS	E-twenty-six
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FPKM	Fragments Per Kilobase Million
FTC	Follicular thyroid cancer
FV-PTC	Follicular variant PTC
GABPB1L	Long isoform of GABPB1
GABPB1S	Short isoform of GABPB1
GPR126	G protein-coupled receptor 126
HDAC	Histone deacetylase
lncRNA	Long non-coding RNA
LUAD	TCGA-Lung Adenocarcinoma
LZD	C-terminal leucine-zipper domain
MAP17	Membrane-associated protein 17
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndromes
MIBC	Muscle-invasive bladder cancer
miRNA	MicroRNA
MTC	Medullary thyroid cancer
mTOR	Mammalian target of rapamycin
NIS	Na+/I− symporter
NMIBC	Non-muscle invasive bladder cancer
OS	Overall survival
PAX8	Paired box 8
PCR	Polymerase chain reaction
PDTC	Poorly differentiated thyroid cancer

PH	Pleckstrin homology
PI3K	Phosphatidylinositide 3-kinases
PI3KR3	Phosphoinositide-3-Kinase Regulatory Subunit 3
PLEKHS1	Pleckstrin homology domain containing S1
PPARγ	Peroxisome proliferator- activated receptor γ
PSA	Prostate-specific antigen
PTC	Papillary thyroid carcinoma
qPCR	Quantitative real-time PCR
RAI	Radioactive iodine
RB	Retinoblastoma
RSEM	RNA-Seq by Expectation Maximization
SNP	Single-nucleotide polymorphism
TC	Thyroid cancer
TCGA	The Cancer Genome Atlas
TDG	Thymine DNA glycosylase
TERC	Telomerase RNA template
TERT	Telomerase reverse transcriptase
TET	Ten-eleven translocation family enzymes
TFs	Transcription factors
THCA	TCGA-Thyroid cancer
THOR	TERT hypermethylated oncological region
TKIs	Tyrosine kinase inhibitors
TSS	Transcription start site
UBC	Urothelial bladder carcinoma
UC	Urothelial carcinoma
UTUC	Upper tract urothelial cancer
WGS	Whole genome sequencing
wt	wild type

1 INTRODUCTION

1.1 CANCER AND ITS MOLECULAR BACKGROUND

Cancer is a disease which originates from some of the body's cells that gain the ability of unlimited proliferation, potentially invade to nearby tissues and/or spread to other parts of the body (1). In healthy multicellular individuals, usually, cells divide to form new cells for cell growth, repair of tissues and formation of germ cells. When cells get old or damaged, they will undergo cell death and be replaced by new ones. The process of cell division and death is strictly and precisely controlled by multiple complicated molecular mechanisms. However, this tightly regulated process may break down occasionally. Cells may proliferate uncontrollably and when these dysregulated cells attempt to break through barriers for survival, they eventually become malignant and a life threatening cancer (2, 3). Cancer research has revealed that dynamic alterations in the genome are involved in the process of cancer development and progression (2, 4-6). It may start with genetic/epigenetic alterations to genes that play essential roles in controlling cell function like cell growth and division. These alterations may be attributed to errors occurred during cell division process or DNA damage caused by detrimental stimulus from the environment such as radiation and toxic chemicals. Normally, abnormal cells will be eliminated or repaired by the body before they become cancerous. However, when the body's ability is insufficient or attenuated to eradicate the abnormal cells, cells with certain genetic/epigenetic changes can evade cell growth control and transfer the genetic errors to their daughter cells. Cells turn more cancerous with accumulation of these kind of genetic/epigenetic alterations and these changes may be inherited by offspring if present in germ cells (3, 6-8).

1.1.1 Types of genes involved in cancer

Generally, proto-oncogenes and tumor suppressor genes are the two main types of genes involved in cancer biology. When genetic/epigenetic changes affect these genes, they may drive the formation of cancer (9).

1.1.1.1 Proto-oncogenes

Proto-oncogenes play crucial roles in normal cell growth and division, inhibiting cell differentiation and cell death. Proto-oncogenes turn into oncogenes upon aberrant alterations, giving rise to cellular immortalization and leading to development of cancer (10). The activation of oncogenes is widely studied to understand how genetic changes of cellular proto-oncogenes would confer survival and growth advantages to the cell. For example, *Ras* gene become oncogenic when suffering from point mutations, resulting in continuously activated protein with enhanced cell proliferation and induced apoptosis, which has been identified in pancreas, lung, colon, thyroid, and other cancers (11, 12).

1.1.1.2 Tumor suppressor genes

Tumor suppressor genes counteract oncogenes by slowing down cell division to keep cells from replicating too quickly. In response to cellular stress, tumor suppressor genes can be activated to trigger cell cycle arrest or even programmed cell death (13). Cancer is commonly

associated with the inactivation of these tumor suppressor genes, for example, the inactivation of *TP53* gene by mutation or deletion has been found in the majority of human cancers, which is normally crucial in cell cycle control and apoptosis induction (14, 15). In addition, some of tumor suppressor genes also have DNA repair functions involved in fixing other damaged genes. Supposedly if DNA damage occur directly in these DNA repair genes, errors in other genes will accumulate over time and lead to formation of cancer (13). For instance, *BRCA1* and *BRCA2* are responsible for repair of DNA double-strand breaks but the occurrence of mutations will increase the risk of breast/ovarian cancer (16).

1.1.2 Genetic alterations in cancer

Genetic alterations result in changes in the DNA sequence, called 'genetic mutations', with 'point mutation' being the smallest change, referring to one-unit nucleotide - the building blocks of DNA – substituted or inserted by another one or deleted entirely. Larger scale alterations include various chromosome rearrangements, in which a long stretch of DNA could be entirely deleted, translocated, inversed or repeated, leading to multiple genes altered at once. These mutations may occur alone or together and generally, cancer cells bear more mutations than normal cells. Of note, each individual's cancer accumulates its unique combinations of genetic changes which explains for the need of personalizing therapeutic strategies to treat the disease (17). Moreover, there could be variability in the magnitude of DNA mutation experienced by cells within a polyclonal tumor, contributing to intratumoral heterogeneity that could be implicated with drug resistance during treatment (18, 19).

1.1.2.1 Small-scale mutations

Small-scale mutations are observed at nucleotide levels with one or a few bases being affected within a gene. The most known mutations occurring in coding regions include three classes: 1) non-synonymous substitution: one single base is substituted by another base leading to change in the amino acid sequence (missense mutation), or giving a premature shorter protein by creating a "stop" codon (nonsense mutation), or producing a longer protein by erasing a stop codon (nonstop mutations) (20). 2) synonymous substitution: base substitution results in no change of amino acid sequence (silent mutation, though they are not always silent) (21). 3) Frameshift mutation: one or more bases are wrongly inserted (insertion mutation) or deleted (deletion mutation) leading to shift of DNA sequence and change of corresponding amino acid sequence (17). Decades of exome-based analyses have revealed innumerable protein-coding somatic mutations, among which the most frequent mutated genes include *TP53*, *PIK3CA*, *APC*, *KRAS*, *PTEN* (22).

Besides, advanced whole genome sequencing (WGS) methods have revealed that mutations are also abundant in the non-coding regions (Figure 1), which are far more larger than the coding exome (23). Non-coding regions, comprising more than 98% of the human genome, are crucial for the regulation of gene transcription and translation via various mechanisms. Particularly, the cis-regulatory elements (e.g. promoters, enhancers, and silencers) are shown to play key roles in regulating the transcription of their neighboring genes (24). In 2013, two seminal studies (25, 26) demonstrated non-coding somatic promoter mutations - *TERT* promoter mutations - can mechanically activate oncogene after years of efforts focus on

protein-coding changes (23, 27). This key finding together with an increasing consensus about non-coding driver mutations have highly improved our understandings of the cancer genomes.

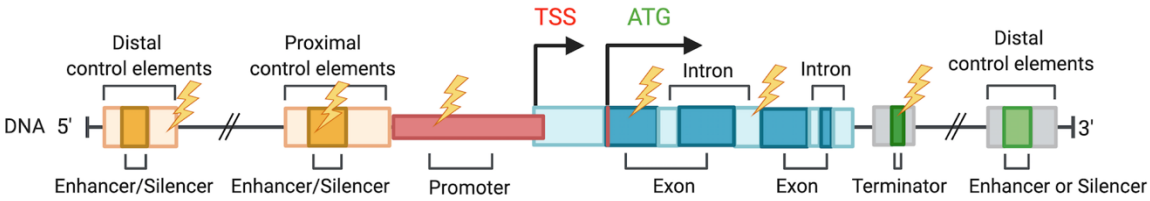

Figure 1. Illustration of gene structure. Gene mutations can occur in any positions of the gene locus. Exons are the sections coding for amino acids of a protein, which can be separated by non-coding introns. TSS: Transcriptional start site. Figure is created with BioRender.com.

1.1.2.2 Large-scale mutations

Large-scale mutations are referred to as chromosome abnormality with alterations of chromosomal structure or number. Structure abnormalities also known as chromosome rearrangements can take place when a portion of chromosome breaks and is deleted (deletion) , is repeated (duplication), flips directions (inversion), or moves to another chromosome (translocation), etc (28). Numerical abnormality called aneuploidy occurs when an individual is missing a chromosome copy from a pair or acquiring more than two copies of a pair (17).

Large-scale mutations are more lethal genetic aberrations compared to small-scale mutations. For example, the Philadelphia chromosome is formed by reciprocal translocation between chromosome 9 and chromosome 22, containing a fusion gene called *BCR-ABL1*, which is a specific genetic abnormality in leukemia cancer cells (especially in chronic myeloid leukemia (CML) cells). The *BCR-ABL1* fusion gene are involved in multiple signaling pathways to achieve limitless proliferation characteristic of CML and a subset of acute leukemias (29).

1.1.3 Epigenetic alterations in cancer

The biology of cancer, however, cannot be fully accounted by genomic instability caused by accumulation of mutations. "Epigenetics" provides one reasonable explanation, which was first introduced by C.H. Waddington in 1939, referring to heritable but reversible changes in phenotype without affecting the DNA sequence (30). Several modes of epigenetic modifications have been known to influence the genome, among which DNA methylation and histone modifications are the two major well-studied epigenetic mechanisms.

1.1.3.1 DNA methylation

DNA methylation is the best-known epigenetic marker and well-established as a critical regulator of gene expression and the architecture of cell nucleus. DNA methylation mostly occurs at 5th carbon of cytosine in Cytosine-phosphate-Guanine (CpG) dinucleotides. These CpG sites are enriched in particular regions called as CpG islands, mostly distributed at 5' end of the regulatory region of genes. The CpG islands are commonly unmethylated in normal cells

while CpG islands in promoter locus can be methylated in normal tissues (30). The main methylation process is shown in Figure 2.

