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DIAGNOSTIC AND PROGNOSTIC MARKERS IN THYROID CARCINOMA WITH FOCUS ON TERT ACTIVATION

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Cover illustration: The shape of the thyroid gland is by many considered as resembling a butterfly. The genetic code in the illustration is in part taken from the DNA sequence of the *TERT* promoter. Illustration by Lucy Bai and computer graphics by Jiwei Gao.

Diagnostic and prognostic markers in thyroid carcinoma with focus on *TERT* activation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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POPULAR SCIENCE SUMMARY OF THE THESIS

The thyroid gland is a small but important organ located in the neck. Its function is to produce hormones that regulate important processes in the body, such as the heart rate and metabolism. Like in most organs in the body, cancer can also develop in the thyroid. There are several types of thyroid cancer of which papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) are the two most common types. Patients with thyroid cancer usually have a good prognosis but some may also develop aggressive disease. Therefore, it is important to find prognostic markers that can be used to detect patients that are more likely to develop an aggressive disease. For FTC, it is currently not possible to distinguish this cancer type from the benign counterpart follicular thyroid adenoma (FTA) before surgery. Thus, diagnostic markers to distinguish FTC from FTA are needed.

Molecular changes that affect DNA, genes and proteins in the cells are major driving forces in cancer development. To study these changes is important since some of these molecular changes could potentially be used to diagnose cancer and predict the outcome of the affected cancer patients. In addition, therapy that targets these specific changes could be developed. In this thesis, the underlying causes for some of these changes and whether they could be used as markers for diagnosis and prognosis in thyroid cancer were investigated.

In Paper I, a protein that is a marker for cell division named Ki-67 was investigated to determine if it could be relevant as a pre-operative diagnostic marker for FTC. We found that high levels of Ki-67 were more common in biopsies from FTC than in benign tumors and concluded that it might help to detect some of the cancers before the patient is undergoing surgery. Still, more markers are needed since Ki-67 alone could not differentiate all FTCs. In Papers II-IV, we focused on studying a gene named *TERT* and other genes that are related to *TERT*. This gene is normally inactivated, but is found activated in cancer since it protects chromosome ends and makes the cancer cells immortal. We found that changes of the *TERT* gene were more common in FTC than in benign tumors. FTC patients with these changes were also more likely to develop aggressive disease. Thus, we concluded that changes that affect the *TERT* gene could be potentially useful markers for diagnosis and prognosis in FTC. To investigate the underlying causes for activation of the *TERT* gene in PTC, we studied a gene named *GABPA*. We found that *GABPA* can activate the *TERT* gene but at the same time have other effects that are more protective against cancer development. Taken together, *GABPA* was found to have stronger protective effects and loss of *GABPA* was found in patients with aggressive PTC. Finally, we found an additional gene named *EHF* that had a strong connection to aggressive disease and the *TERT* gene in PTC. Thus, we speculated that this gene could potentially act together with *TERT* to cause aggressive PTC.

Overall, this thesis investigated potentially useful markers for diagnosis and prognosis in thyroid cancer. In addition, possible underlying molecular changes of aggressive thyroid cancers were explored.

ABSTRACT

Well-differentiated follicular cell-derived thyroid carcinomas are usually categorized into papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), which represent the most common and second most common types of thyroid carcinoma, respectively. PTC patients generally have a good prognosis, although a subset of patients progress in their disease with poor clinical outcome. Also, it remains a diagnostic challenge to differentiate FTC pre-operatively from follicular thyroid adenoma (FTA) and follicular tumor of uncertain malignant potential (FT-UMP). For both PTC and FTC, promoter mutations in the telomerase reverse transcriptase (*TERT*) gene have been identified as an important underlying genetic event. However, other *TERT*-activating alterations are less studied in thyroid carcinoma. Thus, this thesis aimed to investigate potential diagnostic and prognostic biomarkers, and *TERT*-activating alterations in FTC and PTC, respectively.

In Paper I, we identified high cytological Ki-67 proliferation index and larger tumor size as predictors of FTC in follicular thyroid tumors using univariate and multivariate analyses. In the FTC subgroup, extrathyroidal extension and the widely invasive sub-type were associated with higher cytological Ki-67 index. Taken together, cytological Ki-67 index could potentially add pre-operative diagnostic information for a subset of patients.

In Paper II, we found that *TERT* expression was coupled with co-existing *TERT* copy number gain, promoter mutation or hypermethylation in follicular thyroid tumors. Several of these alterations were observed in higher frequencies in FTC/FT-UMP and associated with poor clinical outcome in FTC. Thus, they are promising as biomarkers for diagnosis and prognostication.

In Paper III, the E26 transformation-specific (ETS) transcription factor GABPA was shown to exhibit mutant *TERT* promoter-activating properties in thyroid carcinoma cells. In contrast, GABPA also exhibited tumor-suppressive properties *in vitro* and *in vivo*, highlighting its dual roles. In PTC tumors, low expression of *GABPA* was associated with poor clinical outcome. Moreover, *DICER1*, involved in the microRNA machinery, was identified as a potential downstream target for the GABPA-mediated tumor suppressive effect. We conclude that loss of tumor suppressive functions of *GABPA* is coupled to PTC progression.

In Paper IV, the 28 known ETS transcription factors were analyzed in relation to clinical phenotype in PTC. *EHF* was found to be upregulated in PTC and associated with aggressive clinical features, $BRAF^{V600E}$ and *TERT* promoter mutation or expression. In PTC cells with concurrent $BRAF^{V600E}$ and *TERT* promoter mutations, over-expression of EHF induced an increase in *TERT* expression. Collectively, *EHF* is a potential oncogenic ETS factor in PTC.

In summary, the findings in this thesis add insights into the underlying *TERT*-activating alterations in follicular cell-derived thyroid carcinomas. Further, several potentially clinically relevant biomarkers for diagnosis and prognostication for this patient group were identified.

LIST OF SCIENTIFIC PAPERS

I. **Mu N**, Juhlin CC, Tani E, Sofiadis A, Reihnér E, Zedenius J, Larsson C, Nilsson IL. High Ki-67 index in fine needle aspiration cytology of follicular thyroid tumors is associated with increased risk of carcinoma.
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PMID: 29796987

II. Paulsson JO, **Mu N**, Shabo I, Wang N, Zedenius J, Larsson C, Juhlin CC. TERT aberrancies: a screening tool for malignancy in follicular thyroid tumours.
Endocr Relat Cancer. 2018 Jul;25(7):723-733.
PMID: 29692346

III. Yuan X*, **Mu N***, Wang N*, Stråat K, Sofiadis A, Guo Y, Stenman A, Li K, Cheng G, Zhang L, Kong F, Ekblad L, Wennerberg J, Nilsson IL, Juhlin CC, Larsson C, Xu D. GABPA inhibits invasion/metastasis in papillary thyroid carcinoma by regulating DICER1 expression.
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PMID: 30181547

IV. **Mu N**, Gao J, Wang N, Zedenius J, Nilsson IL, Juhlin CC, Xu D, Larsson C. The ETS transcription factor EHF is upregulated in papillary thyroid carcinoma carrying $BRAF^{V600E}$ and TERT promoter mutations with impact on poor patient outcome.
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* Authors contributed equally

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Stenson G, Nilsson IL, **Mu N**, Larsson C, Lundgren CI, Juhlin CC, Höög A, Zedenius J. Minimally invasive follicular thyroid carcinomas: prognostic factors.
Endocrine. 2016 Aug;53(2):505-11.
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II. Paulsson JO, Wang N, Gao J, Stenman A, Zedenius J, **Mu N**, Lui WO, Larsson C, Juhlin CC. GABPA-dependent down-regulation of DICER1 in follicular thyroid tumours.
Endocr Relat Cancer. 2020 May;27(5):295-308.
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III. Xing X, **Mu N**, Yuan X, Wang N, Juhlin CC, Stråat K, Larsson C, Xu D. PLEKHS1 over-expression is associated with metastases and poor outcomes in papillary thyroid carcinoma.
Cancers (Basel). 2020 Jul 31;12(8):2133.
PMID: 32752127

CONTENTS

LIST OF ABBREVIATIONS

AFTA	Atypical follicular thyroid adenoma
AJCC	American Joint Committee on Cancer
AKT	Protein kinase B
ALT	Alternative lengthening of telomeres
ATC	Anaplastic thyroid carcinoma
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AUC	Area under curve
bp	base pair
BRAF	v-Raf murine sarcoma viral oncogene homolog B
cDNA	complementary DNA
c-Myc	Cellular myelocytomatosis
CN	Copy number
CpG	Cytosine-phosphate-Guanine
CREB3L2	cAMP Responsive Element Binding Protein 3 Like 2
ddNTP	dideoxynucleotide
DEG	Differentially expressed gene
E-boxes	Enhancer box
EHF	ETS homologous factor
ELF1	E74 Like ETS Transcription Factor 1
ELF2	E74 Like ETS Transcription Factor 2
ELF3	E74 Like ETS Transcription Factor 3
ELF4	E74 Like ETS Transcription Factor 4
ELF5	E74 Like ETS Transcription Factor 5
ELK1	ETS Transcription Factor ELK1
ELK3	ETS Transcription Factor ELK3
ELK4	ETS Transcription Factor ELK4
ERF	ETS2 Repressor Factor
ERG	ETS Transcription Factor ERG
ETS	E26 transformation-specific
ETS1	ETS Proto-Oncogene 1, Transcription Factor
ETS2	ETS Proto-Oncogene 2, Transcription Factor
ETV1	ETS Variant Transcription Factor 1
ETV2	ETS Variant Transcription Factor 2
ETV3	ETS Variant Transcription Factor 3
ETV3L	ETS Variant Transcription Factor 3 Like
ETV4	ETS Variant Transcription Factor 4
ETV5	ETS Variant Transcription Factor 5
ETV6	ETS Variant Transcription Factor 6
ETV7	ETS Variant Transcription Factor 7
EU-TIRADS	European Thyroid Imaging and Reporting Data System
FEV	FEV transcription factor
FLI1	Fli-1 proto-oncogene
FNA	Fine needle aspiration

FTA	Follicular thyroid adenoma
FTC	Follicular thyroid carcinoma
FT-UMP	Follicular tumor of uncertain malignant potential
FV-PTC	Follicular variant of papillary thyroid carcinoma
GABPA	GA-binding protein alpha
GRCh37	Genome Reference Consortium Human Build 37
HCA	Hürthle cell adenoma
HCC	Hürthle cell carcinoma
HRAS	Harvey rat sarcoma viral oncogene homolog
Indels	Insertions and deletions
kb	kilobase
KRAS	Kirsten rat sarcoma viral oncogene homolog
lncRNA	Long non-coding RNA
MAPK	Mitogen-activated protein kinase
MEN 2	Multiple endocrine neoplasia type 2
MetI	Methylation index
miRNA	MicroRNA
mRNA	Messenger RNA
MTC	Medullary thyroid carcinoma
ncRNA	Non-coding RNA
NIFTP	Non-invasive follicular thyroid neoplasm with papillary-like nuclear features
NRAS	Neuroblastoma rat sarcoma viral oncogene homolog
PAX8	Paired box 8
PCR	Polymerase chain reaction
PDTC	Poorly differentiated thyroid carcinoma
PI3K	Phosphatidylinositol 3-kinase
PI3KCA	Phosphatidylinositol 3-kinase, catalytic, alpha
PPARγ	Peroxisome proliferator activated receptor γ
pRb	Retinoblastoma protein
PTC	Papillary thyroid carcinoma
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RAF	RAF proto-oncogene
RAS	RAS superfamily
RET	Rearranged-during-transfection
ROC	Receiver operating characteristic
siRNA	Small interfering RNA
Sp1	Specificity protein 1
SPDEF	SAM Pointed Domain Containing ETS Transcription Factor
SPI1	Spi-1 Proto-Oncogene
SPIB	Spi-B Transcription Factor
SPIC	Spi-C Transcription Factor
STR	Short tandem repeat
T3	Triiodothyronine
T4	Thyroxine

TCGA	The Cancer Genome Atlas
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
THOR	TERT hypermethylated oncological region
TNM	Tumor, node, metastasis
TP53	Tumor protein p53
VEGF	Vascular-endothelial growth factor
WHO	World Health Organization

1 INTRODUCTION

1.1 CANCER DEVELOPMENT AND PROGRESSION

1.1.1 Cancer characteristics

Cancer development is a multistep process where normal cells acquire the capabilities for malignant transformation. The hallmarks of cancer, as proposed by Weinberg and Hanahan, describes the characteristics in this multistep process (Figure 1) (1). Originally, they formulated six hallmarks of cancer to describe the features of cancer, namely (1):

