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Behind the Scenes of Thyroid Tumors – Underlying Genetic Mechanisms

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Stockholm 2007

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Published and printed by Larserics Digital Print AB
Landsvägen 65, 17265 Sundbyberg

ISBN 978-91-7357-454-9

May all beings have happiness and the causes of happiness.
May all beings be free from suffering and the causes of suffering.
May all beings never part from the bliss of sorrowless.
May all beings live in equanimity, free from hatred and aversion.

To all sentient beings,
May all be well and happy

CONTENTS

1 ABSTRACT

Thyroid cancer is the most frequently observed malignancy involving endocrine tissues. This group of diseases is also the major cause of death related to neoplasia of the endocrine system. Tumors in the thyroid gland are common in the general population and the majority represents benign follicular adenoma (FTA). Thyroid follicular cell derived tumors encompass FTA, follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), and poorly-differentiated thyroid carcinoma (PDTC). The parafollicular calcitonin-producing C-cells give rise to medullary thyroid carcinoma (MTC). The anaplastic thyroid carcinoma (ATC), is an undifferentiated, highly aggressive cancer largely of unknown etiology. The overall goal of this thesis was to further characterize the molecular genetic mechanisms leading to thyroid tumorigenesis and to explore the possibility of thyroid tumor progression.

MTC can occur in sporadic as well as in heritable forms. Human familial MTC (FMTC) is dominantly inherited**,** either alone or as part of a Multiple Endocrine Neoplasia type 2 (MEN 2) syndrome. Activating germ-line *RET* mutations is the cause of the disease in up to 95% of FMTC and MEN 2. **Paper I** demonstrates the first dog pedigree with familial clustering of thyroid cancer. The affected individuals demonstrated calcitonin positive thyroid cancers, hypothyroidism and chronic dermatitis. Based on the overlapping features with human FMTC, the dog *Ret* was analyzed as a candidate gene after *in silico* prediction of its genomic sequence. The lack of demonstrable *Ret* mutation in this pedigree suggests that the MTC predisposition does not share the genetic etiology commonly underlying FMTC phenotypes in human.

The molecular pathogenesis of ATC is mainly unknown partly as a result of tissue degeneration following pre-operative treatment. Therefore ATC cell lines serve as good models for delineation of genetic mechanisms in the tumor development. In **Paper II**, two novel ATC cell lines (HTh 104 and HTh 112) were presented and together with six other frequently used ATC lines (HTh 7, HTh 74, HTh 83, C 643, KAT-4 and SW 1736) were further characterized cytogenetically. Novel non-random breakpoints at 1p36 and 17q24-25 as well as 3p21-22 and 15q26 that are also implicated in well-differentiated thyroid cancers were revealed. Frequent gain of 20q, including the *UBCH10* gene in 20q13.12 was observed, indicating that this region is important for the pathogenesis of or progression to ATC.

In **Paper III**, bisulphite Pyrosequencing of Long Interspersed Nucleotide Elements-1 and LUminometric Methylation Assay were used to quantify global methylation in a series of 21 FTC and their corresponding normal thyroid tissues. No significant difference of global methylation was detected between FTC and normal tissues. However *RASSF1A* promoter hypermethylation, reduced mRNA expression and allelic loss (AI) were observed in the same material. These findings suggest *RASSF1A* is frequently inactivated in FTC by promoter hypermethylation and AI, but is not coupled to global methylation changes.

In further deciphering the molecular genetic pathogenesis of ATC, array-CGH was performed in a series of 27 primary ATC in **Paper IV**. Amplicons 11q13, 20q11.2 and 20q13.12 were identified and subsequent fluorescence *in situ* hybridization revealed recurrent locus gain of *CCND1* in 11q13 and *UBCH10* in 20q13.12. Homozygous loss encompassing the *CDKN2A* locus in 9p21.3 was detected in one case. Cyclin D1 was found to be expressed in 67% of ATC while p16 protein expression was undetectable in 89% of the tumors. Subsequently, the effect of *CCND1*/*Cyclin D1* on thyroid cell proliferation was assessed *in vitro* in ATC cells by means of siRNA and in thyroid cells after *CCND1* transfection. Furthermore three ATCs harboured the common *BRAF* mutation V600E.

Keywords: Familial medullary thyroid cancer, follicular thyroid cancer, anaplastic thyroid cancer, *CCND1*, *UBCH10*, *p16*, *RASSF1A*, *BRAF*, array-CGH, tumor progression, global methylation, LINE-1, LUMA

ISBN 978-91-7357-454-9

2 PAPERS INCLUDED IN THIS STUDY

This thesis is based on the following papers, which will be referred to by their Roman numerals throughout the text:

I **Lee JJ**, Larsson C*, Lui WO, Höög A, von Euler H.
A dog pedigree with Familial Medullary Thyroid Cancer.
International Journal of Oncology, 2006, 29:1173-1182.

II **Lee JJ***, Foukakis T, Hashemi J, Grimelius L, Heldin NE, Wallin G, Rudduck C, Lui WO, Höög A, Larsson C.
Molecular cytogeneitc profiles of novel and established human anaplastic thyroid carcinoma models.
Thyroid, 2007, 17:289-301.

III **Lee JJ***, Geli J, Larsson C, Wallin G, Karimi M, Zedenius J, Höög A, Foukakis T.
RASSF1A inactivation without global hypomethylation in follicular thyroid cancer.
Submitted.

IV **Lee JJ***, Au A, Foukakis T, Barbaro M, Kiss N, Clifton-Bligh R, Staff J, Borg Å, Robinson B, Wallin G, Höög A, Larsson L.
Array-CGH identifies *Cyclin D1* and *UBCH10* amplicons in primary anaplastic thyroid carcinoma.
Submitted.

* Corresponding author

3 OTHER RELATED PAPERS

1. **Lee JJ***, Juhlin CC, Foukakis T, Robinson B, Zedenius J, Larsson C, Höög A.
   Loss of parafibromin and *HRPT2* genetic alterations in thyroid cancers.
   Manuscript in preparation.

2. Geli J, Kiss N, Karimi M, **Lee JJ**, Bäckdahl M, Ekström TJ, Larsson C.
   Global and regional CpG methylation in pheochromocytomas and abdominal paragangliomas: association to malignant behavior.
   Revision.

3. Ulivieri A, Lavra L, Dominici R, Giacomelli L, Brunetti E, Sciacca L, Trovato M, Barresi G, Foukakis T, **Lee JJ**, Larsson C, Bartolazzi A, Sciacchitano S.
   FZD-1 is specifically downregulated in follicular thyroid tumours and modulates growth and invasiveness.
   Submitted.

4. Fryknas M, Wickenberg-Bolin U, Göransson H, Gustafsson MG, Foukakis T, **Lee JJ**, Landegren U, Höög A, Larsson C, Grimelius L, Wallin G, Pettersson U, Isaksson A.
   Molecular markers for discrimination of benign and malignant follicular thyroid tumors.
   Tumor Biology, 2006, 27:211-220.

4 LIST OF ABBREVIATIONS

AI	Allelic imbalance
ATC	Anaplastic thyroid carcinoma
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridization
DNA	Deoxyribonucleic acid
dATPαS	2'-deoxyadenomsine-5'-O-(1-thiotriphosphate)
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'deoxythymidine 5'-triphosphate
dNTP	Deoxyribonucleotide triphosphates
e.g.	For example
i.e.	Such as
FISH	Fluorescence *in situ* hybridization
FMTC	Familial medullary thyroid carcinoma
FTC	Follicular thyroid carcinoma
gDNA	Genomic DNA
kb	Kilo base
kDa	Kilo Daltons
LOH	Loss of heterozygosity
MB	Mega base
MEN	Multiple endocrine neoplasia
MI	Minimally-invasive
miRNA	Micro ribonucleotide acid
mRNA	Messenger ribonucleotide acid
MTC	Medullary thyroid carcinoma
PCR	Polymerase chain reaction
PDTC	Poorly-differentiated thyroid carcinoma
RNA	Ribonucleotide acid
SKY	Spectral karyotyping
STR	Short tandem repeats
Tg	Thyroglobulin
TSG	Tumor suppressor gene
WDTC	Well-differentiated thyroid carcinoma
WI	Widely-invasive
>	More than

5 INTRODUCTION

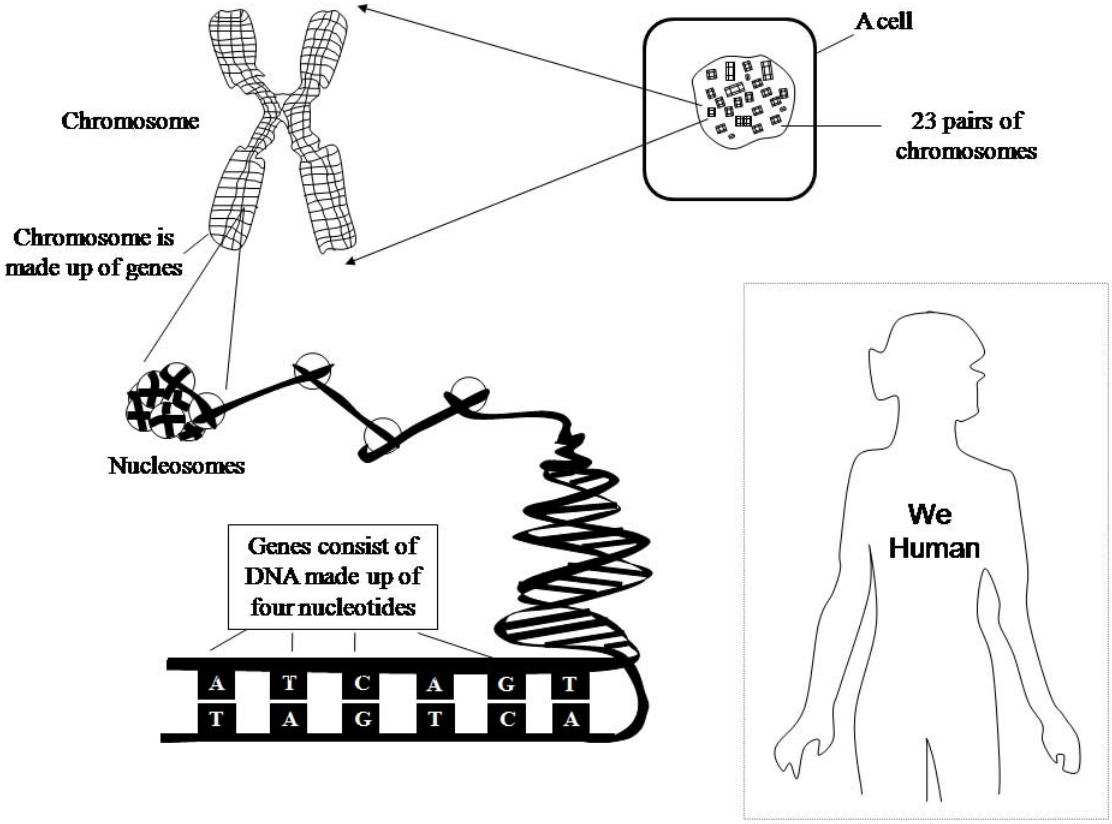


Figure 1. Schematic diagram of genetic make-up in human cells. A normal human cell contains 46 chromosomes. Each chromosome is made up of many genes. Genes are consisting of DNAs that are made up of four nucleotides (Adenine (A), Thymine (T), Cytosine (C) and Guanine (G)).

Cells are small functional units with potential to proliferate. In human, the fusion of an egg and a sperm gives rise to a diploid cell containing 46 chromosomes (Figure 1). This cell will subsequently divide and give rise to new daughter cells, in a process called mitosis. The majority of daughter cells will eventually differentiate to achieve a specialized character and function. In addition, a minority of the cells (referred to as stem cells) will retain the ability of self-renewal.

Under normal conditions, cells will proliferate only when needed which the balance between growth-promoting and growth-restraining signal controls. The duplicated genetic material is controlled before the actual cell division takes place during mitosis. Genetic defects may result from errors of replication or external factors such as carcinogenic chemicals and UV radiation. If DNA defects are detected, cell division will be delayed for

repair to take place. Cells with irreparable damage will have to undergo an internal self-destruction program (apoptosis or programmed cell death). Moreover normal cells have a limited lifespan and will die after a certain number of divisions. In cancer the normal controls are impaired, while the cells can grow without control, become immortal and spread from the site of origin. In contrast, benign tumors show local proliferation without spreading outside the capsule or tissue of origin.

5.1 CANCER AS A GENETIC DISEASE

The suggestion that cancer predisposition can be inherited already proposed in 1866 by Broca who described a family with many members suffering from breast and liver cancers (1). Today it is known that almost all types of cancer may occur in both hereditary and sporadic forms. Furthermore in 1914 Theodor Boveri proposed that cancer is a genetic disease of somatic cells (2). The rapid development of advanced experimental tools over the last three to four decades has contributed to our present view of cancer as a complex genetic disease.

Cancer is a group of diseases exhibiting several distinguishing features as compared to normal cells. Hanahan and Weinberg proposed that cancer cells are characterized by six acquired main features including capability for growth signal autonomy, evasion of growth inhibitory signals, evasion of apoptotic cell death, unlimited replicative potential, formation of new blood vessels (angiogenesis), and invasion and metastasis (3). A comparison of cancer cells and normal cells based on these features is shown in Table 1.

It has been estimated that four to seven genetic events are needed for the development of a common epithelial cancer (4, 5). The occurrence of genetic alterations may follow internal and external environmental exposures or occur as random events at cell division. Mutagens are substances that cause DNA mutations and those that can cause cancer are defined as carcinogens. Furthermore free radicals (from UV and ionizing radiation) can affect the probability of mutational events, and their effects may be modified by normally occurring genetic variations. Viral infections have been associated with human cancer development. Such viral particles encode for oncogenic products that can transform the host cells and interfere with the replication machinery of the host upon infection. Some hormones may also have similar effect as non-mutagenic carcinogens and stimulate cellular proliferation. It has also become evident that tumor cells can evade recognition and elimination by the immune system.

Table 1. Hallmarks of cancer.

Features	Normal cells	Cancer cells
Growth signal autonomy	Need external signals from growth factors to divide.	Independent of growth factor signaling due to mutations disrupting growth factor pathways regulation.
Evasion of growth inhibitory signals	Responsive to inhibitory signals to maintain homeostasis.	Do not respond to growth inhibitory signals.
Evasion of apoptosis	Die in response to DNA damage.	Evade apoptotic signals.
Unlimited replicative potential	Shortening of telomeres during DNA replication for cell senescence.	Cells maintain the length of telomeres and thus keep replicating.
Angiogenesis	Depend on blood vessels for oxygen and nutrients but the vascular architecture is constant in adult.	Cells induce growth of new blood vessels for tumor survival and expansion.
Invasion & metastasis	Cells generally do not migrate.	Cells able to migrate to other parts of body and keep replicating.

5.1.1 The cell cycle and its regulation

The cell cycle and its four phases are illustrated in Figure 2. These phases consist of the DNA synthesis phase (S), during which the DNA is duplicated, the G_1 and G_2 that are gaps before and after the S phase and the M phase in which mitosis takes place and give rise to two identical daughter cells. Under normal conditions most cells are in G1 phase, and depending on the extracellular signals received they can either progress towards mitosis or leave the cell cycle and rest in the G_0 phase. Before the cell can proceed to the S phase, it has to pass a checkpoint, at which its genetic content is controlled to be normal. During the S phase the DNA is duplicated and the cell moves to G_2 phase in preparing for division in the following M phase to give rise to two identical daughter cells.

The progress of the cell cycle is carefully controlled by a set of molecules, some of which are associated with cancer development. Cyclins and cyclin-dependent kinases (CDKs) are two classes of such regulatory molecules. Cyclins and CDKs form complexes that are specific to their target proteins. In these complexes, cyclins are the regulatory subunits and CDKs the catalytic subunits of an activated heterodimer. Following binding with a cyclin, the activated CDK will be phosphorylated and thus activates or inactivates proteins that direct transition to the next phase of the cell cycle. Cell cycle inhibitors also play a crucial role in regulating cell cycle progression. The *cip/kip* family (*i.e. p21*, *p27* and *p57*)

and the *INK4a/ARF* (*In*hibitor of *K*inase *4*/*A*lternative *R*eading *F*rame) (*i.e. p16INK4a* and *p14ARF*) are two groups of genes that can prevent cell cycle progression as reviewed in (6, 7).

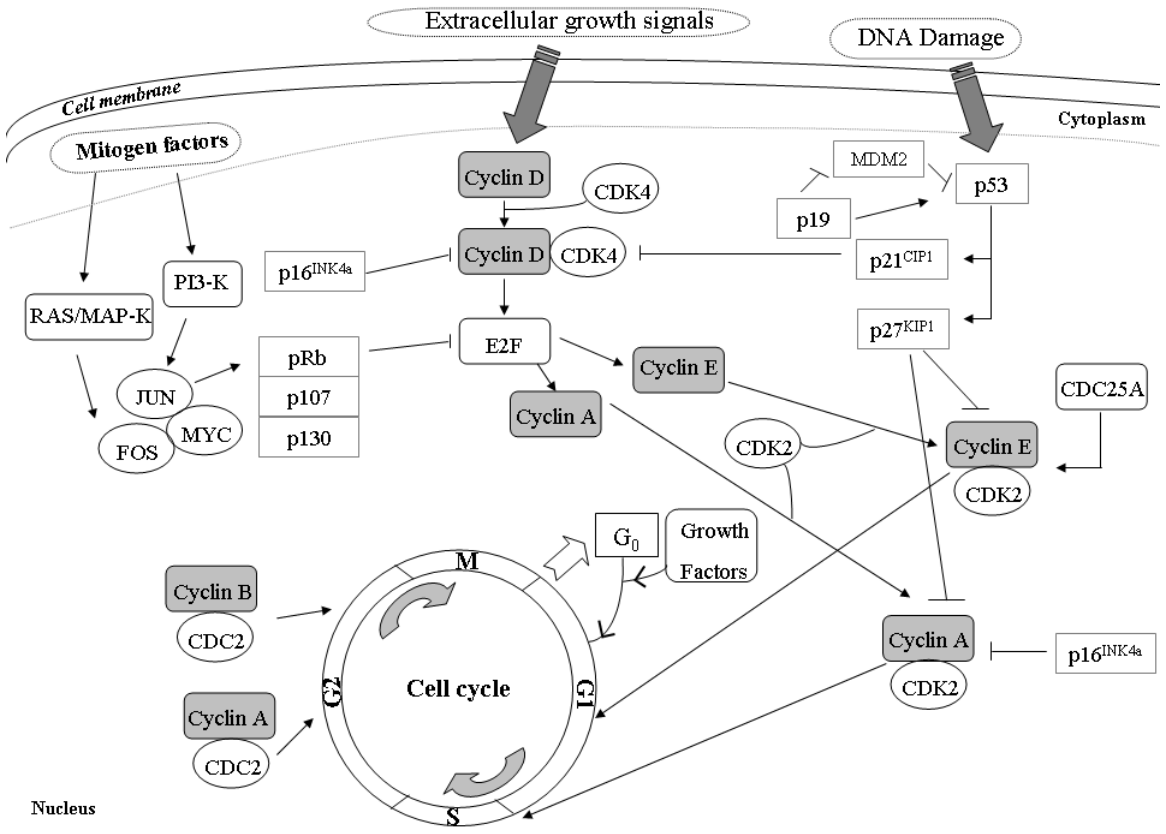


Figure 2. Schematic illustration of the cell cycle regulation based on reviews by Park and Lee (6), and Johnson and Walker (7).

5.1.2 Oncogenes

Proto-oncogenes are a group of genes that are normally present in the human genome and which encode for proteins involved in cell growth and differentiation. Proto-oncogenes are commonly grouped according to their functions such as growth factors, receptor tyrosine kinases, cytoplasmic tyrosine kinases, serine-threonine kinases, regulatory GTPases and transcription factors. The first oncogene, *src* was discovered in 1970 and shown to have oncogenic function in various organisms. This finding was subsequently awarded with the Nobel Prize in 1989. Different types of genetic and chromosomal mechanisms such as point mutations, gene amplifications or chromosomal rearrangements can cause oncogene activation. The result may be over-expression of the normal proto-oncogene, expression of a mutated product or expression of a fusion gene.

Oncogenes are often described as “accelerators” of cell growth.

5.1.3 Tumor suppressor genes (TSGs)

In contrast to oncogenes, TSGs are described as the “brakes” of the cells as their encoded proteins are involved in negative cell cycle regulations or promote cell death and differentiation. Inactivation of TSGs generally follow the “two-hit” model proposed by Knudson according to which both alleles of the gene must be inactivated before tumor growth can be initiated (2). This can be achieved by several types of loss-of-function mutations detected as for example loss of constitutional heterozygosity, or by epigenetic silencing. The first TSG discovered is the retinoblastoma gene (*RB1)*.

5.1.4 DNA repair genes

Our body has a complex system for reparation of discrete DNA damage or molecular lesions that can be caused by *e.g.* normal metabolic activities and environmental factors, or occur randomly (8). Mutations in DNA repair genes cause inefficient repair of DNA, which in turn predispose to genomic instability and subsequent mutations of cancer genes. DNA repair genes are commonly referred as “caretakers”. Well-known examples are repair genes underlying colorectal carcinoma, in which case the tumors show typical patterns of microsatellite instability (MIN). Chromosomal instability (CIN) is another type of genomic instability frequently encountered in cancer cells.

5.1.5 Clonal evolution of cancer

It has long been recognized that the natural course of most tumors is to become clinically more aggressive over time. Fould was the first to report that clinical progression have a tendency to develop step-wise, and Nowell proposed a model for clonal evolution of cancer (9). Subsequently, molecular studies of various cancers have provided experimental support for a multistep process and clonal development of cancer from a single cell that has accumulated necessary mutations. A classical example is the model proposed by Vogelstein and colleagues for genetic and molecular events occurring during development of colorectal cancer (10) (Figure 3).

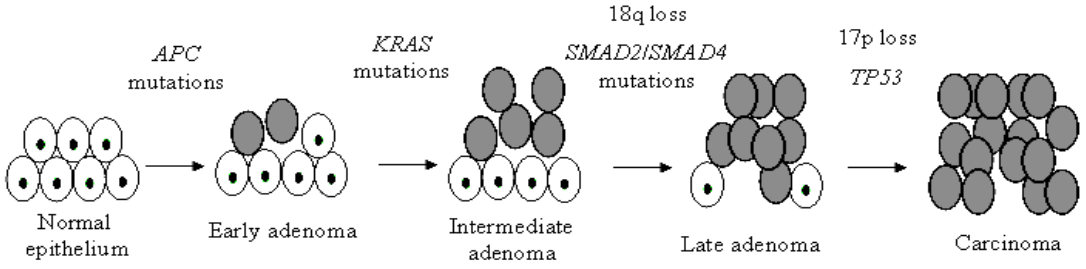


Figure 3. Schematic view of colorectal cancer progression model proposed by Fearon and Vogelstein (10).

5.2 MOLECULAR MECHANISMS OF TUMOR DEVELOPMENT

The chromosomes within our cells contain approximately 30,000 genes, which may be transcribed to RNA and translated to protein. This relationship between DNA-RNA-protein was referred to the Central Dogma as illustrated in Figure 4A (11) and alterations at any of these levels may contribute to the development of cancer. Common types of molecular and genetic alterations associated with cancer are reviewed in the following.

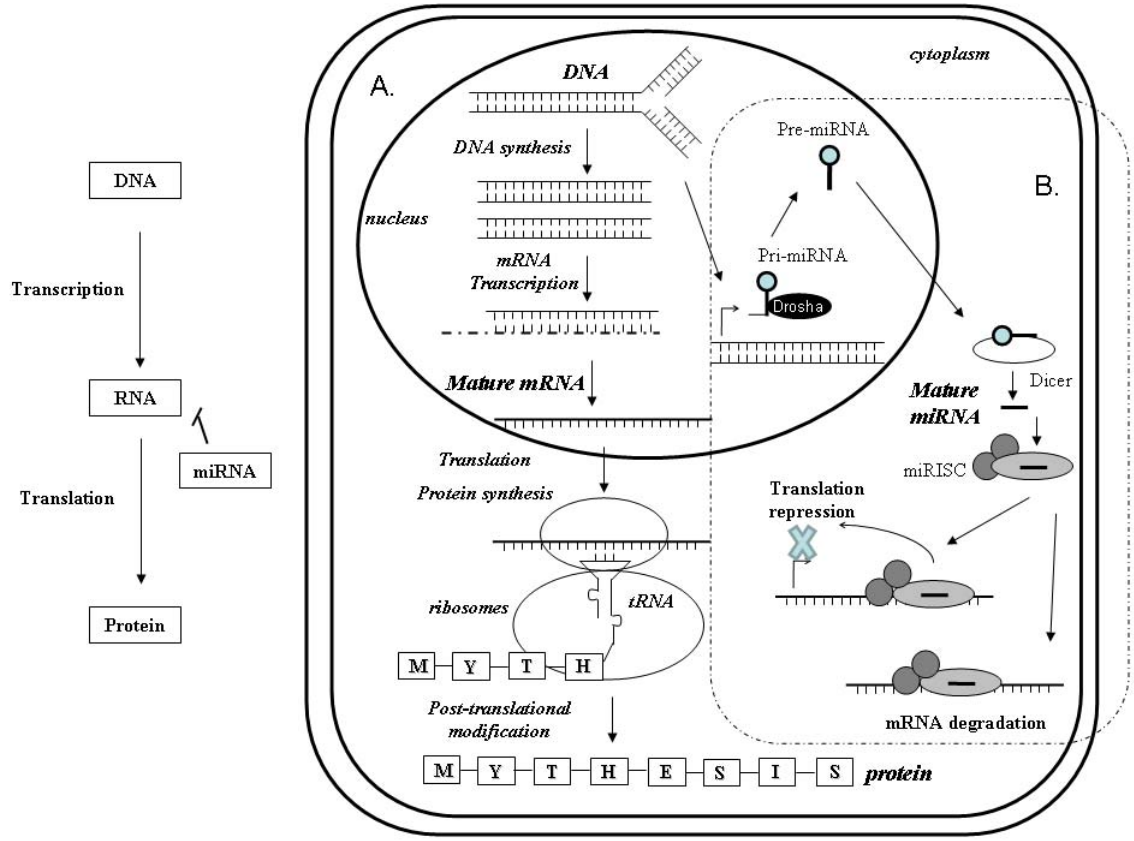


Figure 4. General overview of the relationship between DNA, RNA and protein. A. DNA is transcribed to generate mRNA and mRNA is then translated to protein. The relationship between DNA-RNA-Protein was proposed as the Central Dogma of biology by Francis Crick (11). B. The role of miRNA in RNAi (Section 5.2.4) in regulating gene expression. In the RNAi pathway, primary miRNA (pri-miRNA) is processed by Drosha, a nuclease to become pre-miRNA and subsequently cleaved by dicer to become mature miRNA. miRNA pairs with RISC forming a complex that will either repress translation or degrade mRNA.