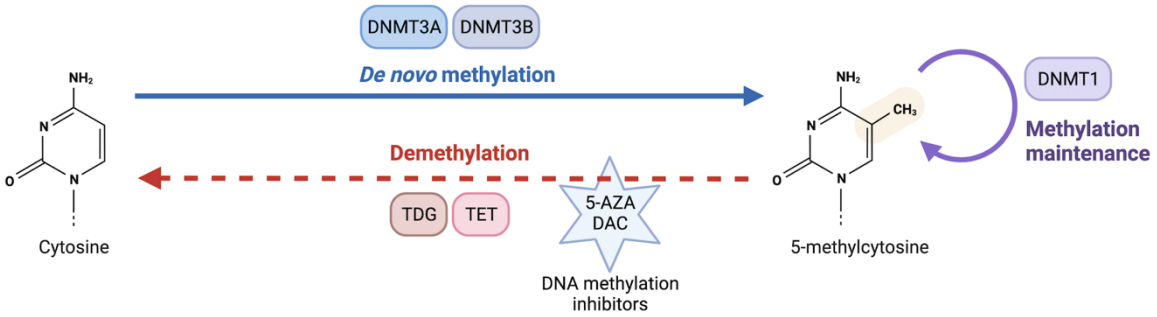

Figure 2. Illustration of DNA methylation. DNA methylation is catalyzed by DNMT family. Canonical DNMT enzymes include DNMT1, DNMT3A, DNMT3B. DNMT3A and DAMT3B are responsible for *de novo* methylation by adding the methyl group to the 5th carbon of cytosine while DNMT1 tends to catalyze hemi-methylation on the single strand of DNA and maintains the methylation state (31). Demethylation is catalyzed by TET and TDG through iterative oxidation and repair (32). Besides, DNA methylation inhibitors (e.g. 5-AZA, DAC) can reduce global methylation level by trapping DNMTs (33). DNMT: DNA methyltransferase; TET: Ten-eleven translocation family enzymes; TDG: Thymine DNA glycosylase; 5-AZA: 5-azacitidine; DAC: 5-aza-2' deoxycytidine. Figure is created with BioRender.com.

DNA methylation accounts for the control of certain tissue-specific genes, such as the germ-lines *MAGE* genes, which are silent in almost all the tissues but expressed in malignant tumors (34). DNA methylation is also involved in genomic imprinting (35), during which one of the two parental alleles of a gene is silenced to ensure monoallelic expression by hypermethylation; similarly, DNA methylation is also a key player in the process of X-chromosome inactivation in females (36). The first discovery indicated that epigenetics was involved in human primary tumors came in 1983 by Feinberg and Vogelstein, showing alteration of methylation to genes in colorectal tumors (37). Both DNA hypomethylation and hypermethylation can contribute to tumorigenesis and irregular methylation have been observed in countless types of neoplasia (38). DNA hypomethylation can activate oncogenes, generate chromosomal instability, reactivate transposable elements and disrupt genomic imprinting; while hypermethylation of the promoter CpG island can inactivate tumor-suppressor genes thereby opening the way to tumor development (30, 39).

Interfering with sustainability of DNA methylation in cancers could reactivate silenced gene and reverse part of its deregulation to accomplish the therapeutically desirable effects, for example apoptosis and improved recognition by immune system. DNA methylation inhibitors have been introduced in the clinic; the most promising application is the use of 5-azacitidine (5-AZA) and 5-aza-2' deoxycytidine (DAC) in the treatment of myelodysplastic syndromes (MDS) (40). AZA and DAC are analogs of cytidine, which can be incorporated into DNA by trapping DNA methyltransferases (DNMTs) and result in hypomethylation in the daughter cells because of the lack of theses enzymes (33, 41). It is noteworthy that there is a therapeutic index in terms of targeting DNA methylation in cancer. Normal cells have a resistance ability to hypomethylation and tend to survive while cancer cells are sensitive to hypomethylation and when this happens, tend to undergo cell death or at least growth arrest. However, the therapeutic dose should be well moderated since AZA and DAC do induce dose-dependent toxicity (33).

1.1.3.2 Histone modifications

Histone modification is another crucial epigenetic mechanism and sometimes mediates a biological process in combination with DNA methylation (42). DNA within cells is packed and twisted by histones, the basic and major proteins in eukaryotic cell nuclei, to form nucleosomes which is the fundamental building block of chromatin. Generally, there are five major histones families, including core histones H2A, H2B, H3 and H4, and linker histones H1/H5. Each nucleosome core consists of two H2A-H2B dimers and a H3-H4 tetramer (39). It is well known that histones are post-translationally modified and these modifications including ubiquitination, phosphorylation, methylation, acetylation and lactylation, occurring in different histone proteins at N-terminal histone tails. These modifications contribute to alterations of the chromatin environment, thus affecting the regulation of gene transcription (43-45). Histone methylation and acetylation have direct effects on various nuclear processes including DNA replication, DNA repair and gene transcription. Generally, histone acetylation is related with transcriptional activation while histone methylation has different functions dependent on the type of amino acid as well as its status (e.g., monomethylation, demethylation, and trimethylation) or its position on the histone tail (46).

Given that histone modifications are reversible, pharmacological intervention could have therapeutic value in the treatment of cancer (47). The most promising application targeting histone modifications is histone deacetylase (HDAC) inhibitors for the treatment of cutaneous T-cell lymphoma (CTCL), which have been approved by Food and Drug Administration (FDA) (40). HDAC inhibitors can induce cell death, apoptosis and cell growth arrest in tumor cells while in contrast, normal cells tend to survive HDAC inhibitors induced cell death (48).

1.1.3.3 Other epigenetic mechanisms

With a boost in epigenetic research in recent years, more epigenetic mechanisms have been elucidated, including RNA transcripts (49), RNA modifications (50), noncoding RNAs (e.g., microRNA (miRNA) (51), long non-coding RNA (lncRNA) (52)), histone variants (53), nucleosome positioning (54), genomic architecture (55) etc. These regulatory mechanisms may interact with each other, generating convoluted coordination in vivo.

1.1.4 Biomarkers in cancer

The remarkable progress of novel technologies, especially those involved in omics research (genomics, transcriptomics, epigenomics, proteomics, and etc.), has contributed to unravel the complicated and precise mechanisms in cancer biology. Cancer being a diverse disease contains various alterations, offering the opportunity for the identification and discovery of potential biomarkers (56, 57). Determining such changes in body tissues or fluids, like blood, saliva or urine, can contribute to early detection, diagnosis, treatment and monitoring of diseases, for example in the early stage of cancer when the tumors may not be detected by conventional methods. By doing so, therapies might be prescribed far earlier, more precisely and more efficiently.

Biomarkers used in precision medicine are categorized into three ways according to their clinical role in guiding treatment decisions, either being predictive, diagnostic or prognostic (58). A predictive biomarker predicts clinical outcomes, reflecting the effect of the therapeutic

intervention. It can be a target for therapy and can be used to optimize treatments. Well known example are estrogen receptor (ER) and HER2/NEU (also known as ERBB2) in breast cancer (59). A diagnostic biomarker can help to narrow down diagnosis along with more specific diagnosis to individual patients. One commonly used diagnostic biomarker is prostate-specific antigen (PSA) in prostate cancer (60). A prognostic biomarker predicts disease outcome (e.g., recurrence, progression, death) independently of therapy (59). For example, *TERT* promoter mutations is a well proved prognostic biomarker in various cancers, including thyroid and urothelial carcinomas (61-63) (See the section below). Certainly, some biomarkers may have the predictive, diagnostic and prognostic value at the same time.

1.2 TELOMERASE, *TERT*, AND *TERT* PROMOTER MUTATIONS

1.2.1 Telomerase and *TERT*

Unlimited proliferation is a hallmark of cancer development, which is mainly attributable to telomerase (*TERT*) reactivation that is present in the majority (up to 90%) of human malignancies (3, 64). Telomerase is an RNA-dependent DNA ribonucleoprotein enzyme that catalyzes telomere elongation by extending the telomeric DNA (TTAGGG repeats) to telomeres at the end of chromosomes, which consists of two main subunits, telomerase reverse transcriptase (*TERT*) and telomerase RNA (*TERC*) (Figure 3). While *TERC* as a RNA template in the telomeric DNA synthesis process is ubiquitously expressed in human normal cells, *TERT* as the catalytic subunit is restricted in stem/progenitor cells, activated lymphocytes and other cells with finite proliferative potential (65, 66).

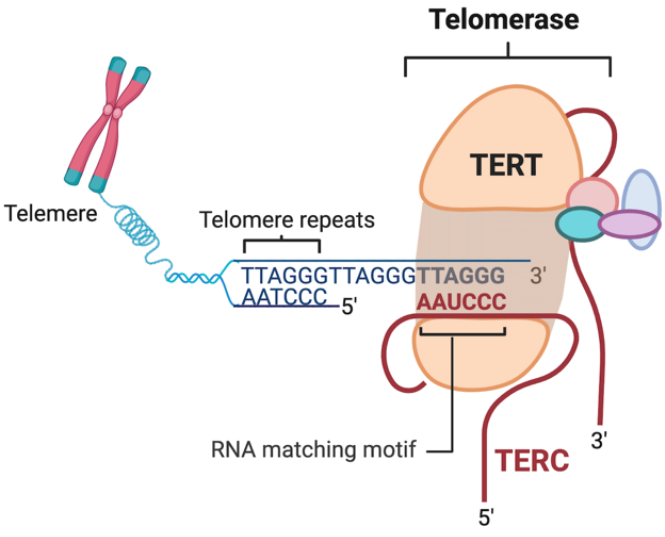

Figure 3. Illustration of telomere and telomerase. Telomerase catalyzes telomere elongation by adding telomere repeats (TTAGGG) to the terminal of chromosome. Figure is created with BioRender.com.

Telomerase is silent in most human differentiated somatic cells because of the tight repression of the *TERT* gene, and these cells will undergo progressive telomere shortening with cell replications. When the length of telomeres reached a certain limit, there is an increased likelihood of triggering the DNA damage response resulting in cell cycle arrest and cellular

senescence in human organs or tissues (67, 68). Therefore, telomere attrition works as a mitotic clock in cell replication process and confers somatic cells a limited lifespan. While during oncogenesis, overcoming senescence barrier by maintaining telomere length is required and it is mainly achieved through telomerase activation/*TERT* derepression in human malignancies (68, 69). Apart from the canonical function of *TERT* that maintains telomere length, *TERT* also takes part in many other activities in cellular processes. Ectopic expression of TERT contributes to carcinogenesis by stimulating cell proliferation, cell survival, mitochondrial function and epithelial-mesenchymal transition (EMT), independently of telomere lengthening (70).

1.2.2 *TERT* regulation

The crucial regulation of *TERT* is at the level of transcription, which is the predominant way to dictate TERT expression and control telomerase activity (71). The *TERT* promoter is GC-rich, containing many binding sites for interacting with transcription factors (TFs), including GC-boxes, E-boxes and E-twenty-six (ETS) binding motifs. Two crucial TFs for activation of *TERT* transcription are c-Myc and Sp1 (72, 73), which binds to E boxes and GC boxes, respectively. Other TFs such as ETS, HIF-1, AP-2, NF-kB can also enhance *TERT* transcription by binding to the promoter region. Negative TFs including p53, WT1, MAD, MZF-2 and Menin have been reported to repress *TERT* transcription (74, 75).

TERT promoter hypermethylation is another way to upregulate TERT expression (76). While promoter hypermethylation is well-defined to repress and silence gene expression, *TERT* promoter methylation is an exception. *TERT* hypermethylated oncological region (THOR) has been described as cancer-associated epigenetic mechanism to upregulate TERT expression and telomerase activity (76-78). THOR is a worse predictor for survival in various cancers probably because of interfering with binding of repressive TFs (75). Besides, *TERT* rearrangements lead to re-positioning of enhancer, through which active histone mark enrichment, chromatin remodeling and *TERT* transcription activation are achieved (79, 80). In addition, alternative splicing of *TERT* mRNA transcripts (81) and post-translational modification (82) also involve in TERT expression.

1.2.3 *TERT* promoter mutations

As described above, ETS TFs also take part in regulation of *TERT* transcription. Interestingly, the hotspot mutations of the *TERT* promoter generate *de novo* ETS binding motifs (Figure 4), recruiting ETS TFs to the mutated promoter, along with mediating long-range chromatin interaction and enriching active histone marks, thereby promoting *TERT* transcription and contributing to tumorigenesis (83-85). *TERT* promoter mutations represent the most common noncoding mutations in cancers, which were first discovered in malignant melanoma in 2013 (25, 26). The two major hotspot mutations, resulting from a cytidine-to-thymidine (C >T) substitution at 1 295 113 (GRCh38.p13), and 1 295 135 (GRCh38.p13), are named C228T and C250T, respectively.