- *Self-sufficiency in growth signals.* In normal physiological conditions, cells respond to exogenous growth signals transmitted through transmembrane receptors before they can enter into proliferation (1). Cancer cells acquire the ability to produce their own growth signals and are thus less dependent of the exogenous growth signaling (1).
- *Insensitivity to anti-growth signals.* Similar to the regulation of growth signals, cell proliferation is regulated by growth inhibitory signals. Normally, cells either enter a quiescent state or permanently lose the proliferative capability upon anti-growth signals (1). In cancer, cells acquire molecular features that enable the evasion of these signals; one example is the disruption of the retinoblastoma protein (pRb) signaling pathway that under normal conditions blocks proliferation (1).
- *Tissue invasion and metastasis.* The natural course for most types of cancer will eventually cause the cancer cells to invade adjacent tissues and vessels and thereby spread to distant sites where they can colonize and form metastases (1).
- *Limitless replicative potential*. Normal cells have an underlying replicative potential that only enables replication a certain number of times before they enter senescence (1). The telomeres, located at the ends of chromosomes, play a key role in the replicative barrier. They protect the DNA from damage during each replication, as the DNA polymerase can not fully replicate the 3’ end of the chromosomes, and are successively shortened during each cell division (1). When the telomeres reach a critical length, the unprotected chromosome ends will lead to end-to-end fusion, which in turn will lead to cell crisis and cell death. Most types of cancers become immortalized by re-activation of telomerase, the enzyme which adds nucleotide repeats to the end of telomeres and which is normally inactivated in differentiated cells (1).
- *Sustained angiogenesis.* All cells require nutrients provided from blood vessels to survive and the formation of new blood vessels in tissues are highly regulated by angiogenesis-initiating and -inhibitory signals. In order for a tumor to grow, they must acquire angiogenic capability (1). This can be achieved by altering gene expression patterns of angiogenic genes. For example, many cancers display over-expression of vascular-endothelial growth factor (VEGF), which is an angiogenesis-inducer (1).
- *Evading apoptosis.* Apoptosis or programmed cell death can be triggered by various abnormalities, including DNA damage and hypoxia, and serves as a barrier to tumorigenesis. A common mechanism for tumors to surpass this barrier is by losing pro-apoptotic components (1). The *TP53* tumor suppressor gene, which plays a crucial

role in sensing DNA damage and other cellular abnormalities, is frequently mutated in cancer, resulting in an inactive p53 protein and impaired regulation of apoptosis (1).

After describing the original six hallmarks, two additional hallmarks and two enabling characteristics were described and summarized by the same authors (Figure 1) (2):

- *Deregulating cellular energetics.* Cancer cells can reprogram their energy metabolism and favor glycolysis even when oxygen is present, which distinguish them from normal cells where glycolysis is favored during anaerobic conditions (2).
- *Avoiding immune destruction.* The immune system serves as a barrier to tumor formation, where it detects most cancer cells and eradicates them before they are able to form a solid tumor (2). This theory is strengthened by studies of immunodeficient mice, which were observed to develop tumors more frequently as compared to their immunocompetent counterparts (2).
- *Genome instability and mutation.* Tumor development and progression is driven by alterations in the genome. Cancer cells often display higher mutation rates as compared to normal cells, since several components of the DNA damage detection and DNA repair system can be affected (2). Another source for altered gene expression are epigenetic modifications, for example altered DNA methylation and histone modifications (2).
- *Tumor-promoting inflammation.* Inflammatory cells are present in virtually all types of tumors (2). It was first believed that this was a way for the immune system to try to eradicate the tumors, but paradoxically, there is also accumulating evidence that tumor inflammation promotes the acquisition of cancer hallmarks (2).

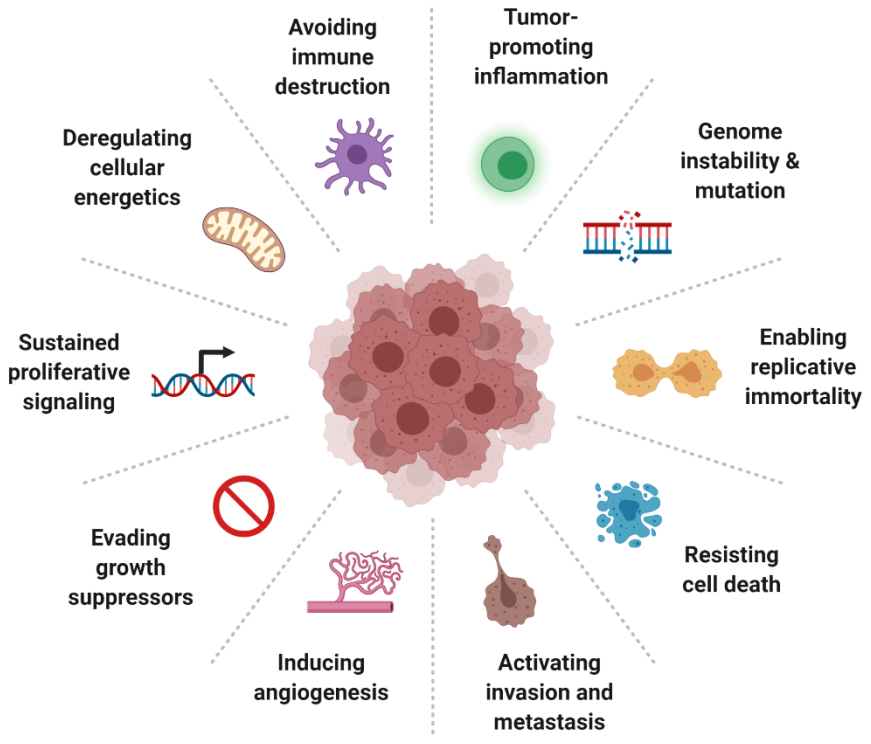

Figure 1. The hallmarks of cancer describe the characteristics of cancer (1, 2). Illustration is modified from Hanahan and Weinberg (2) and created with BioRender.com.

1.1.2 Cancer genes

Cancer development is driven by alterations of the genome. Today, more than 700 genes have been implicated to drive the formation of cancer (3, 4). The genes involved and altered in the process of tumor formation can be categorized into two main types: oncogenes and tumor suppressor genes (5).

1.1.2.1 Oncogenes

In normal cells, proto-oncogenes encode proteins that control cell growth and cell division by receiving, processing and responding to proliferative signals (5, 6). When proto-oncogenes are altered, they can become activated oncogenes which enable continuous cell growth regardless of stimulation from growth signals, thus leading to uncontrolled cell growth and tumorigenesis (5, 6). The genetic alterations causing proto-oncogenes to become activated oncogenes are for example mutations, gene amplifications and chromosomal translocations that lead to gain-of-function (Figure 2) (5). *RAS* and *BRAF* are well known examples of proto-oncogenes that can turn into oncogenes through activating mutations (5, 6).

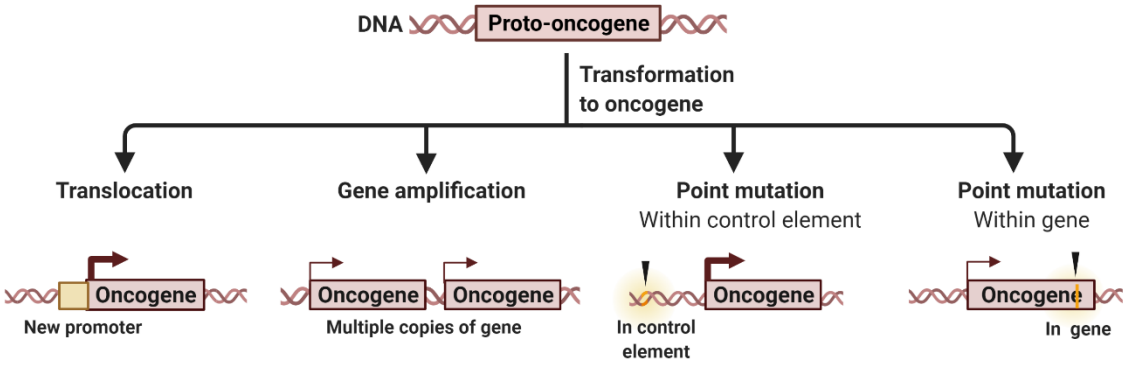

Figure 2. Examples of genetic alterations that can lead to the activation of oncogenes. Created with BioRender.com.

1.1.2.2 Tumor suppressor genes

Tumor suppressor genes are genes that suppress cell proliferation and together with proto-oncogenes they regulate normal cell proliferation to achieve a balance between cell growth and growth suppression (6). In tumor development, these genes can be inactivated leading to disruption of the negative regulation of cell proliferation, thus allowing uncontrolled cell growth (7). Tumor suppressor genes can be inactivated by genetic and epigenetic alterations resulting in loss of function. *TP53* and *RB1* are well known tumor suppressor genes (5-7).

1.1.3 Cancer genetics

Cancer development is coupled to genomic instability and an accumulation of genetic alterations over time (8). The genetic alterations resulting in altered nucleotide sequences are termed mutations. The acquired mutations are classified as somatic mutations while constitutional mutations are inherited from the parents to the offspring. Several types of mutations are found in cancer and they can be classified in several ways. Based on the

consequences of the mutation in cancer formation, mutations can either be “drivers” or “passengers” (8). “Drivers” constitute those mutations that lead to a growth advantage in the evolutionary process of cancer formation and occur in the abovementioned cancer genes (8). “Passengers” are identified in cancer but are not associated with a growth advantage (8). Mutations can further be classified after their size and how they impact DNA structure as described below (9).

1.1.3.1 Single-base substitutions

Single-base substitutions affect one nucleotide leading to a change in the sequence (9). These single-base changes can be synonymous, that do not affect the amino acid sequence, or non-synonymous that lead to an alteration of the amino acid sequence. Non-synonymous mutations can be missense mutations which lead to a change in amino acid or nonsense mutations that result in a premature stop-codon and a shortened protein. In solid tumors, most of the identified mutations are single-base substitutions of which a large majority consist of missense mutations and a smaller proportion are nonsense mutations or affect splice sites (10).

1.1.3.2 Short insertions and deletions (indels)

Indels refers to changes in the nucleotide sequence with either gain of nucleotides (insertion) or loss of nucleotides (deletion). These insertions and deletions are regarded as small-scale mutations (9). In coding regions, indels not occurring in multiples of three lead to frame-shift mutations. These mutations cause a change in the reading frame, resulting in altered translation and proteins (11). Small insertions and deletions are found in cancers causing alterations in cancer genes (8, 12).

1.1.3.3 Structural variants

Structural variants are large-scale mutations and include deletions, insertions, duplications, inversions and translocations (9). These types of alterations are also found in cancer. For example, deletions can occur in tumor suppressor genes while amplifications can occur for oncogenes. In addition, translocations can produce oncogenes by fusing two genes; the *BCR-ABL* fusion gene in chronic myeloid leukemia is a well-known example of this phenomenon (8, 10).

1.1.4 Cancer epigenetics

An epigenetic trait is defined as “a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence” (13). Epigenetic mechanisms play an important role in the regulation of gene expression in normal cells where epigenetic modifications can result in altered gene expression levels. Epigenetic changes are observed in cancer cells and can contribute to cancer development and progression (14-16). Several types of epigenetic mechanisms have been studied in cancer, including DNA methylation, histone modification and RNA-mediated gene regulation as described below (17).

1.1.4.1 DNA methylation

DNA methylation often occur at cytosine guanine dinucleotide sites (CpG sites) by adding a methyl group to the cytosine. Regions that are concentrated with CpG sites are called CpG islands and are usually found in promoter regions. Generally, methylation of these promoter regions will lead to gene silencing while promoters of transcriptionally active genes are unmethylated (18). In cancer, both hypomethylation and hypermethylation are found (16). Global hypomethylation has been observed when comparing tumors with normal tissue, and it is thought that it leads to genomic instability (18). Hypomethylation can also lead to the activation of oncogenes, although this is less frequently observed (18). Hypermethylation, on the other hand, can occur in the promoter region of specific tumor suppressor genes resulting in gene silencing (16, 18).

1.1.4.2 Histone modification

Histones are proteins that DNA wraps around to form nucleosomes, a subunit of chromatin. Histones can be chemically modified by for example methylation, acetylation and phosphorylation, where these modifications can regulate gene transcription (19). In cancer, proteins involved in modifying histones can be identified as dysregulated (15).

1.1.4.3 Non-coding RNA

Non-coding RNAs (ncRNA) have been identified as important epigenetic regulators, involved in many biological systems that can regulate gene expression. Regulation of transcription, translation, RNA processing and chromatin structure are examples of the different functions in which ncRNAs have been identified to play a key role (20, 21). The ncRNAs encompass several different types of RNA with different functions and length, and can be categorized into small ncRNA that are <200 nucleotides, or long ncRNA (lncRNA) (22, 23).