5.2.1 Gain-of-function mutations

Several mechanisms can lead to activation of proto-oncogenes with (over)-expression of normal or aberrant protein that promotes tumor growth.

5.2.1.1 Structural chromosomal alterations

Several types of chromosomal rearrangements may activate a proto-oncogene by juxtaposition of sequences from different chromosomal regions to an active chromatin domain or a strong promoter. The reciprocal translocation in Burkitt lymphoma involving *c-myc* and immunoglobulin regulatory sequences is one good example of this mechanism (12). Alternatively the rearrangement will lead to the creation of a novel fusion-gene. The classical example of this mechanism is the Philadelphia chromosome in which the the nuclear kinase, *c-ABL* is joined to BCR creating a *BCR-ABL* fusion gene (13). Less commonly, chromosomal inversions, deletions and insertions may have similar effects. The fusion protein FIG-ROS associated with astrocytoma is a result of a chromosomal deletion of sequences between FIG and ROS, and has constitutively active kinase function (14).

5.2.1.2 Gene amplifications

An increase in the number of copies of a gene (gene amplifications) may be the result of aberrations at the chromosomal level such as whole chromosome gains or chromosomal rearrangements. Furthermore, redundant replication of genomic DNA may occur which is recognized as double minutes (small fragments of extra-chromosomal DNA without centromere) or homogenously staining regions (HSR). The increased gene expression resulting from the amplification may render a selective advantage to the cancer cells, but may also offer a target for directed therapy. For example, activation of *erb*B by gene amplification is common in breast cancer (15) and allows treatment with an antibody against the over-expressed protein.

5.2.1.3 Gene mutations

Activating mutations include substitutions, deletions and insertions of nucleotides at the promoter or in the coding region, which often change the structure and/or function of the cancer gene. Indeed, a single amino acid substitution as a result of point mutation is the most characterized oncogenic mutation in human cancers. Such activating missense mutations are frequently recurrent involving the same or a few amino acid positions only.

5.2.2 Loss-of-function mutations

The concept of TSG inactivation stems from the two-hit model, postulating that both alleles of the gene should be inactivated by somatic and/or germ-line events. However, there are exceptions to this rule, as exemplified by *TP53*, where a loss of one copy of the gene is sufficient to result in a phenotypic effect. This phenomenon is known as haploinsufficiency.

5.2.2.1 Chromosomal rearrangements

Translocations and inversions may not only lead to activation, but also may delete or disrupt regions harboring TSGs. For example, following the t(11;22)(p13;q13) translocation in desmoplastic small round-cell tumor the trans-activation domain of the EWS protein is fused to zinc-fingers of the Wilm's tumor suppressor (WT1) resulting in its transcriptional repression and induction of platelet-derived growth factor A chain (16).

5.2.2.2 Loss of heterozygosity (LOH)

LOH detected in a tumor DNA sample indicates that one of the two constitutional alleles is missing or under-represented. It is frequently detected as a second "hit" together with a preceding discrete mutation for TSG inactivation. LOH can result from a simple gene copy number loss, a gross deletion or loss at the chromosomal level, or from mitotic recombination.

5.2.2.3 Gene mutations

Gene mutations that occur within a TSG are typically predicted to inactivate the protein by coding for a stop codon (nonsense mutation), an altered amino acid (missense) or a truncated protein. The associated phenotypes are generally recessive in the sense that the other allele remains functional and must also be inactivated. Dominant negative mutations is one exception to this situation, since their resulting altered gene product acts antagonistically to the wild-type allele leading to altered molecular function. Again *TP53* serves as an example for this latter situation.

5.2.3 DNA methylation

DNA methylation is a frequently observed epigenetic mechanism for regulation of gene expression. In normal cells, DNA methylation occurs at CpG sites, which are CG dinucleotides clustered in small stretches of DNA preferentially in promoter regions (17). The actual methylation consists of covalent addition of a methyl group to the 5' carbon of the cytosine ring, resulting in 5'-methylcytosine (18). In the cancer epigenome global hypomethylation and promoter-specific hypermethylation of CpG islands are commonly seen. The present view suggests that DNA hypermethylation contributes to tumorigenesis by predisposing to mutations at methylated CpGs and by silencing of specific genes. CpG sites constitute only about 4% of the genome (19), but are typically found at promoter regions where transcription is initiated. DNA methylation at these regions can therefore repress transcription by inhibiting binding of transcription factors and by recruiting methyl-CpG binding protein (MBD).

Global DNA methylation can also affect mobile repetitive elements such as Long Interspersed Nuclear Elements (LINEs) and Short Interspersed Nuclear Elements (SINEs) that comprise about 45% of the human genome. In normal cells, this ensures that these repetitive elements will replicate late and suppresses potentially harmful retroviral or transposon sequence that may have been integrated into sites containing highly repetitive sequences (17). By contrast, hypomethylation in cancer cells may favor mitotic recombination, leading to LOH and promotion of karyotypically detectable rearrangements (19).

5.2.4 RNA interference (RNAi)

RNAi is a biological mechanism in regulating gene expression by short double-stranded RNA. This discovery of this mechanism was awarded for the Nobel Prize in medicine and physiology 2006. The RNAi pathway is initiated by cleavage of dsRNA to short double-stranded fragments of 20-25 bps by an enzyme called Dicer. Then one of the two strands of each fragment will then be incorporated into the RNA-induced silencing complex (RISC). The RISC paired with strands will induce mRNA degradation and hence post-transcriptional gene silencing. siRNA and miRNA are two types of small RNAs that are derived from this pathway sharing the same precursor. The short RNA fragments that are perfectly complementary to the gene they are suppressing are known as small interfering RNA (siRNA) as they are derived from long dsRNA of that same gene. MicroRNA (miRNA) on the other hand is derived from the intragenic or intronic regions and is just

partially complementary.

miRNA, 21-25 bp non-coding RNA mediates sequence-specific, post-transciptional repression of mRNA targets (Figure 4B). Recent data indicates that miRNAs may impact cellular transformation by modulating oncogenes and TSGs molecular network (20, 21). Furthermore many studies have uncovered specific over- and under-expression of miRNAs in tumors (22). Reduction in miRNA levels following disruption of the miRNA processing machinery has been demonstrated to enhance tumor cell proliferation, which was accompanied by over-expression of *Myc* and *K-Ras* oncogenes. Similarly over-expression of *let-7g* (miRNA that regulates Ras family proteins) reduced colony formation in tumor cells (23). Deregulated miRNA expression can result from impaired miRNA processing, copy number alterations of miRNA transcribing loci, or from methylation of miRNA promoter regions (22). Of note altered miRNA regulated expressions may have either tumor-suppressor-like or oncogene-like consequences (so called oncomir) (22).

5.2.5 Telomere dysfunctions

Telomeres are highly repetitive DNA sequences (TTAGGG) located at the end of chromosomes that have a protective function. The telomerase complex is responsible for maintaining the length of the telomeres, and in cancer the telomerase activity is commonly induced (24). In normal cells telomeres are shortened by 50-200 bp in each round of DNA replication, which is related to the limitations of DNA polymerase. When the telomere length is reduced to a critical limit, the cell growth will be arrested. In cells that avoid this senescence-like stage (*e.g.* as a result of mutation) telomeres will become critically short and possibly dysfunctional. This may cause apoptosis, cell transformation or chromosomal instability with end-to-end chromosomal fusions and anaphase bridges during mitosis that could contribute to genetic instability in cancer cells (25). Telomere dysfunctions have been observed in different types of cancers including colorectal and breast cancers (26, 27).

5.2.6 Metastasis

Malignancy of tumors is frequently accompanied by morphological and phenotypic alterations that resemble immature stages of normal differentiation. In addition there is an increase in the proportion of proliferating cells that have not achieved a fully differentiated phenotype. This concept is well-illustrated in acute promyelocytic leukemia associated with a *PML-RAR*α fusion gene where normal myeloid differentiation is induced by the addition of retinoic acid (28).

Metastasis refers to the processes by which tumor cells can migrate from the primary tumor to other parts of the body and give rise to daughter tumors. The development of metastasis is today the major cause of adverse outcome in affected patients. The resulting cancer morbidity and mortality is mainly a consequence of the shortage of curative therapeutic possibilities for metastatic disease. By contrast, primary tumors can often be successfully treated by surgery and post-operative radiation. The metastatic process is complex and requires the cancer cells that can invade the surrounding tissue, invade and survive in the blood and lymphatic system, arrest and extravasate of the circulatory system and colonize in a new anatomical location (29). It remains unknown why specific cancers have a tendency to metastasize to particular sites, while this remains to be further explored. However, it has been proposed that organ specificity of metastasis is related to factors determining tumor cell trapping, adhesion and niches (30).

5.2.7 Cancer stem cells

Studies of cancer stem cells have emerged as an important new field in cancer research. Cancer stem cells are sub-populations of cells within a tumor that have the capability of reestablishing the tumor in animal models (31). The same cells are also regarded as responsible for aggressive features of cancer in terms of recurrence and establishment of metastases. These features resemble the abilities to self-renewal and differentiations into multiple cell types that are characteristics of normal stem cells. The existence of cancer stem cells was first suggested from studies of hematological malignancies. Bonnet and Dick isolated a sub-population of acute myeloid leukemia cells with the cell surface characteristics $CD34^{+}/CD138^{-}$ from which novel tumors of similar phenotype could be initiated in NOD/SCID mice (32). The ongoing research in this field aims at finding cancer stem cells and their markers in additional types of cancer. The findings are expected to be important for questions related to therapy resistance, disease spreading and recurrence.

5.3 THE THYROID GLAND

Mammals, birds, and many other living organisms harbor an endocrine system that regulates biological processes in the body. This system is made up of glands located throughout the body, producing hormones that are released into the bloodstream with effects on receptors in various organs and tissues. The human endocrine system comprises the hypothalamus, pineal gland, pituitary glands, parathyroid glands, thyroid, thymus, endocrine pancreas, endocrine cells in the gastrointestinal tract, adrenal gland,

kidney and reproductive organs (ovary for female and testicle for male).

5.3.1 Anatomy

The human thyroid gland as illustrated in Figure 5 is situated in the neck, in front of the trachea and below the position of the Adam's apple. The gland consists of two lobes that are connected by the isthmus. In the dog the thyroid has a similar appearance, while *e.g.* in birds the two lobes are isolated (33).

5.3.2 Embryology

The embryonic development of the thyroid starts at three to four weeks of gestation with epithelial proliferation at the base of the tongue where after it descends and migrates to the base of the neck. The calcitonin producing cells originate from the fifth pharyngeal pouch that give rise to the ultimobranchial body. This is later incorporated into the central part of each lobe of the thyroid gland.

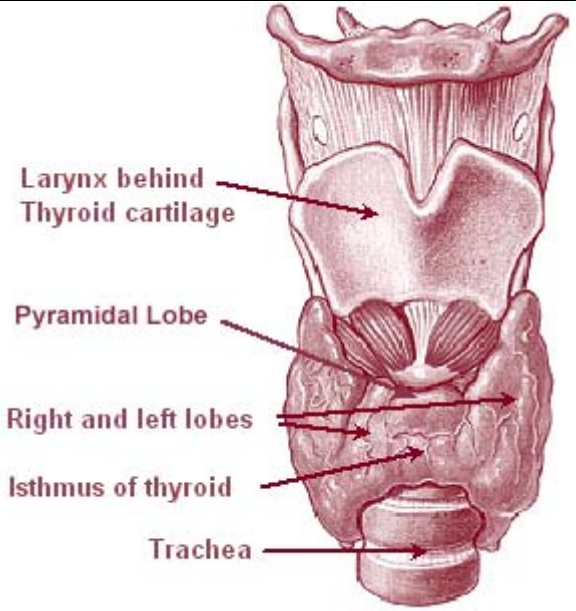


Figure 5. The thyroid gland. (Source: http://training.seer.cancer.gov/)

5.3.3 Histology

The normal thyroid gland (Figure 6A) exhibit a typical histological pattern with spherical sacs (follicles) surrounded by epithelial cells commonly referred to as follicular cells (34). The follicles contain colloids with thyroglobulin (Tg) for production of the thyroid hormones. thyroxine (T_4) and triiodothyronine (T_3). The production of thyroid hormones is regulated by thyroid-stimulating hormone (TSH) from the pituitary gland, which in turn is modulated by various influences such as T_4 and iodine levels, thyrotropin-releasing hormone (TRH) from the hypothalamus, and different other hormones. Differentiated thyroid cancer usually secretes Tg into the blood. For post-operative follow-up, it is a valuable tumor marker. Parafollicular cells located between the follicles produce calcitonin, and are therefore called C-cells. The functions of calcitonin are related to the metabolism of calcium and phosphorous. High levels of calcitonin are common in patients with medullary thyroid carcinoma (MTC), as such it is used as a marker in MTC diagnostics (35).

5.4 THYROID TUMORS

A normal thyroid gland weighs about 15-40 grams. In certain geographical regions thyroid enlargement is frequently encountered in the form of colloid goiter due to iodine deficiency. Nodules in the thyroid gland are common. In regions with sufficient iodine intake, 4-7% of the general population have clinically detectable thyroid nodules, the majority of which are benign (36). Thyroid nodules may reflect a variety of non-neoplastic conditions such as goiter or thyroiditis, as well as benign or malignant tumors.

Figure 6 illustrates the major types of thyroid tumors, recognized in the WHO classification (37). The most common type of thyroid tumor is follicular adenoma (FTA), originating from the follicular epithelium. FTA is an encapsulate solitude tumor, which is in contrast to non-neoplastic thyroid nodules that are not encapsulated and usually multiple. FTA is classified as benign, and the diagnosis requires the absence of vascular and capsular invasion (38). Other subtypes of follicular tumors are; atypical follicular thyroid adenoma (AFTA) and Hürthle cell adenoma (HTA) (37). The diagnosis AFTA refers to adenomas with high cellularity, irregular growth pattern against the capsule and increased mitotic activity. HTA, or oxyphilic cell adenoma, is composed of more than 75% eosinophilic cells with a high content of mitochondria. Malignant thyroid tumors are grossly categorized as well-differentiated (WDTC), poorly differentiated (PDTC) or undifferentiated (anaplastic; ATC) cancer. In general patients with WDTC have superior prognosis as compared to those with PDTC or ATC (39).

Important questions to answer are whether the different types of thyroid tumors are separate entities or can be the result of progression from benign to malignant and undifferentiated forms.

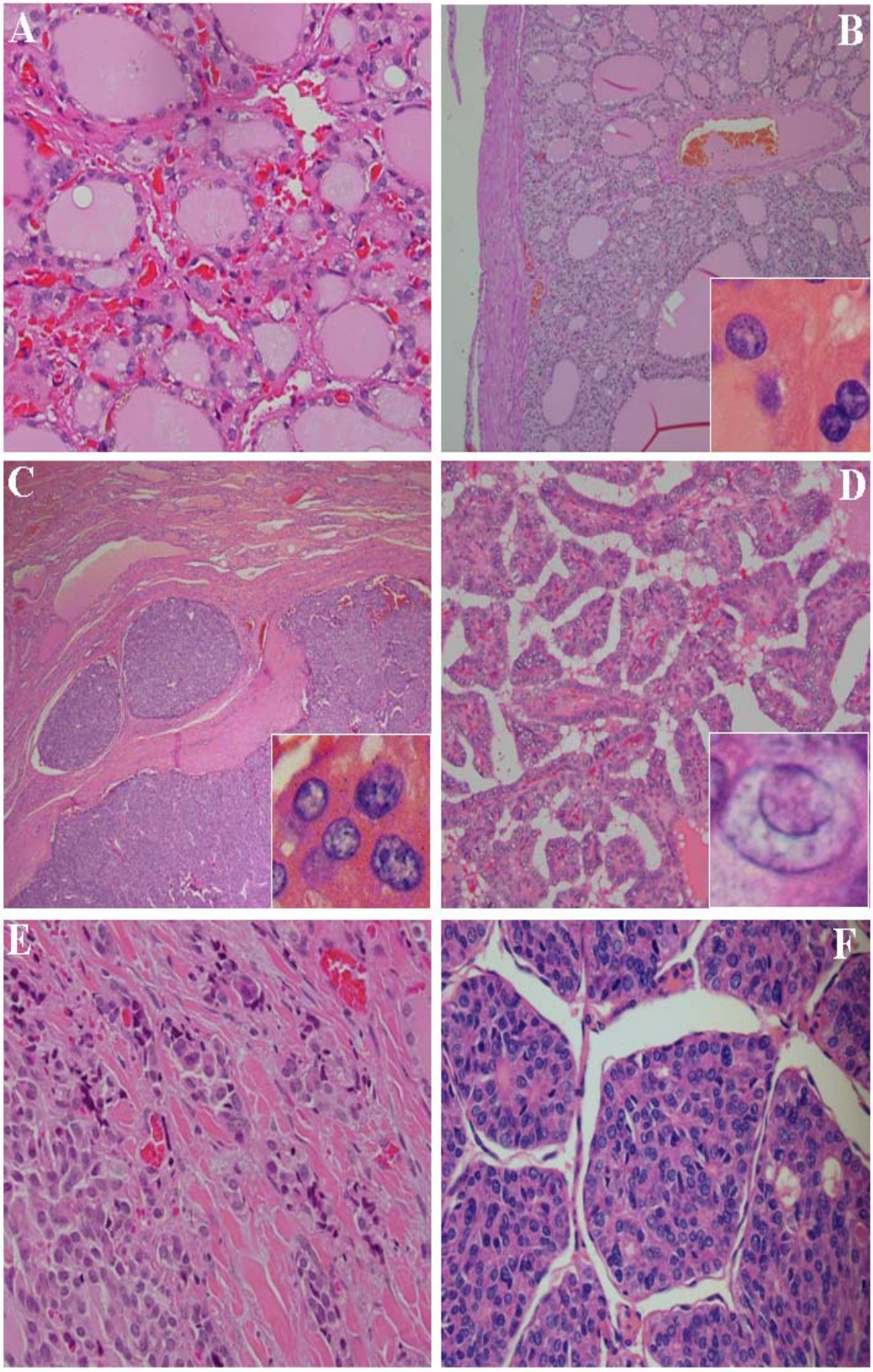
5.4.1 Papillary thyroid cancer (PTC)

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer, constituting 80-90% of all thyroid cancers (40). PTCs are tumors of follicular origin that exhibit papillary and/or follicular growth pattern and characteristic nuclear inclusions and overlapping tumor nucleus (37). The crucial diagnostic feature for a PTC is the typical nuclear changes (also referred to as “ground-glass nuclei”). PTC frequently invades lymphatic vessels and lymph nodes.

5.4.2 Follicular thyroid cancer (FTC)

The second most common type is follicular thyroid cancer (FTC), accounting for about 10% of all cases (40). FTC presents with capsular and/or vascular invasion. Based on the degree of invasion FTCs are further classified as minimally-invasive or widely-invasive (37, 38). Hürthle cell thyroid carcinoma, also known as oxyphillic or oncocytic carcinoma consists of more than 75% oxyphilic cells, is a variant of FTC. FTC metastases are found in the lungs and bones following hematogenous spread. Tg immunostaining is positive in FTCs and can be used to confirm the thyroid origin of a metastasis of FTC (41).

Figure 6. Photomicrographs of haematoxylin & eosin (H & E) stained human thyroid tissues. A. Normal thyroid gland consists of follicles with an average size of 0.2 mm lined by follicular epithelium. B. Follicular thyroid adenoma (FTA), a benign encapsulated tumor with follicular cell differentiation, without capsular or vascular invasion. Inset is a variant of FTA: Hürthle cell thyroid adenoma showing eosinophilic tumor cells rich in mitochondria. C. Follicular thyroid carcinoma (FTC) showing evidence of vascular invasion. Inset is a variant of FTC: Hürthle cell thyroid carcinoma showing eosinophilic evident tumor cells rich in mitochondria. D. Papillary thyroid carcinoma (PTC) showing typical papillary pattern with crowded overlapping tumor cell nuclei and nuclear inclusions (inset). E. Poorly differentiated thyroid carcinoma (PDTC) a tumor type that has microscopical findings and clinical behavior intermediate between WDTC and ATC. F. Medullary thyroid carcinoma (MTC) with typical acellular deposits amyloide (red strands in between tumor cells).


A
B
C
D
E
F

5.4.3 Medullary thyroid cancer (MTC)

Medullary thyroid cancer (MTC) originates from the calcitonin-producing parafollicular C-cells and accounts for about 3% of all thyroid cancers (40). Microscopically these tumors are composed of spindle-shaped, round or polygonal cells separated by fibrous stroma that may contain amyloid (37). An important part of the diagnostic work is the demonstration of immunoreactivity for calcitonin. Metastases to regional lymph nodes are common. Distant metastasis occurs in 20% of the patients and then to the liver, lung and skeleton (37). Notably, MTC may occur in sporadic as well as hereditary forms. The hereditary form of MTC will be discussed in Section 5.4.6.

5.4.4 Poorly differentiated thyroid cancer (PDTC)

PDTC is a rare type of thyroid cancer, accounting for up to 7% of all thyroid cancer (40). However the real frequency is somewhat uncertain, which is due to related inconsistent definition of this entity. Recently a consensus meeting has proposed diagnostic criteria for PDTC (42). According to the WHO classification (37), PDTC is characterized by increased mitotic activity, tumor necrosis, capsular and vascular invasion and at least focal positive staining for Tg. However PDTC lacks several morphologic characteristics that are typical for WDTC, suggesting that it may represent an intermediate entity in the progression from WDTC to ATC (39).

5.4.5 Anaplastic thyroid cancer (ATC)

ATC accounts for only 1-2% of all thyroid tumors and is the most aggressive and lethal form of thyroid cancer (43). The prognosis is extremely poor with a median survival of less than one year after diagnosis (40). This study and others (44) have reported that ATC can occur concurrently with a variety of thyroid disorders, including WDTC (Paper IV) (44) (Figure 7). ATCs are rapid growing unencapsulated tumors that infiltrate the surrounding soft tissues of the neck and into the respiratory tract. Microscopically three types of histologic variants are observed including spindle, giant cell and squamoid cell pattern (39). ATCs are further characterized by frequent mitoses, large areas of necrosis, hemorrhagic, and vascular invasion (37). Staining for Tg is typically negative. In addition, ATC cells do not have thyrotropin receptors, transport iodine, or produce Tg. IHC staining for calcitonin, chromogranin A, carcinoembryonic Antigen and Tg are typically negative. Already at presentation it is commonly accompanied with regional lymph node metastases, local invasion of surrounding tissues (*i.e.* fat, trachea, muscle, esophagus and larynx), as well as distant metastases in the lung, skeleton and brain. If left untreated most

patients will die of ultimate airway obstruction caused by the rapid and extensive tumor growth.

Table 2. Comparison of clinical manifestations and features of MEN 2 syndromes.

	MEN 2		
Clinical features	MEN 2A	MEN 2B	FMTC
MTC	Yes	Yes	Yes
Pheochromocytoma	Yes	Yes	No
Hyperparathyroidism	Yes	No	No
Tall & slender	No	Yes	No
Small benign tumors on lips and tongue	No	Yes	No
Genetic predisposition	*RET*	*RET*	*RET*

5.4.6 Familial thyroid cancers

A well-known example of familial thyroid cancer is MTC, an autosomal dominant condition that is either transmitted alone (FMTC) or as part of multiple endocrine neoplasia type 2A (MEN 2A) or type 2B (MEN 2B) (45). Familial forms of MTC are typically bilateral and multicentric whilst patients with sporadic MTC i usually presented with a single tumor involving one lobe only (37, 38). MTC is a frequent manifestation and also the most common cause of death in patients with MEN 2A, MEN 2B and FMTC (46). In addition to MTC, patients with MEN 2A are predisposed to develop pheochromocytoma and primary hyperparathyroidism, and to a certain degree manifest cutaneous lichen amyloidosis and Hirschprung's disease (46). MEN 2B is the most severe form of MEN 2 where the patients are at risk of MTC and pheochromocytoma, and in addition marfanoid body habitus and mucosal and intestinal ganglioneuromatosis are common features (46). A comparison of clinical manifestations and features are presented in Table 2. FMTC, MEN 2A and MEN 2B are strongly associated with predisposing mutations of the *RET* proto-oncogene (35).

Cowdens syndrome is another well characterized form of hereditary predisposition to thyroid tumors (OMIM 158350) (47). Patients affected by this dominantly inherited syndrome are predisposed to different phenotypic characteristics as well as malignant and benign (hamartomatous) tumors of the breast, thyroid, uterus, skin, brain and mucous membranes. Thyroid tumors diagnosed in these are of non-medullary type, most commonly FTC. Germ-line mutations of the *PTEN* tumor suppressor gene have been established as an underlying cause in most patients with Cowden syndrome (48). The genetics of *PTEN* is discussed in Section 5.6.2.3.

In addition, it has been estimated that 3.5%-6.2% of patients with follicular tumors, (also

known as nonmedullary thyroid cancer NMTC) have a familial form of the disease (49). Familial-NMTC (FNMTC) is considered in kindreds with two or more affected first-degree relatives (50). Although not much is known about FNMTC, the most common histologic subtype observed thus far is PTC.