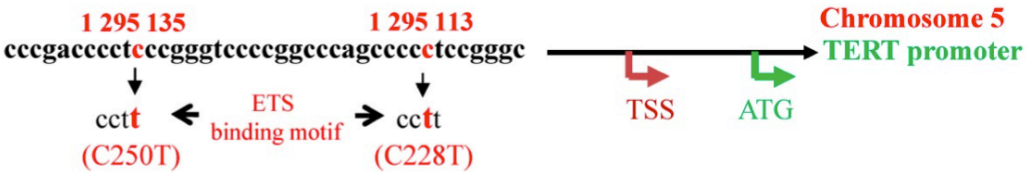

Figure 4. The structure of *TERT* promoter and the position of two hotspot mutations (C250T and C228T) in human malignancies. ETS: E-twenty-six. TSS: Transcriptional start site.

To date, *TERT* promoter mutations have been identified in a variety of human malignancies and the higher frequency is associated with higher tumor stage and worse prognosis. The mutations were frequently detected in glioblastoma, melanoma, bladder, renal, pelvic, liver, thyroid cancer (25, 63, 86), and occasionally in prostate, breast, gastrointestinal, lung cancer and leukemia (87, 88).

1.2.4 Activators of the mutant *TERT* promoter - GABPs

The ETS family is one of the most famous families of transcription factors including 28 genes in human. The discovery of this family was recognized as a gene transduced by the avian leukemia virus, E26 (89). ETS family is implicated in cell differentiation, hormone responses as well as cancer progression. Almost all ETS family members share a highly conserved DNA binding domain, which is a winged helix-turn-helix structure with a central GGA(A/T) DNA sequence (90-92). The ETS family members GABP (GABPA/GABPB) are shown to act as the master drivers for activating mutated *TERT* promoter and induce *TERT* expression (93). GABPA only harbors the DNA binding domain while GABPB bears trans-activation domain. GABPA and GABPB need to be combined together to form heterodimer or heterotetramer to activate *TERT* promoter (94) (Figure 5). GABPB is encoded by two distinct genes, either *GABPB1* or *GABPB2*, while GABPB1 was proved to be more related to the mutant *TERT* promoter (95, 96). GABPB1 has four variants including two short (GABPB1S) isoforms only responsible for dimer formation with GABPA, and two long (GABPB1L) isoforms which harbor C-terminal leucine-zipper domain (LZD) able to mediate tetramerization of two GABPA/GABPB1 heterodimers (95).

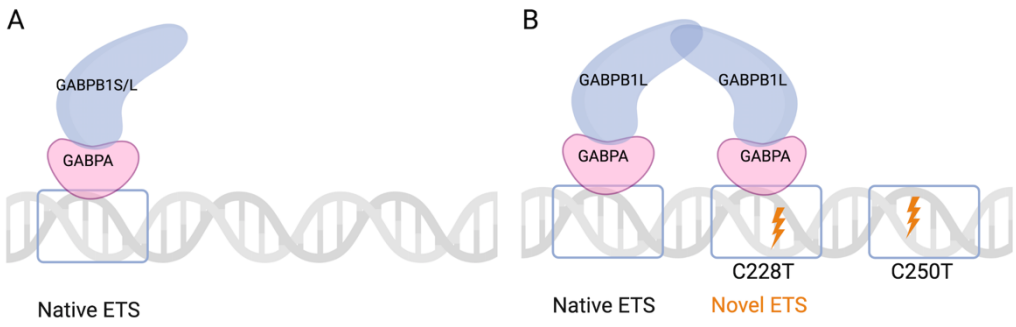

Figure 5. Examples of GABPA/GABPB1 complex in activation of the *TERT* promoter. GABPA and GABPB1 combine together to form heterodimer or heterotetramer to activate the *TERT* promoter. A: Wild type *TERT* promoter; B: Mutant *TERT* promoter. Figure is created with BioRender.com.

More importantly, disruption of GABPB1 in glioma cells led to decreased TERT expression, apoptosis and telomere dysfunction in a mutant *TERT* promoter dependent manner. GABPB1 thus was suggested as a cancer therapeutic target (95, 97). In addition, GABPB1 was

demonstrated to act as downstream target of BRAF through mediating phosphorylation and activations of FOS, by which GABPB1 may theoretically link the two PTC related (BRAF and TERT) oncogenic signal pathways together (98).

Beyond the role in *TERT* regulation, the function of GABPA/GABPB1 complex in oncogenesis remains poorly understood. GABPA has been proven to be a tumor suppressor in several cancers including TC (99-102), where GABPA inhibition was correlated with aggressive signatures by downregulating *DICER1* to enhance metastatic ability in TC (99, 100). However, the role of *GABPB1* in TC pathogenesis remains unclear.

1.3 THE *PLEKHS1* GENE AND *PLEKHS1* PROMOTER MUTATIONS

1.3.1 The *PLEKHS1* gene and PH domains

Pleckstrin homology domain-containing family S member 1 (*PLEKHS1*), also known as *HEL185* and *C10orf81*, is located on chromosome10q25.3. It was previously found that *PLEKHS1* took part in regulation of blood glucose and insulin resistance and could potentially contribute to mild hyperglycemia relevant to obesity in obese rats (103). However, its role in physiological and pathological processes is currently poorly understood (103). Recently, several studies have shown that *PLEKHS1* plays a role in cancers. *PLEKHS1* mRNA was found to be over-expressed in muscle-invasive bladder cancer (MIBC) compared to non-muscle invasive bladder cancer (NMIBC) and its mRNA overexpression could act as an independent predictor for shorter progression-free survival (104). Besides, another bladder cancer study also showed that *PLEKHS1* gene expression was elevated in high grade disease (105). Similarly, higher expression of *PLEKHS1* was correlated with poor outcome in hepatocellular carcinoma (106). On the other hand, the expression of *PLEKHS1* was indicated as a protective prognostic biomarker in gastric cancer (107). Interestingly, these findings suggest the context-dependent effect of *PLEKHS1* on oncogenesis.

In terms of molecular biology, the *PLEKHS1* gene encodes a protein containing pleckstrin homology (PH) domain. PH domains are among the most common modules found in many proteins, which can drive intermolecular interactions in cellular signaling, cytoskeletal rearrangement and other processes (108, 109). PH domains are characterized to bind inositol phospholipids, thereby recruiting their host proteins to cellular membranes (108). PH domains can also be bound to inositol pyrophosphates, involved in various cellular processes including insulin secretion, glucose homeostasis and weight gain (110). PH domains from Akt/PKB identify and bind to phosphoinositide 3-kinase products specifically and drive signal-dependent recruitment to the plasma membrane (108). However, the vast majority of PH domains remain poorly defined and genome-wide studies in yeast have proved that only a small part of PH domains recognize specific inositol phospholipids (111).

1.3.2 *PLEKHS1* promoter mutations

Although the study focus on *PLEKHS1* function in cancer is still in an early stage, the hotspot mutations in the *PLEKHS1* promoter region, are identified in several human cancers which are amongst the most frequent promoter mutations identified by novel sequencing methods (112,

113). These mutations are single-nucleotide substitutions and the two hotspot mutations are G>A at chr.10:113751831 and C>T at chr.10:113751834 (based on the GRCh38.p13, hg19). These two mutations occur at the center of a perfectly palindromic sequence that is flanked by stretches of 9 bp on both sides with a core sequence of TGAACA (112) (Figure 6). Even though it is not clear if the palindrome does have any function, it is known that the TF binding sites can be palindromic sequences (114, 115). The *PLEKHS1* gene shows the second most frequent somatic non-coding mutations within its promoter of the human genome following *TERT* in bladder cancer (113) and the second most significant in hotspot analysis within the promoter following *TERT* in 863 human tumors (112).

Figure 6. Illustration of *PLEKHS1* promoter. Hotspot mutated positions highlighted in blue and its potential secondary structure (hairpin loop).

1.3.2.1 PLEKHS1 promoter mutations in human malignancies.

So far, the *PLEKHS1* promoter mutations have been found in certain cancers (Table 1). In bladder cancer, *PLEKHS1* promoter mutations belong to the most common mutations and can be shed into the urine (104, 105, 112, 113). Besides, Dudley et.al observed 46% of urine samples from UBC patients showed *PLEKHS1* promoter mutations and all mutations clustered at the two described hotspots (113). In TC, about 10% of samples were found to harbor the mutation (112, 116).

Table 1. Summary of *PLEKHS1* promoter mutations in human malignancies.

Tumor type	Mutation frequency	References
Bladder cancer	29.1% - 46%	(104, 105, 112, 113)
Breast cancer	6/172	(112, 115)
Lung cancer	4/64	(112)
Thyroid cancer	1/9	(112)
Differentiated thyroid cancer (DTC)	6/61	(116)
Classic papillary thyroid cancer (PTC)	1/75	(116)
Acute lymphoblastic leukemia	1/1	(112)

1.3.2.2 Consequences of PLEKHS1 promoter mutations

The role of *PLEKHS1* promoter hotspot mutations is poorly characterized in tumorigenesis. Recently, Jeeta et al found that the mutations were highly related with the Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) signature (105). APOBEC enzymes are a family of cytosine deaminases that convert cytosine to uracil. The level of

APOBEC mutational signatures are regulated by APOBEC3B and high APOBEC signature mutation burden is heavily associated with tumor mutation burden (117). APOBEC is a common mutational signature in UC (118, 119), which could potentially explain why these mutations are widespread events in bladder cancer (105). In addition, gene enrichment analysis revealed that the "cell adhesion" pathway was downregulated in *PLEKHS1* mutants and *PLEKHS1* mutations predicted better OS in the entire UBC patient cohort with the same trend in both MIBC and NMIBC (105). Jung et al. showed that *PLEKHS1* promoter mutations was more common in the radioactive iodine (RAI)-refractory DTC cases and the mutation was only detected in one case that had distant metastasis during the follow-up period in an independent cohort, suggesting that *PLEKHS1* promoter mutations could be a potential biomarker of aggressive DTC (116).

To conclude, *PLEKHS1* promoter mutations are particularly frequent in UBC and TC and could be used as a biomarker. While *PLEKHS1* has been characterized as a prognostic biomarker in UBC, its role in TC is still unknown. Moreover, some of these studies showed that there is no clear association between *PLEKHS1* gene expression and its promoter mutation, indicating that the underlying mechanism for its regulation remains to be defined (104, 105, 112).

1.4 THE *GPR126* GENE AND ITS NONCODING MUTATIONS

1.4.1 *GPR126* gene

G protein-coupled receptor 126 (*GPR126*) also known as adhesion G protein-coupled receptor G6 (*ADGRG6*), is located on Chromosome 6q24.2. It encodes the GPR126 protein, which is associated with various biological processes, such as adult height, nerve development and myelination, ear canal and heart development, and angiogenesis (120-125).

1.4.2 *GPR126* intron 6 mutations

Advanced genome sequencing technologies revealed that *GPR126* is one of the major genes bearing most frequently (45.6 - 53%) somatic noncoding mutations in UBC (105, 126, 127), which also exists in breast cancer (115). The mutations are two hotspots of single nucleotide substitution in intron 6 (with enhanced function) of *GPR126* gene (127), indicated as G>A at chr6:142385069 and C>T at chr6:142385072, respectively (assembly to GRCh38.p13). The core motif (TGAACA) is identical to *PLEKHS1* promoter hotspot mutations locus, which is flanked by a similar stretch of 9 bps palindromic sequences on both sides (Figure 7).