MicroRNA (miRNA) is a class of small ncRNA that has been studied and implicated in cancer development and progression (24). The biogenesis of miRNA involves several steps, including nuclear processing of primary miRNA to precursor miRNA, and cytoplasmic processing of precursor miRNA to mature miRNA by the ribonuclease III enzymes Drosha and Dicer, respectively (25). Mature miRNAs are approximately 22 nucleotides in length and function at the post-transcriptional level by binding to complementary sequences of mRNA, leading to its degradation or repression of its translation (25, 26). Dysregulation of miRNA expression profiles has been observed across various types of cancer and specific miRNAs have been shown to act as both oncogenes and tumor-suppressors depending on their target genes (24, 27).

1.2 TUMORS OF THE THYROID GLAND

The thyroid is an endocrine organ which secretes hormones essential for metabolism, growth and development. It is located in front of the trachea and is composed of two lobes interconnected by a medial portion named isthmus (Figure 3). Thyroid tissue constitutes of thyroid follicles surrounded by follicular cells, which produce, store and secrete thyroid

hormones (28). The secreted hormone is predominantly thyroxine (T4), that mainly acts as a prohormone and is converted to the biologically active triiodothyronine (T3) outside the thyroid. The follicles contain colloid, which in turn consist of mainly thyroglobulin. Parafollicular cells (C-cells), located in between the follicles, produce and secrete the hormone calcitonin, which is involved in calcium metabolism (Figure 3) (28).

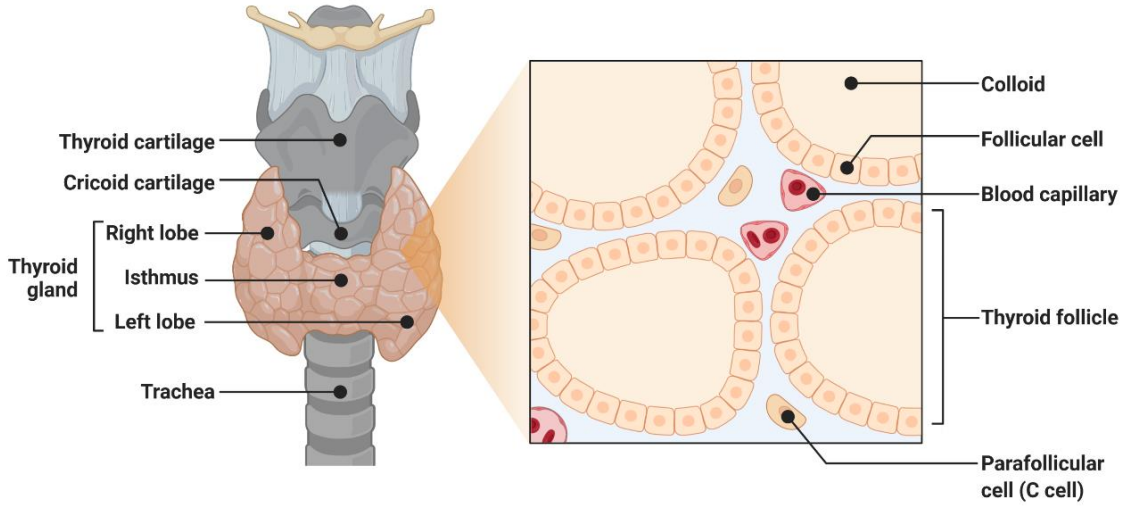

Figure 3. The anatomy and histology of the thyroid gland. Created with BioRender.com.

1.2.1 Classification of thyroid tumors

There are two main types of thyroid tumors: benign adenomas and malignant carcinomas (29). Although thyroid carcinoma is less common than adenoma, it is the most common cancer of endocrine organs with an increasing incidence reported during the last three decades (29-31). Tumors of the thyroid can be classified according to their cellular origin, where the majority originate from the follicular cells (29, 32).

Follicular thyroid adenoma (FTA) is a benign follicular cell-derived thyroid tumor while follicular tumor of uncertain malignant potential (FT-UMP) is a tumor entity which is histologically equivocal, although the majority of patients are relapse-free (29). Follicular cell-derived thyroid carcinomas have traditionally been categorized into the well-differentiated carcinomas - papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) - and the undifferentiated anaplastic thyroid carcinoma (ATC) (29). Poorly differentiated thyroid carcinoma (PDTC) serves as an intermediate between the well-differentiated and undifferentiated carcinomas both histopathologically and clinically, and is regarded as a separate tumor type (29). Thyroid carcinoma originating from parafollicular cells is called medullary thyroid carcinoma (MTC) (29, 32).

1.2.2 Papillary thyroid carcinoma (PTC)

PTC is the most common thyroid malignancy, accounting for 80-90% of all manifest thyroid carcinomas (29, 33, 34). The increased incidence of thyroid cancer is mainly due to an observed increase of PTCs, largely attributed to improved diagnostic methods and intensified screening (30, 31, 34). Microscopically, the two distinct features of conventional PTC are specific nuclear

features and a papillary growth pattern (29). Several variants of PTC exist, including papillary microcarcinoma, follicular variant and tall cell variant (29). The prognosis for patients with PTC is generally excellent, with a 10-year survival rate at approximately 95% (35-37). However, clinical staging greatly influences the prognosis, with 99.8% survival rate reported for stage I and 41% survival rate for stage IV at 10-year follow-up (38). Other important clinical prognostic factors include older age at diagnosis, larger tumor size, extrathyroidal growth, distant metastasis and tumor type/variant (39). Recurrence rate is estimated to approximately 15% and occur most frequently within the first decade of diagnosis (40). Predictive factors of recurrence include incomplete surgical excision, lymph node metastasis and extranodal extension (41).

1.2.3 Follicular thyroid carcinoma (FTC)

The second most common type of thyroid carcinoma is FTC, accounting for approximately 6-10% of the manifest thyroid malignancies (29, 42, 43). The incidence of FTC has also been increasing during the last decades, however, to a much lesser extent than PTC (34, 42-44). By definition, FTC lacks the specific nuclear features distinctive of PTC and display capsular and/or vascular invasion in contrast to FTA (29). Traditionally, FTC has been sub-classified as either minimally or widely invasive (32). However, in the 2017 WHO classification system, a subgroup of FTCs displaying vascular invasion but with no or only limited capsular invasion are being referred to as encapsulated angioinvasive FTC (29). The 10-year survival rate is approximately 80% for FTC (45) and prognostic factors include the widely invasive sub-type, older age at diagnosis, larger tumor size, extrathyroidal extension and distant metastasis (46-50).

1.2.4 Hürthle cell carcinoma (HCC)

Hürthle cell tumors have traditionally been regarded as a histopathological variant of follicular thyroid tumors, where >75% of the cells are Hürthle cells, also referred to as oncocytic or oxyphilic cells (32). Since the implementation of the 2017 WHO classification, HCC and Hürthle cell adenoma (HCA) are now separate tumor types (29). Similar to FTC, HCC exhibits capsular and/or vascular invasion as opposed to its non-invasive benign counterpart HCA (29). It has been reported that HCC is coupled to a more aggressive behavior, including older age at diagnosis, larger tumors, higher disease stages and shorter overall and disease-specific survival as compared to other well-differentiated thyroid carcinomas (51, 52). Furthermore, HCC does not respond as well to radioiodine treatment as conventional FTCs, limiting the treatment options (53-55).

1.2.5 Poorly differentiated thyroid carcinoma (PDTC)

As mentioned, PDTC serves as an intermediate between the well-differentiated and the undifferentiated thyroid carcinomas (29). Although many PDTCs are a product of dedifferentiation from FTC or PTC, some have also been suggested to arise *de novo* (56-58). Depending on geographical region, the prevalence of PDTC is reported to be approximately 0.8-6.7% of all thyroid malignancies (56, 57). PDTC are aggressive carcinomas, where lymph

node metastasis is observed in 15-65% and distant metastasis in 40-70% of the patients (56, 59-61). PDTC has a less favorable outcome compared to the well-differentiated carcinomas, with 5-year overall survival rate estimated to 50-70% (29, 56, 59-61).

1.2.6 Anaplastic thyroid carcinoma (ATC)

ATC is an uncommon but highly aggressive, undifferentiated form of thyroid carcinoma, accounting for around 1-2% of all thyroid carcinomas (58, 62). ATC can have a variety of histological features including spindle cells and giant cells, and mitotic figures and necrotic areas are plentiful (29). Most often, ATC arises from a previously well-differentiated carcinoma or PDTC (62). ATC usually presents clinically as a fast-growing tumor mass with infiltrative growth and symptoms such as difficulty in breathing, dysphagia, hoarseness and neck pain (62). It is by far the most aggressive type of thyroid carcinoma with a high mortality rate at >90% and the majority of patients die within the first year (62, 63). At the time of diagnosis, around 50% of the patients present with distant metastasis, usually to the lung, bone or brain (62).

1.2.7 Medullary thyroid carcinoma (MTC)

In contrast to the abovementioned carcinomas, which all derive from follicular cells, MTC originates from parafollicular cells of the thyroid (29). It is an uncommon form of thyroid carcinoma, accounting for approximately 2 % (33). Approximately 25-30% of all manifest MTC are hereditary and a part of the multiple endocrine neoplasia type 2 (MEN 2) tumor syndromes (64, 65). The MEN 2 tumor syndromes exhibit autosomal dominant inheritance, and the causative germline mutations are found in the *RET* proto-oncogene in these patients (66).

1.2.8 Follicular thyroid adenoma (FTA)

FTAs are common benign, non-invasive and encapsulated follicular thyroid tumors (29). Its incidence has been estimated to 3-5% based on autopsies (67, 68). Microscopically, FTAs are surrounded by a fibrous capsule and capsular and vascular invasion is absent (29). The only way to distinguish FTA from FTC is by histopathological evaluation excluding capsular and vascular invasion (29). Fine needle aspiration (FNA) cytology cannot distinguish these features, posing a pre-operative diagnostic challenge for follicular thyroid tumors (69, 70). Hence, a diagnostic lobectomy has to be performed to allow for a histopathological diagnosis of FTA or FTC (71). In cases of FTA, no additional treatment is required, while a completion lobectomy is performed for most cases of FTC (71). Taken together, additional diagnostic markers are needed to improve the pre-operative diagnosis for this group of patients.

1.2.9 Follicular tumor of uncertain malignant potential (FT-UMP)

In cases where capsular and vascular invasion is dubious and the distinction between FTA and FTC is indeterminate after thorough sampling, the tumors are termed as “follicular tumor of uncertain malignant potential” (FT-UMP) (29). Previously, this group was referred to as atypical follicular thyroid adenoma (AFTA) (32). Similar to FTA and FTC, FT-UMP cannot

be distinguished pre-operatively from other follicular thyroid tumor types (29, 70), and are usually treated in the same way as FTA (72).

1.2.10 Clinical management of thyroid tumors

1.2.10.1 Clinical presentation and diagnosis

Patients with thyroid tumors are typically rather asymptomatic, euthyroid and present with a thyroid nodule either detected by the patient or by a physical examination. The enlargement of the thyroid nodule can cause local symptoms such as dysphagia, vocal changes, swelling and hoarseness. With the increased usage of imaging techniques in the past decades, more patients with subclinical thyroid tumors are also incidentally discovered (73).

Apart from patient medical history and physical examination, the initial diagnosis of suspected thyroid nodules should include ultrasonography and FNA cytology (71, 72). Ultrasonography of the thyroid and cervical lymph nodes is performed to characterize the thyroid gland and the nodule regarding size, location and sonographic features (71). The sonographic features can be reported according to thyroid imaging and reporting data systems (e.g. EU-TIRADS) for malignancy risk stratification and further provide guidance in the decision making of proceeded FNA (74).

FNA cytology has a central role in the initial diagnosis of thyroid nodules and is reported after the established and standardized Bethesda system into six diagnostic categories: (I) non-diagnostic or unsatisfactory; (II) benign; (III) atypia of undetermined significance or follicular lesion of undetermined significance; (IV) follicular neoplasm or suspicious for a follicular neoplasm; (V) suspicious for malignancy; and (VI) malignant (70). The risk of carcinoma is substantially increased in higher Bethesda categories (70). However, as mentioned, FNA cannot distinguish FTC or HCC from benign follicular lesions and these tumors are most often classified in the indeterminate Bethesda categories III or IV (69, 70). The final diagnosis of thyroid tumors is based on the WHO classification system after a histopathological examination of the tumor specimen (29).

1.2.10.2 Staging and treatment of thyroid carcinomas

Staging is usually performed according to the American Joint Committee on Cancer (AJCC) staging manual to predict prognosis and determine the best treatment option. The TNM staging system is based on a standardized classification to describe the extent of the cancer disease in primary tumor (T), lymph node metastasis (N) and distant metastasis (M) (75). Based on the AJCC staging and risk-stratification in current national and international guidelines, different treatment options are available for patients with thyroid carcinoma (71, 72, 75, 76).