Figure 7. Photomicrographs of H & E staining of PTC and anaplastic thyroid carcinoma (ATC). The PTC dedifferentiated tumor cells to undifferentiated/ATC with large pleomorphic tumor cells.

PTC
ATC
PTC
ATC
PTC
ATC
PTC
ATC
ATC

5.5 THYROID EPIDEMIOLOGY, DIAGNOSIS & TREATMENTS

5.5.1 Epidemiology and risk factors

Overall thyroid tumors account for approximately 1% of all human malignancies (37), however the incidence is influenced by several factors such as gender, ethnic, origin and geographical location. Women are more frequently affected than men, and on a worldwide basis. Between 1.2 to 2.6 men and 2.0 to 3.8 women per 100,000 individuals are diagnosed with thyroid cancer each year (51). The geographical variation is exemplified by *e.g.* the two-fold higher incidence in Iceland and Hawaii as compared to other North European countries, Canada, U.S.A and Israel (35). Furthermore, thyroid cancer is also more common among certain ethnic groups, *e.g.* Chinese men, Filipino women in Hawaii (52) and Caucasian men and women (53).

Epidemiological studies have suggested that the risk of developing thyroid cancer is influenced by a variety of internal and external factors. Predisposing genetic alterations contribute to familial clustering of thyroid cancer as discussed in Section 5.6. Additional determining parameters are iodine deficiency, diet, and exposure to ionizing radiation (54), sex hormones (55), age and gender (35). Individuals with dietary iodine deficiency frequently develop goiter as a result of compensatory thyroid proliferation. Interestingly FTC is more prevalent in areas of iodine deficiency, and ATC often coincides with long-standing goiter (56). Radiation is another well-established risk factor for development of in particular PTC, which have become evident in individuals exposed to radiation from nuclear weapon (*e.g.* Hiroshima and Nagasaki) or nuclear power plant (*e.g.* Chernobyl) (54).

5.5.2 Diagnosis and treatments

When a patient is diagnosed in the clinic with a thyroid nodule the first diagnostic procedure is to perform a fine needle aspiration biopsy (FNAB). In most the cytological analyses of the FNAB will reveal a benign diagnosis such as colloid goiter, or thyroiditis. Some types of thyroid malignancy can be readily diagnosed pre-operatively such as PTC and ATC. However, in the case of follicular tumors, FNAB cannot be used to distinguish benign and malignant forms. This is related to the diagnostic criteria of FTC requiring the demonstration of capsular and/or vascular invasion. Therefore, all follicular thyroid tumors will be treated by surgery, most commonly by removal of the involved lobe (*i.e.*

lobectomy). If the histopathological investigation of the surgically removed tumor shows FTC, a second operation is often performed (*i.e.* total thyroidectomy). It is expected that the increased molecular and genetic knowledge of these tumors will lead to the development of diagnostic tools for pre-operative distinction of these entities.

There is presently an active debate as to whether lobectomy or total thyroidectomy should be recommended for patients with thyroid cancer. The recommendation of total thyroidectomy is supported by reports showing lower recurrence (57), and mortality (58) and improved survival (59) as compared to lobectomy. Total thyroidectomy also offers the possibility of effective I^{131} treatment and subsequent recurrences can be identified by measurements of Tg or calcitonin. However total thyroidectomy has the drawback of requiring lifelong substitution with thyroid hormone. On the other hand lobectomy has the advantage of decreased risk for surgical complications such as hypoparathyroidism and damage to local nerves.

Treatment of ATC remains a challenge in clinical practice. Since the tumors are large, locally infiltrative giving air-way obstructions radiotherapy and chemotherapy are commonly given to decrease the tumor mass. This procedure has both positive effects on the outcome of surgery as well as reduces the local symptoms in the neck (43).

5.6 MOLECULAR GENETICS OF THYROID TUMORS

Development of thyroid tumors is associated with a variety of molecular and genetic mechanisms related to *e.g.* oncogene activation, fusion oncogenes, tumor suppressor inactivation, genomic imbalances, expression profiles of genes, proteins and microRNA, epigenetic modifications, and possibly mitochondrial DNA alterations. Several of these modifications are associated with certain types of thyroid tumors or related to familial and sporadic forms of disease as reviewed in the following. The expanding knowledge of thyroid cancer molecular genetics continuously adds to our understanding of thyroid tumor etiology and possible multistage tumorigenesis.

5.6.1 Oncogenes

Activating oncogene mutations are frequently encountered in thyroid tumors and may be observed in germ-line and more commonly on the somatic level. Well-known examples of recurrently involved oncogenes are *RET*, *BRAF*, the three *RAS* genes, *β-catenin* and *PIK3CA* which are described below.

5.6.1.1 RET

The *RET* (*rearranged during transfection*) proto-oncogenes (60) located in chromosomal region 10q11.2 encodes for a tyrosine kinase receptor that is expressed in the cell membrane of different tissues including the thyroid, adrenal medulla, parathyroid, and enteric ganglion (61). The RET receptor consists of an extracellular, a transmembrane, and two intracellular domains (62). Activation is achieved by binding of the complex formed by the glial cell line-derived neurotrophic factor (GDNF) ligand and the GDNF family receptors-α (GDNFα). Binding of the ligand induces dimerization of the receptor and autophosphorylation in the intracellular domain, whereafter downstream signaling cascades can be activated (62, 63). The *RET* gene has been strongly associated with the development of thyroid cancer (61). Chromosomal rearrangements involving *RET* will be discussed in the following under Section 5.6.3.1. In MTC *RET* mutations are frequently encountered in exons 10,11,13,14,15 and 16 (61). These mutations typically involve a single base-pair substitution leading to a shift in amino acid often involving a cysteine residue. MEN 2A or FMTC patients harbor constitutional mutations that are commonly affecting the cysteine-rich part of the extracellular domain or the intracellular tyrosine kinase domain (64, 65). A subset of apparently sporadic MTC cases also carry germ-line mutations of *RET* (64, 65). MEN 2B is most commonly caused by a mutation in exon 16 that affects codon 918 and the intracellular substrate recognition pocket in the catalytic core (61). Sporadic MTC frequently exhibit the same codon 918 mutation on the somatic level although other mutations may also occur (64, 65).

5.6.1.2 BRAF

BRAF mutations are presently known as the most common genetic abnormalities in PTC. The *BRAF* gene is located in chromosome 7, and its encoded protein is a member of the RAF family of serine-threonine kinases (66). *BRAF* normally functions as an effector of the MAPK (mitogen-activated protein kinases) pathway. Binding of Ras leads to activation and recruitment of BRAF to the cell membrane for subsequent phosphorylation and activation of members of the MAPK signaling cascade with effects on cellular differentiation, proliferation and survival (66). Studies of benign thyroid cells have documented an important function for *BRAF in vitro* studies (67). *BRAF* is frequently mutated in a variety of different human cancers such as melanoma, ovarian cancer and colorectal cancer as reviewed in Dhomen and Marais (66). One of the hitherto reported mutations constitutes approximately 90% of all mutations. This “hotspot” mutation involves a 1799T-A single

nucleotide transition in exon 15, predicted to give an amino acid substitution of a valine by a glutamic acid at the position 600 (V600E) (68). The missense mutation has been reported in approximately 45% of PTC (69), thus making it the most common genetic abnormality of this entity. Although V600E is by far the most common type of mutation, activating *BRAF* mutations have also been reported at codons 599 and 601 (70, 71). Furthermore, V600E has also been found in approximately 10% of PDTC and a third of ATC (40). In ATC, *BRAF* mutations have been reported to be more prevalent in cases presenting with a PTC component as compared to regular ATC (40). Based on these results, it has been hypothesized that PTCs with a *BRAF* mutation would be more aggressive and could possibly progress to ATC. However the possible relationship between V600E and aggressive tumor phenotypes is controversial and remains to be further clarified (72).

5.6.1.3 Ras

Point mutations in any of the three *RAS* genes *H-RAS*, *K-RAS*, and *N-RAS* are common findings in human cancers and present estimates suggest that approximately 30% of all human tumors harbor mutations in one of these genes (73). *RAS* mutations are also commonly observed in follicular thyroid tumors including both FTA and FTC, while in PTC it is less frequent with the exception of PTC of follicular variant (40). *RAS* mutations have also been reported in PDTC (18-27%) and ATC (20-60%) (40). These findings could possibly suggest a progression of *RAS* mutated follicular tumors to ATC, but may also be coincidental following the overall high frequency of *RAS* mutations in human cancer. *RAS* mutations are typically missense alterations that affect two different locations in the gene including: exon 1 and codons 12 or 13 of the GTP binding part; or exon 2 and codon 61 of the GTP-binding domain (73). The normal function or Ras molecules are to convey signals from membrane-bound tyrosine kinase receptors to the MAPK cascade to activate transcription of target genes. Following activation of Ras by mutation, downstream targets will be continuously stimulated with effects *e.g.* apoptosis and proliferation, and in addition contribute to genomic instability, mutations and malignant transformation (73).

5.6.1.4 β-catenin (CTNNB1)

β-catenin encoded by the *CTNNB1* gene is a cytoplasmic protein with several functions. These involve regulation of cell-cell adhesion by binding to cadherins, mediation of the wingless (Wnt) signaling pathway, and transcriptional up-regulation of different oncogenes such as *Cyclin D1* (*CCND1*), *C-MYC* and *C-JUN* (40, 74). Mutations of *CTNNB1* have

been reported in the most aggressive forms of thyroid cancer including 0-25% of PDTC and up to 66% of ATC (40). *CTNNB1* mutations, especially those in exon 3, stabilize β-catenin so that its normal degradation by the adenomatous polyposis coli (APC) complex is avoided (74). This will in turn lead to over-expression of β-catenin and constitutive activation of target gene expression.

5.6.1.5 PIK3CA

The phosphatidylinositol-3-kinase (PI3K)/Akt signaling involved in regulation of cell growth, proliferation, and survival, has an established role in human tumor development (75). Oncogenic mutations or gene amplifications have been demonstrated in the catalytic subunit of the PIK3CA isoform (76). In thyroid cancer, *PIK3CA* mutations and copy gain have been reported (77-80), with a preferential involvement of *PIK3CA* mutations in FTC and ATC, and of copy number gains in ATC (80). In a recent study, significant correlation of *PIK3CA* copy gain with increased PIK3CA protein expression was demonstrated (80). In addition, activation of the PI3K/Akt pathway has also been implicated in thyroid cancers (81-83). Notably PIK3CA is activated by Ras, whilst the *PTEN* tumor suppressor (Section 5.6.2.3) has a phosphatase activity antagonizng the signaling of the PI3K/Akt pathway as reviewed in (75). These observations collectively suggest that PI3K/Akt/MAPK pathway is strongly associated with thyroid tumorigenesis.

5.6.2 Tumor Suppressors

5.6.2.1 p53

p53 is the tumor suppressor gene most frequently mutated in human cancer and it plays a central role in cell cycle regulation, DNA repair and apoptosis. In thyroid, mutations of *p53* are generally restricted to the entities of PDTC (17-38%) and ATC (67-88%) (40). Furthermore in a study of tumors with both WDTC and ATC, *p53* mutations were only detected in the ATC component (84). In animal models, transgenic mice with *RET/PTC* rearrangements that developed PTC were crossed with *p53*-/- mice, giving rise to progeny with rapid development of PDTC and ATC (84). Taken together, these observations indicate that *p53* inactivation is an important step for the dedifferentiation and progression of thyroid cancers.

5.6.2.2 p27

p27 or kinase inhibitor protein 1 (KIP1) encodes a nuclear protein member of the CDK

inhibitors (CDKIs). These molecules negatively regulate cyclin activity and thus control the transition from the G1 phase to the S phase in the cell cycle as reviewed in Park and Lee (6). Several studies have compared p27 levels in thyroid tumors with those of normal thyroid tissues, and reported recurrent decrease in levels of p27, with lowest intensity detected in PDTC (85, 86). Moreover, under-expression of p27 in PTC has been reported as an independent predictor of lymph node metastasis (87).

5.6.2.3 PTEN

PTEN is a dual-function lipid phosphatase that exerts its tumor suppressor effects by acting on the PI3K and the Akt/PKB pathways (75, 88). Somatic mutations in *PTEN* are rare in primary epithelial thyroid tumors. However hemizygous deletion occurs in 10-20% of FTAs and thyroid carcinomas as reviewed in (89). Moreover, progressive loss or reduction of PTEN protein expression is observed in thyroid cancers (90, 91). Recently aberrant promoter methylation for *PTEN* was also shown in thyroid cancers (91).

5.6.2.4 RASSF1A

The *Ras Association Domain family I* (*RASSF1A*) gene is located in chromosomal region 3p21.3 that is frequently loss in many human cancers including thyroid. It is a Ras effector possessing tumor suppressor properties through its involvement in apoptotic signaling, stabilization of microtubule and mitotic progression (92). *RASSF1A* promoter hypermethylation and LOH have been demonstrated to be the main mechanism to inactivate this gene, whilst mutation is common in many human cancers. *RASSF1A* methylation but not LOH has been reported in all types of thyroid cancer, mostly in FTC (93-95).

5.6.3 Chromosomal rearrangements

Chromosomal rearrangements leading to oncogenic activation contributing to cancer development have been frequently described in hematological malignancies and sarcomas. In solid tumors of epithelial origin such rearrangements are less well characterized and have previously been regarded as of minor importance. However thyroid tumors serve as a good example of the importance of chromosomal rearrangements and associated cancer genes in solid tumors. Approximately twenty different such rearrangements are presently known that are described below.

5.6.3.1 RET/PTC rearrangements

Under normal conditions *RET* is highly expressed in C-cells but not in follicular cells of the

thyroid. In thyroid tumors *RET* is frequently activated by mutational events that contribute to tumor development. The common missense mutations that are characteristics of in familial and sporadic MTC have been described in Section 5.6.1.1. In PTC *RET* is recurrently activated by chromosomal rearrangement such as translocation or inversion. Following the rearrangement *RET* and the partner gene are juxtaposed leading to fusion of the 3' part of *RET* gene with the 5' part of one of several unrelated genes, known as *RET/PTC* rearrangements. To date, at least 11 different RET/PTC rearrangements have been reported in varying frequencies of PTC cases (63). Overall *RET/PTC* rearrangements have been reported in varying frequencies of PTC cases (63). Overall *RET/PTC* has been more frequently reported in pediatric patients (up to 80%) as compared to adult patients (2-34%) (84). *RET/PTC1* and *RET/PTC3* are the most commonly observed variants among the pediatric patients, which has been proposed to reflect an association with radiation exposure (84). *RET/PTC* rearrangements are regarded as early events in development of PTC but unrelated to progression from PTC to PDTC or ATC (96). Santoro and colleagues found less than 10% of *RET/PTC* positive PDTCs suggesting that *RET/PTC* carrying PTCs have a low risk of progressing (97).

5.6.3.2 NTRK1 rearrangements

The known chromosomal rearrangements in PTC involve either *RET* or *NTRK1*, which are fused with a variety of different partner genes. The *NTRK1* gene (neurotrophic tyrosine kinase receptor 1) is located on chromosome 1q21-22 and its encoded product is the nerve growth factor receptor. Three different variants of rearrangements involving *NTRK1* have been reported in PTC (98) especially in those associated with previous radiation. *NTRK1* rearrangements have been reported in frequencies from 3 to 12% making it less common than *RET/PTC* (40, 99, 100).

5.6.3.3 BRAF rearrangement

BRAF presents a similar situation as *RET* in that both activating mutations and chromosomal rearrangements may render an oncogenic function. A novel fusion gene between *AKAP9* (*A-kinase anchor protein 9*) and *BRAF* was recently reported in a small subset of PTCs (101). The underlying chromosomal mechanism is an intrachromosomal rearrangement of chromosome 7 involving a paracentric inversion of the long arm. This resulted in the formation of a fusion gene with in-frame fusion of the N-terminus of *AKAP9* with the C-terminal of *BRAF*. In support of its oncogenic function *AKAP9-BRAF* was shown to have constitutive kinase activity, to stimulate MAPK pathways and to promote transformation of NIH3T3 cells (101).

5.6.3.4 PPARγ rearrangements

Chromosomal translocations giving rise to fusion oncogenes are recognized in both PTCs and follicular thyroid tumors. The first fusion gene identified in FTC results from a chromosomal translocation t(2;3)(q13;p25). This leads to fusions of the *PAX8* (*paired domain 8*) gene in 2q13 with the *peroxisome proliferator-activated receptor γ* (*PPARγ*) gene in 3p25 as originally reported by Kroll *et al.* (102). *PAX8-PPARγ* has been identified in FTC in significant but varying frequencies, and in addition it has been reported in a subset of FTAs (84). Chromosomal region 3p25 has also been found involved in other translocation events in follicular thyroid tumors, suggesting that it could be a hot spot breakpoint region (103, 104). In two such cases has the 3p25 breakpoint been shown to involve the *PPARγ* gene locus, including one primary FTC (104) and one FTA cell line (103). Interestingly reduced expression of the RAS effector *NORE1A* (*RASSF5A*) in FTCs has been associated with the presence of *PAX8-PPARγ* (104), suggesting a link to the *RAS* signaling pathway.

5.6.3.5 THADA rearrangement

Cytogenetic investigations of FTA have revealed frequent rearrangements involving chromosomal regions 19q13 and 2p21, of which the latter has been associated with the *THADA* (thyroid adenoma-associated) gene (105). The identification of *THADA* was achieved by detailed characterization of two FTA cell lines carrying the translocations t(2;20;3)(p21;q11.2;p25) and t(2;7)(p21;p15), respectively (105). In these FTA cells *THADA* fused with sequences from chromosomes 3 and 7. The exact identification of the possible partner genes remains to be clarified, although the 3p25 breakpoint was assigned to the location of *PPARγ* (103). The function of the THADA protein is largely unknown but has been suggested to be related to the death receptor pathway (105).

5.6.4 Mitochondrial DNA (mtDNA)

The mtDNA is relatively small and encode for some of the components that are involved in mitochondrial energy-production. The possible involvement of mtDNA in thyroid cancer is by far less well studied than the nuclear DNA. Nevertheless, studies of the mtDNA have revealed deletions and/or point mutations oncocytic thyroid tumors (40). Notably, oncocytic thyroid tumors are characterized by a high content of mitochondria.

5.6.5 miRNAs

Studies of miRNA in human cancer constitute a relatively new field of cancer research. Nevertheless miRNAs have been shown to have regulatory functions as oncogenes and TSGs in different types of cancer and has also been associated with thyroid cancer. Two years ago, He and colleagues reported up-regulation of five miRNA species, especially miR-221, miR-222 and miR-146 in PTC as compared to normal thyroid (106). Following this original observation, Pallante *et al.* confirmed the involvement of miR-221 and miR-222 in addition to miR-181b (107). miRNA-221 is a putative oncogene proposed to play a role in an early stage of PTC tumorigenesis (106). In the same study, up-regulation of miR-221, -222 and -146 was associated with loss of *KIT* gene and protein expression. Interestingly PTCs with loss of KIT expression harbored germline single-nucleotide alterations of *KIT* that involved the recognition sequences for miR-221 and miR-222. In another follow-up study by Visone and coworkers, enforced expression of miR-221 and miR-222 resulted in reduced p27(Kip1) protein levels but not corresponding mRNA levels (106), and in addition gave progression to the S phase in PTC cells (108). Profiling of microRNAs has also been carried out in follicular thyroid tumors, which revealed significant over-expression of miR-197 and miR-346 in FTCs as compared to FTAs and normal thyroid tissues (109).

5.6.6 CGH and LOH studies

Gains and losses of chromosomal regions have been described in most types of thyroid tumors including MTCs (110, 111), FTAs (112-115), FTCs (111-115), PTCs (111, 116-121), PDTCs (114, 122) and ATCs (111, 114, 122-124). Taken together the detected abnormalities have suggested that FTCs, PDTCs and ATCs are genetically less stable as compared with PTCs, FTAs and MTCs (125).

PTC is the best studied type of thyroid tumor using CGH. It has been found that less well differentiated PTCs display significantly more abnormalities as compared to other PTCs (117, 118). In addition, gains in 1p are associated with aggressive PTCs (117, 120) and *MUC1* (1q22) was subsequently identified to be amplified as well as over-expressed in PTCs of aggressive type (120). Of note, the frequency of LOH is generally low in PTC (126, 127), with the exception of two studies that detected LOH in 50% of cases frequently involving 4q, 5p and 7p (125, 128).

Cytogenetic analyses of FTA have revealed frequent rearrangements involving chromosomes 2 and 19. Furthermore, trisomies of chromosomes 4, 5, 7, 12 and 22 have

been recurrently observed in this tumor group (129-133). In contrary, loss of 7q has been reported in FTC (134-136). Deletions in 3p in particular the 3p21-25 region have been recurrently observed, indicating putative TSGs for FTC tumorigenesis. Notably, two major TSGs, *PPARγ* (Section 5.6.3.4) and *RASSF1A* (Section 5.6.2.4) implicated in FTC reside in this region. Loss of 22 is particularly common in FTCs, and it is associated with the widely-invasive type (111). Other studies also demonstrated LOH at chromosomes 3p, 3q, 10q, 11p, 11q, 13q, 17p and 22q in FTC (125, 137-139).

The reported studies of CGH analyses in PDTC and ATC include a total of 33 PDTCs and 54 ATCs (111, 114, 122-124). Of note these studies utilized conventional CGH. Wreesmann *et al.* identified gains at 1p34-p36, 6p21, 9q34, 17q25 and 20q and losses at 1p11-p31, 2q32-q33, 4q11-13, 6q21 and 13q21-q31 as recurrent alterations in PDTC (114). Loss at 13q was also reported in PDTC by Rodrigues *et al.* (122). Several alterations have been identified in this type of tumor. Hemmer *et al.* identified frequent gains of 7p22-pter, 8q22-qter and 9q34-qter in ATC, whilst Wilkens *et al.* demonstrated gains of 5p and alterations of chromosome 8 are responsible for ATC tumorigenesis (111, 123). Interestingly, gains at 3p13-p14 and 11q13, and loss of 5q11-q31 were proposed to be involved in ATC progression from PDTC by Wreesmann *et al.* (114). On the other hand, Rodrigues *et al.*, suggested that gains at 3q and 20q are associated to ATC transformation from FTC, while losses of 7, 12q and 13q play an important role in ATC development from PDTC (122). In this study, gains of 11q13 and 20q in ATC cell lines were observed and subsequently three amplicons of the two regions (11q13, 20q11.2, and 20q13.12) were identified in ATC primary tumors (Papers II and IV).

5.6.7 Gene-specific promoter and genome-wide methylation

Aberrant methylation at the promoter region of some TSGs *i.e. RASSF1A* and *PTEN* (Section 5.6) is commonly observed not only in thyroid cancer but also in adenomas, suggesting its role is early in thyroid tumorigenesis. Methylation of several TSGs may also be associated with thyroid tumorigenesis. For example *TIMP3* (*tissue inhibitor of metalloproteinase*), *SLC5A8* (a member of the sodium solute symporter family) and *DAPK* (*calcium/calmodulin-dependent serine threonine kinase*) promoter hypermethylations were reported to be associated with poor pathological characteristics of PTC (140). Recently Hoque *et al.* revealed hypermethylation of *TSHR*, *RAR-β2*, *CDH1* and *TGF-β* in addition to *RASSF1A*, *DAPK* and *TIMP3* in a panel of thyroid tumors (141). Promoter hypermethylations were observed for thyroid hormone receptors-α and -β (TRs) mainly in

FTCs and in some PTCs and FTAs (142). *Fibroblast growth factor receptor 2* (*FGFR2*) is down-regulated in neoplastic thyroid cells through DNA promoter methylation (143). However, little is presently known about genome-wide methylation changes in thyroid tumors. The only published study is by Galusca and colleagues who demonstrated a lower level of global methylation in thyroid cancer tissues as compared to benign tumors or adjacent normal thyroid based on analyzed using 5-methylcytidine antibodies (144).

5.6.8 Expression studies

Several studies have applied cDNA microarray analyses for global gene expression profiling in thyroid tumors (120, 145-153). Some of the genes identified in these studies have been verified using immunohistochemistry such as *FN1* (encoding fibronectin), *LGALS3* (encoding galectin 3), and *KRT19* (encoding cytokeratin 19), suggesting that they could be developed into tools for thyroid cancer diagnostics (154, 155). Gene expression profiles are distinctly different in FTC as compared to PTC. Over-expression of *Cbp/p300-interacting transactivator* (*CITED1*), *claudin-10* (*CLDN10*), and *insulin-like growth factor binding protein 6* (*IGFBP6*) was seen in PTC, while e.g. *caveolin-1* (*CAV1*) and *-2* (*CAV2*) were expressed at similar levels in the two tumor types (145). Using a similar approach Fryknäs *et al.* observed significant under-expression of the *four and a half LIM domains 1* (*FHL1*) gene in FTCs as compared to FTAs (156). Molecular profiling studies have also been applied with the aim to clarify molecular events in thyroid tumor dedifferentiation. Montero-Conde *et al.* found that genes related to the MAP kinase and TGF-β-signaling pathways, focal adhesion, cell motility, activation of actin polymerization and cell cycle were up-regulated in ATC as compared to WDTCs (152). Similarly a recent study also revealed that up-regulation of a set of genes involved in cell cycle progression and chromosome segregation is a unique feature of ATC (153).

6 GENERAL CONSIDERATIONS

The discoveries and findings of molecular and genetic studies in the thyroid research field over the last decade, no doubt have immensely aided our understanding of thyroid tumors, however some issues in the field remain to be addressed.