GPR126 mutations are related with increasing stage and grade, predicting worse outcome in UBC (105, 127). Moreover, the mutations have been reported to enhance APOBEC enzymatic activity (23) and frequently co-occur with mutations in *PLEKHS1*, *TBC1D12*, *LEPROTL1,* and *WDR74* (105). Although there is an inconsistent correlation between *GPR126* mutations and expression in different UBC cohorts (105, 126, 127), *GPR126* mutations are common and exist across stages and grades of UBC. It may be used as a valuable biomarker for bladder cancer detection.

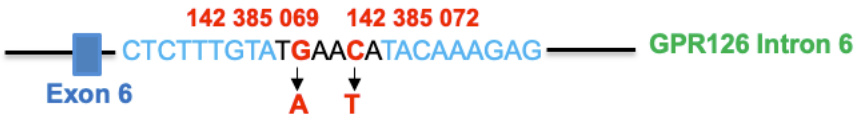

Figure 7. Illustration of *GPR126* intron 6 hotspot mutations. Hotspot mutation sites are highlighted in red and palindromic sequence are highlighted in blue.

1.5 THYROID CARCINOMAS (TC)

1.5.1 Overview

TC is the most common type of endocrine malignancy, making up ~2.1% of all diagnosed cancer worldwide, and the incidence is rapidly increasing (128). The majority (95%) of thyroid tumors originate from the follicular cell type, including papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC). PTC and FTC are collectively referred to as differentiated thyroid cancer (DTC). In addition, there remains a small group called medullary thyroid cancer (MTC) arising from parafollicular C cells (Table 2).

Generally, exposure to ionizing radiation is the best-described risk factor (128). Patient age, gender and family history serve as strong predictors of TC development. Women are 3 times more likely to have TC while men have a worse outcome (129). Environmental factors revealed by epidemiological studies, such as obesity and cigarette smoking, are also contributive to an increased risk (130, 131).

Table2. Subtypes of TC (132-134).

Subtypes	Prevalence (%)	Cell type	10- year survival (%)	Common mutations	Treatment
PTC	80-85	Follicular cell	95-98	BRAF, RAS, RET/PTC	(DTC) Surgery, RAI therapy, Locoregional therapy, Systemic therapy, TKIs[#1]
FTC	10-15	Follicular cell	90-95	RAS, PAX/PPAR, PIK3CA	
PDTC	<2	Follicular cell	~50	TERT, RAS, TP53, BRAF	Surgery, RAI therapy, radiotherapy, systemic therapy
ATC	1-2	Follicular cell	<10	TP53, TERT, CTNNB1, RAS, BRAF	Surgery, radiotherapy, systemic therapy, TKIs[#2]
MTC	3-5	Parafollicular C cell	60-80	RET, RAS	Surgery, systemic therapy, TKIs[#3]

DTC: differentiated thyroid cancer, including PTC and FTC. RAI: Radioactive iodine; TKIs: Tyrosine kinase inhibitors. [#1] multikinase inhibitors (Sorafenib, lenvatinib) for RAI refractory DTC, [#2] multikinase inhibitors (dabrafenib, trametinib) for BRAF V600E-positive ATC, [#3] multikinase inhibitors (Cabozantinib and Vandetanib) for MTC.

1.5.1.1 PTC

PTC is the most common type of TC, which can occur at any age but mostly it affects people aged 30 to 50 years (135). The rising incidence of thyroid cancer is mainly due to the increased detection of small-volume PTC (136, 137). Most PTCs are indolent with good outcome and with high 10-year survival rate. However, there are still a minority of patients that will eventually metastasize, relapse or evolve into undifferentiated TC, and develop treatment resistance, leading to disease-specific mortality.

1.5.1.2 ATC

ATC is the least common but the most aggressive type, which only accounts for 1 - 2% of all thyroid cancers but attributable to more than 50% of all deaths of TC (138). ATC is an undifferentiated cancer, which may develop from a differentiated thyroid cancer or occur as a *de novo* cancer. It usually affects people older than 60 years, growing rapidly and leading to poor outcomes (132).

1.5.2 Genetic alterations in TC

The current most popular diagnostic criteria is the American Joint Commission on Cancer (AJCC) staging system, including age, tumor size, extrathyroidal extension, presence and location of lymph node metastases and distant metastases. The AJCC and similar staging systems can correctly predict only a proportion of patients with risk for death and remain insufficient to predict the risk of recurrence. It may be problematic since the surveillance and treatment are guided by individualized estimation of recurrence risk (136). Molecular markers are promising for cancer prognostication as suggested by 2015 American Thyroid Association guidelines (139).

TC can be characterized by the type of mutations acquired. Most TCs bear mutations along the mitogen-activated protein kinase (MAPK) signaling pathway (also known as RAS-RAF-MEK-ERK pathway), which plays a key role in mediating cell proliferation, differentiation, senescence and survival (140). The presence of other additional mutations, such as *TERT* promoter mutations, may attribute to more aggressive disease.

1.5.2.1 Mutations along the MAPK (RAS-RAF-MEK-ERK) signaling pathway

BRAF is suggested as the strongest activator of MAPK signaling pathway, which is an isoform of RAF, participating in cell growth signal transmission inside cells. The most common *BRAF* mutation is $BRAF^{T1799A}$ point mutation, resulting in $BRAF^{V600E}$ mutant protein which can elevate its kinase activity and thereby activate the MAPK pathway. $BRAF^{V600E}$ mutation is the most frequent mutation in TC originated from the follicular cell type. It is exclusive to PTC and PTC-derived ATC. About 50-70% PTCs bear a $BRAF^{V600E}$ mutation. Patients with PTCs bearing a *BRAF* mutation had increased clinically aggressive disease and worse outcome (140-142). Mutations in the *RAS* family also frequently occur in TC, including *HRAS*, *KRAS*, *NRAS* genes. *RAS* mutations can act as activators of the RAS-RAF-MERK-ERK and phosphatidylinositol 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway, leading to alterations of gene expression, thereby contributing to cell proliferation, loss of differentiation and cell growth. It is exclusive to FTC and follicular variant PTC (FV-PTC)

(134, 143). *RET* mutation is considered as the major genetic alteration in MTC, which is a receptor for neurotrophic growth factors and its oncogenic activation is attributed to the gain-of-function mutation (134, 144). *RET* mutations lead to overactive RET protein which triggers the MAPK signaling pathway through a series of adaptor proteins (134, 145). *RET* mutation can occur as an inherited germline event and predicts a poor outcome (134).

1.5.2.2 TERT promoter mutations

TERT promoter mutations C228T and C250T are crucial events in TC. *TERT* C228T is a more common mutation than C250T and taken together, their prevalence is 0%, 11.3%, 17.1%, 43.2%, 40.1% in benign thyroid tumor, PTC, FTC, PDTC, and ATC, respectively (146). *TERT* promoter mutations are significantly associated with poor prognosis in TC (61, 147-149) and concurrence of *TERT* and *BRAF* mutations worsened the outcome of patients (147, 148).

1.5.2.3 Other gene mutations

Gene Mutations in *TP53*, *PTEN*, *PIK3CA*, *AKT1*, *CTNNB1*, *IDH1*, *ALK*, *EGFR*, *NDUFA13* are also present in thyroid cancers, which are believed to occur in advanced thyroid cancer and promote tumor progression (134, 150).

1.5.2.4 Chromosomal translocations

Chromosomal rearrangements cause expression of fusion oncogenes, which initiate oncogenesis as reported in many cases of thyroid cancer. The paired box 8 (*PAX8*) peroxisome proliferator- activated receptor γ (*PPARγ*) translocation, resulting in *PAX8-PPAR γ*, is the main (about 30%) genetic alteration in FTC (150). Rearrangement of the *RET* gene, also known as *RET-PTC*, occurs in about 7% PTC patients (151). Chromosomal translocations are also present in other genes, including *BRAF*, *NTRK* gene family, *ALK,* and *THADA*. These rearrangements, though rare, can implicate poor response to therapy and outcome (152).

1.5.3 Epigenetic alterations in TC

In addition to the well-known genetic alterations in the development of TC, numerous genes have been found to be epigenetically dysregulated, which have enriched the molecular landscape of TC (153). In contrast to the limited studies on histone modifications, aberrant DNA methylation is found as a common event in regulating gene transcription of (proto-) oncogene and tumor suppressor genes in TC (154). DNA methylation is mostly reported as a repressive marker predominantly silencing expression of tumor suppressors by hypermethylation, especially in distal regulatory elements region (e.g. promoter) (Figure 8). Promoter methylation may repress gene transcription by disturbing the binding of transcription factors and /or enrolling enzymes which modify chromatin structure (154).

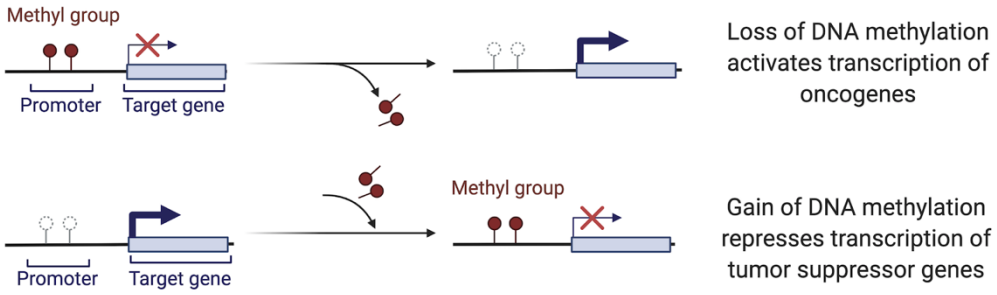

Figure 8. Aberrant DNA methylation in the promoter region. Figure is created with BioRender.com.

1.5.3.1 Hypermethylation of tumor suppressor genes

Gene-specific DNA methylation pattern has been widely studied in TC, accordingly hypermethylation of tumor suppressor genes have received more attention. Mostly studied tumor suppressor genes in TC include *RASSF1A*, $P16^{INK4A}$ and *DAPK*. *RASSF1A* is the tumor-suppressive Ras effector, encodes a homologous protein to RAS and is associated with modulating RAS signaling pathways, which inactivation is mainly attributed to promoter methylation. RASSF1A hypermethylation is suggested as a biomarker of aggressive tumors (154, 155). $P16^{INK4A}$ (*CDKN2A*) is a cyclin-dependent inhibitor to induce G1 phase arrest and its functional loss by promoter hypermethylation is common in TC (156). *DAPK* is a death-associated protein kinase promoting tumor necrosis factor-induced apoptosis. *DAPK* promoter hypermethylation is associated correlated with aggressive features and poor prognosis (157). In addition, the methylated profiles of thyroid-specific genes, such as Na+/I− symporter (*NIS*), has also been broadly explored. *NIS* is a gene mediating active transport of iodide to follicular thyroid cells. *NIS* downregulation by promoter hypermethylation leads to abolished ability of cancer cells to concentrate iodine, resulting in resistance to RAI therapy (158). Other genes silenced by aberrant methylation include $p27^{KIP1}$, *p14 (ARF)*, and Retinoblastoma (*RB*), *TIMP*, *REC8*, *E-cadherin*, and *PTEN* (159, 160).

1.5.3.2 Hypomethylation of oncogenes

DNA hypomethylation in oncogenes also plays a crucial role in carcinogenesis, such as membrane-associated protein 17 (*MAP17*). *MAP17* overexpression is caused by hypomethylation in its promoter region, which contributes to tumor growth in ATC. Other interesting hypomethylated genes include *DPPA2*, *INSL4*, *NOTCH4,* and *TCL1B,* which have been suggested to play a role in tumorigenesis (161).