In most patients with thyroid carcinoma, a total thyroidectomy is performed as the primary treatment. However, exceptions can be made for uncomplicated cases of PTC $\leq$ 1 cm and minimally invasive FTC < 2 cm, where lobectomy is sufficient (72). Lymph node dissections are not performed routinely, but is performed when lymph node metastases are detected and in

PTC cases with advanced tumor stages (T3 and T4). Radioactive iodine treatment can be given to patients with FTC, PTC and PDTC as remnant ablation meant to facilitate the detection of recurrent disease by measurement of thyroglobulin or scintigraphy, and to decrease the risk of relapse. In addition, external radiation and systemic treatment with tyrosine kinase inhibitor are treatment options for patients with advanced thyroid cancer (72).

1.3 GENETIC BACKGROUND OF FOLLICULAR CELL-DERIVED THYROID CARCINOMAS

In recent research, it has become more apparent that the underlying forces that drives thyroid carcinoma development and progression are genetic and epigenetic changes in some key signaling pathways. Important signaling pathways implicated in thyroid cancer development include the RAS/RAF/MEK/ERK/MAP kinase (MAPK) pathway which plays a crucial role in cell proliferation, and the PI3K-AKT pathway which is thought to promote invasion (77). Alterations affecting these pathways such as mutations, gene amplifications and translocations can have a synergistic effect on tumor progression and are interesting targets for diagnostic, prognostic and therapeutic purposes (77). Recurrent genetic alterations in follicular-cell derived thyroid carcinomas are described below.

1.3.1 Genetic alterations in papillary thyroid carcinoma

Recently published data by The Cancer Genome Atlas (TCGA) Research Network, demonstrated that the somatic mutation burden of PTC is lower compared to carcinomas arising from other organs (78). Most of the genetic alterations found in PTC activate the MAPK-signaling pathway, which regulates the expression of genes associated with cell proliferation, apoptosis and cell metabolism (77). Mutations in *BRAF* and *RAS* are common, generally mutually exclusive and both can activate the MAPK-signaling pathway (77).

BRAF is one of three *RAF* genes that code for serine/threonine kinases involved in the MAPK-pathway. Approximately 30-90% of all PTC display a *BRAF* mutation, most commonly the driver mutation $BRAF^{V600E}$ (77, 79). The mutated *BRAF* results in elevated expression and continuous activation of the kinase (80). The $BRAF^{V600E}$ mutation in PTC has been reported to have prognostic and predictive value in some studies but not in others (79, 81-85).

The three *RAS* genes (*NRAS, HRAS, KRAS*) code for small GTPases involved in signal transmission in both the MAPK and PI3K-AKT pathways (86). In PTC, activating *RAS* hotspot mutations are most common in the follicular variant (15-35%), but also occur in classic/conventional PTC (0-15%) (29, 78, 87).

The proto-oncogene *RET* encodes a receptor tyrosine kinase. Chromosomal rearrangements of the *RET* gene lead to fusion genes with continuous activation of the tyrosine kinase, which can in turn activate both the MAPK and PI3K-AKT pathways (77). Over ten different variants of *RET* fusion genes in PTC have been identified, termed *RET/PTC*, and they constitute the most common chromosomal rearrangements in PTC, with a prevalence of 5-35% (29, 77, 88).

The two hotspot mutations C250T and C228T in the *TERT* promoter are also found in PTC with a prevalence of 10-30% (89). The mutations are reported to be mutually exclusive and associated to an aggressive phenotype with poor clinical outcome (78, 89).

1.3.2 Genetic alterations in follicular thyroid carcinoma

The most common somatic mutations in FTC are *RAS* mutations (30-50%), which are also found in the benign FTA (20-40%) (29, 77, 90), suggesting that these mutations are early genetic events (77). The mutant *NRAS* has been reported as the most prevalent form among the three isoforms (87).

The second most common genetic event in FTC is the paired box 8 *(PAX8)* - peroxisome proliferator activated receptor γ *(PPARγ)* chromosomal rearrangement, occurring in approximately one third of FTC (91-93). *PAX8* codes for a transcription factor important for the differentiation of follicular thyroid cells, while *PPARγ* codes for a steroid/thyroid nuclear receptor. This fusion gene has also been reported in FTA, although the frequency is much lower (94). Gene fusion of *CREB3L2-PPARγ* has also been identified in FTC, although with lower frequency compared to *PAX8- PPARγ* (95).

Other genetic events related to FTC are commonly affecting and activating the PI3K-AKT pathway (77). For example, *PI3KCA* mutations (5-15%), which are also found in FTA but with lower estimated prevalence (0-5%) and *PTEN* mutations (10-15%) which inactivates the *PTEN* gene and activates the PI3K-AKT pathway (77).

The hotspot *TERT* promoter mutations are also found in approximately 20% of FTCs and are, similar to PTC, associated to a more clinically aggressive phenotype (96-98).

1.3.3 Genetic alterations in poorly differentiated thyroid carcinoma and anaplastic thyroid carcinoma

Compared to the well-differentiated thyroid carcinomas, PDTC and especially ATC carries a higher mutation burden (99, 100). This is suggestive of a progression model in which well-differentiated thyroid carcinoma successively accumulate genetic events leading to dedifferentiation and progression to PDTC and ATC (99, 100).

Except for the early genetic driver events such as *RAS* and *BRAF* mutations, PDTC and ATC both exhibit higher frequencies of *TERT* promoter mutations (40% and 75% respectively) (99, 100). In addition, 10-35% of PDTC and 40-80% of ATC carry *TP53* mutations, which is considered as a major indicator of progressive thyroid cancer and extremely rare events in well-differentiated thyroid carcinomas (29, 100).

1.4 TELOMERASE REACTIVATION

1.4.1 Telomere

The first evidence of the existence of telomeres and their function as protectors of chromosomal end degradation is dated to the 1930s (101-103). The telomere structure consists of telomeric

DNA that is bound to associated binding proteins. The most important telomere binding protein complex is shelterin, which consists of six proteins that either bind directly to telomeric DNA or interact to form a complex (104). The human telomeric DNA is formed by double stranded short tandem repeats (TTAGGG) with single stranded G-rich overhangs towards the 3' end. The 3' overhangs can form a T-loop by binding to the double stranded DNA repeats, thereby stabilizing the chromosome (101).

The main function of the telomeres and their associated binding protein complex are to protect the chromosomal ends from damage by end-to-end fusions, double-strand breaks and degradation, since the DNA polymerase is not able to fully replicate the chromosomal ends at the lagging strand (105, 106). In most normal human cells, the telomeres are successively shortened during each cell division. When the telomeres are shortened to the point where they no longer can protect chromosomal DNA, the cell will reach a critical point and enter senescence or crisis (105). The telomeres are thus critical for life-span control of the cells, preventing uncontrolled growth and immortalization (107).

1.4.2 Telomerase

Telomerase was discovered in the 1980s by Greider and Blackburn, for which they received the Nobel Prize in 2009 (108). Telomerase is an enzyme with reverse transcriptase activity that can synthesize telomeres by adding DNA (TTAGGG) repeats to the 3' end of the chromosomes. Telomerase is a ribonucleoprotein complex and consist of a telomerase RNA component (*TERC*) which acts as a template, and telomerase reverse transcriptase (*TERT*), the catalytic subunit of the enzyme (108, 109). As *TERT* is the rate-limiting subunit of telomerase, *TERT* expression is most often associated to telomerase activity (110, 111). While *TERC* is generally expressed in normal cells, *TERT* is expressed in germ cells, stem cells and activated lymphocytes but generally not expressed in normal differentiated cells (112, 113).

1.4.3 Telomerase reactivation in cancer

Telomerase reactivation is an important mechanism for cancer cells to obtain, as they have to overcome the proliferative barrier posed by the successive shortening of telomeres (Figure 4) (1). Telomerase activity is detectable in approximately 90% of all cancers (114). The telomerase-independent mechanism of alternative lengthening of telomeres (ALT) is also present in approximately 10% of cancers with telomere maintenance functions, but in a much lower frequency as compared to telomerase reactivation (113). Activation of telomerase is highly dependent on the activation of *TERT*, as the induction of *TERT* has been shown to be sufficient to induce telomerase activity (113). In addition to its telomere maintenance functions, telomerase and *TERT* activation have also been implicated to have other tumor-promoting effects related to gene expression regulation, cell proliferation, apoptosis and migration (115, 116). As telomerase activity is highly correlated with *TERT* induction (111), *TERT* gene regulation and alterations serve as important underlying mechanisms for telomerase reactivation (117).

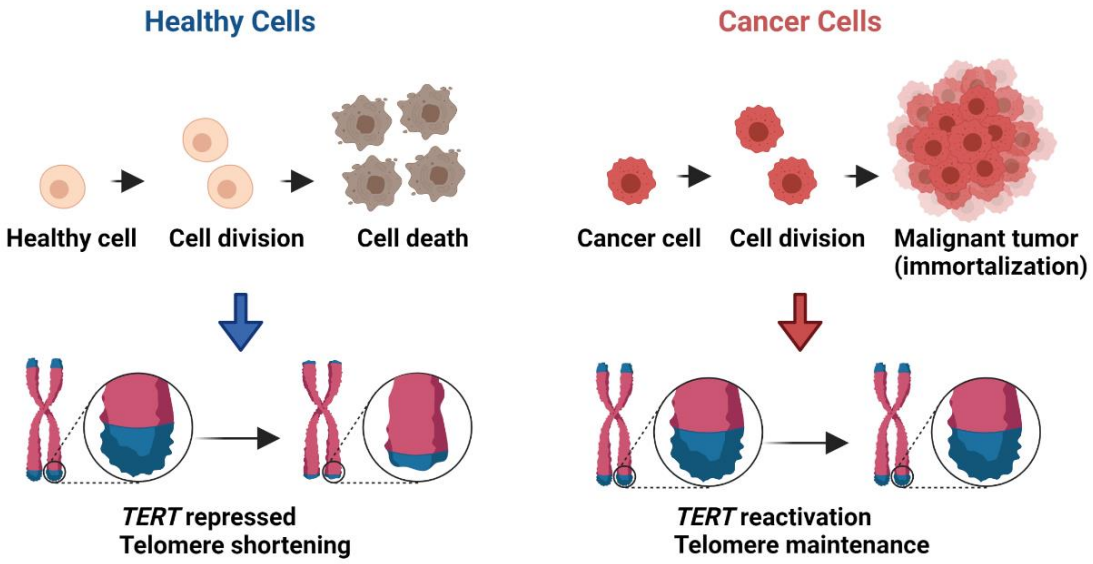

Figure 4. Telomerase and *TERT* reactivation is important for cancer development and progression. Created with Biorender.com.

1.4.4 Regulation of TERT

1.4.4.1 Transcriptional regulation

The *TERT* gene expression can be regulated at the transcriptional level (118). In humans, the single copy *TERT* gene is 42 kb long, consist of 16 exons and 15 introns and is located at chromosomal region 5p15.33 (119, 120). The *TERT* gene has one single promoter that is GC-rich and contains several E-boxes and GC-boxes that are binding motifs for transcription factors involved in its regulation (121). For example, the E-boxes have been shown to be direct binding sites for the oncogenic transcription factor c-Myc, which can activate and upregulate *TERT* (122). Moreover, the zinc finger transcription factor Sp1 has been demonstrated to bind to the GC-boxes on the *TERT* promoter to activate *TERT* transcription (123). Several repressors of *TERT* transcription have also been identified, for example Mad and TP53 (124, 125).

1.4.4.2 Alternative splicing

TERT is also regulated at the post-transcriptional level by alternative splicing (126). Over 20 alternative splice variants of *TERT* have been identified, but the function for many of these remains largely unknown (126-129). Of the more well-studied transcripts, the α and β splice sites generate a 36 bp and 182 bp deletion, respectively. None of these two transcripts leads to telomerase activity (130), and the activity of telomerase has been largely attributed to the expression of full-length *TERT* transcript (131-133).

1.4.5 Genetic alterations of TERT

1.4.5.1 Hotspot TERT promoter mutations

Another important mechanism for telomerase reactivation is the two hotspot *TERT* promoter mutations (134-136). The two mutations are referred to as C228T and C250T since they are located at chromosome 5, positions 1,295,228 and 1,295,250 (GRCh37) or -124 bp and -146 bp from the ATG site, respectively (134, 135). The mutations are cytidine-to-thymidine transitions and occur in a mutually exclusive manner, where C228T is more prevalent than C250T (136). Their occurrence creates *de novo* binding sites for E26 transformation-specific (ETS) transcription factors, which when binding promotes *TERT* transcription and subsequently results in telomerase activity (134, 135, 137).