There has been a long-standing debate of whether thyroid cancer progression from well-differentiated to poorly differentiated and undifferentiated (ATC) represents a biological continuum. The *RET/PTC* and *PAX8/PPARγ* rearrangements that are typical of PTC and FTC respectively, have not been observed in PDTC and ATC, suggest that ATC arise *de novo* (40). This notion was supported by an investigation on aneuploid DNA pattern of ATC containing histologically well-differentiated tumor foci within or adjacent to the ATC (157). In contrary other genetic alterations such as mutations of *RAS*, *BRAF*, and *PIK3CA* are observed in both ATCs and WDTCs implying the existence of thyroid progression (40, 79). Intriguingly, a novel hypothesis of thyroid carcinogenesis known as the “fetal cell carcinogenesis” hypothesis has been recently proposed by Takano (158) (Figure 8). Takano proposed that thyroid cancer cells are derived from the remnants of three types of fetal thyroid cells in lieu of normal thyroid follicular cells by proliferation without differentiation, while oncogenes prevent fetal cells from differentiating.

It is critical to understand thyroid tumorigenesis, in particular ATC, which remains one of the most aggressive human cancers with poor prognosis and survival to date. If WDTC could progress to ATC, total thyroidectomy and follow-up examination would be strategically given to WDTC patients harboring certain genetic alterations that would be part of the ATC entity. However the approach of administering total thyroidectomy to WDTC patients who account for 98% of the thyroid cancer patients remain aggressive to prevent ATC, which has only 1-2% of prevalence rate.

Other primary goals in delineating the molecular and genetic mechanisms of thyroid tumorigenesis are to identify therapeutic targets especially for patients with metastasis and aggressive tumors as well as accurate diagnostic and prognostic markers.

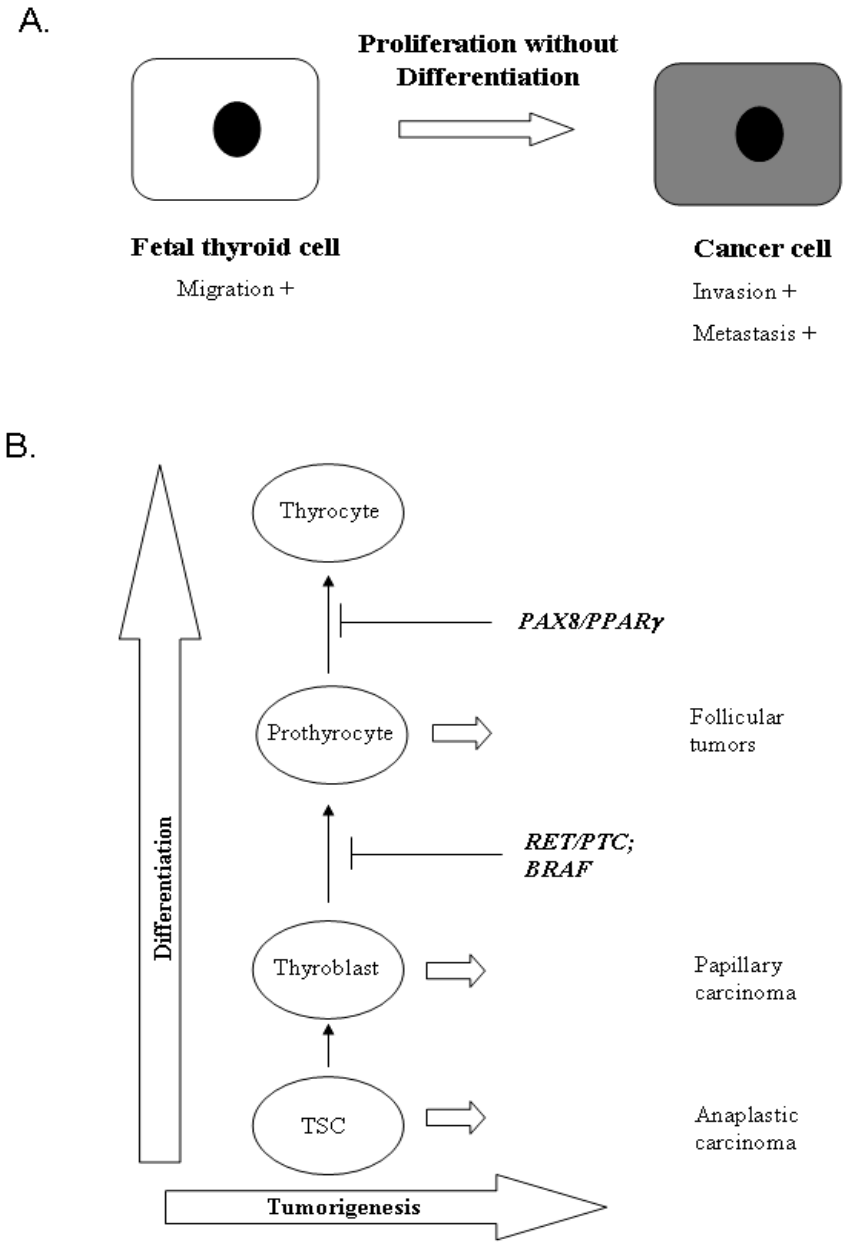


Figure 8. Fetal cell thyroid carcinogenesis model. A. Fetal thyroid cell proliferates without differentiation to cancer cells. B. The role of oncogenes in fetal cell thyroid carcinogenesis, where ATC, PTC and FTC are derived from thyrocyte, thyroblasts and prothyrocytes, respectively. *BRAF* mutations and *RET* rearrangements prevent the differentiation of thyroblasts into prothyrocytes, resulting in the generation of PTC. The *PAX8-PPARγ* rearrangement prevents differentiation of prothyrocytes into thyrocytes, resulting in the generation of follicular tumors. Figure adapted from Takano (158).

7 AIMS OF THE STUDY

The overall goal of this thesis was to further characterize the molecular genetic mechanisms leading to thyroid tumorigenesis and to explore the possibility of thyroid tumor progression.

The specific aims were to:-

1. Explore the possible involvement of *RET* mutations in a dog pedigree with FMTC.
2. Identify novel pathogenic chromosomal alterations in ATC established cell lines.
3. Investigate the DNA copy number alterations in ATC cell lines and primary ATC.
4. Determine the involvement of candidate genes (*CCND1*, *UBCH10* and $p16^{INK4A}$) located in areas of recurrent changes that are involved in ATC tumorigenesis.
5. Illumine the silencing mechanisms of *RASSF1A* in FTC tumorigenesis.
6. Delineate the role of global hypomethylation in FTC and investigate its association with *RASSF1A* promoter hypermethylation in FTC.

8 MATERIALS AND METHODS

8.1 MATERIALS

This thesis is based on canine and human thyroid tissues (Papers I-IV), dog serum (Papers I) and established human cancer cell lines (Papers II and IV). The dog familial medullary thyroid carcinomas and serum were collected at Small Animal University Hospital in Uppsala, Sweden (Paper I). The novel human ATC cell lines were established from surgical samples of ATC patients from Karolinska University Hospital and Uppsala University Hospital, Sweden (Paper II). All FTCs as well as normal thyroids were obtained from Karolinska University Hospital, Sweden (Papers III). ATC tumors were collected from Karolinska University Hospital, Sweden and Royal North Shore Hospital, Sydney, Australia (Paper IV) from 1986-2006. The use of all samples in these studies was approved by ethical committees (approval numbers C23/4 for Paper I, Dnr 00-128 for Paper II, Dnr 03-517 and Dnr 91:86/9104 for Papers II-IV and 0603-030M(SP) for Paper IV).

8.1.1 Patients and tumor materials

8.1.1.1 Dog Tissues and Blood Specimens

The pedigree of a mixed Alaskan malamute breed as a major influence that was investigated in this study is shown in Figure 9. The proband, also the father (II:1) first presented in 1998 with symptoms of itching and loss of luster to the hair coat. He also demonstrated severe para- and hyperkeratosis, chronic dermatitis and hypothyroidism. His three daughters were subsequently admitted with similar symptoms. Thyroid cancer was diagnosed in all four affected members. The fourth littermate (III:4) did not demonstrate any symptom of the disease at clinical, biochemical or radiological examinations. Blood and tissue samples collected for this study are illustrated in Figure 9.

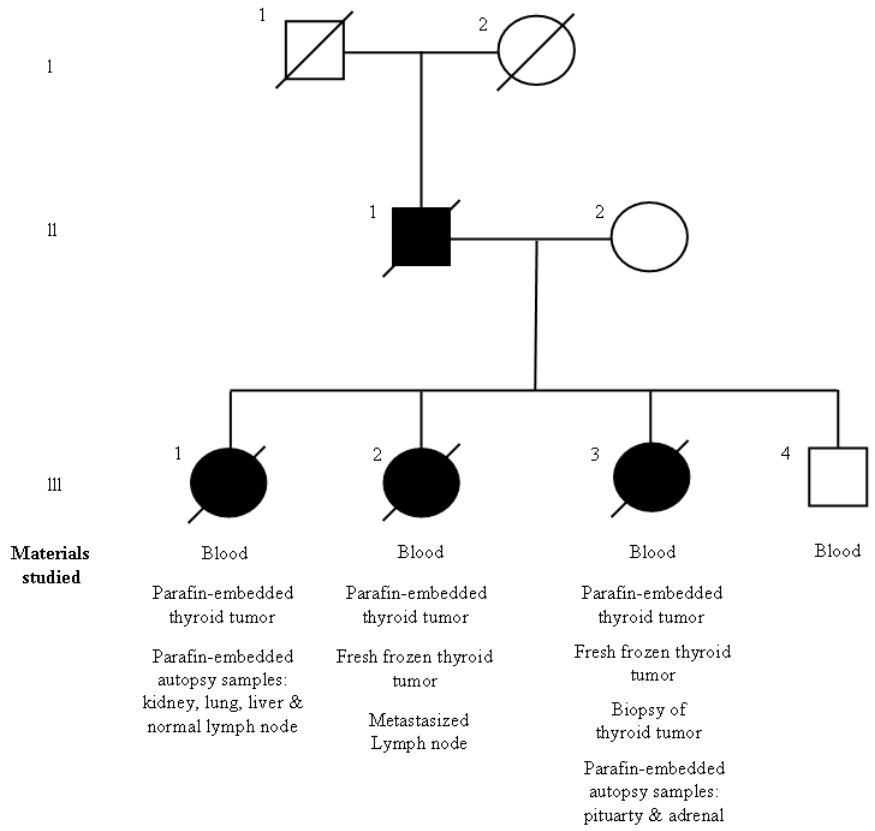


Figure 9. Dog pedigree with FMTC and the materials studied (Paper I). Affected individuals are denoted in black and a slash represented deceased individuals.

8.1.1.2 Human Tissues

All tissue samples were snap-frozen in liquid nitrogen after surgery and stored at -80 ^{o}C in the endocrine biobank until used. Frozen tissues of primary FTC and corresponding normal thyroid tissues obtained from 21 patients who underwent total thyroidectomy at the Karolinska University Hospital, Stockholm, Sweden, were included in Paper III. For Paper IV, primary tumors from 28 cases of ATC were collected of the Karolinska University Hospital and Royal North Shore Hospital, Sydney, Australia. Twenty-three of the ATC patients had received pre-operative treatment with radiotherapy and/or chemotherapy according to standard treatment protocols (159). In this study, normal thyroids, parathyroid adenoma and MTC collected from Karolinska University Hospital were included as relevant controls. Representative sections of all human tissues were subjected to histopathological examination whereby the accurate cellular content was verified and the diagnosis confirmed according to WHO classification (37). Only tumor samples with high proportion of tumor cells were included while necrotic tumor tissue was avoided for further

analyses.

8.1.2 Established Human Cell Lines

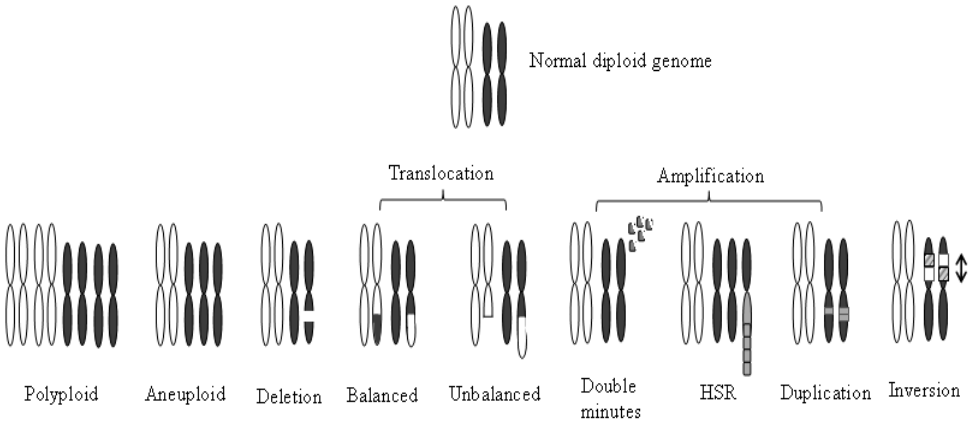
Two novel ATC cell lines, HTh 104 and HTh 112 were established from ATC tissues obtained from two patients who underwent thyroidectomy at the Karolinska University Hospital, Stockholm, Sweden and Uppsala University Hospital, Uppsala, Sweden respectively. Both patients had received chemotherapy and radiotherapy before surgery, in agreement with the recommended treatment for this condition. The tumor tissue was first minced and enzymaticaly digested where after cells were washed and seeded in an appropriate culture medium. Colonies from the culture were selected for further culturing and hence a cell line was established.

In addition to the two novel ATC lines, other human thyroid cell lines used in this study were HTh 7, HTh 74, HTh 83, C 643, SW 1736, KAT-4, ARO and HTh 83 and sub-clones (HTh 83 Clone 7, HTh 83 Clone 8 and HTh 83 Clone 11) (Papers II and IV). MCF7 (breast cancer cell line) and SAOS-2 (sarcoma cell line) were used as controls in p16 protein expression analysis in Paper IV.

8.2 METHODS

8.2.1 Karyotypic and Genomic analyses

Genomic imbalances or translocation breakpoints are important observations from which cancer-causing genes can be located and isolated. Cytogenetic and molecular cytogenetic techniques have played an important role in the characterization of such alterations. The tools applied in deciphering the genetic basis of cancer including thyroid tumors which is the theme of this study, are discussed in the following. It is worth to mention that since each technique has advantages and limitations, several techniques are applied to complement each other (Figure 10).



Methods	Detection								
G-banding	+	+	+/-	+	+	+	+	-	+
SKY	+	+	-	+	+	+	+	-	-
FISH	+	+	+	+	+	+	+	+	+
Conventional CGH	-	+	+	-	+	+	+	+	-
Array-CGH	+/-	+	+	-	+	+	+	+	-
LOH	-	+	+	-	+	+	+	+	-

Figure 10. Applications of several cytogenetic techniques applied in this thesis. These techniques have been extensively reviewed in (160, 161). Modified from Albertson and Pinkel (161).

8.2.1.1 Metaphase preparation

Cells of interest are first grown according to standard cell culture conditions. When a sufficient proportion of cells undergo mitotic division, a mitotic inhibitor (*i.e.* colcemid) is added to stop cell division at mitosis and gives an increased yield of mitotic cells for analysis. Cells are then placed in a hypotonic solution, repeatedly fixed, and dropped onto slides whereby chromosomes will spread. The fixative serves to lyse red blood cells and to harden the nuclei of the remaining white blood cells. After storing the slides in an oven or by waiting a few days (ageing), chromosomes can be banded and analyzed. Metaphase slides on ATC cell lines were prepared in Paper II.

8.2.1.2 Banding by Giemsa-staining (G-banding)

Several banding techniques that differentially stain chromosomes have been developed, allowing the distinction of normal chromosomes of similar appearance as well as elucidation of rearrangements such as the breakpoints and constituent chromosomes involved in translocations. These types of banding techniques are commonly used in cytogenetics laboratories. In this study, Giemsa staining after trypsination creating unique banding patterns on the chromosomes, banded metaphase chromosomes and from the resulting karyotype chromosomal aberrations were identified. Giemsa bands constitute a series of lightly and darkly stained bands. The dark regions tend to be heterochromatic, late replicating and rich in Adenine (A) and Thymine (T) nucleotides, while the light regions are mainly euchromatic, early replicating and Guanine-Cytosine (GC) rich. This staining technique is also known as G-banding. Giemsa stain is a mixture of methylene blue and eosin that is specific for the phosphate groups of DNA. The resolution is on the level of 550 bands, allowing identification of deletions or duplications and inversions if they are of sufficient size (161). The karyotype of ATC cell lines was analyzed by G-banding in Paper II.

8.2.1.3 Fluorescence in situ hybridization (FISH)

FISH is used to detect and localize specific DNA sequences in individual chromosomes and cells. FISH utilizes labeled DNA probes targeting single loci, repetitive sequences and individual chromosomes or their sub-bands. Since the establishment of this technique, several types of probes have been developed and applied. These include chromosome-painting probes, probes specific for certain DNA repeats, cDNA probes that are specific to a single gene, and locus specific genomic clones. The latter represent the widely used bacterial artificial chromosome (BAC) and P1-derived artificial chromosome (PAC) clones that carry large genomic fragments in the range of 10^5 bps. In this study, FISH probes were indirectly labeled by nick translation and in parallel interphase or metaphase chromosome slides were prepared and hybridized after denaturation. Figure 11 illustrates the methodology of FISH. FISH was frequently used in this study to validate both CGH and array-CGH results. This technique was applied in Papers II and IV.

8.2.1.4 Spectral Karyotyping (SKY)

SKY allows differential visualization of all human or mouse chromosomes in distinct colors with a single hybridization (162) (Figure 12). For this purpose, chromosome specific

libraries are labeled with different combinations of five fluorophores to create probes for detection of the 24 different chromosomes. Repetitive sequences are blocked with addition of excess Cot-1 DNA before hybridization onto metaphase chromosomes. The result is visualized using an epifluorescence microscope and the chromosomes are identified by analysis of the emission spectra using a “spectral-cube” and subsequently assigned individual pseudo-colors. SKY was applied in identifying structural and numerical chromosome aberrations in ATC lines of this study in Paper II.

8.2.1.5 Comparative genomic hybridization (CGH)

CGH is used to determine relative copy number alterations between two samples of DNA, following competitive hybridizing of differentially labeled DNA to normal metaphases (163). gDNA from tumor tissue and from normal control tissue (used as reference) are labeled with different fluorochromes, frequently Fluorescein (FITC) and Rhodamine or Texas Red. Tumor and reference DNA are mixed with unlabeled human Cot-1 DNA to suppress repetitive DNA sequences, and hybridized to normal metaphase chromosomes. The ratio of fluorescence color is measured along the chromosomes to identify regions of DNA gain or loss in the tumor sample. Metaphase-CGH gives a good overview of copy number variations in a test sample. However, it does not enable the detection of balanced aberrations, such as reciprocal translocations or imbalances between allele representations. The resolution is approximately 3–10 Mb (160). In Paper II, 11 ATC lines including three sub-clones were analyzed by CGH.

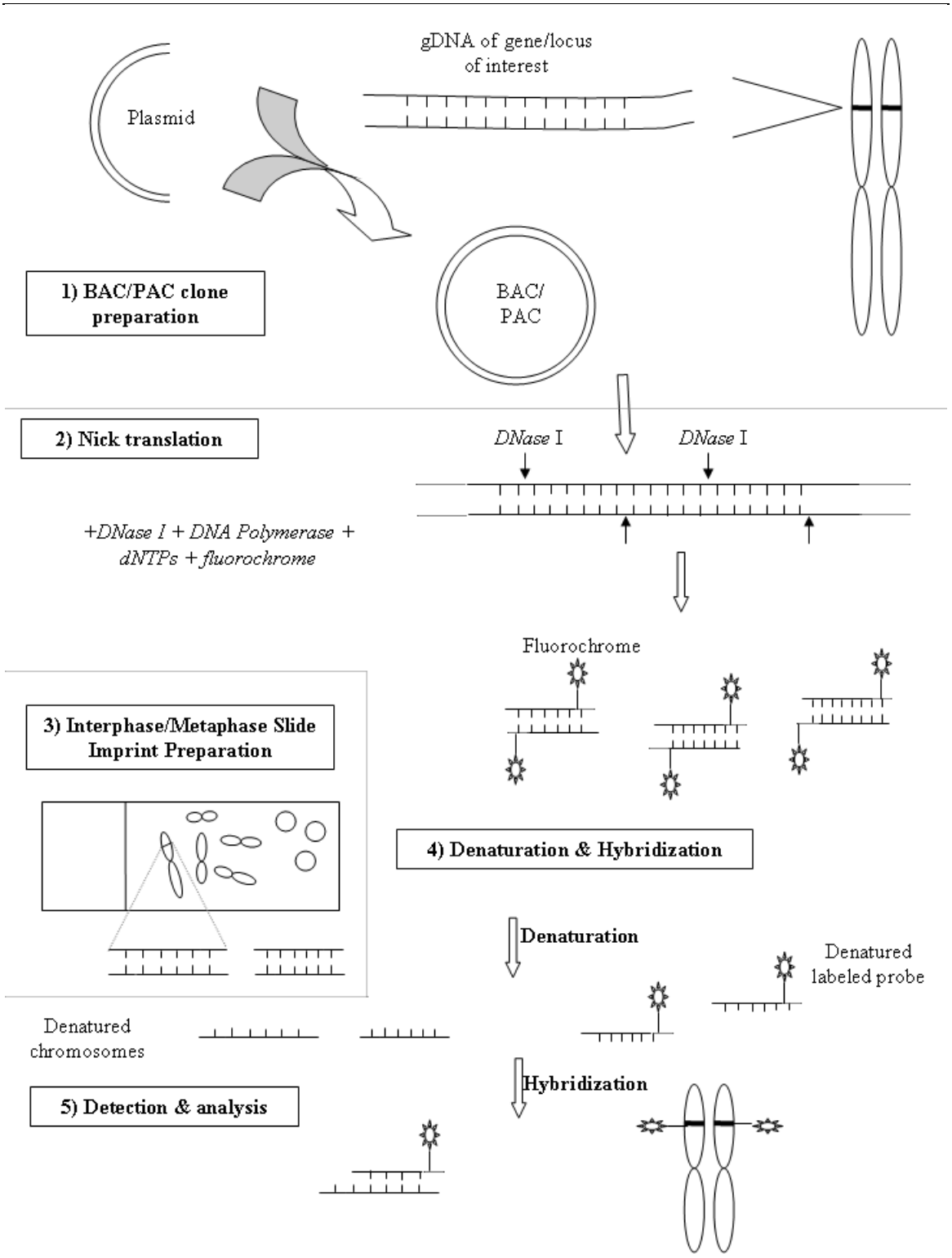


Figure 11. Schematic diagram of FISH. The method started with isolation of bacterial artificial chromosome (BAC) or P1-derived artificial chromosome (PAC) harboring regions of interest. The DNA is then nick-translated, a process of labeling the digest fragments with designated fluorochrome, while at the same time Interphase or metaphase slides are prepared. The labeled probe(s) and slides will then be denatured and hybridized overnight and then followed by detection and analysis.

8.2.1.6 Microarray based CGH (Array-CGH)

Array-CGH represents a development of the conventional CGH technique allowing detection of chromosomal copy number changes at a resolution level of 30 kb-1 Mb depending on the density of the array (160). Array-CGH and metaphase CGH shares the similar principle of competitive co-hybridization with the exception that in array-CGH immobilized BAC or PAC clones are used instead of metaphase chromosomes (Figure 13). Array-CGH has proved to be valuable technique for detection of copy number changes including discrete amplifications and micro deletions. However, array-CGH has limited ability to reveal chromosomal rearrangements and structural configuration of the abnormal chromosomes, in particular concerning the order and orientation of the rearranged segments. At present it is recommended that copy number changes detected by array-CGH are validated by other techniques *e.g.* FISH (Section 8.2.1.3) or Multiplex ligation-dependent probe amplification (MLPA) (Section 8.2.2.5). In Paper IV, we continued to examine a panel of 27 ATC tumors by 33K microarray produced by Swegene DNA Microarray Resource Centre, Lund University, Sweden. In Paper II, HTh 102 and HTh 7 were analyzed by 1Mb microarray produced by Spectral Genomics.

8.2.2 Genetic analyses

8.2.2.1 DNA sequencing

DNA sequencing is used to precisely determine genetic changes on the nucleotide level, such as mutations, polymorphisms and rearranged sequences. Primers targeting specific sequences that flank the region of interest first amplify genomic DNA. Cycle sequencing (linear sequencing) of the amplified product is then performed in a subsequent PCR reaction using fluorescently-labeled dideoxynucleotides (ddNTPs) and chain terminator (dye-terminator). Due to the incorporation of a ddNTP at the 3'-end the sequencing products will have varying lengths and can be size-fractionated in gel based or capillary systems. The result can be visualized in sequencing chromatograms in which each nucleotide is represented as an individual peak. Sequencing was performed on *Ret* in Paper I, and on *BRAF* in Papers II and IV.