1.6 UROTHELIAL CARCINOMA (UC)

1.6.1 Overview

UC, also known as transitional cell carcinoma, is a type of cancer occurring in the urinary system, consisting of lower (bladder and urethra) and upper (ureter and renal pelvis) urinary tract urothelial carcinomas. Urothelial bladder carcinoma (UBC) is the most common urinary tract urothelial carcinoma, making up 90-95% of urothelial carcinomas; while upper tract

urothelial carcinoma (UTUC) is uncommon, including renal pelvic carcinoma and ureter carcinoma, constituting only 5-10% of urothelial carcinomas (162).

Cigarette smoking and occupational carcinogens are typical risk factors to all UCs, regardless of the location. Smoking is the major risk factor, contributing to about 50% of cases. The elderly and men are more likely to develop UC (163, 164). In addition, UTUC has unique etiological risks compared to UBC. Aristolochic acid (AA) exposure is a well-characterized risk factor for UTUC, which is a component of Aristolochia-based herbal medicines widespread in East Asia (165, 166). Suffering from kidney stones may also contribute to UTUC by chronic irritation and inflammation to urothelial cells (167).

1.6.1.1 UBC

UBC is mostly diagnosed after appearance of symptoms such as hematuria, frequent or painful urination. However, 15-25% of UBC patients already have metastatic disease when first showing the clinical manifestations (162). Currently, diagnosis and surveillance of UBC is highly dependent on cystoscopy and biopsy, which are invasive and uncomfortable. While cystoscopy is still the mainstay method for diagnosis, urinary molecular analysis could be a complementary non-invasive approach to further aid disease detection and follow-up (168).

1.6.1.2 UTUC

Most of UTUCs have invasive or metastatic features when diagnosed, principally because of the lack of early clinical symptoms and efficient diagnostic methods (162, 169). In contrast to UBC, tumor tissue biopsy is sometimes inadequate and limited for UTUC (170, 171). Hence, searching for reliable biomarkers and developing accurate diagnostic approaches are needed to improve early diagnosis, treatment and outcome of UTUCs.

1.6.2 Genetic signatures in UC

UBCs and UTUCs share certain molecular pathological events since they both originate from urothelium. Genomic studies have revealed the molecular landscape of UBCs and UTUCs showing that they share certain genetic mutations in similar genes but at different frequencies, such as *FGFR3*, *TP53*, *HRAS,* and *TERT* (172). Patients with UTUC are more likely to bear *FGFR3* and *HRAS* mutations whereas patients with UBC tend to have *TERT* and *TP53* mutations. These differences potentially contribute to their metachronous recurrences and they should be regarded as distinct yet related diseases. It is noteworthy that mutational patterns in UC differ largely in relation to tumor grade and stage. For example in UTUC, low-grade disease is more likely to harbor *FGFR3* mutations while high-grade disease appear more likely to have *TP53* mutations (171, 172).

1.6.3 Novel and potential prognostic biomarkers

TERT promoter mutations have proved to be present in UTUCs and UBCs, predicting worse outcomes (62, 63, 173, 174). *TERT* promoter mutations are detected at a high frequency (48-85%) in UBC (175, 176) while relatively lower in UTUCs, with 43% and 19% in primary renal pelvic carcinomas and ureter carcinomas, respectively (63). Since over 50% of renal pelvic

carcinomas and 80% of ureter carcinomas are carrying a wt *TERT* promoter, other biomarkers are needed to predict the outcome of these patients.

Recently, non-coding mutations of *PLEKHS1* and *GPR126* were observed across stages and grades in UBCs, suggesting that they could be potential biomarkers in UBC (105, 113, 126). Given the high prevalence of these mutations in UBC as well as the genetic similarity between UTUC and UBC, it will be meaningful to investigate if the mutations also occur in UTUC. And if so, determining whether they can overlap with *TERT* promoter mutations and whether they can act as biomarkers in UTUC will be of great clinical interest.

2 RESEARCH AIMS

Overall, the aim of the study is to define the oncogenic function and implications of molecular alterations in non-coding regions, with a focus on promoter loci in thyroid and urothelial carcinomas. The ultimate aim is to contribute to a better understanding of thyroid and urothelial carcinomas pathogenesis and define new biomarkers for disease diagnostics and surveillance.

The specific aims are:

1. To identify *PLEKHS1* promoter mutations/methylation in thyroid cancer and to evaluate if PLEKHS1 expression could influence clinical outcome of thyroid cancer (**Paper I**).

2. To decipher how *TERT* promoter mutations and its activator GABPB1 contribute to the pathogenesis of thyroid cancer (**Paper II**).

3. To determine the mutations of *TERT*, *PLEKHS1* and *GPR126* non-coding regions in urothelial carcinomas and evaluate whether these can serve as urinary biomarkers for disease diagnostics and monitoring (**Paper III**).

3 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS AND PATIENT SAMPLES (PAPERS I - III)

In the studies, tumor tissue and/or urine samples as well as the clinical information such as gender, age, tumor size, metastasis status, pathological diagnosis and other related laboratory test results from patients were included. All research subjects participated voluntarily in the study including both oral and written informed consent.

Specially, in studies I and II, tumor samples from 93 PTCs and 18 ATCs were collected at Karolinska University Hospital. The patients were diagnosed according to the 2004 World Health Organization (WHO) classification. We further excluded the patients diagnosed as FV-PTC to avoid potential inclusion of non-invasive follicular thyroid neoplasms with papillary-like nuclear features according to the more recent WHO classification from 2017 (177). The main approval was from 2015 (Dnr: 2015/959-31) and an additional approval was applied for new patient information from 2020 (Dnr: 2020-00281). In study III, tumor tissue and/or urine samples were collected from 164 UTUCs and 106 UBCs. The project was approved by the ethics committee of Shandong University Second Hospital. All samples were frozen at -80 °C until further use.

3.2 CELL LINES AND CELL CULTURE (PAPERS I AND II)

TC cells line MDA-T32, MDA-T41, U-hth-74, U-hth-104, and SW1736 were used in papers I and II. Cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100ug/ml streptomycin and 4mM L-glutamine (Thermo Fisher Scientific, Waltham, MA).

3.3 SIRNA AND PLASMID TRANSFECTION (PAPERS I AND II)

PLEKHS1 expression and empty control plasmids, GABPA and GABPB1 siRNAs were commercially available and were stored at -20 °C after received. Cells were transfected with plasmids using Lipofectamine 3000 or transfected with siRNA using Lipofectamine 2000 according to the provided protocol.

3.4 RNA EXTRACTION, REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME PCR (QPCR) (PAPERS I AND II)

Total RNA was extracted from cell lines and patient samples using Trizol™ Reagent (Thermo Fisher Scientific) and mirVana miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA), respectively. RNA was then reversely transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qPCR was performed in QuantStudio 7 Flex Real-Time PCR System using SYBR Green or Taqman PCR Master Mix (Thermo Fisher Scientific).

3.5 WESTERN BLOT (PAPERS I AND II)

Total protein was extracted from cell lines and tumor samples using RIPA lysis buffer with 1% Phenylmethanesulfonyl fluoride with or without Protease Inhibitor Cocktail (Sigma-Aldrich, Darmstadt, Germany). Thirty micrograms of proteins were loaded into Mini-PROTEAN TGX Gels (Bio-Rad Laboratories, Hercules, CA) and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% non-fat milk diluted in TBST for 1 hour at room temperature, and then incubated with primary antibodies and secondary antibodies before being imaged.

3.6 DNA EXTRACTION (PAPERS I - III)

Genomic DNA was extracted from patients' samples or cell lines using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) or QIAamp DNA blood Mini Kit (Qiagen) and kept in -20 °C until further sequencing.

3.7 SANGER SEQUENCING (PAPERS I - III)

Sanger sequencing also known as "chain termination method", is a method for determination of nucleotide sequences of DNA. It has been the most widely used method for DNA sequencing in the past several decades. Generally, genomic DNA was first amplified using PCR with specific primers. The PCR product was further used as template for amplification using either a forward or reverse primer with the addition of dye-terminator reagent (BigDye Terminator V3.1, Applied Biosystems). The reaction mixture was purified, precipitated with ethanol and EDTA, resolved in distilled water, and followed by electrophoresis in an ABI 3730 DNA analyzer machine. The PCR products were all sequenced in both (forward and reverse) directions and analyzed by visual inspection of chromatograms. The main principle is shown in Figure 9.

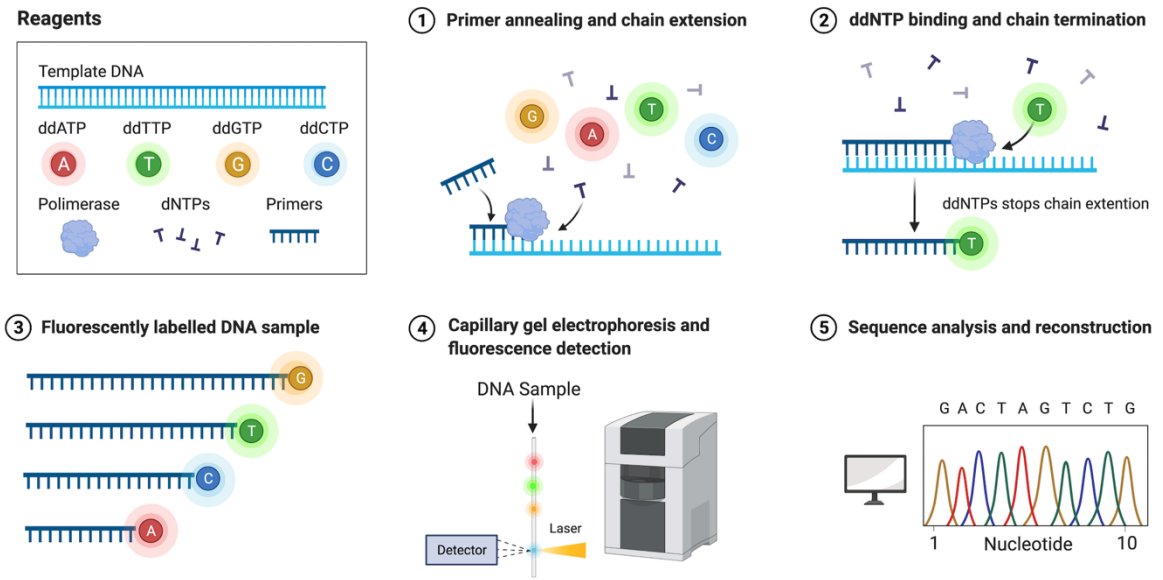

Figure 9. Illustration of Sanger sequencing. Figure is created with BioRender.com.

3.8 PYROSEQUENCING (PAPERS I AND II)

Pyrosequencing is a method for DNA sequencing, which uses enzyme-coupled reactions and bioluminescence to monitor the releasing of pyrophosphate following nucleotide incorporation in real-time. The method is widely used for single-nucleotide polymorphism (SNP) analysis, DNA methylation analysis, sensitive genetic testing and accurate genotyping. Generally, genomic DNA was converted by sodium bisulfite using EpiTect Bisulfite Kit (Qiagen). The region of interest was amplified with a biotinylated primer using the PyroMark PCR Kit (Qiagen). Then the PCR product was purified using PyroMark Q96 vacuum workstation (Qiagen). Thereafter, the purified product was annealed to the specific sequencing primer and followed by Pyrosequencing on a PyroMark Q96 MD instrument (Qiagen). The main principle is shown in Figure 10.