In 2013, the mutations were initially discovered in malignant melanoma (134, 135) and have since been identified as a recurrent genetic event across multiple types of cancer (136, 138). The overall frequency of *TERT* promoter mutation in cancer was 27% based on analyses of 31 cancer types (136). Thus, these mutations represent the most common non-coding driver mutations in cancer that has been identified so far (117, 136, 139). Glioblastoma, melanoma, bladder cancer and hepatocellular carcinoma are among cancer types that exhibit the highest *TERT* promoter mutations frequencies (117, 136, 140). In addition, *TERT* promoter mutations have been associated with poor clinical outcome in several types of cancer, including melanoma (141), glioma (142) and thyroid cancer (97, 143). A schematic illustration of the two hotspot *TERT* promoter mutations is shown in Figure 5.

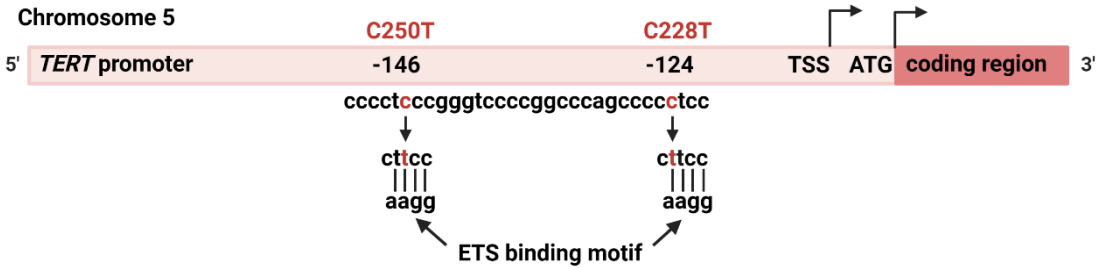

Figure 5. The location of the two hotspot *TERT* promoter mutations C250T and C228T. TSS: transcription start site. ATG: translation start site. ETS: E26 transformation-specific.

1.4.5.2 Gene amplifications

Genetic aberrations of the *TERT* include amplifications of the *TERT* gene, which have been demonstrated in many types of human cancer and are associated with *TERT* induction and telomerase activity (144, 145). In a large pan-cancer analysis of 31 cancer types, *TERT* amplifications were observed in 4% of all cancers, with the highest rates in ovarian cancer (22%), lung cancer (14%), esophageal carcinoma (14%) and adrenocortical carcinoma (15%) (136). In this large cohort, *TERT* amplification was coupled with *TERT* expression, thus serving as an important genetic mechanism for *TERT* reactivation in cancer (136).

1.4.5.3 Chromosomal rearrangement

The *TERT* locus can also be affected by chromosomal rearrangements, which have been observed and reported in some cancers (136, 146, 147). Such *TERT* rearrangements are often found to cause the reposition of enhancer elements to the vicinity of *TERT* coding sequence, leading to *TERT* transcription (136, 146, 147). In neuroblastoma, these rearrangements have been coupled with aggressive tumors with poor outcome (146, 147).

1.4.6 Epigenetic alterations of TERT

1.4.6.1 DNA methylation

Epigenetic alterations of the *TERT* gene locus, such as altered DNA methylation in the *TERT* promoter have been identified (136, 148). DNA methylation is most commonly associated with gene silencing. Paradoxically, hypermethylation of the *TERT* promoter is coupled with *TERT* expression and telomerase activity in various types of cancer (136, 148-151). For example, *TERT* promoter hypermethylation was found in glioma (150) and prostate cancer (152) while normal or benign tissues were generally not hypermethylated (150, 152). Recently, a 433 bp region in the *TERT* promoter consisting of 52 CpG sites named *TERT* hypermethylated oncological region (THOR) was described as regulatory of *TERT* expression independently or together with *TERT* promoter mutation in various types of cancer (148). Interestingly, THOR methylation was more common among cancer types, such as prostate cancer and breast cancer, where *TERT* promoter mutations are rare (148).

1.4.6.2 Other epigenetic mechanisms

In addition to the abovementioned mechanisms that can activate or regulate *TERT*, several other epigenetic mechanisms have been described to be involved. For example, histone acetylation (153) and histone methylation (154) have been implicated to play a role in *TERT* regulation through interaction with other *TERT* regulating factors such as transcription factors and *TERT* promoter mutation and methylation, respectively. Moreover, miRNAs may play a role in *TERT* regulation (155).

1.4.7 TERT in follicular cell-derived thyroid carcinomas

Telomerase reactivation and *TERT* expression has been studied in thyroid carcinomas, where 20-100% of PTC and 30-100% of FTC and ATC express *TERT* or display telomerase activity (156-163). However, some of these studies also identified *TERT* expression or telomerase activity in benign thyroid lesions, although generally occurring in lower frequencies (156-163). The possible explanation for this wide range of variation and occurrence in benign lesions has been attributed to contaminating lymphocytes in the thyroid specimen, which generally express *TERT* (157-161, 163). As for alternative splicing of *TERT*, although the number of studies is limited, the full-length *TERT* transcript has been associated with telomerase activity and found to be more abundant in follicular cell-derived carcinomas than in benign thyroid lesions (133).

After the identification of *TERT* promoter mutations, numerous studies have shown their prevalence in follicular-cell derived thyroid carcinomas (78, 96, 98, 138, 143, 164). The association of *TERT* promoter mutations with aggressive phenotype has consistently been shown in PTC and FTC concerning: higher recurrence and mortality rates; older age; larger tumor sizes; higher stages; distant metastasis and extrathyroidal extension (61, 98, 165). The mutations are also more frequent in PDTC and ATC as compared to the less aggressive PTC and FTC, supporting their role in tumor progression (61, 98, 100, 166). The *TERT* promoter mutations are thus promising targets for diagnostic and prognostic purposes (98).

Moreover, it has been shown that PTCs with concurrent $BRAF^{V600E}$ and *TERT* promoter mutations have the least favorable outcome (167-172). Although limited in number, studies in PTC and other cancer types, such as melanoma and glioma, have proposed a possible mechanistic link between $BRAF^{V600E}$ and *TERT* promoter mutations. This suggested mechanism involves the BRAF-induced activation of the MAPK pathway which may lead to the upregulation of some of the ETS transcription factors, such as *ETS1*, *ETV1*, *ETV4* and *ETV5*, which in turn can bind to the mutant *TERT* promoter (173-176).

Other genetic *TERT* alterations that have been reported in follicular cell-derived thyroid carcinomas are copy number amplifications and *TERT* rearrangements (177, 178). However, these alterations occur in much lower frequencies than *TERT* promoter mutations and are less extensively studied (177, 178). Similarly, *TERT* promoter hypermethylation has been identified in subsets of thyroid carcinomas, but remains to be further explored (148, 179).

1.4.8 ETS transcription factors

The ETS factors constitute one of the largest transcription factor families with 28 members identified in humans (180). They are well-conserved and share homologous DNA-binding domains named ETS domains, which can recognize and bind to 5′-GGA(A/T)-3′ DNA sequences (180, 181). Based on the ETS domain homology, they can be further sub-classified into twelve sub-families (Table 1) (180, 181). Due to the similarity of the ETS domain, many of the ETS factors have partially overlapping target genes and functions, although specificity for the ETS factors also exist (180, 182). They can act as both transcriptional activators and repressors and have a wide range of target genes (182). Through their target genes, they are involved in regulating many important biological processes including proliferation, differentiation and apoptosis (181). Thus, disruption in their expression and functions have been associated with cancer development and progression (183).

Several underlying mechanisms for the dysregulation of ETS factors in cancer have been identified (183). For example, chromosomal rearrangement and amplifications of ETS factors have been found in Ewing sarcoma and prostate cancer, and melanoma, respectively (184-186). ETS factors are also frequently observed as either overexpressed or coupled with loss of tumor suppressive functions across many cancer types (183). Moreover, as mentioned, the ETS transcription factors can serve as activators of the mutant *TERT* promoter. To date, several ETS factors have been implicated in activating the mutant *TERT* promoter, including *GABPA*,

ETV1, *ETV4*, *ETV5*, *ELF1*, *ELF2*, *ETV6*, *ETS1* and *ETS2* (137, 175, 176, 187, 188). The ETS transcription factors, their role in regulating mutant *TERT* promoter and examples of their role in various types of cancer are presented in Table 1 (180, 181, 183).

Table 1. List of ETS transcription factors that could potentially bind mutant *TERT* promoter and are implicated in cancer (137, 175, 183, 186-212).

Sub-family	ETS factor	Shown to bind mutant *TERT* promoter in	Example of implication in cancer type	Associated alteration
SPI	SPI1		Hematological malignancies	Fusion gene
	SPIB		Hematological malignancies	Oncogenic
	SPIC			
TEL	ETV6	Melanoma	Thyroid carcinoma	Fusion gene
	ETV7		Hematological malignancies	Oncogenic
ESE	ELF3		Colorectal cancer	Oncogenic
	EHF		Thyroid carcinoma	Oncogenic
	ELF5		Breast cancer	Tumor suppressive
ELF	ELF4		Ovarian cancer	Oncogenic
	ELF2	Melanoma	Hematological malignancies	Tumor suppressive
	ELF1	Melanoma	Prostate cancer	Tumor suppressive
PDEF	SPDEF		Breast cancer	Tumor suppressive
PEA3	ETV1	Thyroid	Melanoma	Amplification
	ETV5	Thyroid	Prostate cancer	Fusion gene
	ETV4	Thyroid	Prostate cancer	Fusion gene
TCF	ELK3		Breast cancer	Oncogenic
	ELK4		Prostate cancer	Fusion gene
	ELK1		Prostate cancer	Oncogenic
ETS	ETS1	Glioblastoma	Breast cancer	Oncogenic
	ETS2	Glioblastoma	Breast cancer	Oncogenic
ETV	ETV2		Melanoma	Oncogenic
ERF	ETV3		Breast cancer	Amplification
	ETV3L			
	ERF		Prostate cancer	Tumor suppressive
ERG	ERG		Ewing sarcoma	Fusion gene
	FLI1		Ewing sarcoma	Fusion gene
	FEV		Ewing sarcoma	Fusion gene
GABP	GABPA	Glioblastoma	Prostate cancer	Oncogenic

2 RESEARCH AIMS

The general aim was to identify potential genetic and molecular markers for diagnosis and prognostication of follicular-cell derived thyroid tumors and to elucidate how *TERT*-related alterations affect progression of these tumors.

The specific aim of each study was:

I. To explore if Ki-67 proliferation index in fine needle aspiration cytology adds diagnostic value in follicular thyroid tumors

II. To establish the occurrence and role of *TERT* alterations in follicular thyroid tumors in relation to diagnosis and prognosis

III. To elucidate the role of the ETS transcription factor GABPA in relation to *TERT* promoter mutation and clinical phenotype in PTC

IV. To investigate the role of the known ETS transcription factors in relation to clinical phenotype, $BRAF^{V600E}$ and *TERT* promoter mutation in PTC

3 MATERIALS AND METHODS

3.1 PATIENT COHORTS AND TUMOR SAMPLES

Patient and tumor tissue cohorts from the Karolinska University Hospital were used for all included studies. The Karolinska University Hospital has been collecting blood and tumor tissue samples from patients operated for endocrine tumors at Karolinska University Hospital since 1986. After surgery and routine histopathological examination of tumors, parts of the tumor specimens were snap frozen in liquid nitrogen and stored at -80 °C in the biobank until use for research purposes. All tumor tissue samples used in the studies were collected from the local biobank and reviewed by an experienced pathologist for tumor cell representation before inclusion in the studies. All patients gave their informed consent prior to sample collection.

3.1.1 Follicular thyroid tumor cohorts (Paper I and Paper II)

In Paper I, two separate patient cohorts of follicular thyroid tumors (and Hürthle cell tumors), diagnosed and operated at Karolinska University Hospital during different time periods, were analyzed. The first cohort (Cohort A) consisted of 234 patients diagnosed between 2006 – 2017 and were identified through either the Scandinavian Quality Register for Thyroid, Parathyroid and Adrenal surgery or a local database at the Department of Pathology. A second cohort (Cohort B) was included as a validation cohort, consisting of a total of 109 patients. Cohort B was previously characterized and included patients were diagnosed between 1987-2005 (213). Clinical data including age at diagnosis, gender, cytological and histopathological information were collected for both cohorts.

In Paper II, a total of 171 tumor samples from patients with follicular thyroid tumors (and Hürthle cell tumors) were analyzed. In addition, 12 samples of multi-nodular goiter were analyzed as non-tumorous reference. All tumor tissue samples were collected between 1986-2017. Clinical information was retrieved from medical records.