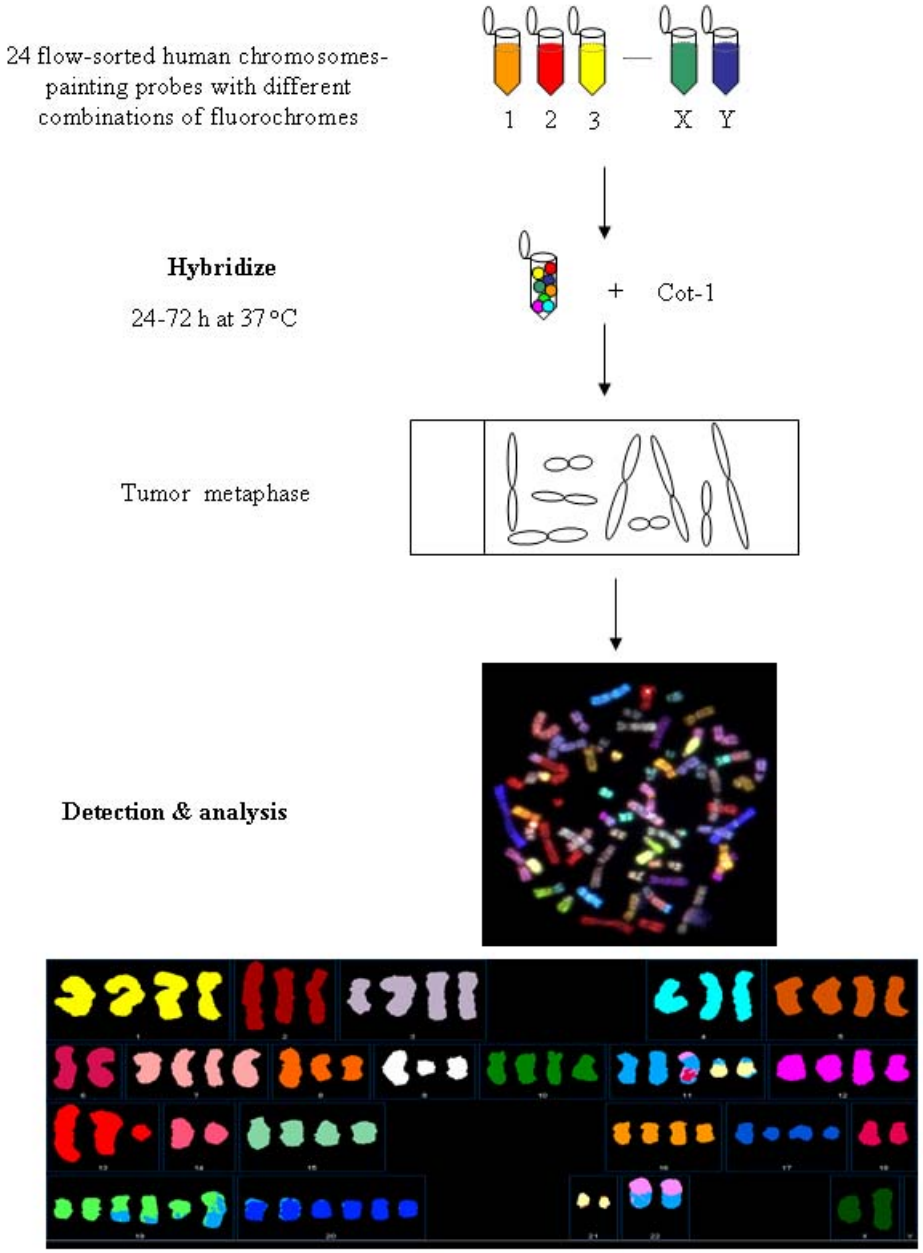


Figure 12. Schematic representation of the methodology of SKY. Chromosome-specific probe pools are generated from flow-sorted chromosomes, amplified and fluorescently labeled by DOP-PCR with different combinations of five fluorescent dyes (Rhodamine, Texas Red, FITC, Cy5 and Cy5.5). A combination of differentially labeled probes and Cot-1 DNA cocktail is hybridized to tumor metaphase preparations. After wash and detection, the metaphases are imaged using a fluorescent microscope.

8.2.2.2 Single strand comformation polymorphism (SSCP)

The SSCP methodology utilizes the tendency of single-stranded DNA (ssDNA) to fold into a tertiary structure under non-denaturing conditions. In practice, double-stranded PCR products are denatured to form ssDNA which is subsequently subjected to non-denaturing electrophoresis, during which a tertiary structure will be formed that determine the mobility. This method has been frequently used to screen for sequence alterations in large sample series. Advantages are its low cost and commonly available equipment. However the method has low sensitivity and the laboratory conditions, such as temperature needs to be optimized. This method was used to screen the *Ret* gene of the canine pedigree by comparing the mobility patterns of DNA from affected dogs with those of a healthy dog in Paper I.

8.2.2.3 Loss of Heterozygosity (LOH)

LOH is the somatic conversion from constitutional heterozygosity to somatic homozygosity which is a common mechanism for TSGs inactivation (164). Several methodologies have been applied to detect LOH, by genotyping of DNA markers previously in the form of restriction fragment length polymorphisms (RFLPs) and variable number tandem repeats (VNTRs), and currently also using array-based analyses of single nucleotide polymorphisms (SNPs). In this study, PCR-based detection of microsatellite polymorphisms was utilized, which is commonly applied in the field. The alleles of the locus of interest are amplified by PCR, and then separated by polyacrylamide gel or capillary based systems. The alleles are detected by autoradiography for radioactively-labeled primers, or by GeneScan for fluorescently-labeled primers. In Paper III, the amplified products from the tumor tissues were compared to those of the matched normal thyroid tissues of the same individual. If a tumor sample has undergone LOH, the peak height of one of the two alleles will decrease in relation to the normal allele peak heights.

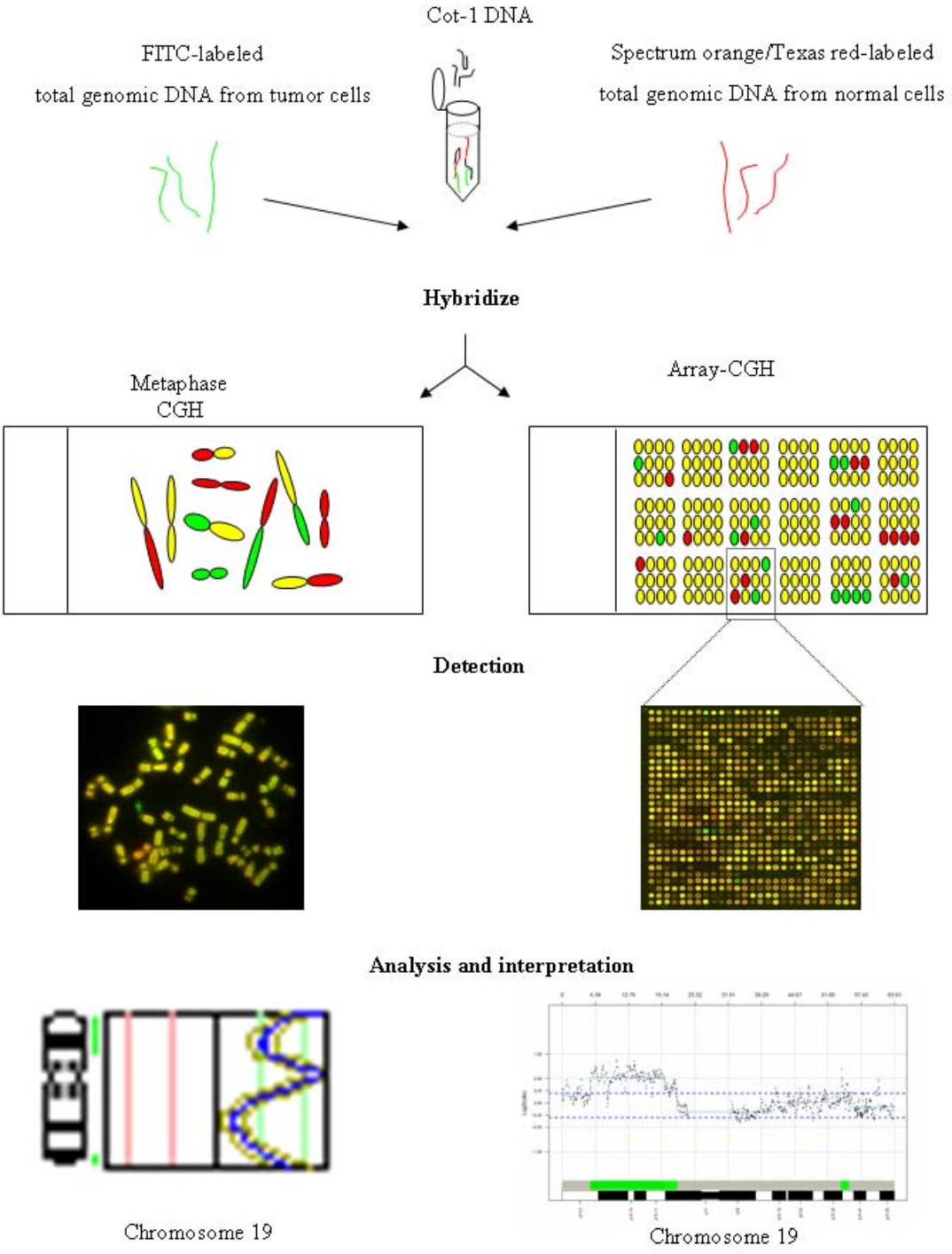


Figure 13. Schematic illustration of metaphase-CGH and array-CGH. An example of gain of chromosome 19 profile demonstrated in one ATC case that was included in Paper IV.

8.2.2.4 Short tandem repeat (STR) profiling

STR markers, also known as microsatellite markers, are polymorphic DNA loci that contain a repeated nucleotide sequence, which is usually between two to seven nucleotides in length. The majority of repeats within an STR locus consist of repeat units of equal length. However, within a population the number of repeat units for a given STR locus varies between individual, thus creating multiple alleles. As such genotyping of polymorphic STR loci is a useful technique for human identification purposes (165). In this study, STR profiling was applied to provide an identification fingerprint for ATC cell lines established in several laboratories. STR loci can be amplified by PCR, using primers from the flanking unique sequences. The PCR products are then subjected to electrophoresis to separate the alleles according to size. PCR-amplified STR alleles can be detected using various methods involving *e.g.* fluorescent dye or silver staining. In this study, the AmpFISTR Profiler Plus kit was used (Applied Biosystems, Foster City, CA). This PCR-based kit uses primers labeled with 5'FAM, JOE and NED fluorophores to amplify nine STR markers and a gender marker, and which is also used for identification of commercially available cell lines. The amplicons were then analyzed in an ABI 377 DNA Automated Sequencer using GeneScan version 3.1 (Applied Biosystems).

8.2.2.5 Multiplex ligation-dependent probe amplification (MLPA)

MLPA (166) involves amplification of multiple sequence targets by a PCR based approach. As such MLPA have become valuable for detection of *e.g.* gene deletions smaller than the resolution of FISH but larger than the resolution of regular sequencing. In this study MPLA was used to verify some of the copy number alterations identified by array-CGH in Paper IV. The method involves hybridization of gDNA in a solution containing a series of primer pairs for different parts of the target sequence (Figure 14). The target sequence of the probe is split so that one half of the probe consists of a target specific sequence (20-30 nucleotides) flanked by a universal primer sequence. The other half has a target-specific sequence at one end (25-43 nucleotides), which is tagged with a DNA sequence of predefined length (stuffer sequence) and a universal primer sequence at the other end. Multiple target amplicons are separated in the gel at electrophoresis based on the variable length of stuffer sequence. The amounts of ligated probe are proportional to the target copy number, while the relative peak heights indicate the relative representation of the target sequence. In Paper IV, MLPA products were run in an ABI 3100 genetic analyzer of capillary electrophoresis system and the peaks were analyzed using the GeneMapper v4.0 software.

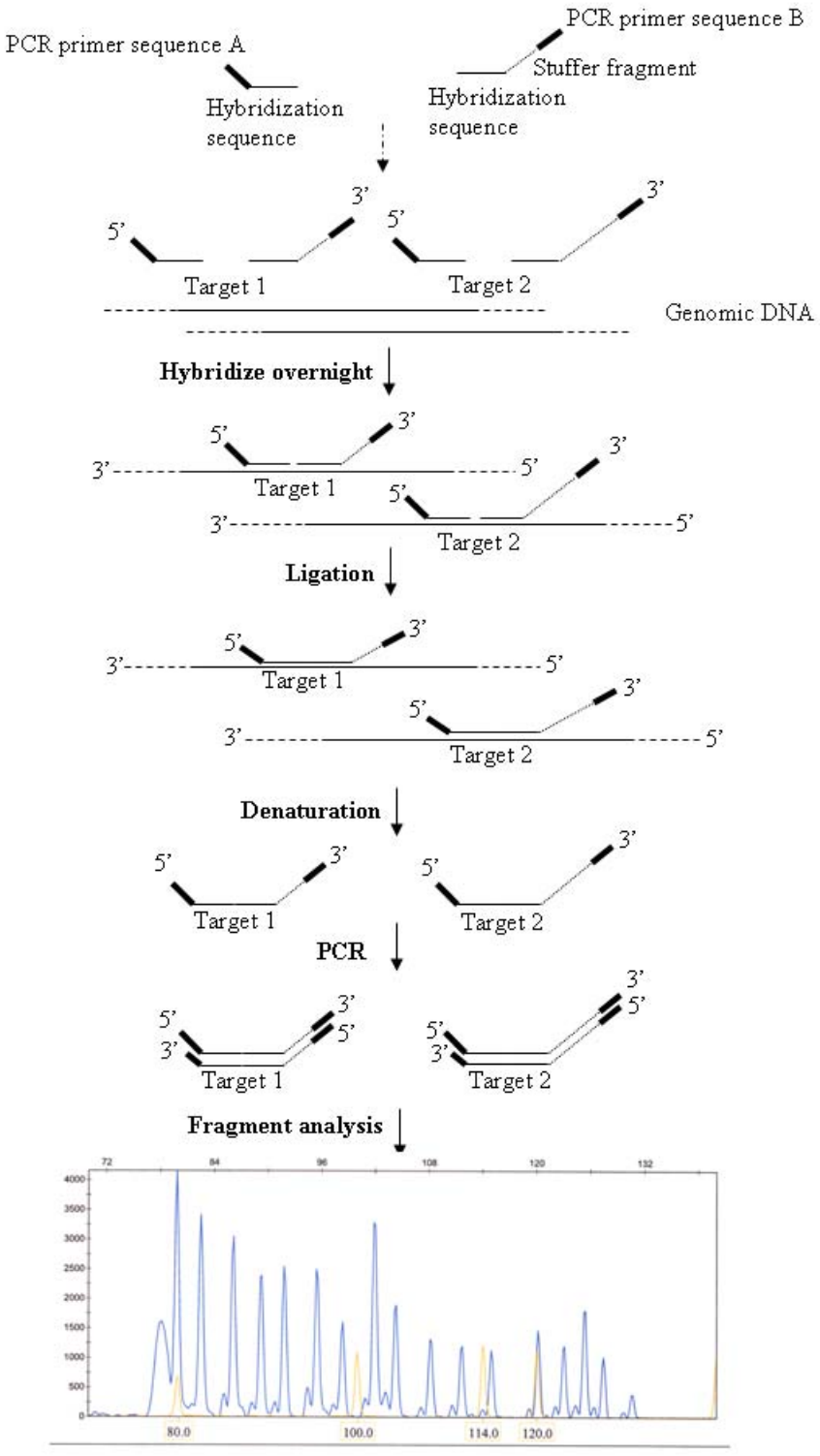


Figure 14. Schematic illustration of multiplex ligation–dependent probe amplification (MLPA). Essentially the designed probes targeting gene(s)/area(s) of interest are hybridized to gDNA overnight. Subsequently ligation, denaturation and PCR are performed before proceeding to fragment analysis.

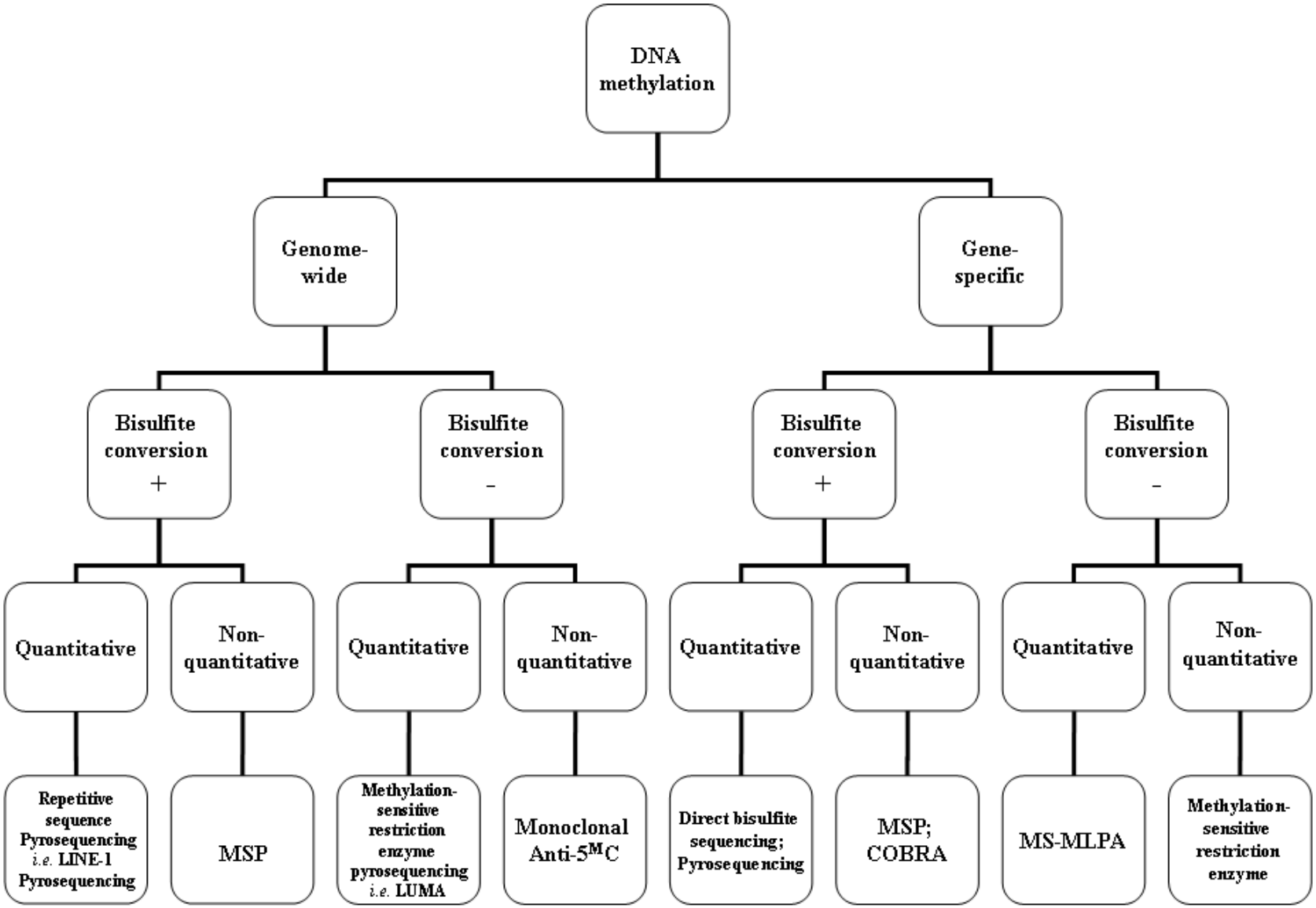
8.2.3 Analyses of epigenetic modifications

Methylation of cytosines in the 5' position of the pyrimidic ring is the most important epigenetic alteration in eukaryotes. DNA methylation involves the addition of a methyl group to the number 5 carbon of the cytosine pyrimidine ring (commonly known as CpG). This mechanism plays a key role in the regulation of gene expression. Two forms of DNA methylation changes have been associated with cancer development: genome-wide hypomethylation and localized promoter hypermethylation (19). Major advances have been made over the last 20 years in developing techniques for detection of DNA methylation on the global as well as gene-specific level. Figure 15 illustrates some of the methods available today, which may be either quantitative or qualitative involving chemical and sequence modification. However each technique has its own applications and limitations, and the choice depends upon the goal of the study.

8.2.3.1 Sodium bisulfite modification

Sodium bisulfite modification involves treatment of gDNA with bisulfite, whereby unmethylated cytosines are converted to uracil, whilst methylated cytosines remain unchanged (Figure 16A). This method thus allows discrimination between methylated and unmethylated DNA providing the basis for further DNA methylation analysis, by *e.g.* methylation-specific PCR (MSP) (Section 8.2.3.2) and sequencing (Section 8.2.3.3). This method thus allows discrimination between methylated and unmethylated DNA providing the basis for further DNA methylation analysis.

Figure 15. Various techniques for genome-wide as well as gene-specific DNA methylation analyses.


DNA methylation
Genome-wide
Gene-specific
Bisulfite conversion +
Bisulfite conversion -
Bisulfite conversion +
Bisulfite conversion -
Quantitative
Non-quantitative
Quantitative
Non-quantitative
Quantitative
Non-quantitative
Quantitative
Non-quantitative
Repetitive sequence Pyrosequencing i.e. LINE-1 Pyrosequencing
MSP
Methylation-sensitive restriction enzyme pyrosequencing i.e. LUMA
Monoclonal Anti-5MC
Direct bisulfite sequencing; Pyrosequencing
MSP; COBRA
MS-MLPA
Methylation-sensitive restriction enzyme

8.2.3.2 Methylation-specific PCR (MSP)

MSP has been widely used in studying methylation of CpG islands of promoter regions. Following bisulfite modification, PCR is performed using two sets of primers designed to amplify either methylated or unmethylated alleles, as illustrated in Figure 16B. MSP is a sensitive technique and can be used on DNA samples of limited quantity and/or poor quality. However, MSP is not quantitative and may give false positive results if the PCR conditions are not optimal. Similarly, the high sensitivity may lead to over-interpretation of positive findings in cases with low levels of methylation. In Paper III MSP was applied to detect *RASSF1A* promoter methylation.

8.2.3.3 Pyrosequencing

Pyrosequencing is a technique based on the "sequencing by synthesis" principle(167). Bisulfite Pyrosequencing technique consists of a PCR reaction that uses a biotinylated primer or a tailed primer. This primer is combined with a biotin-labeled universal primer during the reaction after the bisulfite conversion (167) (Figure 16C). The amplified PCR is hybridized with primers for sequencing with the following enzymes: DNA polymerase, ATP sulfurylase, luciferase and apyrase, adenosine 5' phosphosulfate (APS) and luciferin. The DNA polymerase acts as a catalyst by integrating the dNTPs into the DNA strand that accompanies the template. The Pyrophosphate (PPI) is released in the same concentration of the incorporated nucleotide. ATP sulfurylase is converted to ATP from PPI when APS is present. ATP converts luciferin to oxyluciferin and visible light is produced based on the quantity of ATP. The visible light generated from the luciferase reaction will be detected with a charge coupled device (CCD) camera that converts the data into a visible peak, which can be observed in Pyrogram™. The number of integrated nucleotides is relative to the height of each peak. The degradation of ATP and unincorporated dNTPs will switch the light off and allows the reaction solution to be restored. As dNTP is added, the reaction creates more complementary DNA strand where the signal peaks from the Pyrogram™ will reveal the sequence (Figure 16D). This technique is highly sensitive and has controls for unconverted cytosines, which permit quantitation of multiple CpG methylation sites in the same reaction. The number of CpG sites is limited because only 25-30 bps can be sequenced in each reaction. Pyrosequencing was applied to examine methylation of LINE-1 (surrogate marker for genome-wide methylation) and *RASSF1A* promoter in matched pairs of FTCs and normal thyroid in Paper III.