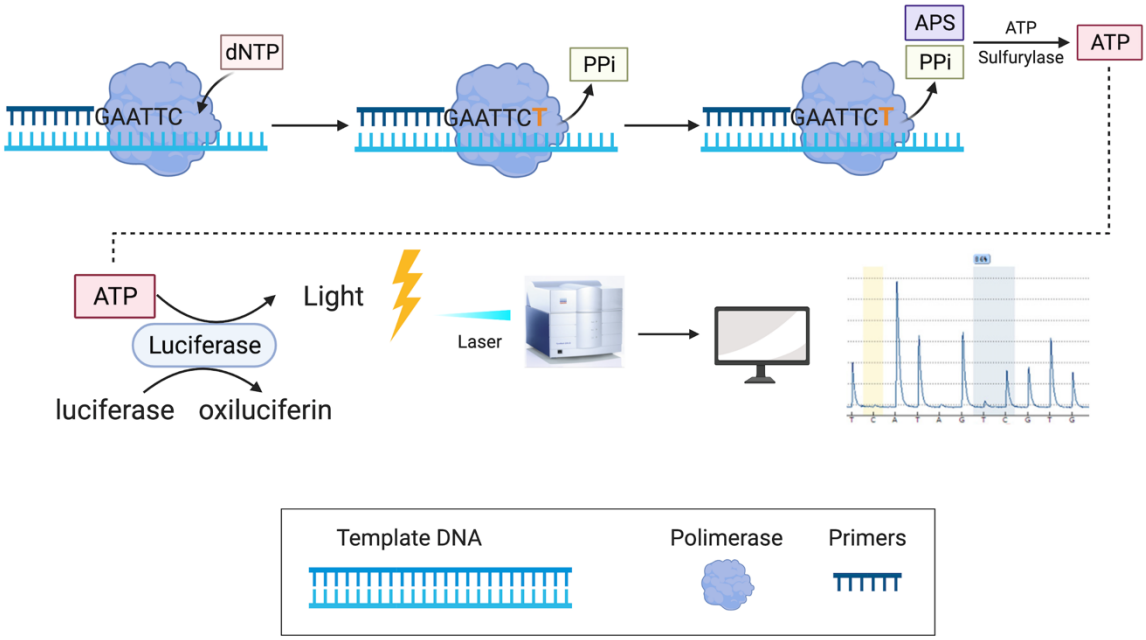

Figure 10. Main principle of Pyrosequencing. Figure is created with BioRender.com.

3.9 TRANSWELL ASSAYS FOR MIGRATION AND INVASION (PAPERS I AND II)

For the migration assay, cells were seeded in the upper chamber in the absence of FBS and the lower chamber contained RPMI-1640 medium with 20% FBS. The migrated cells were stained with crystal violet, photographed and counted 24/48 hours later. For invasion assay, 50 µL matrigel (Corning Life Sciences, Flintshire, UK) was first loaded into the bottom of the upper chamber followed by the same procedure as described in the migration assay.

3.10 CELL PROLIFERATION ANALYSES (PAPERS I AND II)

In Paper I, cell proliferation was determined by cell counting using a hemocytometer 48 hours post-transfection. In Paper II, cell proliferation was monitored and analyzed for 72 hours by an IncuCyte S3 Live-Cell Analysis System (Essen Bioscience, Ann Arbor, MI, USA), in which cell proliferation was represented by the changes of phase area confluence.

3.11 TCGA DATASET ANALYSIS (PAPERS I AND II)

The clinico-pathological information, genetic data, mRNA and methylation expression data for PTC (FV-PTC subtype was excluded) and their non-tumorous adjacent thyroid tissues were exported from the TCGA datasets via cBioPortal (https://www.cbioportal.org) or The Cancer Genome Atlas (TCGA) (http://cancergenome.nih.gov/). mRNA abundances were expressed as RSEM (RNA-Seq by Expectation Maximization) in cBioPortal or FPKM (Fragments Per Kilobase Million) in TCGA. For TCGA pan-cancer analysis, batch-effects normalized gene expression (log2 expression) and methylation expression data were exported from UCSC Xena browser (xena.ucsc.edu). Similarly FV-PTC tumor subtype was excluded from thyroid carcinoma (THCA). DNA methylation was expressed as beta value (β value, the ratio of signal intensity between methylated and unmethylated CpGs).

3.12 STATISTICAL ANALYSES (PAPERS I - III)

Unless stated, all statistical analyses in Papers I - III were performed using IBM SPSS Statistics version 24 (IBM, Armonk, NY, USA) or GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Student's t-test, Mann-Whitney U-test, Kruskal Wallis test and Chi^2-test or Fisher's exact test were used for comparison between groups according to the data distribution. Spearman's Rank-Order or Pearson's Correlation coefficient was applied to determine correlation coefficients r. Survival analyses were performed with log-rank test and visualized with Kaplan-Meier plots. Univariate or multivariate analysis was performed using Cox regression. *p*-values below 0.05 was considered as statistically significant.

In Paper II, methylation densities across 33 cancer types were shown in unclustered heatmap using "pheatmap" R package. Tukey boxplots were created with customized codes from "ggplot" R package.

In Paper III, visualization of different proportions of mutations in samples was shown by annotated pie chart and analysis was performed by permutation test using SPICE 6.0 (NIH, Bethesda, Maryland, USA).

4 RESULTS AND DISCUSSION

This section presents the main findings and discussion of studies included in the thesis. More details can be found in the corresponding articles or manuscript at the end of this thesis.

4.1 PAPER I: THE ROLE OF *PLEKHS1* IN THE PATHOGENESIS OF TC

4.1.1 *PLEKHS1* promoter mutations in PTCs and ATCs

A previous study (112) identified *PLEKHS1* promoter mutations in one out of nine TCs, suggesting that this genetic alteration might be recurrent in TC. Therefore, we determined the *PLEKHS1* promoter hotspot status in 5 TC cell lines, 93 tumors from patients with PTC, and 18 tumors from patients with ATC by Sanger sequencing. The results showed that only one PTC tumor was mutation-positive while all other tumors and all 5 cell lines were of wild-type (Figure 11). This result demonstrated that *PLEKHS1* promoter mutation was rare in PTCs and ATCs.

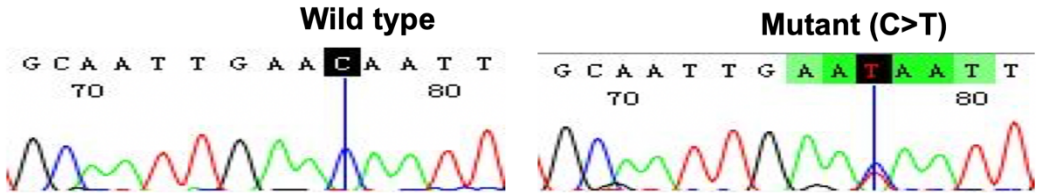

Figure 11. Chromatograms of wt and mutant *PLEKHS1* promoter. A representative figure showing the tumor with a wt *PLEKHS1* promoter (left). The only PTC tumor carrying mutant *PLEKHS1* promoter (right) with a C>T substitution.

4.1.2 Aberrant PLEKHS1 expression in PTCs and ATCs

We next determined PLEKHS1 expression in TC cell lines and primary tumors. PLEKHS1 expression was shown higher in 3 ATC cell lines than that in 2 PTC cell lines both at mRNA and protein levels, with the highest expression in the SW1736 cell line. Comparing *PLEKHS1* mRNA expression in PTC tumors and non-cancerous thyroid tissues from TCGA dataset revealed a significant increase of *PLEKHS1* mRNA in PTC tumors. In addition, in our cohort, *PLEKHS1* mRNA levels were higher in ATCs than those in PTCs. Because ATC is clinically more aggressive than PTC, higher *PLEKHS1* expression could be a signal for aggressive tumors. Taken together, PLEKHS1 expression was aberrantly upregulated in PTCs and ATCs.

4.1.3 Correlation between *PLEKHS1* expression and metastases & survival in PTCs and prognostic value of *PLEKHS1*

Given the above results, we sought to explore the relationship between *PLEKHS1* expression and clinical outcome in PTC. *PLEKHS1* mRNA was significantly higher expressed in patients with lymph node metastasis and/or distant metastases in our cohort. Besides, patients with higher *PLEKHS1* showed significantly shorter overall survival (OS) and disease-free survival (DFS) (Figure 12). There were no relationship between *PLEKHS1* expression and patient age, gender, tumor size, or extrathyroidal extension.

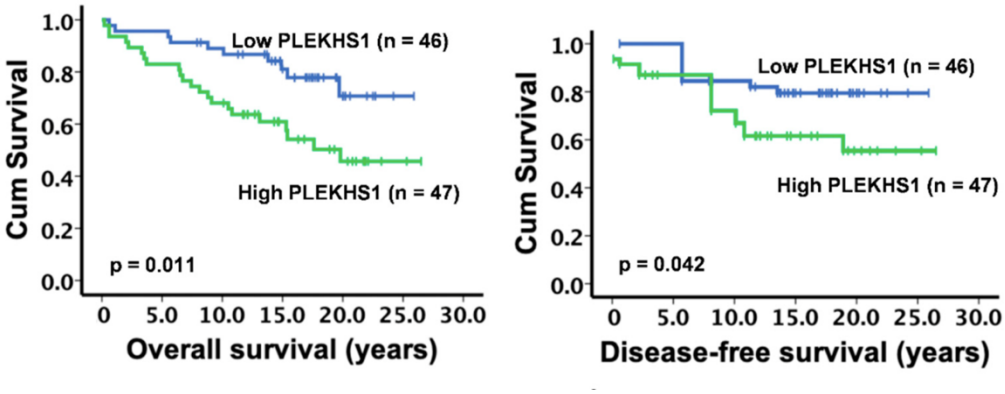

Figure 12. Higher *PLEKHS1* expression predicted shorter OS and DFS in PTC patients.

In addition, a previous study (100) has shown that *TERT* promoter mutations are associated with worse patient survival in PTCs but most PTCs carry a wt promoter. Thus, it is important to find new markers to improve prediction of outcome in this group. In order to verify if *PLEKHS1* is qualified for this purpose, we grouped PTC patients by their *TERT* promoter status and determined the association between *PLEKHS1* expression and survival respectively. No significant difference for *PLEKHS1* expression was found between tumors with wt or mutant *TERT* promoter and *PLEKHS1* expression was not associated with survival in the group with mutant *TERT* promoter. However, higher *PLEKHS1* expression predicted significantly shorter OS and showed a propensity towards shorter DFS in patients with a wt *TERT* promoter (Figure 13).

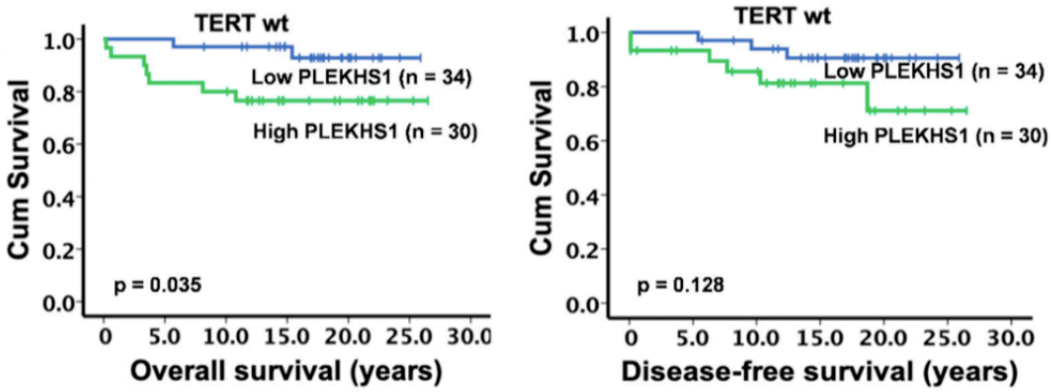

Figure 13. Higher *PLEKHS1* expression predicted shorter OS but not DFS in PTC patients with a wt *TERT* promoter.

In multivariate analyses, higher *PLEKHS1* expression, age (≥ 55 years old), and larger tumor size (>4 cm) were independently correlated with shorter OS. However, higher *PLEKHS1* expression failed to have effect on DFS while larger tumor size (>4 cm) remained a negative predictor for DFS.