3.1.2 Papillary thyroid carcinoma cohort (Paper III and Paper IV)

In Paper III and IV, a cohort consisting of 93 PTC tumor samples were included and analyzed. Tumor specimens were collected for patients operated between 1987-2005 and clinical data was retrieved from medical records. Cases with follicular variant of PTC (FV-PTC) were excluded to avoid inclusion of non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) according to the newer version of the WHO classification of endocrine tumors from 2017 (29).

3.1.3 Anaplastic thyroid carcinoma cohort (Paper III)

A cohort of 18 cases with ATC operated between 1989-2007 was included for analyses in Paper III. Tumor specimens were collected and clinical data was retrieved.

3.1.4 The Cancer Genome Atlas cohort (Paper III and Paper IV)

The Cancer Genome Atlas (TCGA) public data sets of PTCs were used in Paper III and IV. Genomic, transcriptomic and clinical data was retrieved from public sources and analyzed. Cases with FV-PTC were excluded from analysis, in accordance with the Karolinska cohort of PTC.

3.2 CELL LINES

The established human thyroid cancer cell lines used in this thesis are summarized in Table 2. The *BRAF*^{V600E} and *TERT* promoter mutation status of cell lines were previously reported (143, 214-217) and verified in this thesis. Cell lines were authenticated by short tandem repeats (STR) profiling or purchased from the American Type Culture Collection (ATCC).

Table 2. Established cell lines used in this thesis.

Cell line	Origin	Source	*BRAF*^{V600E}	*TERT* promoter mutation	Paper
U-hth-74	ATC	Uppsala University	Wild-type	C228T	III, IV
U-hth-104	ATC	Uppsala University	Mutant	C228T	III, IV
U-hth-112	ATC	Uppsala University	Wild-type	C228T	III, IV
SW1736	ATC	Uppsala University	Mutant	C228T	III, IV
U-hth-7	ATC	Uppsala University	Wild-type	C250T	III
LUTC-1	ATC	Lund University	Wild-type	Wild-type	III
KAT4	Colon cancer	ATCC	Mutant	Wild-type	III
ARO	Colon cancer	ATCC	Mutant	Wild-type	III
MDA-T32	PTC	ATCC	Mutant	C228T	IV
MDA-T41	PTC	ATCC	Mutant	Wild-type	IV

3.2.1 Anaplastic thyroid carcinoma cell lines (Paper III and Paper IV)

The established ATC derived cell lines U-hth-74, U-hth-104, U-hth-112 and SW1736 were used in Paper III and Paper IV. In addition, ATC derived cell lines U-hth-7 and LUTC-1 were

included in Paper III. The KAT4 and ARO cell lines were included as additional *TERT* promoter mutation negative cell lines in Paper III.

3.2.2 Papillary thyroid carcinoma cell lines (Paper IV)

Two established PTC derived cell lines, MDA-T32 and MDA-T41, were used in Paper IV. Both cell lines were purchased from the ATCC.

3.3 METHODS

Various methods were used in this thesis. This section contains the most frequently used methods throughout Paper I-IV. The specific methodologies for each study are summarized in the methods part in each paper.

3.3.1 DNA, RNA and protein preparations

DNA, RNA and protein lysates were prepared from fresh frozen tumor samples and cell lines. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) while total RNA was extracted using mirVana miRNA Isolation Kit (Invitrogen) in Papers II-IV. For Paper III and IV, protein lysates from tumor tissues or cell lines were extracted using Pierce RIPA Buffer (Thermo Scientific).

3.3.2 Polymerase Chain Reaction (PCR)

PCR and PCR-based techniques are commonly used molecular biological methods that are fundamental to genetic research and have a broad range of applications. It was first introduced by Kary Mullis in 1983, for which he was awarded the Nobel Prize in Chemistry in 1993. The method is used to rapidly amplify target regions of DNA sequences and is based on the principle of thermal cycling with repetitive changes in temperature for each cycle. The main steps in each cycle are denaturation which separates double stranded DNA into single stranded DNA, followed by annealing where primers bind to the DNA template and finally elongation where new DNA is synthesized from the template DNA. PCR or PCR-based methods were used in Papers II-IV.

3.3.3 Quantitative PCR (qPCR)

Real-time or quantitative PCR (qPCR) is a widely used method to amplify and quantify specific nucleic acid sequences. For quantification of gene expression, total RNA is first converted to complementary DNA (cDNA) by reverse transcription before qPCR is performed. Quantification is then performed by measuring the amount of fluorescence emitted during PCR amplifications in real time. The fluorescence can be detected by using non-specific fluorescent dyes that bind to double-stranded DNA formed during PCR, an increase of PCR product in each cycle will thus yield an increase of fluorescence emitted. SYBR Green is one commonly used fluorescent dye. Another commonly used method for qPCR is using fluorescent probes. The TaqMan assay (Applied Biosystems) is one example of this method. The chemistry of this method is based on an oligonucleotide probe containing a fluorescent dye and a quencher. When the quencher is in proximity of the fluorescent reporter dye, it inhibits fluorescence, but

when the probe binds to target sequences, it is cleaved by DNA polymerase and the intensity of fluorescence is increased as the fluorescent reporter dye is separated from the quencher. Both SYBR Green and TaqMan probe-based assays were used to determine different gene expressions in Paper II-IV. Relative expressions were obtained by normalization to a stably expressed housekeeping gene.

In Paper II, the gene copy number of *TERT* was assessed by a qPCR-based method using TaqMan copy number assay (Applied Biosystems). Genomic DNA samples were used for the analysis. The *RNase P* gene served as a diploid control and was run simultaneously with the target gene in a duplex qPCR reaction. Results were analyzed with the CopyCaller software (Applied Biosystems).

3.3.4 Sanger sequencing

Sanger sequencing was developed in 1977 by Frederick Sanger *et al* and is still a widely used method for DNA sequencing even after the development of next-generation sequencing technologies. The method is run in a similar way as a PCR but with selective incorporation of chain-terminating dideoxynucleotides (ddNTP) which are labeled with base-specific fluorescent dyes. When ddNTPs are incorporated by DNA polymerase at different positions, the extension of the oligonucleotide chain will stop, thereby creating sequences of different lengths. The sequences are then separated by capillary electrophoresis based on their lengths which enables sequence reading of the incorporated base-specific fluorescent dyes and generation of chromatograms. Sanger sequencing was performed at the KIGene Core Facility at Karolinska Institutet in Paper II-IV to detect point mutations in the *TERT* promoter region and/or $BRAF^{V600E}$.

3.3.5 Pyrosequencing

Pyrosequencing is a DNA sequencing method that was developed at the Royal Institute of Technology in Stockholm. The method uses the principle of sequencing by synthesis and is a commonly used method to quantify DNA methylation. For methylation analysis, DNA is first treated with sodium bisulfite which selectively converts un-methylated cytosine to uracil. A PCR amplification is then performed with a biotinylated primer to enable hybridization of the amplified single-stranded DNA to pyrosequencing primers. The pyrosequencing reaction is then run where nucleotides are added sequentially, if the nucleotide is incorporated by DNA polymerase, a pyrophosphate is released and converted to ATP. ATP in turn acts like a substrate for luciferase which generates light signals that can be detected and analyzed. The light intensity corresponds to the quantity of pyrophosphate and incorporated nucleotides. Unused nucleotides are degraded before the addition of the next nucleotide. In Paper II, bisulfite Pyrosequencing was used to quantify methylation densities at the *TERT* promoter using PyroMark Q24 (Qiagen).

3.3.6 Western blot

Western blot or immunoblot is a common method used for the detection and semi-quantification of proteins. Protein lysates are usually prepared with lysis buffer and then separated with electrophoresis on a SDS polyacrylamide gel. Proteins are separated based on their molecular weight and subsequently transferred from the gel to a membrane. Proteins of interest can then be detected using antibodies. The membrane is first incubated with a primary antibody specific to the target protein, then with a secondary antibody that is conjugated with an enzyme. Finally, the protein can be visualized when adding a substrate that reacts with the enzyme-conjugated secondary antibody. Western blot was used in Paper III and IV for detection of proteins.

3.3.7 Transfection

Transfection is a method used for introducing nucleic acid into eukaryotic cells. The method can be used to study gene function and regulation by either enhancing or inhibiting the gene expression of the transfected cells. In this thesis, the lipid-mediated transfection technique was used with reagents that pack nucleic acids into lipid complexes. The lipid complexes fuse with the cell membrane, allowing the nucleic acids to be delivered into the cells. In Paper III and IV, transfections with siRNA and/or DNA plasmids were performed and transfection efficiency was verified with Western blot.

3.3.8 Statistical analyses

For statistical analyses of categorical variables, Chi-square or Fisher's exact test was used. Since normal distribution could not be assumed for most of the analyses, non-parametric tests were used for comparison of differences between groups; Mann-Whitney U was used for comparison between two groups and Kruskal Wallis was used when more than two groups were compared. Spearman's rank correlation coefficient (ρ) or Pearson correlation coefficient (r) was used for bivariate correlations between continuous variables depending on the distribution of data. Survival analyses were performed with log-rank test, visualized in Kaplan-Meier plots, and by using univariate or multivariate Cox regression. In addition, binary logistic regression was used for multivariate analyses while receiver operating characteristic (ROC) curves and area under curve (AUC) were used for evaluation of diagnostic ability in Paper I.

3.4 ETHICAL CONSIDERATIONS

In this thesis, clinical information and tumor tissue samples from patients with thyroid tumors were used as the main study material. Ethical approvals were obtained for collecting tumor tissue samples and clinical data from the patients prior to conducting the studies in this thesis.

When including patients in the studies, we always follow some basic principles to ensure the patients integrity and autonomy is respected. Prior to inclusion in the studies, both oral and written information about the studies were given to the patients. After the patients accepted inclusion in the studies and consented to the use of removed tissue for research, they had the right to withdraw their participation at any time without any reason provided. The patients who

consented to participate in research projects were not rewarded in any way that could influence their decision. The diagnosis or treatment of the patients were not affected by their participation in studies. The data, information and samples collected from the patients were also de-coded to assure their integrity.

Although the ultimate goal of the research is to improve clinical diagnostics and prognostication for the affected patients, the individual patients' needs, autonomy and integrity must always be respected.

4 RESULTS AND DISCUSSION

4.1 PAPER I. THE DIAGNOSTIC VALUE OF CYTOLOGICAL KI-67 PROLIFERATION INDEX IN FOLLICULAR THYROID TUMORS

It remains diagnostically challenging to differentiate FTC from FTA preoperatively with FNA cytology (70). Hence, these patients need to undergo a lobectomy for diagnostic purposes. In the majority of patients with FTC, a completion thyroidectomy is required (71). Additional diagnostic markers are thus needed to improve diagnosis and treatment to avoid repeated surgical procedures. In this study, we investigated if the Ki-67 proliferation index in FNA material adds diagnostic value to follicular thyroid tumors.

Two separate cohorts of follicular thyroid tumors and Hürthle cell tumors, namely Cohort A and Cohort B, were analyzed for comparison of Ki-67 proliferation index in FNA material and clinical parameters such as age at diagnosis and tumor size. Cohort B was previously published by Sofiadis *et al* (213) and was re-analyzed to validate the results from Cohort A.

In Cohort A, 92% of the analyzed cases were classified as Bethesda category III (atypia or follicular lesion of undetermined significance) or IV (follicular neoplasm or suspicious for a follicular neoplasm) as determined by routine FNA cytology. For a subset of patients, Ki-67 index was also determined in post-operative specimens which correlated significantly to cytological Ki-67 index. Examples of the Ki-67 immunocytochemistry in pre-operative specimens is shown in Figure 6.

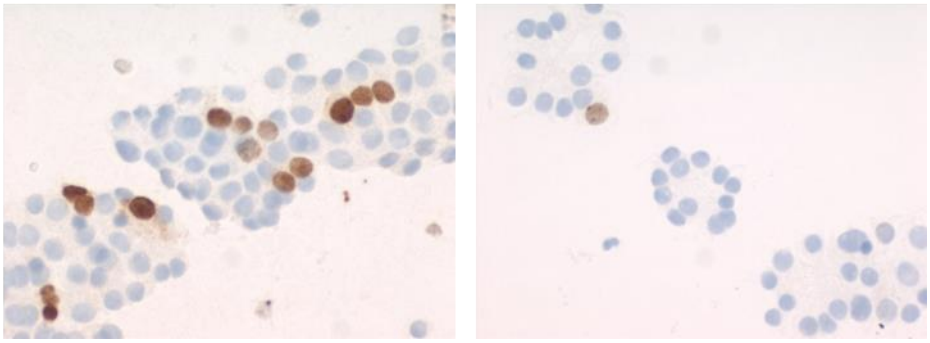

Figure 6. Immunocytochemistry of Ki-67 proliferation index in pre-operative specimens. A high Ki-67 index at 10% from a patient with FTC (left panel) and a low Ki-67 index at 1% from a patient with FTA (right panel) is shown at x20 magnification. Modified from Paper I.