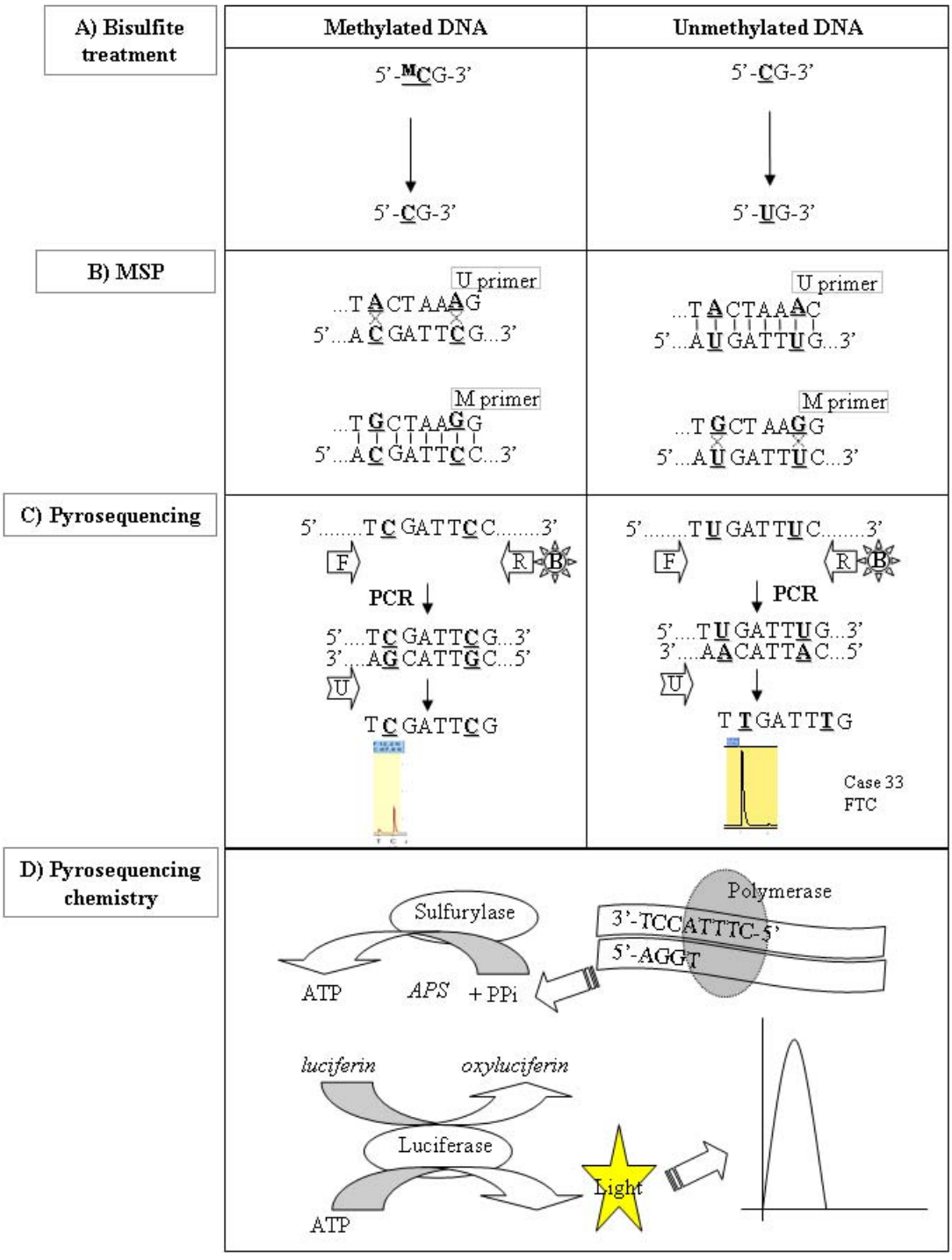
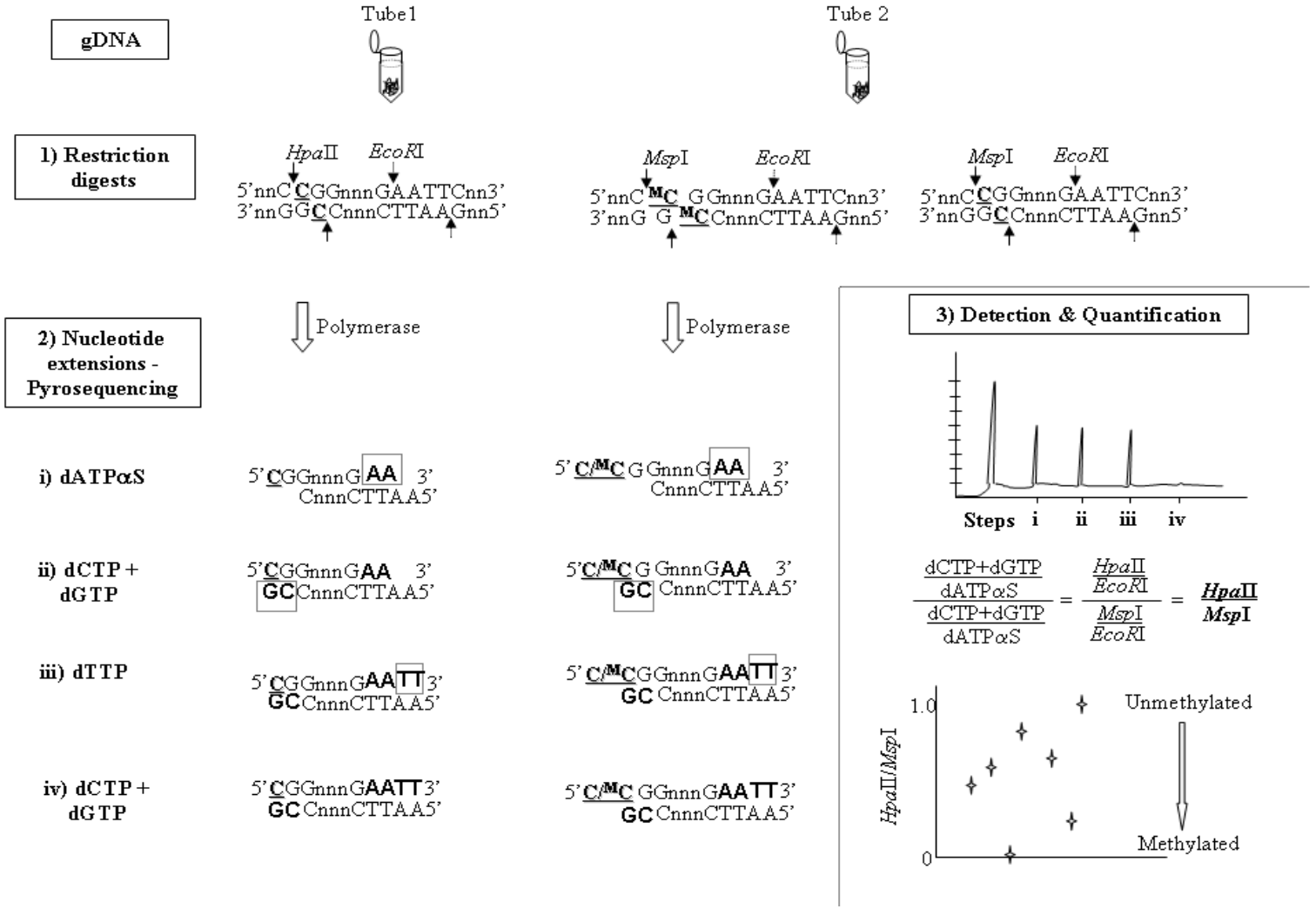


Figure 16. Schematic illustration of DNA methylation methodologies. A. Bisulfite conversion. B. Methylation specific-PCR (MSP). C. Pyrosequencing for gene-specific methylation. Pyrograms from quantitation of *RASSF1A* promoter methylation for Case 33 is exemplified. D. Pyrosequencing chemistry.

8.2.3.4 LUminometric methylation assay (LUMA)

LUMA is a novel method to assess genome-wide methylation by quantifying methylation density at CCGG restriction sites throughout the genome (168) (Figure 17). The three main steps of LUMA comprises: (1) Cleavage of gDNA with the isoschizomer enzymes *Hpa*II and *Msp*I, (2) DNA polymerase extension assay using Pyrosequencing technology with incorporation of dNTP nucleotides in the overhangs that result from the restriction cleavage, and (3) Analysis of peak-height and calculation of *Hpa*II/*Msp*I ratios. Each test sample is subjected to two separate cleavage reactions by *Hpa*II + *EcoR*I or *Msp*I + *EcoR*I. Notably *Msp*I cuts CCGG regardless of its methylation status, while *Hpa*II will only cleave if the sequence is unmethylated. *EcoR*I serves as an internal control. The *Hpa*II/*Msp*I ratio is calculated as (*Hpa*II/*EcoR*I)/(*Msp*I/*EcoR*I) and is used as an indicator of the degree of methylation in the genome. If the DNA sample is fully methylated the ratio will be close to zero, while a ratio at 1 indicates that the samples is unmethylated. Global methylation profiles of normal thyroid and FTC pairs were examined by LUMA in Paper III.

Figure 17. LUminometric Methylation Assay (LUMA) for genome-wide methylation. gDNA is first digested with isoschizomer enzymes *Hpa*II+*EcoR*I and *Msp*I+*EcoR*I respectively. Nucleotides are incorporated in four steps sequentially to the digested DNA by DNA polymerase extension assay. A peak will be reflected in the pyrosequencing run for each incorporation. The *Hpa*II/*Msp*I ratio obtained from the peaks will indicate the level of methylation.


gDNA
Tube1
Tube 2
1) Restriction digests
HpaII EcoRI
5'nnCCGGnnnGAATTCnn3'
3'nnGGCCnnnCTTAAGnn5'
MspI EcoRI
5'nnCMCGGnnnGAATTCnn3'
3'nnGGMCCnnnCTTAAGnn5'
MspI EcoRI
5'nnCCGGnnnGAATTCnn3'
3'nnGGCCnnnCTTAAGnn5'
2) Nucleotide extensions - Pyrosequencing
Polymerase
Polymerase
i) dATPαS
ii) dCTP + dGTP
iii) dTTP
iv) dCTP + dGTP
3) Detection & Quantification
Steps i ii iii iv
dCTP+dGTP / dATPαS = HpaII/EcoRI / MspI/EcoRI = HpaII/MspI
HpaII/MspI
1.0
0
Unmethylated
Methylated

8.2.4 Gene and protein expression analyses

8.2.4.1 Western blot analysis

Western blot, also known as immunoblot, is a common method to detect a specific protein in a cell or tissue homogenate or extract. By comparison to a reference protein and standard size markers, the method can also provide information about the amount and size of a protein. Native or denatured proteins are first separated using gel electrophoresis. This can either be done under denaturing conditions according to length of the polypeptides, or by the three-dimensional structure of the protein under native/non-denaturing conditions. The proteins are then transferred to a membrane and incubated with antibodies specific to the target protein. Western blot analysis was used in Paper IV for the detection of cyclin D1 and p16 proteins.

8.2.4.2 Immunohistochemistry (IHC)

IHC allows cellular localization of proteins in *e.g.* a tissue section using antibodies. Direct detection and indirect detection are two main strategies used for IHC detection of antigens in tissue. At direct detection, only one antibody is utilized. This is a simple and rapid procedure. However it often encounters problems in relation to sensitivity due to lack of signal amplification. Indirect detection, as illustrated in Figure 18, involves an unlabeled primary antibody and a labeled secondary antibody that reacts with the primary antibody. The secondary antibody can be labeled with a fluorescent dye or an enzyme. In this study, a biotinylated secondary antibody that couples with streptavidin-horseradish peroxidase was applied. The complex is then reactivated with 3,3'-Diaminobenzidine (DAB) to produce a brown staining wherever the primary and secondary antibodies are attached in a process known as DAB staining. In Paper I, IHC was applied to tumor tissue sections from the dog family using markers for tumor classification such as chromogranin A, chromogranin B, synaptophysin, RET, calcitonin, neuron-specific enolase (NSE), PTH, S-100, and and Tg. Parallel analyses of normal dog thyroid were carried out to determine which antibodies gave cross-reactivity with the dog protein and could thus be reliably used in the analyses.

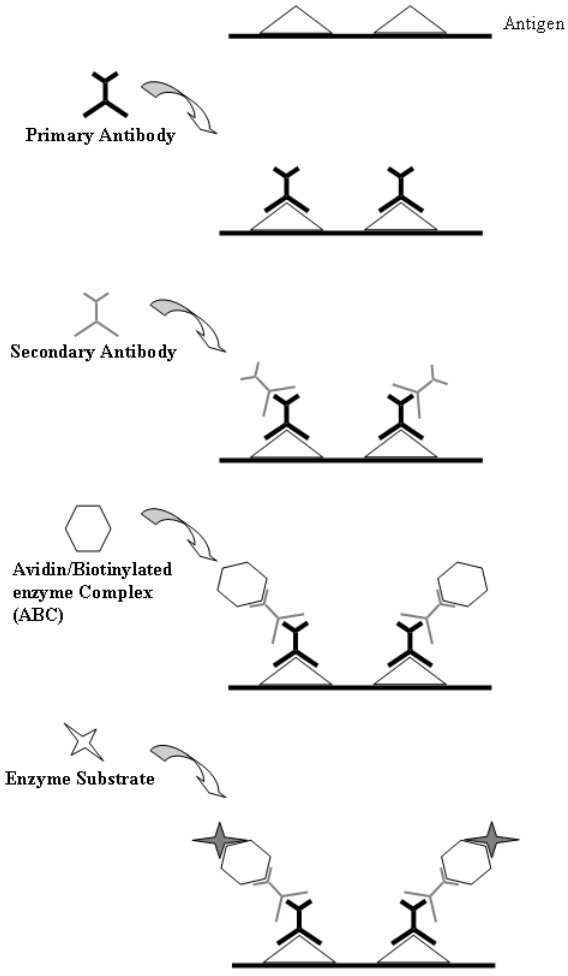


Figure 18. Schematic illustration of the chemistry for immunohistochemistry (IHC) indirect detection method. Primary antibody targeting the protein of interest is applied and a secondary antibody (biotinylated) targeting the primary antibody is added. The avidin/biotinylated enzyme complex (ABC) is formed upon binding of streptavidin-horseradish peroxidase with the biotinylated secondary antibody. This complex further reacts with an enzyme substrate DAB (3,3'-Diaminobenzidine) in producing a brown staining wherever the primary and secondary antibodies are attached.

8.2.4.3 mRNA in situ hybridization

mRNA *in situ* is used to localize specific mRNA species within individual cells in tissue sections or whole cell preparations. This technique involves labeling of a probe that is complementary to the mRNA of the gene of interest. cDNA clones and synthetic oligonucleotides are commonly used as probes and detected using radioactivity or fluorescence. In this study, a probe targeting dog *Ret* mRNA was synthesized and tagged with biotin at its 3' end. After hybridization, and an additional step of biotinylated

amplification to enhance the sensitivity of the signal, the result was detected as illustrated in Figure 19. Figure 20 illustrates the different applications of IHC and mRNA *in situ* hybridization.

8.2.4.4 Quantitative real-time PCR (qRT-PCR)

qRT-PCR is used for quantification of a specific transcript in a given RNA sample. Thus qRT-PCR reflects the average expression level of a transcript in all cells within a sample, but does not give information about its cellular distribution. This method is widely used and has largely replaced the previously applied RT-PCR method which allows semi-quantitative measurements only. In practice, isolated RNA is first converted to cDNA using reverse transcriptase. In the subsequent amplification the amount of accumulated PCR products is proportional to the starting amount of cDNAs, assuming a doubling in each cycle. TaqMan® and SYBR® Green are the two common systems for detection. In this study, TaqMan® was used, in which fluorescence signal is generated by Förster Resonance Energy Transfer (FRET) (Figure 21). The TaqMan® probe used is designed to hybridize to one of the PCR strands, and contains a fluorescent that serves as a "Reporter" at the 5'-end and a quencher at the 3'-end. When a new strand is replicated with the forward and reverse primers, the nuclease activity of the polymerase will cleave the probe and release the reporter from the quencher. Since the fluorescence is proportionate to the probe cleaved, it will increase after every PCR cycle.

A standard curve is generated from parallel analysis of a serially diluted cDNA sample and used for quantification and as an indicator of the PCR efficiency. Normalization is done against an endogenous control gene to correct for differences in RNA quality and quantity, as well as for any experimental biases. The endogenous control gene should optimally show consistent expression across the test samples. Different controls could therefore be used for different tissue types, and in addition the average of a set of different control genes can be used. In Papers II and III of this study, *18S* was selected as a relevant endogenous control, as it has been demonstrated to be consistently expressed in relevant samples. qRT-PCR was used to quantify the mRNA expressions of *PTEN* in Paper II and of *RASSF1A* in Paper III.

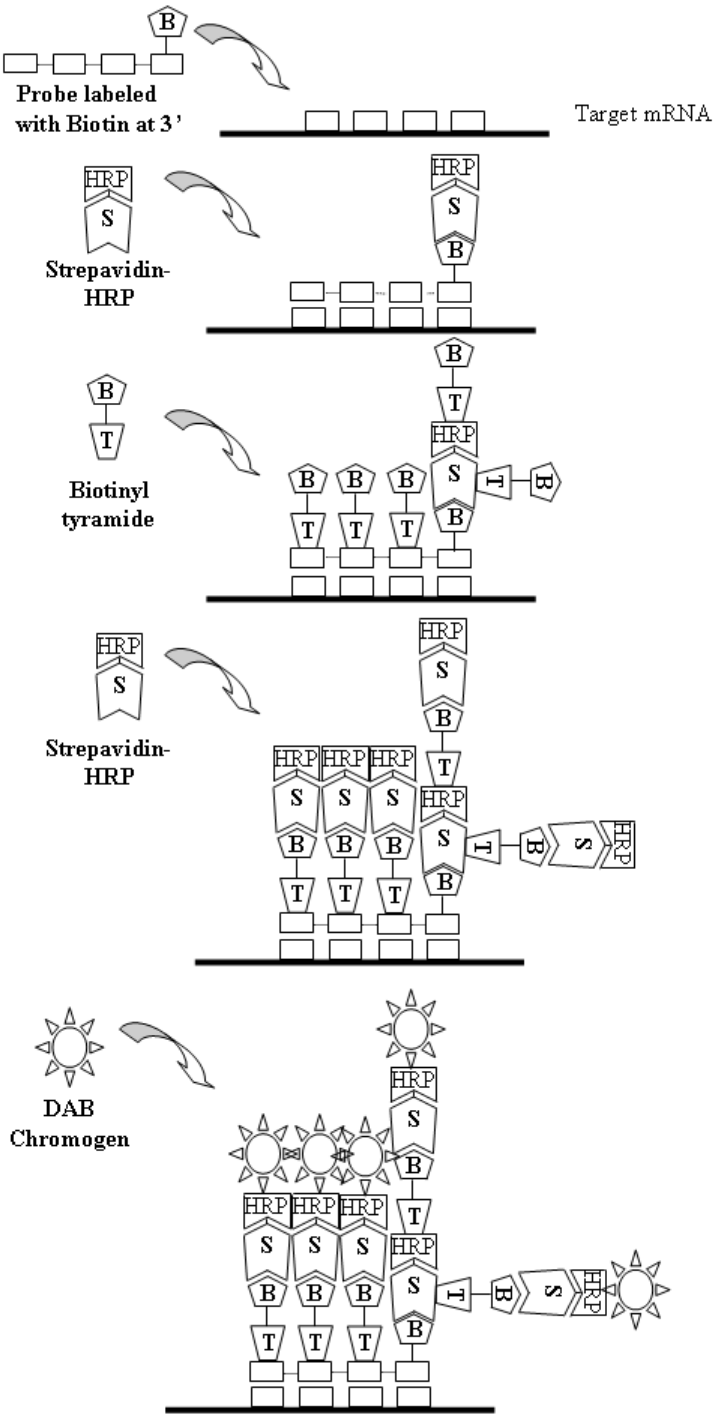
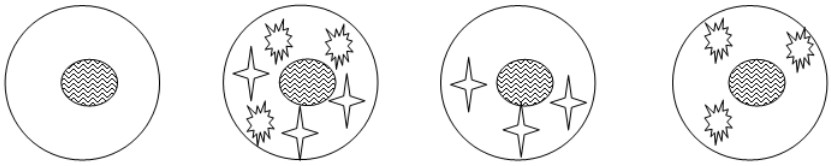


Figure 19. Schematic illustration of the chemistry of mRNA *in situ* hybridization. The probe targeting the mRNA of interest is designed and labeled with biotin at the 3'-end. Strepavidin-HRP will hybrize to where the probe is bound. An amplification step could be performed by adding biotinyl tioramide in increasing the signal for detection. Strepavidin-HRP will couple with biotinyl tioramide and further react with DAB chromogen in generating a brown signal of where the probe.



Observations				
1)mRNA *in situ*	No detectable mRNA	mRNA detectable	mRNA detectable	No detectable mRNA
2)Immunohistochemistry	No detectable peptide	Peptide detectable	No detectable peptide	Peptide detectable
Interpretations	•No synthesis of mRNA & hence no peptide	•Synthesis & storage of peptide	•No storage of peptide, active secretion •Only synthesis of mRNA but not peptide •Synthesis of peptides in abnormal forms	•Uptake or storage of peptide

Figure 20. Applications of IHC and mRNA *in situ* hybridization. Modified with permission from Höög A's doctoral thesis, Karolinska Institutet 1997.

8.2.5 Functional analysis of candidate genes

The possible role of a gene in cancer development can be evaluated *in vitro* or *in vivo*. *In vitro* studies commonly involve (1) introduction of the gene into a model system or (2) silencing of the gene in a model system. The former can be achieved by introducing an expression vector containing the gene of interest to a cell model (*i.e.* transfection). The latter frequently involves inhibition of the gene in a cell with known high-expression using siRNA.

8.2.5.1 Transfection

The functional consequences of a suggested cancer gene can be studied *in vitro* by different methods commonly involving introduction of the gene of interest to cultured cells used to model the disease in question. The procedure involves cloning of the gene of interest into an expression vector (*i.e.* plasmid), and subsequent introduction into the cell system by "transfection" using one of several available methodologies. In Paper IV, a novel non-viral method (Nucleofection manufactured by Amaxa Biosystem) was applied to introduce cyclin D1 expression into normal human follicular thyroid cells (Nthy-ori 3-1). This method is based on a unique combination of electrical parameters and cell-type specific solutions as illustrated in Figure 22. Cyclin D1 function was subsequently

assessed from transiently transfected cells. Nucleofection was also used to introduce the *CCND1* siRNA probe that will be discussed in Section 8.2.5.2.

8.2.5.2 siRNA

The discovery of RNA interference (RNAi) has led to the development of frequently employed and powerful tools to study gene function. This can involve silencing in a cell model system of the disease with documented expression of the gene in question. RNAi is in itself a normal biological mechanism for gene silencing involving targeting and degradation of complementary mRNA by dsRNA. Figure 23 illustrates how the RNAi mechanism can be triggered directly by a small interfering RNA (siRNA typically 21 bp RNA molecules) or by using DNA-based vectors to express short hairpin RNA (shRNA) that will be processed by Dicer into siRNA molecules. In an attempt to silent *Cyclin D1* in Paper IV, a commercially available (Qiagen) siRNA probe specifically targeted against *Cyclin D1* (Figure 23B) was transfected to the ATC cell line HTh 7 over-expressing cyclin D1.

8.2.5.3 MTS assay

With the understanding that cancer cells are aggressive, invasive and have a potential to metastasize, it is essential to investigate the role of a candidate gene in cell viability and growth. There are several assays available to serve this purpose. Amongst them, MTS assay is one of the common ones applied in functional studies in cancer models (169). MTS is a tetrazolium compound, 3-(4,5-dimethylathiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, which gives a water-soluble formazan product in the presence of phenazine methosulfate (PMS). MTS assay is a colorimetric method measuring the formation of the soluble formazan product that is directly proportional to the number of live cells in culture.

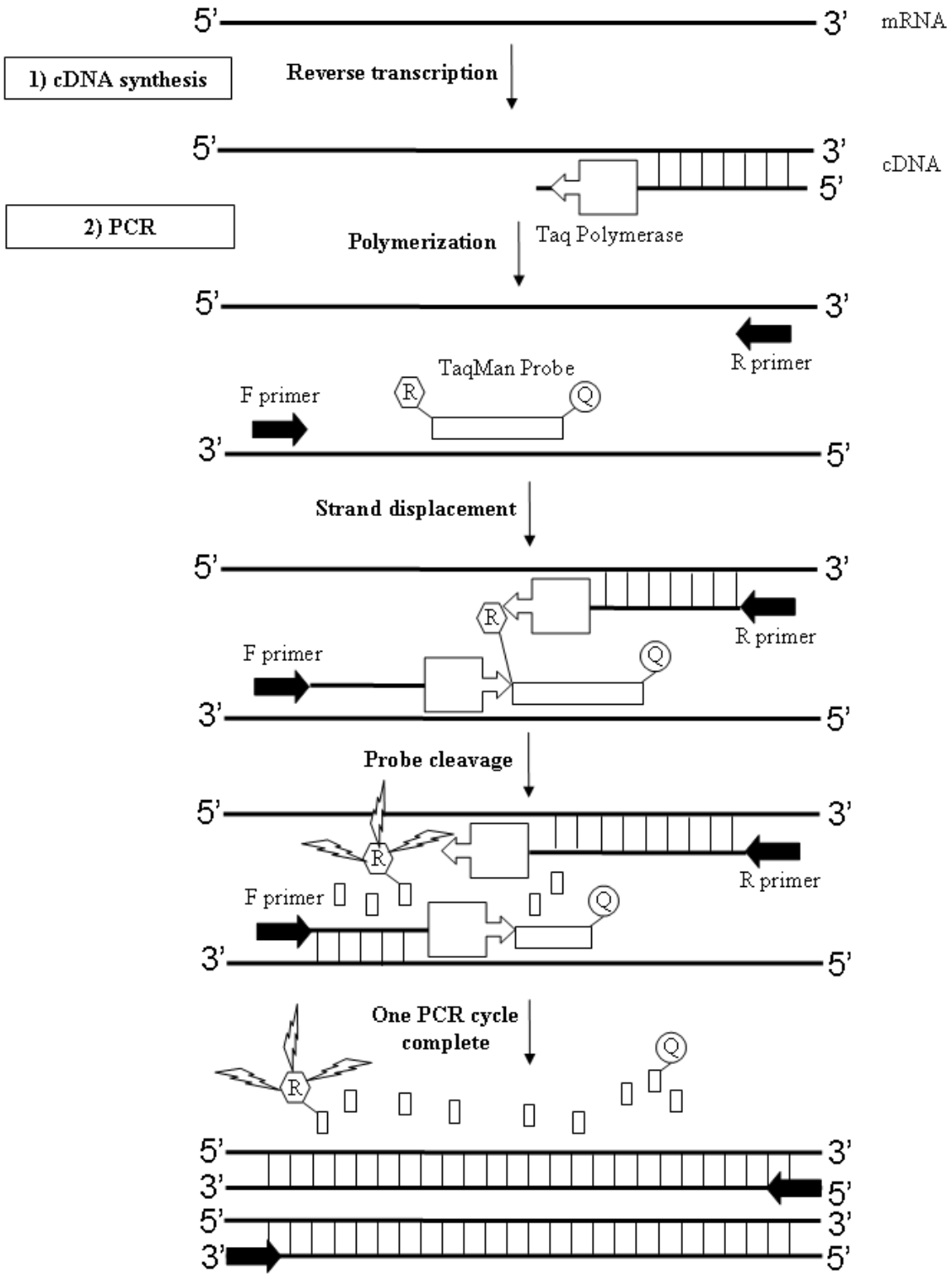


Figure 21. Schematic illustration of the chemistry of quantitative real-time PCR (qRT-PCR). cDNA is synthesized from the isolated RNA with reverse transcriptase. Two primers (forward and reverse) are designed to amplify the gene of interest. The nuclease activity of the DNA polymerase will cleave Taqman® probe and thus the fluorescence molecule, also known as the reporter (R) molecule will be released from the quencher (Q) upon completion of one PCR cycle.