We also analyzed the association between *PLEKHS1* expression and clinicopathological variables in TCGA cohort. Higher *PLEKHS1* expression was significantly associated with lymph node metastasis but not distant metastasis, probably because of only 4 of 249 patients

with distant metastasis in this cohort. There was no association between *PLEKHS1* expression and patient survival, likely due to low number of deaths and progression in TCGA cohort.

4.1.4 *PLEKHS1* overexpression-induced AKT phosphorylation, proliferation and invasiveness

Because *PLEKHS1* contains PH domains and can interact with Phosphoinositide-3-Kinase Regulatory Subunit 3 (PI3KR3) (178), we hypothesized that PLEKHS1 may activate the PI3K-AKT pathway. To verify this, we overexpressed *PLEKHS1* in TC cells and then determined AKT phosphorylation, which is an activation signal of the PI3K/AKT pathway. *PLEKHS1* overexpression significantly increased the phosphorylated AKT without effect on the total AKT level in TC cells. Since activation of the PI3K/AKT pathway will promote tumor cell proliferation, cell growth and invasion or metastasis (179), we further explored if these phenotypes occurred in PLEKHS1 over-expressed TC cells. Indeed, PLEKHS1 over-expressed TC cells were observed to have more cell numbers 48 hours post-transfection. More importantly, PLEKHS1 over-expressed TC cells achieved enhanced abilities in migration and invasion. These results were highly in accordance with the phenotype we found in clinical analyses namely that metastatic PTC patients expressed higher *PLEKHS1*.

4.1.5 Relationship between DNA methylation and mRNA expression of *PLEKHS1* in PTCs

Because of the rarity of *PLEKHS1* promoter mutations, we hypothesized epigenetic regulation might be involved in *PLEKHS1* expression. We thus analyzed the methylation status of *PLEKHS1* in the TCGA database, including 393 PTC samples and 56 adjacent normal thyroid tissue samples. Indeed, the total methylation level was decreased in PTC tumors compared to non-tumor tissue counterparts and the methylation level was significantly inversely correlated with *PLEKHS1* mRNA expression. Furthermore, we identified 3 specific CpG sites differentially methylated at the *PLEKHS1* locus and they were all located at 5' regulatory region of the *PLEKHS1* gene, including cg10627429, cg11204562, and cg22618337. The methylation level of all these three CpG sites were inversely correlated with *PLEKHS1* mRNA levels with cg22618337 observed at a borderline significance.

Additionally, we further determined the methylation level of cg10627429 and cg11204562 in our PTC cohort using Pyrosequencing. Consistent with the TCGA analysis result, a significant inverse association was observed between *PLEKHS1* mRNA expression and methylation of each CpG site, or the average methylation status.

4.1.6 Discussion

In this study, we identified that *PLEKHS1* promoter mutation was a rare genetic event while PLEKHS1 was aberrantly expressed in PTCs and ATCs. More importantly, we first investigated the association between *PLEKHS1* expression and clinical outcome in patients with PTC. The key finding presented in this study is that *PLEKHS1* overexpression is associated with both lymph node and distant metastases, and is a prognostic biomarker for shorter OS and DFS in PTC patients. Besides, higher levels of *PLEKHS1* still predict poor outcomes in a subset of PTC patients with a wt *TERT* promoter. *TERT* promoter mutations are independent predictors for a worse outcome in PTC (61, 146), but the application of it is

relatively insufficient since the majority of PTCs harbor a wt promoter. Our finding presented here showed that *PLEKHS1* can potentially act as a prognostic predictor in patients with the wt *TERT* promoter.

Our results were consistent with the present finding in bladder cancer. *PLEKHS1* overexpression was observed to be a negative predictor of progression- free survival and its overexpression promoted progression from NMIBC to aggressive MIBC (104). Therefore, it is conceivable that *PLEKHS1* has an oncogenic role in TC and bladder cancer. In addition, our in vitro cell experiments revealed that overexpression of PLEKHS1 enhanced the proliferative, metastatic and invasive ability of TC cells, which was consistent with the ectopic expression of PLEKHS1 and metastatic features in poor-prognosis TCs. We further showed that such a phenotype might be mediated by activation of AKT phosphorylation.

Furthermore, we also explored the regulation mechanism of *PLEKHS1* expression. In the previous study on bladder cancer, Pignot et.al identified almost half of the bladder tumors to harbor *PLEKHS1* promoter mutations but the mutations were not associated with its mRNA expression (104), suggesting a minimal effect on *PLEKHS1* transcription. However, Weinhold et al. observed that tumors with a mutated *PLEKHS1* promoter had lower expression of PLEKHS1 (112). Given the inconsistent findings reported along with the high prevalence of *PLEKHS1* promoter mutations in human malignances, further investigations are needed to determine how *PLEKHS1* is regulated and which function *PLEKHS1* promoter mutations mediate in cancer. In this study, since the genetic event of *PLEKHS1* is nearly absent in TCs, we supposed that epigenetic regulation might be involved in *PLEKHS1* expression. By analyzing the TCGA cohort, we found DNA methylation density at the *PLEKHS1* locus to be downregulated in PTC tumors compared with its adjacent normal tissues. More importantly, *PLEKHS1* gene methylation and its mRNA expression were significantly and inversely correlated, which was also observed in our PTC cohort. Similarly, such an inverse correlation was also identified in UBC (180). Collectively, these results suggested that hypomethylation of the *PLEKHS1* promoter promoted the transcription of *PLEKHS1* during the pathogenesis of PTC and UBC. Further studies are needed to explain the underlying mechanisms of *PLEKHS1* promoter demethylation in TC.

4.2 PAPER II: THE ROLE OF *GABPB1* IN TC

4.2.1 *GABPB1* knockdown-induced *TERT* downregulation and enhanced invasive capacity

Previous study revealed that GABPA depletion resulted in impaired *TERT* expression, but promoted invasion ability in TC cells (100). To explore if this was also true for GABPB1, we depleted GABPB1 in U-hth-74 and U-hth-104 cells using RNAi. *TERT* expression was significantly downregulated following reduction of GABPB1 in both cell lines. However, the decreased *TERT* expression did not affect the proliferation of these cells. On the other side, these same set of cells obtained stronger invasive capacity as determined by transwell assays. Since GABPA suppressed the invasive phenotype of TC cells via promoting *DICER1* transcription (99, 100), we further determined *DICER1* expression in GABPB1-depleted cells.

Indeed, *DICER1* significantly decreased in GABPB1-reduced cells. In addition, we also identified that GABPB1 was significantly correlated with *DICER1* expression both in our and TCGA PTC cohorts, which was the same case for GABPA (100). This result indicated that GABPB1 is required for *TERT* expression while itself may inhibit the invasive phenotype in TC cells. Moreover, there was a functional similarity between GABPB1 and GABPA in regulating *TERT* and *DICER1* expression.

4.2.2 The correlation between *GABPB1* expression and aggressiveness in TC

To further determine the role of *GABPB1* in TC, we compared *GABPB1* mRNA between PTC and ATC tumors. *GABPB1* expression was significantly lower in the ATC cohort than in the PTC. Because the long variant of GABPB1 (*GABPB1L*) was reported to mediate a mutant *TERT* promoter in glioblastoma (95, 97), we also investigated *GABPB1L* expression in these tumors and found a similar downregulation of *GABPB1L* in ATC tumors. Our clinical analysis suggested that downregulation of *GABPB1* could possibly lead to tumor progression, which highlighted the need to reveal the regulatory mechanism of its gene expression.

4.2.3 Association between hypermethylation of *GABPB1* promoter and GABPB1 reduction & shorter disease-free survival

Epigenetic regulation may be one potential explanations for GABPB1 reduction in aggressive TCs. Hence, we first explored the relationship between *GABPB1* methylation status and its expression in the TCGA dataset. Across 33 cancer types in TCGA, only one site (cg14821257) showed high variability. THCA (TCGA-Thyroid Cancer) ranked second following ACC (TCGA-Adrenocortical Carcinoma) when using the median of β value threshold of cg14821257. THCA ranked second when using the average β value following LUAD (TCGA-Lung Adenocarcinoma). Moreover, THCA is one of the cancer types with lower *GABPB1* mRNA expression. This data suggested hypermethylation of cg14821257 may have crucial role in regulation of *GABPB1* expression within thyroid cancer.

We then further identified the effects of *GABPB1* hypermethylation in PTC. Indeed, cg14821257, within *GABPB1* promoter, was the predominant one negatively related to *GABPB1* expression. The methylation density at cg14821257 in tumors was significantly higher than that in adjacent tissues. More importantly, a hypermethylated *GABPB1* promoter at cg14821257 was observed to significantly correlate with shorter DFS and a tendency to shorter OS when using a β value threshold at 0.8 (Figure 14).

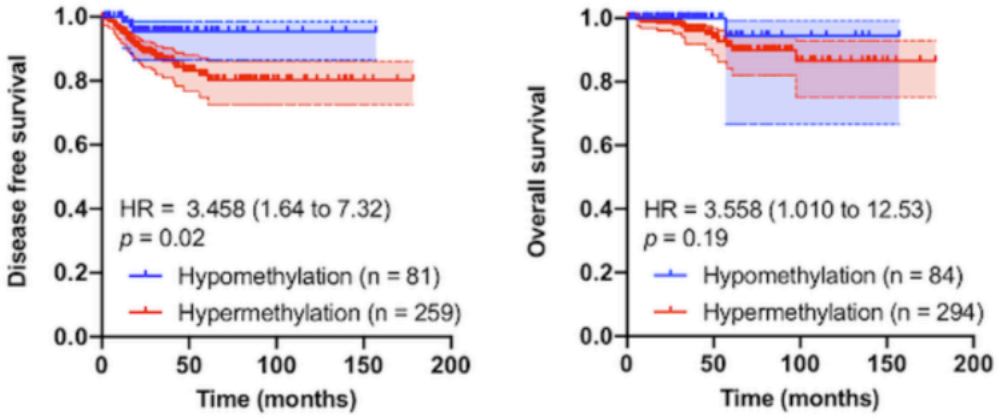

Figure 14. Hypermethylation of *GABPB1* (cg14821257) predicted shorter DFS but not OS in PTC patients. Log-rank test was used to determine the survival distribution at a cutoff of 0.8. The confidence interval for HR is 95%. HR: Hazard ratio.

To directly verify the causal relationship between promoter methylation and expression of *GABPB1*, we treated U-hth-74 and U-hth-104 cells with the DNA methylation inhibitor 5'-Aza and then determined the methylation expression of cg14821257 as well as *GABPB1* mRNA expression. Increased *GABPB1* expression coupled with reduced methylation at cg14821257 status were observed after 5'-Aza treatment. Taken together, this data indicated that the increased DNA methylation was responsible for the downregulation of *GABPB1* in TCs.

4.2.4 Discussion

While it was suggested that GABPB1 knockout-mediated *TERT* downregulation would induce apoptosis in glioblastoma cells in a mutant *TERT* promoter dependent manner (95), in our study, GABPB1 knockdown led to reduced *TERT* expression whereas it enhanced the invasiveness of TC cells. This phenomenon induced by disruption of GABPB1 was similar to its interacting partner GABPA. Previous study has shown GABPA downregulation inhibited *TERT* expression while increased invasive phenotypes and was associated with aggressive disease and worse survival in TC (100). Mechanistically, GABPA facilitated cellular invasion by stimulating *DICER1* through which metastasis was suppressed in TC (99, 100). Similarly, GABPB1 knockdown led to reduced *DICER1* expression and moreover, *GABPB1* was significantly positively correlated with *DICER1* expression in PTC cohorts. GABPA and GABPB1 likely work together to inhibit invasive behaviors by regulating *DICER1* expression.