The cytological Ki-67 index and tumor size was identified as significantly higher in FTC as compared to FTA/FT-UMP. Results were similar for Hürthle cell tumors, where HCC exhibited significantly higher Ki-67 index and tumor sizes as compared to HCA/FT-UMP with Hürthle cell differentiation. Interestingly, Hürthle cell tumors had higher cytological Ki-67 index than follicular thyroid tumors. However, when analyzing follicular thyroid tumors in pooled analyses with Hürthle cell tumors, carcinomas still exhibited higher cytological Ki-67 and larger tumor sizes as compared to adenomas and FT-UMP. To validate these results, Cohort B was re-evaluated. Similarly, the carcinomas exhibited higher Ki-67 index and larger

tumor sizes in pooled analyses of follicular thyroid tumors and Hürthle cell tumors in Cohort B, confirming the findings from Cohort A.

In subgroup analyses of FTCs in Cohort A, we found that FTCs with extrathyroidal extension exhibited higher cytological Ki-67 index as compared to FTCs without extrathyroidal extension. Based on the 2017 WHO classification, the widely invasive subtype of FTC demonstrated significantly higher cytological Ki-67 index than the minimally invasive subtype.

To further investigate whether cytological Ki-67 was an independent predictor of carcinoma, multivariate analysis was performed using binary logistic regression with the variables cytological Ki-67, tumor size, age at diagnosis and gender in the model. In Cohort A, cytological Ki-67 index and tumor size were identified as independent predictors of FTC. Similar findings were obtained when including Hürthle cell tumors in the analysis and results were consistent in pooled analyses of Cohort A + Cohort B, respectively. Then, the predictive value of Ki-67 index for carcinomas was evaluated with ROC-analysis where the AUC was 0.722. With a cut-off value at Ki-67 index >5%, the specificity was 93% and sensitivity 31%, respectively.

Taken together, we have identified cytological Ki-67 index and large tumor size as predictors of malignancy in follicular thyroid tumors. Cytological Ki-67 index could potentially add diagnostic information for some of the patients with follicular thyroid tumors.

4.2 PAPER II. THE DIAGNOSTIC AND PROGNOSTIC VALUE OF TERT ALTERATIONS IN FOLLICULAR THYROID TUMORS

The two hotspot *TERT* promoter mutations C228T and C250T are recurrent genetic events in FTC and have been associated with poor clinical outcome (143). *TERT* promoter mutation is an important mechanism for *TERT* reactivation and *TERT* expression (134, 135, 218). However, not all *TERT*-expressing tumors have an identifiable *TERT* promoter mutation (96). Hence, we aimed to determine the frequencies of *TERT* promoter mutations and other *TERT*-related genetic and epigenetic alterations in relation to *TERT* expression, and investigate whether these alterations could have a diagnostic and prognostic value in follicular thyroid tumors.

Analyses of *TERT* mRNA expression, *TERT* gene copy number (CN) and *TERT* promoter mutation and methylation were performed in a cohort of follicular thyroid tumors consisting of FTA, FT-UMP and FTC. *TERT* expression was grouped based on detectable or undetermined expression. *TERT* CN gain was defined as >2 copies while CN loss was defined as <2 copies, respectively. Promoter methylation density was determined by calculating the methylation index (MetI) at the investigated *TERT* promoter region. Hypermethylation was defined as MetI>18% based on the distribution of MetI in FTA.

In our analyzed cohort, 43% of FTC, 39% of FT-UMP and 14% of FTA expressed *TERT*. Of the *TERT*-expressing FTCs, 24 of the 28 cases harbored at least one other *TERT* alteration (promoter mutation, promoter hypermethylation or CN gain) that could possibly explain the

positive *TERT* expression (Figure 7). In this group, *TERT* expression was significantly associated with *TERT* promoter mutation, hypermethylation and CN gain. Of the FT-UMPs that exhibited detectable *TERT* expression, eight of the nine cases harbored at least one of the other abovementioned *TERT* alterations (Figure 7).

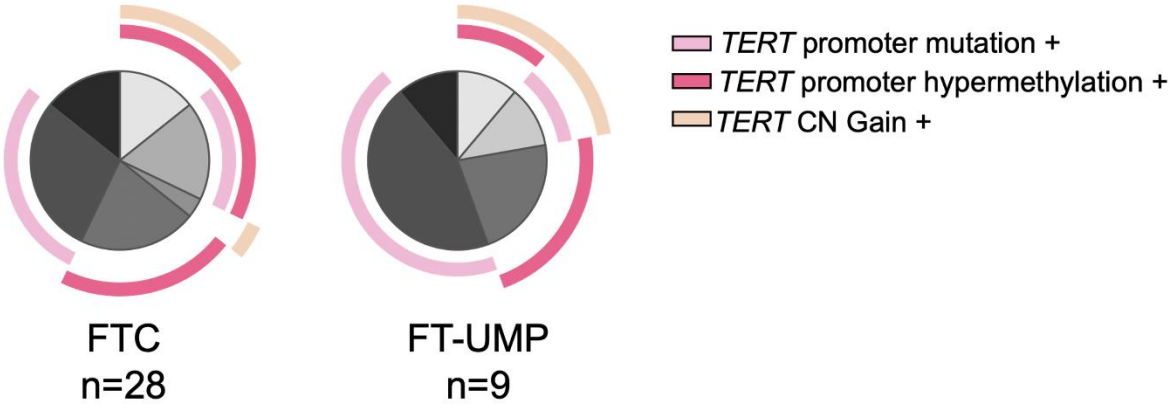

Figure 7. The distribution of other *TERT* alterations in *TERT*-expressing FTC and FT-UMP. 24 of the 28 FTC cases and eight of the nine FT-UMP cases with *TERT* expression harbored at least one other *TERT* alteration (CN gain, promoter mutation and hypermethylation) that could possibly explain the positive *TERT* expression. CN: copy number.

The frequencies of *TERT* expression, *TERT* promoter mutation and hypermethylation, respectively, were significantly higher in FTC and FT-UMP as compared to FTA. Results were consistent in analyses combining all *TERT* alterations together, showing a higher frequency of alterations in FTC and FT-UMP as compared to FTA. No difference in the frequency of CN variations were observed for the analyzed patient groups. Interestingly, we found no difference in the distribution for any of the *TERT* alterations between FTC and FT-UMP. When analyzing whether these *TERT* alterations could be diagnostically relevant to distinguish FTC and FT-UMP from FTA, we found generally high values of specificities and positive predictive values for all analyzed *TERT* alterations. These results indicate a high risk of FTC/FT-UMP if *TERT* alterations are present.

We then proceeded by investigating whether these *TERT* alterations were associated with poor clinical outcome. *TERT* expression, *TERT* promoter mutation and all *TERT* alterations combined were significantly associated with shorter time to relapse in univariate analysis. Multivariate Cox regression analyses were then performed, adjusting for the covariates age, gender, tumor stage and FTC-subtypes, where *TERT* expression, promoter hypermethylation and CN gain were identified as independently associated with shorter time to relapse. When assessing the prognostic utility of the analyzed *TERT* alterations in relation to relapse in FTC, high specificities and negative predictive values were observed, indicating a lower risk of relapse if *TERT* alterations are absent.

In summary, *TERT* expression was associated with the occurrence of other *TERT* alterations, such as *TERT* CN gain, *TERT* promoter mutation and hypermethylation in follicular thyroid tumors. FTC and FT-UMP had significantly higher frequencies of *TERT* alterations as compared to FTA, and these alterations were also associated with poor clinical outcome in FTC. *TERT* genetic and epigenetic alterations could thus serve as potential biomarkers for both diagnosis and prognosis for follicular thyroid tumors.

4.3 PAPER III. THE ROLE OF THE ETS TRANSCRIPTION FACTOR GABPA IN PAPILLARY THYROID CARCINOMA IN RELATION TO TERT PROMOTER MUTATION AND CLINICAL PHENOTYPE

The two recurrent hotspot *TERT* promoter mutations are important genetic events in PTC and are associated with poor clinical outcome (98, 219). It was previously reported that the occurrence of these mutations give rise to novel binding sites for ETS transcription factors, leading to the upregulation of *TERT* (134, 135). More specifically, the ETS transcription factor family member GABPA was reported to selectively bind to the mutant *TERT* promoter (137). Thus, we aimed to explore the role of GABPA in relation to *TERT* promoter mutation and clinical phenotype in PTC by analyzing patient cohorts of PTC and ATC primary tumors, public datasets from TCGA and cell lines.

Firstly, the relationship between GABPA and *TERT* was investigated in thyroid cancer cell lines. When inhibiting GABPA with siRNA, a significant decrease in *TERT* mRNA expression in both *TERT* promoter mutant and wild-type cells was observed (Figure 8). Analyses of *TERT* promoter activity showed that GABPA inhibition led to decreased activity in mutant *TERT* promoter but no significant decrease was observed for the wild-type *TERT* promoter. These results suggested that GABPA can regulate *TERT* expression, with effects prominent in *TERT* promoter mutated cells. However, in PTC tumors, low expression of *GABPA* was associated with the presence of *TERT* promoter mutation and correlated inversely with *TERT* expression. Analysis of the TCGA cohort of PTC confirmed the association of *TERT* promoter mutation with low expression of *GABPA*.

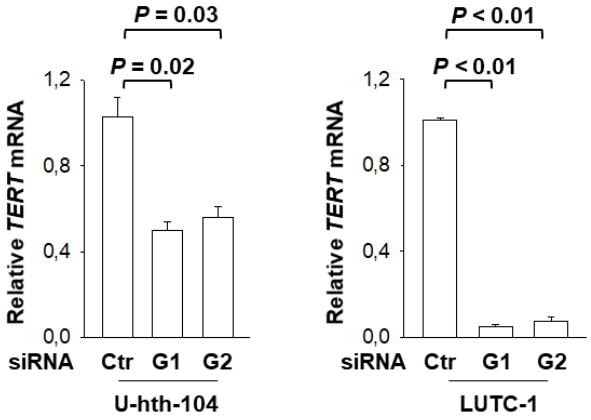

Figure 8. *TERT* mRNA is decreased upon GABPA-inhibition with siRNA in both *TERT* promoter mutant (U-hth-104) and wild-type (LUTC-1) thyroid cancer cell lines. Ctr: control siRNA. G1: GABPA siRNA 1. G2: GABPA siRNA 2. Modified from Paper III.

Given these paradoxical findings, we proceeded by investigating the invasive properties of GABPA, and surprisingly found that GABPA inhibition led to a significant increase in invasive potential in both *TERT* promoter mutant and wild-type thyroid cancer cells. Consistently, over-expression of GABPA was observed to decrease the invasive potential. In PTC tumors, lower levels of *GABPA* expression was significantly correlated with older at diagnosis and larger tumor sizes, and associated with distant metastasis and shorter overall and disease-free survival, respectively. Analyses of TCGA cohort of PTCs showed similar results where lower *GABPA* expression was coupled with higher disease stages, shorter disease-free survival and older age at diagnosis. Also, the clinically more aggressive ATCs exhibited lower levels of *GABPA* expression than PTCs. Collectively, these results indicated a more tumor-suppressing role of GABPA as opposed to its *TERT*-activating properties.

To further investigate the underlying mechanism of the invasive potential of GABPA, a PanCancer Progression Panel assay, consisting of 770 genes, was analyzed to identify differentially expressed genes between control and GABPA-depleted cells. In both mutant and wild-type *TERT* promoter carrying cell lines, *DICER1* was identified as the top candidate gene. Consistently, inhibition of GABPA led to a decrease in DICER1 expression. Furthermore, over-expression of DICER1 in GABPA-depleted cells diminished the invasive potential of GABPA-depletion alone. The GABPA-mediated regulation of *DICER1* was further explored using prediction tools where eight potential binding sites of GABPA on the *DICER1* promoter were identified. In cell lines, the promoter activity of *DICER1* increased when over-expressing GABPA, but diminished when the predicted GABPA binding site at -417 was mutated. In PTC tumors, the mRNA and protein expression of DICER1 and GABPA was significantly correlated with each other. In addition, TCGA patient cohorts of PTC as well as eight other types of cancer showed similar correlations between *DICER1* and *GABPA* mRNA expression. The positive correlation of *GABPA* and *DICER1* in PTC is shown in Figure 9. Taken together, these results indicate a strong association between *GABPA* and *DICER1*, in line with the findings obtained from cell experiments.