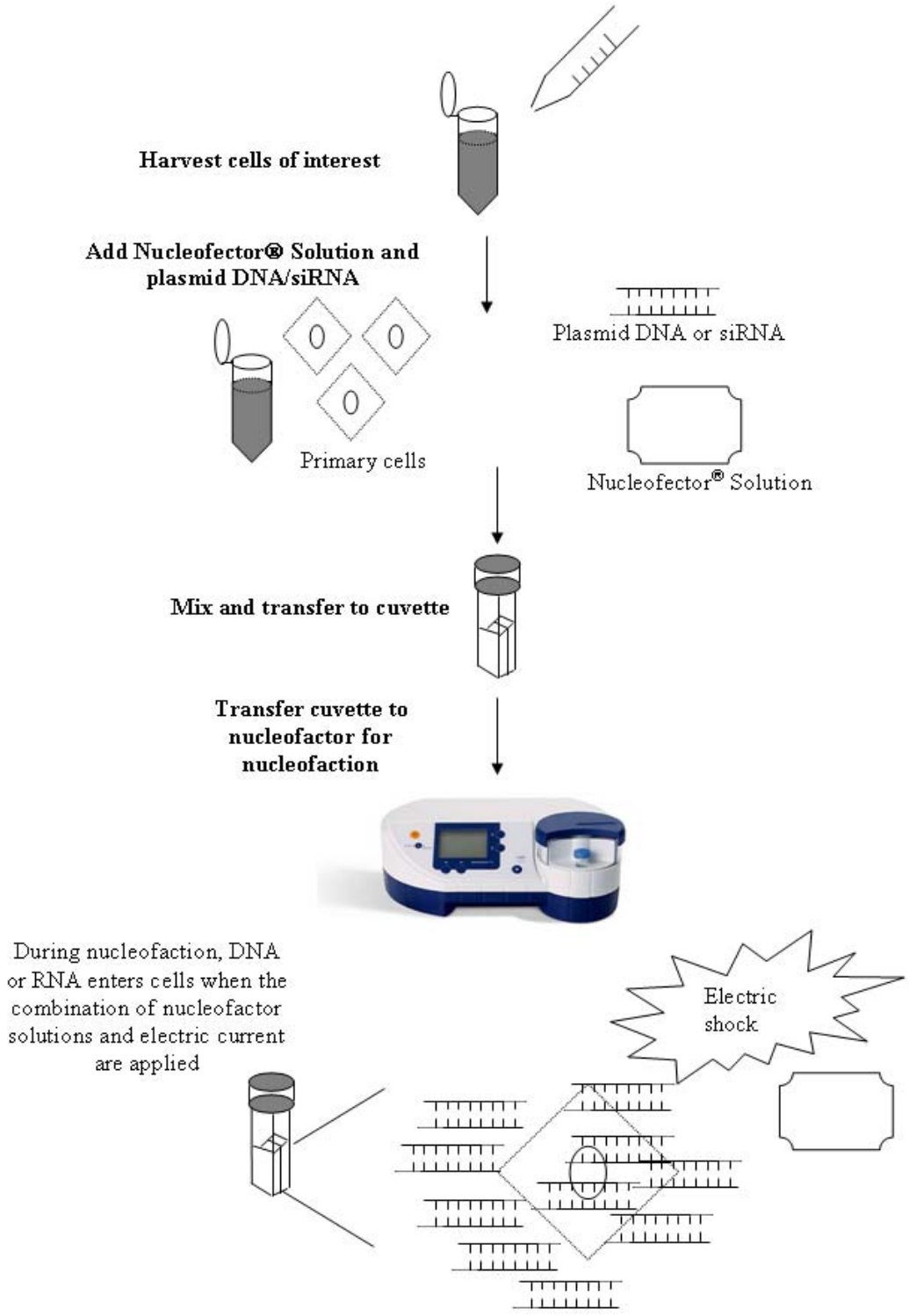


Figure 22. Schematic diagram of transfection by Nucleofection technique (Amaxa Biosystem: www.amaxa.com/technology). Cells of interest are harvested when reaching optimum confluent for transfection. Nucleofector® solution and plasmid DNA or siRNA of interest are added to the cells. The mixture is then transferred to the cuvette and subsequently to the nucleofactor for nucleofaction. DNA or RNA will enter the cells when the nucleofector® solution and electric current are applied. This process is referred as nucleofaction.

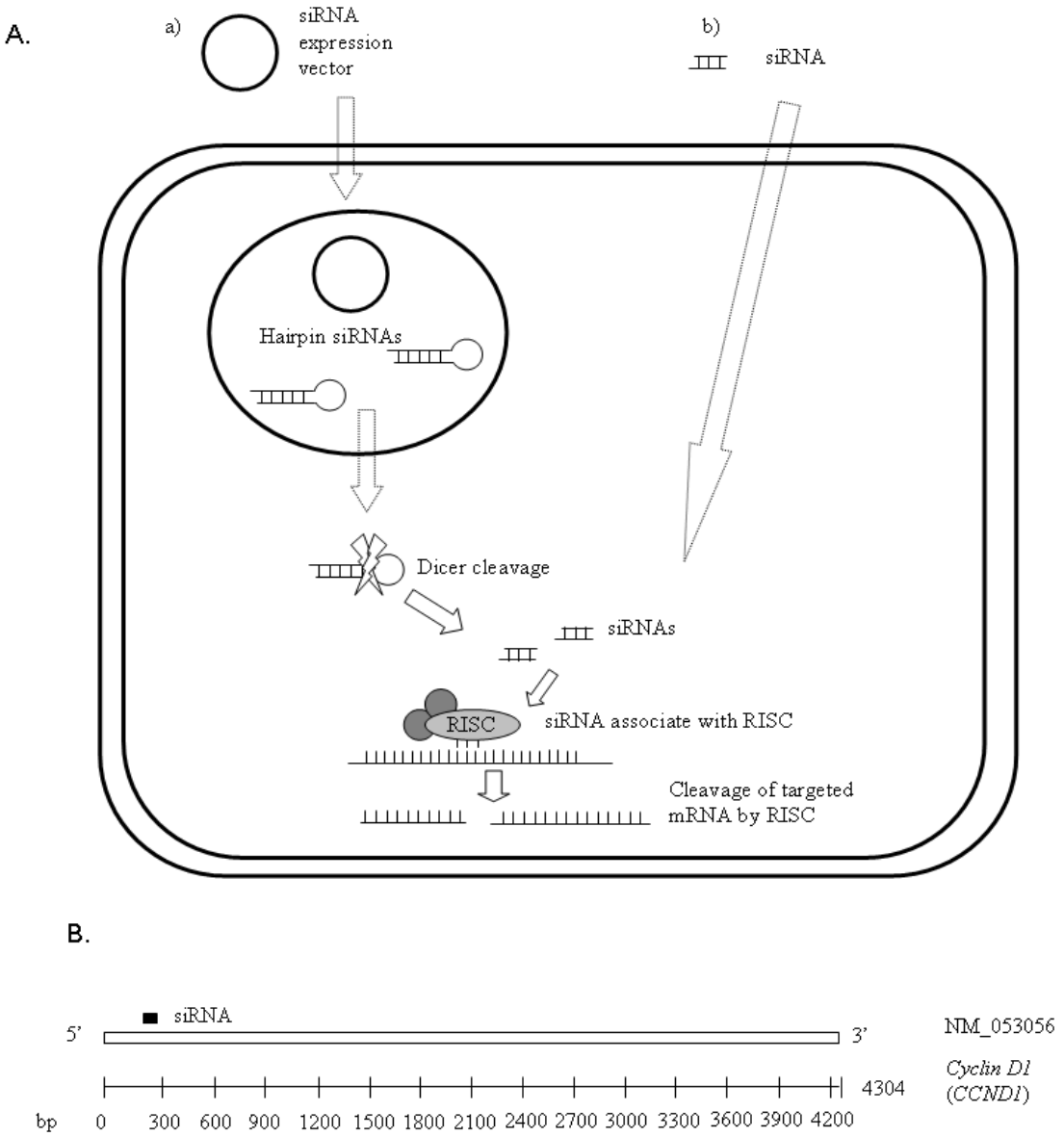


Figure 23. siRNA as a gene silent tool. A. Targeted mRNA can be silent by introducing siRNA expression vector that are processed by Dicer into siRNA or by siRNA directly. B. siRNA probe targeting *CCND1* mRNA applied in Paper IV was designed by Qiagen (https://www1.qiagen.com).

9 RESULTS AND DISCUSSION

9.1 FAMILIAL MTC IN A DOG PEDIGREE (PAPER I)

9.1.1 Identification of the first dog pedigree with familial MTC

A dog pedigree with several members affected by thyroid cancer was identified and characterized concerning tumor phenotype and candidate gene involvement (Figure 9). Clinical, biochemical and immunohistochemical examinations of four affected and one clinically unaffected member were performed. The affected individuals presented symptoms of fatigue, weight loss, muscle atrophy, and neck oedema in addition to chronic dermatosis. Furthermore they were found to have severe anemia and hypothyroidism with low levels of serum T4, increased level of TSH and positive for Tg autoantibody. One of the affected dogs also exhibited high levels of serum calcium. Neck ultrasound showed bilateral highly vascularised tumor masses. These findings suggested that the tumors represented thyroid cancer, which was later confirmed at autopsy. In all dogs examined by autopsy differentiated thyroid cancers were found with infiltrative local growth and/or metastases in lymph nodes and other tissues.

The tumors were microscopically characterized at histopathological and immunostaining examinations. Thus, all tumors generally exhibited compact cell growth pattern or follicular pattern with intramuscular infiltration. All three tumors were positive for calcitonin (a marker for MTC) and NSE, and two tumors were positive for Tg, whilst all were negative for parathyroid hormone (PTH) and S-100 stainings. Collectively the tumors were classified as highly malignant differentiated thyroid cancer, possibly of medullary (calcitonin positive) or mixed follicular-medullary (calcitonin and Tg positive for 2 tumors) subtype. Other diagnoses were also considered such as thyroid paraganglioma, parathyroid carcinoma and FTC. However, the negative staining results for S-100 protein (marker for paraganglioma) and PTH (parathyroid tumor marker) made these alternatives less likely. Taken together, the observations suggested a familial form of MTC without involvement of tumors in other endocrine organs such as the pituitary or the adrenal medulla. This finding overlaps with the condition FMTC in human, which is associated with activating germ-line mutations of the *RET* proto-oncogene.

9.1.2 *Ret* genetic screening of FMTC in dog

The possible involvement of the *Ret* gene in this dog pedigree was subsequently explored. Since only partial *Ret* sequence was reported in the database the complete cDNA *Ret* sequence was first predicted *in silico*. This was achieved by comparing the human *RET* gene sequence with a genomic clone from dog. The dog *Ret* gene was predicted to consist of 23 exons resulting in a 3711 bp open reading frame (ORF). Furthermore a putative exon 24 was predicted for the dog *Ret* gene that matched with exon 20 of human *RET*. The predicted structure and exon-boundaries of dog *Ret* were in agreement with human *RET*, showing 86% identity at the nucleotide level.

Mutation screening was carried out by sequencing or SSCP in three affected and one unaffected member. Sequencing analysis was performed for exons 12-21 of the dog *Ret* gene, that correspond to human *RET* exons 8-17 where all known MEN 2A and MTC related mutations are located. The screening of the remaining exons was performed by SSCP, followed by sequencing of observed variations. Three single nucleotide variants (R493R, I821I and IVS21-44) were found in the pedigree but did not segregate with the disease suggesting that they are polymorphisms of no clinical relevance to the phenotype.

The *RET* gene is well established as the causative gene for FMTC and MEN 2 in human. The lack of demonstrable *Ret* mutation in this dog pedigree suggests that the MTC predisposition does not share the genetic etiology commonly underlying FMTC phenotypes in human. Several possibilities can be considered for this difference. Mutations in the dog *Ret* gene may be located outside the human hotspots thus escaping detection in the screening performed. The possible involvement of other causative genes than *Ret* should also be considered. Interestingly, in about 5% of human MEN 2 kindreds reported genetic alterations of *RET* have not been identified (170). This dog pedigree could be one such example. Alternatively, this dog pedigree could harbor a novel familial MEN syndrome. It is possible that the observed familial MTC is part of an unrecognized syndrome related to another gene than *Ret*. The observation of multinodular hyperplasia in the adrenal cortex of lll:3 at autopsy could suggest a form of hereditary MEN not restricted to FMTC. Recently MEN X, a MEN-like syndrome without involvement of *Ret*, was described in rats with inborn cataract and development of multiple tumors affecting the neuroendocrine system (171). It can therefore be speculated that the unique occurrence of familial MTC makes this pedigree an important model in further delineating the genetic basis of MTC or even possibly a novel MEN-like syndrome.

9.2 CYTOGENETIC ANALYSES OF ESTABLISHED ATC CELL LINES (PAPER II)

9.2.1 Initial characterization and establishment of novel ATC cell lines

A number of thyroid tumor cell lines representing different pathological entities have been developed as for example reported in Heldin, *et al.*, Dahlman *et al.*, Ain *et al.*, and Fabien *et al.* (172-175). Established cancer cell lines serve as good *in vitro* models to delineate molecular genetic events in tumor development, as well as for functional studies and initial therapeutic studies. Studies of established cell lines are especially useful for ATC owing to the limitations and possible technical difficulties in analyzing primary surgical specimens from patients who have undergone pre-operative radiotherapy and chemotheraphy. In this study, two novel ATC cell lines, HTh 104 and HTh 112, were established from surgically removed samples of primary ATCs of giant cell type. These novel lines and six existing main ATC lines (HTh 7, HTh 74, C 643, KAT-4, SW 1736, and HTh 83) were initially characterized by STR profiling for identification purposes. All cell lines showed distinct STR profiles, indicating that they were unique and not cross-contaminated. All lines were also screened for *BRAF* mutations by sequencing of “hot spot” exons, which revealed a missense mutation V600E in three of the lines. Furthermore, reduced levels of *PTEN* expression was demonstrated in most lines using qRT-PCR. The subsequent molecular cytogenetic characterization was carried out using SKY analyses and G-banding, CGH analyses, and FISH.

9.2.2 Identification of chromosomal aberrations in ATC lines

Isochromosome 13 and i(5)(p10) were the most common recurrent aberrations observed, and C 643 and SW 1736 both carried a translocation t(15:22). It is possible that t(15:22) is an early event in the malignant transformation of thyroid neoplasms since similar translocations have been reported in goiter patients (176) and in FTC (177). Several nonrandom breakpoints were identified in this study, such as 3p21-22 and 15q26 which have also been previously reported in WDTC (178). On the other hand, the recurrent breakpoints at 1p36, 17q24-25 and 20q13.3 observed in this study, have not been reported in other types of thyroid cancer implicating that these regions could harbor genes important for ATC development. Thus far, structural chromosomal aberrations have only been reported in PTC and FTC, but not in other types of thyroid cancer. It is likely that chromosomal abnormalities are still present in primary ATC cells but have not been

revealed due to the limited number of reported ATC karyotypes. Established ATC cell lines could instead be used as models for identification of chromosomal aberrations, which could subsequently be verified in primary ATC.

9.3 GAIN OF CHROMOSOMAL REGION 20q IS FREQUENT IN ATC CELL LINES (PAPER II) AND PRIMARY ATC (PAPER IV)

The most common copy number alteration recorded in ATC cells was gain of 20q. This alteration was observed in six out of eight ATC cell lines, of which three showed high-level amplification. Two lines (HTh 112 and HTh 7) were further analyzed by 1Mb array-CGH whereby the region of gain was found to involve the interval 20q11.1-qter. In support of the findings, previous studies of gene copy number imbalances in ATC using conventional CGH have reported recurrent gains of 20q (114, 122).

Array-CGH analysis performed on 27 primary ATC revealed gain in 20q as the most frequent aberration (n=14). Furthermore 52% of the ATCs in the panel showed 20q gains or amplifications involving the 20q11.2 or 20q13.12 regions suggesting 20q gain play a significant role in dedifferentiation of thyroid tumors. Gain of 20q was not observed in the ATC patient who was relapse free in the study. Moreover the translocation t(X;20) identified in KAT-4 in Paper II is similar to the observations in an ATC clone previously reported (179), indicating a breakpoint at 20q. Observations from Paper II and Paper IV collectively suggest that 20q11.2 and 20q13.12 amplicons could harbor putative oncogene(s) or breakpoints for fusion genes responsible for ATC tumor development.

9.3.1 Gain of *UBCH10* in ATC

One candidate target gene for the frequent gains of 20q in ATC is *UBCH10*, which is located at 20q13.12 and thus overlaps with the distal of the two regions of gain in this chromosome. Over-expression of *UBCH10* has been reported in ATCs and ATC cell lines including KAT-4, and it has been shown to affect thyroid cell proliferation (180). *UBCH10* is a member of the E2 gene family, and it encodes a 19.6 kDa protein involved in the ubiquitin-dependent proteolysis (180). Seven of the eight ATC cell lines and 25% of the ATC primary tumors exhibited increased copy number of *UBCH10*. These observations support that *UBCH10* plays a role in ATC tumorigenesis, and suggest that gain in copy number can be associated with its increased expression.

9.3.2 Increased copy numbers in 11q13 and functional studies of the candidate gene *CCND1* encoding cyclin D1 (Paper IV)

Gains involving the 11q13 region were observed in about 50% of the ATC tumors and in two ATC cell lines. Increased copy numbers of the *CCND1* locus in 11q13 was recurrently found by FISH in tumor cells of half of the ATC panel. These findings prompted further investigation of the known oncogene *CCND1* encoding the cyclin D1 protein. Using Western blot analysis cyclin D1 expression was demonstrated in 67% of primary ATCs. However, cyclin D1 expression was not observed in normal thyroid tissue, suggesting that its induction is involved in ATC development.

Gain of 11q13 and/or *CCND1* has been commonly observed in advanced stages of other types of human cancer (181) and in metastatic PTC (182). Interestingly cyclin D1 over-expression in this study was observed in ATCs with as well as without copy number gain in 11q13. It is likely that pathogenic activation of cyclin D1 can also occur via other mechanisms than copy number gains, such as transcriptional and post-transciptional regulatory signals.

Cyclin D1 has been shown to promote cell proliferation and drive tumorigenesis in several human cancer models (183). However, since little is presently known about its role in thyroid cancer possible effects of cyclin D1 expression on thyroid cell proliferation was functionally assessed *in vitro*. Cyclin D1 was introduced into normal thyroid cells (Nthy-ori 3-1) that resulted in modestly increased cell population as compared to control cells transfected with empty vector. Furthermore, HTh 7 cells were transfected against *CCND1* siRNA, which resulted in reduced proliferation as compared to control cells. This could be due to the swift restoration of *CCND1* knockdown within 24 hours of transfection against *CCND1* siRNA. Collectively, the results suggest that cyclin D1 has a modest stimulatory effect on thyroid cell proliferation but is in itself not a strong determining factor.

9.3.3 Homozygous loss of *CDKN2A* and lack of p16 expression in primary ATCs (Paper IV)

In one case of primary ATC the array-CGH analyses revealed a homozygous loss in chromosomal region 9p21, which included the C*DKN2* locus encoding for $p16^{INK4A}$. This ATC was subsequently found to lack p16 protein expression as determined by Western blot analysis. In addition, only four of the 27 primary ATCs revealed p16 expression.

Intriguingly normal thyroid tissues used as control in this study was also negative for p16 expression. This observation concurs with previous findings by Ball *et al.* who reported that the vast majority of normal thyroid samples lacked p16 immunostaining (184). However p16 expression was frequently observed in WDTC in this and other studies (184, 185). From these observations it was hypothesized that p16 is induced in differentiated thyroid cancer but suppressed during progression towards the undifferentiated phenotype.

9.3.4 *BRAF* mutations in a subset of ATCs (Paper II and Paper IV)

Three ATC cell lines (HTh 104, KAT-4 and SW 1736) and three ATCs harbored the common *BRAF* mutation V600E involving nucleotide 1799 in exon 15. The same mutation has also been frequently observed in PTC, which has been interpreted in support of a developmental link between PTC and ATC (40). The *BRAF* mutation frequencies observed in our studies are comparable of the overall reported frequency of 10-35% in ATC (40).

9.4 LACK OF GLOBAL HYPOMETHYLATION IN FTCS (PAPER III)

Two independent methods, bisulphite Pyrosequencing of LINE-1 elements and LUMA, were used to quantify global methylation levels in matched pairs of FTCs and normal thyroid tissues from 21 patients. Overall, there was no significant difference in the LINE-1 methylation level between FTCs and corresponding normal thyroid. Similarly, LUMA revealed no significant difference between *Hpa*II/*Msp*I ratios in tumor samples and normal tissues. These findings indicate that there was lack of hypomethylation between FTCs and their corresponding normal tissues. However, the observations are in contrast with Galusca and colleagues who showed a significantly lower level of global methylation in thyroid cancer tissues as compared with benign tumors or adjacent normal thyroid (144). The different conclusions reached in the two studies could be related to the different methodologies applied. Galusca *et al.* used an immunohistochemical approach to detect methylated cytosines in interphase nuclei, while in our study genome-wide methylations were measured at retrotransposon LINE-1 elements and at CCGG sequences.

9.4.1 Inactivation of *RASSF1A* in FTCs

RASSF1A promoter hypermethylation and reduced expression are frequently seen in all histological types of thyroid tumors and in particular FTC (95, 104). These findings demonstrate that *RASSF1A* plays an important role in FTC tumorigenesis. Therefore the inactivation mechanisms of this gene and the association of this gene with global

methylation were further explored in this study. Pyrosequencing revealed increased methylation levels of the *RASSF1A* promoter methylation in tumor tissues. In addition allelic imbalance (AI) at the *RASSF1A* locus was observed in 71% of the tumors. There was also significantly reduced *RASSF1A* expression in FTCs compared to corresponding normal tissues. Unexpectedly no obvious correlation between *RASSF1A* hypermethylation, AI and mRNA reduction was demonstrated in this series. This could suggest that *RASSF1A* is down-regulated by other mechanisms at transcriptional and post-transcriptional levels in FTCs. Alternatively a larger sample size study is required in drawing statistically significant conclusion.

Genome-wide methylation and *RASSF1A* promoter hypermethylation are two independent events, whereby *RASSF1A* is a frequent and possibly an early event in FTC tumorigenesis.

10 CONCLUSIONS

This study was undertaken to investigate the different genetic mechanisms and genetic alterations involved in thyroid tumor development and progression, with the focus in exploring the possibility of thyroid tumor progression. The main findings conclude:

1. The first dog pedigree was diagnosed with familial medullary thyroid carcinoma based on clinical, biochemical and histopathological examinations. The lack of demonstrable *Ret* mutation in this pedigree makes this potentially an important model in further defining the genetic basis of familial MTC.
2. Novel chromosomal alterations are detected by karyotypic analysis of novel (HTh 104 and HTh 112) and established human ATC cell lines. The cytogenetic characterization of these alterations may serve as starting point for the identification of novel fusion genes in ATC, which could be verified in primary ATC later.
3. *RASSF1A* is frequently inactivated in FTC by promoter hypermethylation and allelic loss. However genome-wide methylation change is not coupled to *RASSF1A* promoter hypermethylation.
4. Amplicons of 11q13, 20q11.2 and 20q13.12 are recurrent findings in ATC. These regions are likely to harbor candidate genes in ATC tumorigenesis. Lack of p16 expression, over-expression of cyclin D1, and increased copy numbers of *CCND1* and *UBCH10* are characteristics of ATCs.
5. Genetic and cytogenetic methodologies provide insight into the mechanisms driving thyroid tumor progression. *BRAF* mutations and the array-CGH profiles and karyotyping findings indicated the existence of the progression pathway of thyroid cancer from well-differentiated to ATC.

11 ACKNOWLEDGMENTS

In pursuit of my dreams, **Siddhartha Gautama** has inspired my journey through this unknown path. My adventure in Sweden would not be as exciting, invaluable and unforgettable without the following mentioned people. I would like to express my deepest sincere gratitude to:

Dr. Anders Höög, my main supervisor, who was abundantly helpful and thoughtful in offering valuable assistance, support and guidance throughout my education training. Thank you for your interest in my projects, always assuring me that I have done a great job, being always able to find time out of your extremely busy schedule to check on my progress, and also for being concerned of my overall well-being during my stay here in Sweden, which was something I sincerely appreciate.

Professor Catharina Larsson, my co-supervisor (who was also my main supervisor for the first half of my degree) for not only giving me the opportunity to pursue my research training and my career aspiration in your laboratory, but the freedom and complete independence in developing my scientific skills. Thank you for spending late hours and even weekends to discuss research projects and manuscripts with me. Your talent in science (and cooking), intelligence as well as ability in balancing career and family life have truly inspired me.

Dr. Theodoros Foukakis, my co-supervisor in passing the torchlight of thyroid research to me and sharing with me tips of being efficient. Thank you for inviting me to Greek parties and dinners, and also for being my tour-guide in Buenos Aires.

Dr. Virpi Thohonen, my mentor, for your encouragement, advice, tips on mRNA *in situ* hybridization and the fun chat we had. Your positive attitude and enthusiasm about life have always lifted me up.

Professor Andrew Fire for inviting me to visit your laboratory that has coincidentally turned out to be a remarkable eye-opener. It was a great pleasure for being able to witness and join the celebration of Nobel Prize in 2006.

Dr. Weng-Onn Lui for introducing me to the worlds of cytogenetic and microRNA, and also for hosting my visit in Stanford University. Thank you for following up my Swedish student visa issue before my arrival and for many delicious Asian dinners.

Professor Lars Grimelius, for your expertise in endocrine pathology and your effort in following up the patients' slides included in my studies.

Dr. Jan Zedenius for your expertise in endocrine surgery and for organizing the Molecular Genetics Wine Seminars. Thank you for the Swedish dinner invitation and your witty comments that have provided me with a good dose of laughter. **Dr. Göran Wallin** for your expertise in endocrine surgery in particular ATC. Thank you for making sure I was not left out during the International of Thyroid Congress in Buenos Aires.

Professor Bertil Hamberger, Professor Lars-Ove Farnebo & **Dr. Martin Bäckdahl** for your great effort in establishing the endocrine biobank.

Dr. Günther Weber for organizing the Molecular Genetics Wine Seminars and some fun badminton time.

Professor Bruce Robinson from the University of Sydney, Australia, for your keen interest in the ATC project and a wonderful collaboration. **Dr. Roderick Clifton-Bligh,** my Australian collaborator for your expertise in the functional studies.

Dr. Nils-Erik Heldin from Uppsala University for fantastic collaboration, establishing the ATC lines and for taking time in answering all my questions about ATC cell culture.

Mr. Johan Staaf & **Professor Åke Borg** for hosting my visit to Lund Universitet and forming a nice collaboration.

Ms. Monika Patersson for taking time in answering all technical questions on pyrosequencing promptly as well as for a pleasant collaboration.

Dr. Henrik von Euler from the Swedish University of Agricultural Sciences for an interesting collaboration that itself deserves a Guinness World record; the first dog family with thyroid cancer being studied.

Dr. Helen Billman-Jacobe, my undergraduate supervisor and **Professor Francis Carbone**, my undergraduate course coordinator from the University of Melbourne, Australia for introducing me to the world of research.