We also investigated the potential mechanism of GABPB1 downregulation in TC. It is well-known that aberrant hypermethylation of gene promoters is a common way to silence tumor suppressive genes. And here we found hypermethylation of *GABPB1* occurs in TC besides its inhibitory effect on TC cell invasion, supporting a tumor suppressive function of GABPB1 in TCs. Moreover, patients with a hypermethylated *GABPB1* promoter at cg14821257 had a shorter disease-free survival, a finding which may function as a prognostic biomarker for PTC patients.

Given the oncogenic role of *TERT*, GABPA and GABPB1 inhibition-mediated *TERT* down-regulation is expected to suppress tumor progression. However, GABPA or GABPB1 loss contributes to aggressive disease in TC despite decreased *TERT* expression. This suggests GABPA and GABPB1 may have a tumor suppressive role beyond its role as activators in *TERT*

regulation. However, Liu *et al* demonstrated FOS inhibitors-induced GABPB1 and TERT reduction led to apoptosis of TC cells and other cancer cells (181). In contrast, we did not observe any difference in proliferation when inhibited GABPB1 directly. Therefore, further studies are needed to explain these inconsistent results.

Taken together, GABPB1 can act as an activator of *TERT* expression while itself has a tumor suppressive activity in TC pathogenesis, which is similar to its partner GABPA. Therefore, caution is needed when considering targeting GABPB1 for anti-telomerase cancer therapy since it may counteract the tumor suppressive function of GABPB1, thereby promoting TC progression.

4.3 PAPER III: THE CLINICAL VALUE OF *TERT*, *PLEKHS1*, AND *GPR126* REGULATORY REGION MUTATIONS IN UC

4.3.1 Mutations of *TERT*, *PLEHKS1* promoters and *GPR126* intron 6 in UC tumors

We comprehensively determined the regulatory regions mutation status of *TERT*, *PLEKHS1* and *GPR126* in UTUCs. Tumors derived from 164 UTUCs were screened by Sanger sequencing. The mutation frequencies at regulatory regions in UTUCs were 28%, 5.8% and 11% for *TERT*, *PLEKHS1* and *GPR126*, respectively. For most tumors, these mutations occurred separately, while in a small subset (less than 3%) the mutations co-existed.

The results showed that the frequencies of these mutations were extremely lower than that in the reported UBC studies (104, 105, 113, 126, 127, 170, 176). Hence, we further analyzed tumor samples from 106 UBC patients from the same hospitals to exclude potential confounders including region, diet habit or race. The mutation frequencies for *TERT*, *PLEKHS1* and *GPR126* in this cohort were 44.3%, 26.4%, and 31.4%, respectively, which indicated that these mutations are indeed less common in the UTUC cohort (Figure 15).

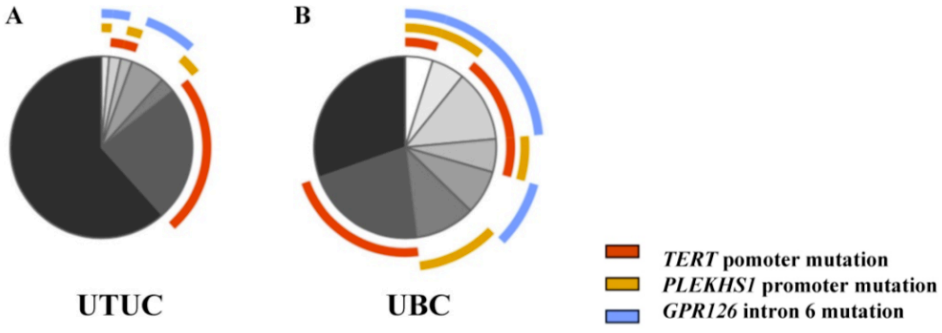

Figure 15. Frequencies of different mutations in UTUC and UBC cohorts.

4.3.2 Detection of regulatory mutations of *TERT*, *PLEKHS1* and *GPR126* in urine from UTUCs and UBCs

Previous studies have demonstrated that *TERT* mutant promoter fragments were detectable in urine samples from patients with UTUCs and UBCs, which indicated the clinical value as a urinary biomarker (62, 63, 176). Here, we aimed to explore whether all these mutant fragments could be detected in urine samples by Sanger sequencing method. We found that all these mutations were readily detected in patient urine samples collected from 13 UTUCs and 76 UBCs before surgery. In addition, we also checked the sensitivity, specificity and accuracy of urine detection compared to tumor samples. We defined the presence of any mutation as MT while absence of all mutations as WT. Among 59 MT tumor samples we only detected 36 with mutations in urine samples. However, in 30 WT tumor samples, we surprisingly found 3 urine samples with mutation. Although the sensitivity for urine detection was only 61%, the specificity is satisfactory (90%) which indicated a high consistency between tumors and urine (Table 3).

Table 3. Consistency between urine and tumor samples.

Urine samples	Tissue samples				
	MT (n)	WT (n)	Sensitivity (%)	Specificity (%)	Accuracy (%)
MT (n)	36	3	61%	90%	70.7%
WT (n)	23	27			
Total	59	30			

MT: presence of any mutations; WT: absence of all mutations; n: numbers.

4.3.3 Biomarker value of *TERT*, *PLEHKS1* promoters and *GPR126* intron 6 mutations in UC

We also compared the mutant DNA sequences in preoperative and postoperative urine samples collected from 20 patients. A significant reduction of mutated fragments was observed in post-operative urine samples (Figure 16). Ten of them had mutant DNA sequences before surgery while the mutated DNA fragments disappeared in 6 of them 1 week after surgery, suggesting that this analysis may be used in the clinical monitoring of patients with UTUC or UBC.

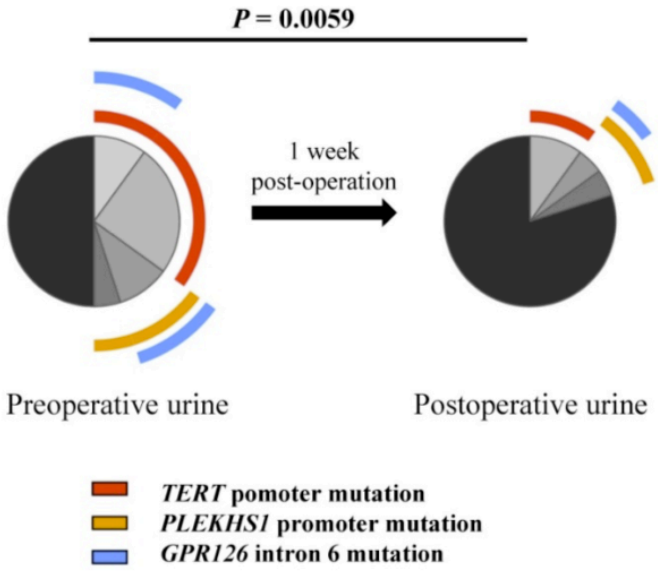

Figure 16. Different frequencies of mutant fragments in pre-operative and post-operative urine samples.

4.3.4 Discussion

The failure of early detection in most UTUC patients is due to lack of reliable methods and hence it would be of great value if reliable disease biomarkers were to be developed (163). In this study, we comprehensively demonstrated the occurrence of *TERT* and *PLEKHS1* promoter, and *GPR126* intron 6 mutations in UTUC but with lower frequencies compared to UBC. These mutations have all been identified in UBC previously and may serve as predictive biomarker (105, 113, 126, 127, 173, 176). While *TERT* promoter mutations have been investigated and shown as urinary biomarker in UTUC (63, 182), to our knowledge, it is the first time that the occurrence of *PLEKHS1* promoters and *GPR126* intron 6 mutations were reported in UTUC. In addition, all these mutant fragments were detectable in urine samples from patients bearing these mutations and disappeared quickly after tumor removal in most patients, which suggested their clinical value in UC diagnosis and surveillance.

Urine-based tests are becoming more popular since it is non-invasive and urine may accumulate more tumor-derived material with low background noise compared with other tissues obtained from patients, however, urine tumor cells or cell-free DNA may have low mutant alleles (170). In this study, the mutation status of the three genes were determined by Sanger sequencing, a gold standard method for DNA sequencing. However, its detection threshold is at least 10% for *TERT* promoter mutant alleles (63). In this case, a more sensitive detection method is required to provide more accurate disease information (170). In fact, digital droplet PCR, which is of high sensitivity and specificity, has been developed to analyze mutant *TERT* promoter abundance in patient urine (183, 184). The same approaches should be developed for detecting *PLEKHS1* and *GPR126* mutation too.

5 SUMMARY AND CONCLUSIONS

Overall, the studies presented in this thesis gain insights into the biological and clinical implications of the regulatory region mutations and/or aberrant methylation in *TERT*, *GABPB1*, *PLEKHS1* and *GPR126* genes in TCs and UCs. More specifically:

PLEKHS1 is aberrantly expressed in PTCs and ATCs, predicting a worse prognosis in patients. The higher expression of *PLEKHS1* involves demethylation of its promoter and it may enhance the invasive capacity via AKT phosphorylation. Given the fact that high expression of *PLEKHS1* is associated with lethal ATCs as well as metastatic PTCs, *PLEKHS1* expression can potentially act as a promising biomarker in surveillance of PTC (**Paper I**).

GABPB1 inhibition led to downregulation of *TERT* expression in cells with a mutant *TERT* promoter but significantly promoted invasive behaviour of TC cells. *GABPB1* expression was inhibited in aggressive TCs and low expression of *GABPB1* was mediated by hypermethylation of its promoter, which in turn predicted shorter disease-free survival. *GABPB1* may act as a tumor suppressor in TC (**Paper II**).

TERT, PLEKHS1 promoter and *GPR126* intron 6 mutations occur in UTUCs but with lower frequencies compared to UBCs. These mutant DNA fragments can be voided into urine and disappeared quickly after surgery; they may serve as specific urinary biomarkers for UTUC early diagnosis and disease surveillance (**Paper III**).

6 POINTS OF PERSPECTIVE

The boost of genetic and epigenetic research has improved our understanding of the complicated landscape of tumor biology, however, it is still challenging for us to identify and translate clinically relevant discoveries within thousands of studies. In my thesis, we explored several potential biomarkers for disease prediction, diagnosis, and surveillance in TC and UC. Yet, the studies involved in this thesis need to be further extended.

Specifically, in **Paper I**, the function of *PLEKHS1* in TC still needs to be further explored. Further mechanistic studies can be performed followed by comprehensive analysis of RNA-seq. An animal model would also give more information compared with *in vitro* experiments. Moreover, hypomethylation of *PLEKHS1* promoter was found to enhance transcription of *PLEKHS1* in TC. Further studies are needed to elucidate the mechanisms underlying hypomethylation of the *PLEKHS1* promoter and determine if the promoter hypomethylation also is of prognostic value in TC.

In **Paper II**, GABPB1 was shown to exert a similar function to GABPA in regulating *DICER1* and *TERT*. As discussed earlier, the role of *GABPB1* could be context-dependent given its tumor-promoting or suppressive functions in different cancers. Therefore, it may be of interest to further dissect these conflicting functions of *GABPB1*.

In **Paper III**, since Sanger sequencing has limited capacity to detect low mutational burden, more sensitive methods (e.g. digital droplet PCR) should be developed to improve the detection of *TERT* and *PLEKHS1* promoter or *GPR126* intron 6 mutations. Additionally, given that *PLEKHS1* and *GPR126* have already shown an oncogenic role in UBC, investigating the function of these genes in UTUC will be meaningful since UTUC share many molecular characteristics with UBC.

Generally, in the TC and UC field, current prognostic or diagnostic methods/biomarkers are not optimal. I hope our efforts will contribute to better understanding of the intricate biological landscape and brighten the prospects of biomarkers in the clinical management.

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