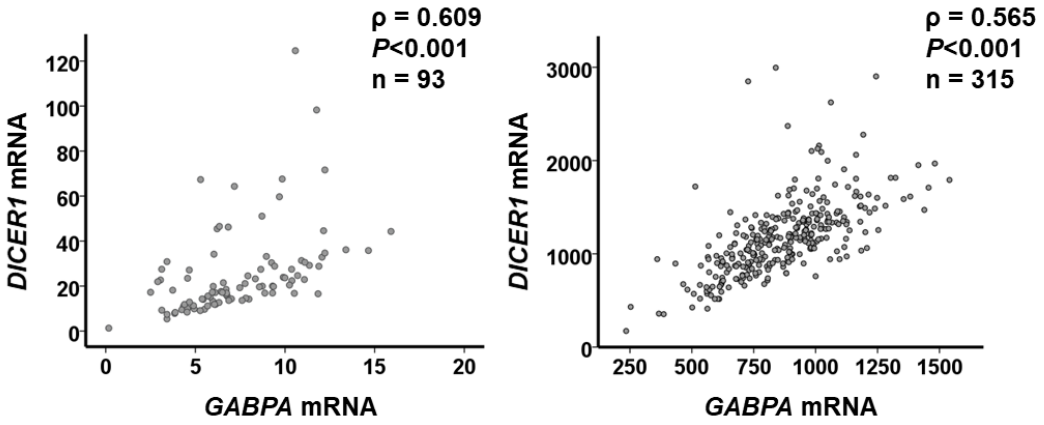

Figure 9. The positive correlation of *GABPA* and *DICER1* mRNA expression in a local cohort (left panel) and TCGA cohort (right panel) of PTC, respectively. ρ: Spearman's rank correlation coefficient. n: number of cases. Modified from Paper III.

Since DICER1 is known to have a key role in the maturation of miRNA, we analyzed the miRNA profiles of the TCGA cohort of PTCs to identify potential miRNA that could be affected by the regulatory effect of GABPA on DICER1. Nine miRNAs were identified as negatively correlated with *GABPA* but positively associated with relapse and two of them (*hsa-mir-15a* and *hsa-mir-767*) were validated as increasing when inhibiting GABPA in cells.

Finally, to investigate the *in vivo* effects of GABPA, thyroid cancer cells stably over-expressing GABPA were injected through tail vein into nude mice. Tumor colonies were formed in the mouse lungs when injecting both control and GABPA over-expression cells. However, the number of tumor colonies were fewer for the group that were injected with GABPA over-expression cells.

Conclusively, even though GABPA has been shown to exhibit mutant *TERT* promoter-activating properties, in PTC tumors, its low expression was paradoxically associated and negatively correlated with *TERT* promoter mutation and *TERT* expression, respectively. This divergence highlights the complex relationship between *TERT* promoter mutations and GABPA. In addition, the results indicate that GABPA exhibits tumor-suppressive properties, as demonstrated both *in vitro* and *in vivo*. Consistently, in patient cohorts, our findings suggest that loss of the tumor suppressive properties of *GABPA* is associated with a more aggressive phenotype. Further, we identified *DICER1* as a potential downstream target for the GABPA-mediated tumor suppressive effect.

4.4 PAPER IV. THE ROLE OF THE ETS TRANSCRIPTION FACTOR EHF IN PAPILLARY THYROID CARCINOMA IN RELATION TO CLINICAL PHENOTYPE, TERT PROMOTER MUTATION AND BRAF^{V600E}

Concurrent *BRAF*^{V600E} and *TERT* promoter mutations have been identified as associated with aggressive clinical features and poor clinical outcome in PTC (169, 171, 172). The proposed underlying mechanism of this effect is the *BRAF*^{V600E}-induced MAPK-pathway activation that upregulates ETS transcription factors which in turn bind to the mutant *TERT* promoter and activates *TERT* (175). Several of the ETS transcription factors have been implicated to play a role in thyroid cancer development and progression (175, 176, 220, 221), but a thorough exploration of their role in relation to clinical phenotype in PTC is warranted. Hence, we sought to explore the 28 ETS transcription factors in relation to clinical phenotype, *BRAF*^{V600E} and *TERT* promoter mutation in PTC by analyzing publicly available TCGA datasets and our local cohort of PTC primary tumors.

Differentially expressed genes (DEG) analysis of the TCGA cohort of PTC was performed to identify differentially expressed ETS transcription factors in relation to disease stage according to 7th edition of AJCC. We identified *SPDEF*, *EHF* and *ETV6* as upregulated, while *GABPA*, *ETS1*, *FLI1* and *ERG* were downregulated, respectively, in stage III + IV as compared to stage I + II (Figure 10). *GABPA* was excluded from further analyses as it was subjected for analysis already in Paper III.

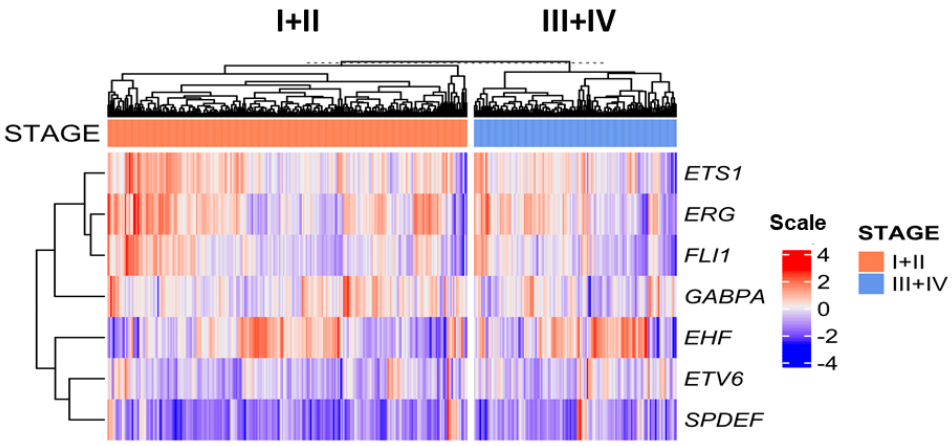

Figure 10. Heatmap showing the differentially expressed ETS transcription factors in disease stages I+II as compared to III+IV in the TCGA cohort of PTC. *ETS1*, *ERG*, *FLI1*, *GABPA*, *EHF*, *ETV6* and *SPDEF* were identified as significantly differentially expressed across the disease stages. Scale: scaled mRNA expression. Modified from Paper IV.

We proceeded by analyzing the expression profiles of *SPDEF*, *EHF*, *ETV6*, *ETS1*, *FLI1* and *ERG* in relation to clinicopathological features and mutational status of $BRAF^{V600E}$ and *TERT* promoter in the TCGA cohort of PTC. Except for *SPDEF*, all other five ETS transcription factors demonstrated a significant association to at least one clinical variable (age, disease stage, tumor stage, lymph node metastasis, distant metastasis or extrathyroidal extension) and either $BRAF^{V600E}$, *TERT* promoter mutation or *TERT* expression.

Given these findings, a local validation cohort of PTC was used to analyze the gene expressions of *EHF*, *ERG*, *FLI1*, *ETV6* and *ETS1*. High *EHF* expression was observed as consistently associated with aggressive clinical features, $BRAF^{V600E}$ and *TERT* promoter mutation or expression in both analyzed cohorts. Thus, our results indicated *EHF* as the most promising candidate of the analyzed genes. In the TCGA cohort, its high expression was significantly associated with more advanced disease stages and tumor stage, as well as lymph node metastasis and extrathyroidal extension. The presence of $BRAF^{V600E}$ and *TERT* expression was also associated with higher *EHF* expression. In addition, comparison between PTC and adjacent normal thyroid tissue in TCGA data demonstrated an upregulation of *EHF* in PTC. In our local cohort of primary PTC tumors, high *EHF* expression was coupled with significantly shorter disease-free survival and correlated with higher age at diagnosis. Similarly, the association of high *EHF* expression with $BRAF^{V600E}$ and *TERT* expression was confirmed. Moreover, high *EHF* expression was coupled with *TERT* promoter mutation in our local PTC cohort.

Based on the abovementioned findings and the proposed mechanistic link between $BRAF^{V600E}$, ETS transcription factors and *TERT* promoter mutation, we sought to further investigate the relationship between these in relation to prognosis in PTC. In both analyzed cohorts, the *EHF* expression was lower in the patient group with neither $BRAF^{V600E}$ nor *TERT* promoter mutation as compared to those with $BRAF^{V600E}$ only or co-existing $BRAF^{V600E}$ and *TERT* promoter mutation. High *EHF* expression in $BRAF^{V600E}$ mutated cases was also coupled with shorter

disease-free survival. In survival analysis stratifying for both *BRAF*^{V600E} and *TERT* promoter mutation status, the group with both mutations and high *EHF* expression was associated with the shortest disease-free survival. These findings in PTC cohorts are consistent with the hypothesized link between *BRAF*^{V600E}, ETS transcription factors and *TERT* promoter mutation and indicates that the adverse clinical effect of *EHF* could be mediated by this mechanistic axis. Furthermore, over-expression of EHF was coupled with increased *TERT* expression in PTC cells with concurrent *BRAF*^{V600E} and *TERT* promoter mutation, but no significant difference was observed in PTC cells with *BRAF*^{V600E} only.

Collectively, we found several of the ETS transcription factors as potentially related to disease progression in PTC, where *EHF* was identified as the top candidate. *EHF* expression and its adverse clinical effect was highly associated with both *BRAF*^{V600E} and *TERT* promoter mutation, indicating a mechanistic link between these factors that calls for further investigations.

5 CONCLUSIONS

This thesis provides insights into potential diagnostic and prognostic markers in follicular-cell derived thyroid tumors and explores the genetic and molecular background related to *TERT* activation in disease progression of these tumors.

Paper I: Cytological Ki-67 proliferation index can potentially add diagnostic information for patients with follicular thyroid tumors pre-operatively.

Paper II: *TERT* alterations are recurrent and potential biomarkers for diagnosis and prognostication in follicular thyroid tumors.

Paper III: The ETS factor GABPA can act as a tumor suppressor in PTC even though it has mutant *TERT* promoter-activating properties.

Paper IV: *EHF* is a potential oncogenic ETS factor in PTC with strong associations to $BRAF^{V600E}$ and *TERT* promoter mutations.

6 POINTS OF PERSPECTIVE

Although much advances in cancer research has been made, there is still a lot of clinical challenges left in terms of accurate diagnosis, precise risk-stratification, optimized treatment and timely follow-up. Specifically, for thyroid cancer, there is still improvements to be made in the pre-operative diagnosis of FTC. Accurate prognostic markers are needed for patients with well-differentiated thyroid carcinoma, such as PTC and FTC, for timely surveillance and optimized treatment. Also, an effective treatment is still lacking for ATC patients. Thus, continued investigations of potential biomarkers are needed. In addition, a deep understanding of the underlying molecular and genetic background of these tumors is required to identify potential targets for therapy. In this thesis, some of these issues were explored, but these findings need to be further investigated or validated.

In Paper I, a high cytological Ki-67 proliferation index could possibly contribute to the pre-operative identification of FTC, but the results need to be validated in larger cohorts prospectively. Preferably the validation should include every patient with an indeterminate cytology in the Bethesda categories III and IV to evaluate its efficiency as a diagnostic marker in an everyday clinical context. Further, combining additional molecular markers, such as those described below, should be considered to increase the sensitivity since cytological Ki-67 alone is clearly not enough to identify all patients with FTC.

In Paper II, several *TERT* alterations were found to be promising potential biomarkers. Testing of *TERT* promoter mutation is for now the most feasible approach in a clinical setting, as it is relatively cost-effective and easy to perform methodologically. For expanded use in a clinical setting, the next step would be to perform the testing on cytological material to provide information pre-operatively. However, optimization of the methodological aspects of this type of testing is needed to increase the sensitivity as the cytological material is sparse. In addition, further investigations of *TERT* expression, CN gain and promoter methylation are needed before clinical implementation can be attempted.

In Paper III, GABPA was shown to exhibit both mutant *TERT* promoter-activating as well as tumor-suppressive properties, highlighting its diversity and possible context-driven functions. The diverse functions of GABPA, including its other potential targets, and whether it differs between cancer types are of interest in future investigations to gain deeper knowledge of the underlying biological processes. In addition, continued investigations of other possible activators of the mutant *TERT* promoter is of importance to identify potential targets for therapy. Moreover, the miRNAs found as potentially altered by the GABPA-DICER1 axis and their role in PTC pathogenesis remains to be explored in detail.

In Paper IV, *EHF* was found as possibly related to aggressive PTC features, $BRAF^{V600E}$ and *TERT* promoter mutation. However, it remains to investigate whether there is a regulatory effect of $BRAF^{V600E}$ and MAPK activation on *EHF* gene expression and to determine whether EHF is an activator of the mutant *TERT* promoter.

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