Ms. Dephi Post & **Ms. Catarina Zannin** for being so cheerful every mornings I walked in CMM, **Ms. Britt-Marie Witasp**, **Ms. Helena Nässen**, **Ms. Majalena Granqvist**, **Ms. Christina Bremer** & **Ms. Yvonne Stridsberg** for superb administrative support. **Ms. Lisa Ånfalk** for collecting tumor samples and organizing the biobank. **Mr. Lennart Helleday** for providing IT support in a timely manner.

Dr. Emma Flordal-Thelander, for your generous help and the interesting girls' talks we had in between work breaks, especially for sending me cheerful well-wishes and encouragement during thesis writing. **Dr. Andrea Villablanca**, for picking me up at the airport during my Stanford University visit. **Dr. Andrei Alimov**, for teaching me SSCP. **Dr. Petra Kjellman,** for being part of thyroid team. **Drs. Mattias Berglund & Lars Forsberg**, for fun chats. **Dr. Srinivasan Thoppe** for delicious Indian food, **Dr. Stiina Välimäiki** for organizing lab dinner in 2004. **Dr. Wendy Weng** for great time in Cambridge 2004 workshop and yummy Chinese meals. **Dr. Dolors Costa** for a lively and fun time in the lab. **Dr. Christina Ruddock** for teaching me cytogenetics nomenclature. **Ms. Hanne Bergius**, my attachment student for allowing me to gain experience in teaching. **Dr. Junko Takagi** for your advice and impressive Japanese meals. **Dr. Chen Zhao** and **Dr. Shasha Lu** for teaching me genotyping, friendship and authentic Chinese meals. **Dr. David Velazquez Fernandez** for good laughs and excellent Mexican lunch. **Dr. Janos Geli** for always cheering me up and generous offer to help, fruitful collaborations and dinner invitations. **Mr. Nimrod Kiss** for wonderful discussions about philosophy and friendship. **Dr. Anastasios Sofiadis** for being an excellent organizer in organizing lab activities and lab-related matters, making the lab a conducive place to work at. Special thanks for making excellent frappé! **Mr. Christofer Juhlin** for joyful chats, for teaching me IHC and also for a fruitful collaboration. **Mr. Felix Haglund**, **Mr. Stefano Caramuta, Drs. Kawa Ali** & **Andrii Dinets** for making the lab lively. **Dr. Jamileh Hashemi** for a great time in Lund and Anaheim, and for giving me a ride home from the airport. **Dr. Svetlana Langercrantz** for setting a role model in staying optimistic and cheerful always.

Fire lab members and labs members from other research groups in the whole CMM, in particular L8:01 and L8:02, for being a nice bunch of people to work with particularly **Drs. Biying Zheng**, **Zheng Ge**, **Vladana Vukojevic**, **Petra Tollet-Egnell**, **Tianling Wei**, **Feng Qing**, **Baoxia Wong**, **Zahidul & Mounira Khan, Emma Tham, Tzvetomir Philipova, Jin Hu** & **Ming Lu**, **Ms. Clara Ibel Chamoro**, **Ms. Carolina Gustavsson, Ms. Roxana Merino Martinez, Ms. Cecilia Dahlgren** & **Mr. Johan Ökinger**. **Drs. Fayezeh Yari**, **Yu Ming** & **Ghazal Zaboli** for joyful afternoon tea, especially to **Dr. Fayezeh Yari** for delicious cakes. **Ms**. **Agneta Gunnar** for being so cheerful even during late evenings/weekends at work. **Mr. Mohsen Karimi** for intriguing scientific discussions during and outside book club. **Dr.**

Jacqueline Schoumans for useful tips in running FISH and array. **Ms. Anna Maria Marino** for your friendliness and help in preparing my dissertation party.

Drs. Keng-Ling & **Erik Wallin** for inviting me to your summer house in 2004, an enjoyable West Coast / Norway trip and social activities, especially to **Keng-Ling** for being a kind sister.

Dr. Michela Barbaro for being a great friend and your hospitality to your Italian parties and gatherings. Thank you for teaching me MLPA too and those late-evening fun chats.

Mr. Yin-Choy Chuan for always being helpful. Thank you for bringing me to fix my banking and personal number applications, helping me moved as well as assembling my furniture.

Ms. Louisa Cheung & **Mr. Daniel Eklöf** for great friendship, help with my IKEA shopping and also many dinner invitations. Special thanks to **Louisa** who have helped me in many ways from Swedish translations to giving me insightful advice.

Ms. Lillian Chan for your amazing creativity in designing such a fabulous front cover for this thesis!

Dr. Yun Chau Long & **Ms. Sue Chin Lwee**, for being really good friends in helping and giving me advice whenever I needed and also for inviting me over for lunch / dinner making me feel like home. I am especially indebted to **Dr. Yun Chau Long** who serendipitously introduced Karolinska Institutet to me and hence my serendipitous adventure here.

Dr. Amy Au for being such a great friend outside work and a fantastic collaborator at work. Knowing a bosom friend (with familiar Australian accent) upon my arrival to this foreign land has already convinced me that my decision of coming here a wise one. Thank you for being my sounding board.

Ms. Chin Chin Lee my best friend since childhood, for constantly being very supportive in whatever I am pursuing. Thank you for the bonded friendship that remains strong over the years without being affected by the distance apart.

My family, the Lee Family who has always been supportive to me. My grandmother, **Mdm. Foon Kwan** for being the greatest grandmother in the world in taking good care of me. My aunts, **Ms. Poh Peng Cheah** & **Ms. Poh Kheng Cheah** for always checking on me in making sure I eat well and always stay happy. My sisters, **Jia Wei**, **Jia Heng**, **Jia Ee** & **Jia Xuan**, and my brother, **Zong Han** for constantly being proud of me and endless love. My father, **Mr. Chee Kong Lee** & my mother **Mdm. Poh Kwai Cheah** for support throughout these years and unfailing love and care. Especially to my father, who has always been my inspirational and motivational source, and my role model in striving my best towards my dreams and goals in life. Thank you to my family for excusing my absence residing in three countries that are thousand miles away from home over the last ten years.

Sze Ching Chan, my fiancé and companion in life for your understanding, patience, respect and love. Thank you for being extremely supportive during the process of the completion of my degree and always be there for me whenever I need you.

This study was supported by **Swedish Cancer Society**, **Swedish Research Council**, **Göran Gustafsson Foundation for Research in Natural Sciences and Medicine**, **Gustav V Jubilee Foundation**, **Cancer Society in Stockholm**, **Stockholm County Council**, **Swedish Medical Association** and **Karolinska Institutet**.

The merit of this work is dedicated to **all patients** participated in this study.

12 REFERENCES

1. van de Vijver MJ. The pathology of familial breast cancer: The pre-BRCA1/BRCA2 era: historical perspectives. Breast Cancer Res 1999;1(1):27-30.
2. Knudson AG. Two genetic hits (more or less) to cancer. Nat Rev Cancer 2001;1(2):157-62.
3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57-70.
4. Renan MJ. How many mutations are required for tumorigenesis? Implications from human cancer data. Molecular carcinogenesis 1993;7(3):139-46.
5. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nature medicine 2004;10(8):789-99.
6. Park MT, Lee SJ. Cell cycle and cancer. Journal of biochemistry and molecular biology 2003;36(1):60-5.
7. Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. Annual review of pharmacology and toxicology 1999;39:295-312.
8. Lodish H, Berk A, Matsudaira P*, et al.* 5th ed. New York: WH Freeman; 2004.
9. Nowell PC. Tumor progression: a brief historical perspective. Seminars in cancer biology 2002;12(4):261-6.
10. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61(5):759-67.
11. Crick F. Central dogma of molecular biology. Nature 1970;227(5258):561-3.
12. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM. Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proceedings of the National Academy of Sciences of the United States of America 1982;79(24):7824-7.
13. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature 1973;243(5405):290-3.
14. Charest A, Lane K, McMahon K*, et al.* Fusion of FIG to the receptor tyrosine kinase ROS in a glioblastoma with an interstitial del(6)(q21q21). Genes, chromosomes & cancer 2003;37(1):58-71.
15. Seshadri R, Matthews C, Dobrovic A, Horsfall DJ. The significance of oncogene amplification in primary breast cancer. International journal of cancer 1989;43(2):270-2.
16. Gerald WL, Rosai J, Ladanyi M. Characterization of the genomic breakpoint and chimeric transcripts in the EWS-WT1 gene fusion of desmoplastic small round cell tumor. Proceedings of the National Academy of Sciences of the United States of America 1995;92(4):1028-32.
17. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM. Predicting aberrant CpG island methylation. Proceedings of the National Academy of Sciences of the United States of America 2003;100(21):12253-8.
18. Bird A. DNA methylation patterns and epigenetic memory. Genes & development 2002;16(1):6-21.
19. Lettini AA, Guidoboni M, Fonsatti E, Anzalone L, Cortini E, Maio M. Epigenetic remodelling of DNA in cancer. Histology and histopathology 2007;22(12):1413-24.
20. He L, Thomson JM, Hemann MT*, et al.* A microRNA polycistron as a potential human oncogene. Nature 2005;435(7043):828-33.
21. Johnson SM, Grosshans H, Shingara J*, et al.* RAS is regulated by the let-7 microRNA family. Cell 2005;120(5):635-47.
22. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer 2006;6(4):259-69.
23. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nature genetics 2007;39(5):673-7.
24. Masutomi K, Hahn WC. Telomerase and tumorigenesis. Cancer letters 2003;194(2):163-72.
25. Hackett JA, Greider CW. Balancing instability: dual roles for telomerase and telomere dysfunction in tumorigenesis. Oncogene 2002;21(4):619-26.
26. Chadeneau C, Hay K, Hirte HW, Gallinger S, Bacchetti S. Telomerase activity associated

with acquisition of malignancy in human colorectal cancer. Cancer research 1995;55(12):2533-6.
27. Loveday RL, Greenman J, Drew PJ, Monson JR, Kerin MJ. Genetic changes associated with telomerase activity in breast cancer. International journal of cancer 1999;84(5):516-20.
28. Grignani F, Ferrucci PF, Testa U*, et al.* The acute promyelocytic leukemia-specific PML-RAR alpha fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 1993;74(3):423-31.
29. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nature medicine 2006;12(8):895-904.
30. Palmieri D, Chambers AF, Felding-Habermann B, Huang S, Steeg PS. The biology of metastasis to a sanctuary site. Clin Cancer Res 2007;13(6):1656-62.
31. Tang C, Ang BT, Pervaiz S. Cancer stem cell: target for anti-cancer therapy. Faseb J 2007.
32. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nature medicine 1997;3(7):730-7.
33. De Felice M, Di Lauro R. Thyroid development and its disorders: genetics and molecular mechanisms. Endocrine reviews 2004;25(5):722-46.
34. Mauchamp J, Mirrione A, Alquier C, Andre F. Follicle-like structure and polarized monolayer: role of the extracellular matrix on thyroid cell organization in primary culture. Biology of the cell / under the auspices of the European Cell Biology Organization 1998;90(5):369-80.
35. Schlumberger M PF. Thyroid Tumors. Paris: Editions Nucleon; 1999.
36. Mazzaferri EL. Management of a solitary thyroid nodule. The New England journal of medicine 1993;328(8):553-9.
37. DeLellis RA LR, Heitz PU, Eng C. Pathology and genetics of tumours of endocrine organs. Lyon: IARC Press; 2004.
38. Rosai J CM, De Lellis RA. Tumors of the thyroid gland. Atlas of tumor pathology. Washington DC: AFIP; 1992.
39. Patel KN, Shaha AR. Poorly differentiated and anaplastic thyroid cancer. Cancer Control 2006;13(2):119-28.
40. Kondo T, Ezzat S, Asa SL. Pathogenetic mechanisms in thyroid follicular-cell neoplasia. Nat Rev Cancer 2006;6(4):292-306.
41. Mizukami Y, Michigishi T, Nonomura A*, et al.* Distant metastases in differentiated thyroid carcinomas: a clinical and pathologic study. Human pathology 1990;21(3):283-90.
42. Volante M, Collini P, Nikiforov YE*, et al.* Poorly differentiated thyroid carcinoma: the Turin proposal for the use of uniform diagnostic criteria and an algorithmic diagnostic approach. The American journal of surgical pathology 2007;31(8):1256-64.
43. Are C, Shaha AR. Anaplastic thyroid carcinoma: biology, pathogenesis, prognostic factors, and treatment approaches. Annals of surgical oncology 2006;13(4):453-64.
44. McIver B, Hay ID, Giuffrida DF*, et al.* Anaplastic thyroid carcinoma: a 50-year experience at a single institution. Surgery 2001;130(6):1028-34.
45. Ashworth M. The pathology of preclinical medullary thyroid carcinoma. Endocrine pathology 2004;15(3):227-31.
46. Lakhani VT, You YN, Wells SA. The multiple endocrine neoplasia syndromes. Annual review of medicine 2007;58:253-65.
47. Eng C. Genetics of Cowden syndrome: through the looking glass of oncology. International journal of oncology 1998;12(3):701-10.
48. Eng C. Role of PTEN, a lipid phosphatase upstream effector of protein kinase B, in epithelial thyroid carcinogenesis. Annals of the New York Academy of Sciences 2002;968:213-21.
49. Sippel RS, Caron NR, Clark OH. An evidence-based approach to familial nonmedullary thyroid cancer: screening, clinical management, and follow-up. World journal of surgery 2007;31(5):924-33.
50. Sturgeon C, Clark OH. Familial nonmedullary thyroid cancer. Thyroid 2005;15(6):588-93.
51. Franceschi S, Boyle P, Maisonneuve P*, et al.* The epidemiology of thyroid carcinoma. Critical reviews in oncogenesis 1993;4(1):25-52.
52. Goodman MT, Yoshizawa CN, Kolonel LN. Descriptive epidemiology of thyroid cancer in Hawaii. Cancer 1988;61(6):1272-81.
53. Spitz MR, Sider JG, Katz RL, Pollack ES, Newell GR. Ethnic patterns of thyroid cancer

incidence in the United States, 1973-1981. International journal of cancer 1988;42(4):549-53.
54. Schlumberger MJ. Papillary and follicular thyroid carcinoma. The New England journal of medicine 1998;338(5):297-306.
55. Negri E, Dal Maso L, Ron E*, et al.* A pooled analysis of case-control studies of thyroid cancer. II. Menstrual and reproductive factors. Cancer Causes Control 1999;10(2):143-55.
56. Majima T, Komatsu Y, Doi K*, et al.* Anaplastic thyroid carcinoma associated with Graves' disease. Endocrine journal 2005;52(5):551-7.
57. Taylor T, Specker B, Robbins J*, et al.* Outcome after treatment of high-risk papillary and non-Hurthle-cell follicular thyroid carcinoma. Annals of internal medicine 1998;129(8):622-7.
58. Mazzaferri EL, Kloos RT. Clinical review 128: Current approaches to primary therapy for papillary and follicular thyroid cancer. The Journal of clinical endocrinology and metabolism 2001;86(4):1447-63.
59. Cooper DS, Specker B, Ho M*, et al.* Thyrotropin suppression and disease progression in patients with differentiated thyroid cancer: results from the National Thyroid Cancer Treatment Cooperative Registry. Thyroid 1998;8(9):737-44.
60. Takahashi M, Ritz J, Cooper GM. Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell 1985;42(2):581-8.
61. Machens A, Dralle H. Multiple endocrine neoplasia type 2 and the RET protooncogene: from bedside to bench to bedside. Molecular and cellular endocrinology 2006;247(1-2):34-40.
62. Runeberg-Roos P, Saarma M. Neurotrophic factor receptor RET: structure, cell biology, and inherited diseases. Ann Med 2007:1-9.
63. Ciampi R, Nikiforov YE. RET/PTC rearrangements and BRAF mutations in thyroid tumorigenesis. Endocrinology 2007;148(3):936-41.
64. Romei C, Elisei R, Pinchera A*, et al.* Somatic mutations of the ret protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. The Journal of clinical endocrinology and metabolism 1996;81(4):1619-22.
65. Zedenius J, Wallin G, Hamberger B, Nordenskjold M, Weber G, Larsson C. Somatic and MEN 2A de novo mutations identified in the RET proto-oncogene by screening of sporadic MTC:s. Human molecular genetics 1994;3(8):1259-62.
66. Dhomen N, Marais R. New insight into BRAF mutations in cancer. Current opinion in genetics & development 2007;17(1):31-9.
67. Mitsutake N, Knauf JA, Mitsutake S, Mesa C, Jr., Zhang L, Fagin JA. Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer research 2005;65(6):2465-73.
68. Michaloglou C, Vredeveld LC, Mooi WJ, Peeper DS. BRAF(E600) in benign and malignant human tumours. Oncogene 2007.
69. Xing M. BRAF mutation in thyroid cancer. Endocr Relat Cancer 2005;12(2):245-62.
70. Trovisco V, Vieira de Castro I, Soares P*, et al.* BRAF mutations are associated with some histological types of papillary thyroid carcinoma. The Journal of pathology 2004;202(2):247-51.
71. Moretti S, Macchiarulo A, De Falco V*, et al.* Biochemical and molecular characterization of the novel BRAF(V599Ins) mutation detected in a classic papillary thyroid carcinoma. Oncogene 2006;25(30):4235-40.
72. Espinosa AV, Porchia L, Ringel MD. Targeting BRAF in thyroid cancer. British journal of cancer 2007;96(1):16-20.
73. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 2007;7(4):295-308.
74. Nikiforov YE. Genetic alterations involved in the transition from well-differentiated to poorly differentiated and anaplastic thyroid carcinomas. Endocrine pathology 2004;15(4):319-27.
75. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2(7):489-501.
76. Samuels Y, Ericson K. Oncogenic PI3K and its role in cancer. Current opinion in oncology 2006;18(1):77-82.
77. Wu G, Mambo E, Guo Z*, et al.* Uncommon mutation, but common amplifications, of the PIK3CA gene in thyroid tumors. The Journal of clinical endocrinology and metabolism

2005;90(8):4688-93.
78. Garcia-Rostan G, Costa AM, Pereira-Castro I*, et al.* Mutation of the PIK3CA gene in anaplastic thyroid cancer. Cancer research 2005;65(22):10199-207.
79. Hou P, Liu D, Shan Y*, et al.* Genetic alterations and their relationship in the phosphatidylinositol 3-kinase/Akt pathway in thyroid cancer. Clin Cancer Res 2007;13(4):1161-70.
80. Wang Y, Hou P, Yu H*, et al.* High prevalence and mutual exclusivity of genetic alterations in the phosphatidylinositol-3-kinase/akt pathway in thyroid tumors. The Journal of clinical endocrinology and metabolism 2007;92(6):2387-90.
81. Ringel MD, Hayre N, Saito J*, et al.* Overexpression and overactivation of Akt in thyroid carcinoma. Cancer research 2001;61(16):6105-11.
82. Miyakawa M, Tsushima T, Murakami H, Wakai K, Isozaki O, Takano K. Increased expression of phosphorylated p70S6 kinase and Akt in papillary thyroid cancer tissues. Endocrine journal 2003;50(1):77-83.
83. Vasko V, Saji M, Hardy E*, et al.* Akt activation and localisation correlate with tumour invasion and oncogene expression in thyroid cancer. Journal of medical genetics 2004;41(3):161-70.
84. Patel KN, Singh B. Genetic considerations in thyroid cancer. Cancer Control 2006;13(2):111-8.
85. Tallini G, Garcia-Rostan G, Herrero A*, et al.* Downregulation of p27KIP1 and Ki67/Mib1 labeling index support the classification of thyroid carcinoma into prognostically relevant categories. The American journal of surgical pathology 1999;23(6):678-85.
86. Resnick MB, Schacter P, Finkelstein Y, Kellner Y, Cohen O. Immunohistochemical analysis of p27/kip1 expression in thyroid carcinoma. Mod Pathol 1998;11(8):735-9.
87. Khoo ML, Freeman JL, Witterick IJ*, et al.* Underexpression of p27/Kip in thyroid papillary microcarcinomas with gross metastatic disease. Archives of otolaryngology--head & neck surgery 2002;128(3):253-7.
88. Davies MA, Lu Y, Sano T*, et al.* Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis. Cancer research 1998;58(23):5285-90.
89. Segev DL, Umbricht C, Zeiger MA. Molecular pathogenesis of thyroid cancer. Surgical oncology 2003;12(2):69-90.
90. Frisk T, Foukakis T, Dwight T*, et al.* Silencing of the PTEN tumor-suppressor gene in anaplastic thyroid cancer. Genes, chromosomes & cancer 2002;35(1):74-80.
91. Alvarez-Nunez F, Bussaglia E, Mauricio D*, et al.* PTEN promoter methylation in sporadic thyroid carcinomas. Thyroid 2006;16(1):17-23.
92. Dammann R, Schagdarsurengin U, Seidel C*, et al.* The tumor suppressor RASSF1A in human carcinogenesis: an update. Histology and histopathology 2005;20(2):645-63.
93. Dammann R, Schagdarsurengin U, Strunnikova M*, et al.* Epigenetic inactivation of the Ras-association domain family 1 (RASSF1A) gene and its function in human carcinogenesis. Histology and histopathology 2003;18(2):665-77.
94. Schagdarsurengin U, Gimm O, Hoang-Vu C, Dralle H, Pfeifer GP, Dammann R. Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. Cancer research 2002;62(13):3698-701.
95. Xing M, Cohen Y, Mambo E*, et al.* Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. Cancer research 2004;64(5):1664-8.
96. Tallini G, Santoro M, Helie M*, et al.* RET/PTC oncogene activation defines a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes. Clin Cancer Res 1998;4(2):287-94.
97. Santoro M, Papotti M, Chiappetta G*, et al.* RET activation and clinicopathologic features in poorly differentiated thyroid tumors. The Journal of clinical endocrinology and metabolism 2002;87(1):370-9.
98. Alberti L, Carniti C, Miranda C, Roccato E, Pierotti MA. RET and NTRK1 proto-oncogenes in human diseases. Journal of cellular physiology 2003;195(2):168-86.
99. Beimfohr C, Klugbauer S, Demidchik EP, Lengfelder E, Rabes HM. NTRK1 re-arrangement in papillary thyroid carcinomas of children after the Chernobyl reactor accident. International journal of cancer 1999;80(6):842-7.

100. Musholt TJ, Musholt PB, Khaladj N, Schulz D, Scheumann GF, Klempnauer J. Prognostic significance of RET and NTRK1 rearrangements in sporadic papillary thyroid carcinoma. Surgery 2000;128(6):984-93.
101. Ciampi R, Knauf JA, Kerler R*, et al.* Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. The Journal of clinical investigation 2005;115(1):94-101.
102. Kroll TG, Sarraf P, Pecciarini L*, et al.* PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]. Science (New York, NY 2000;289(5483):1357-60.
103. Drieschner N, Belge G, Rippe V, Meiboom M, Loeschke S, Bullerdiek J. Evidence for a 3p25 breakpoint hot spot region in thyroid tumors of follicular origin. Thyroid 2006;16(11):1091-6.
104. Foukakis T, Au AY, Wallin G*, et al.* The Ras effector NORE1A is suppressed in follicular thyroid carcinomas with a PAX8-PPARgamma fusion. The Journal of clinical endocrinology and metabolism 2006;91(3):1143-9.
105. Rippe V, Drieschner N, Meiboom M*, et al.* Identification of a gene rearranged by 2p21 aberrations in thyroid adenomas. Oncogene 2003;22(38):6111-4.
106. He H, Jazdzewski K, Li W*, et al.* The role of microRNA genes in papillary thyroid carcinoma. Proceedings of the National Academy of Sciences of the United States of America 2005;102(52):19075-80.
107. Pallante P, Visone R, Ferracin M*, et al.* MicroRNA deregulation in human thyroid papillary carcinomas. Endocr Relat Cancer 2006;13(2):497-508.
108. Visone R, Russo L, Pallante P*, et al.* MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. Endocr Relat Cancer 2007;14(3):791-8.
109. Weber F, Teresi RE, Broelsch CE, Frilling A, Eng C. A limited set of human MicroRNA is deregulated in follicular thyroid carcinoma. The Journal of clinical endocrinology and metabolism 2006;91(9):3584-91.
110. Frisk T, Zedenius J, Lundberg J, Wallin G, Kytola S, Larsson C. CGH alterations in medullary thyroid carcinomas in relation to the RET M918T mutation and clinical outcome. International journal of oncology 2001;18(6):1219-25.
111. Hemmer S, Wasenius VM, Knuutila S, Franssila K, Joensuu H. DNA copy number changes in thyroid carcinoma. The American journal of pathology 1999;154(5):1539-47.
112. Wada N, Duh QY, Miura D, Brunaud L, Wong MG, Clark OH. Chromosomal aberrations by comparative genomic hybridization in hurthle cell thyroid carcinomas are associated with tumor recurrence. The Journal of clinical endocrinology and metabolism 2002;87(10):4595-601.
113. Roque L, Rodrigues R, Pinto A, Moura-Nunes V, Soares J. Chromosome imbalances in thyroid follicular neoplasms: a comparison between follicular adenomas and carcinomas. Genes, chromosomes & cancer 2003;36(3):292-302.
114. Wreesmann VB, Ghossein RA, Patel SG*, et al.* Genome-wide appraisal of thyroid cancer progression. The American journal of pathology 2002;161(5):1549-56.
115. Castro P, Eknaes M, Teixeira MR*, et al.* Adenomas and follicular carcinomas of the thyroid display two major patterns of chromosomal changes. The Journal of pathology 2005;206(3):305-11.
116. Singh B, Lim D, Cigudosa JC*, et al.* Screening for genetic aberrations in papillary thyroid cancer by using comparative genomic hybridization. Surgery 2000;128(6):888-93;discussion 93-4.
117. Kjellman P, Lagercrantz S, Hoog A, Wallin G, Larsson C, Zedenius J. Gain of 1q and loss of 9q21.3-q32 are associated with a less favorable prognosis in papillary thyroid carcinoma. Genes, chromosomes & cancer 2001;32(1):43-9.
118. Bauer AJ, Cavalli LR, Rone JD*, et al.* Evaluation of adult papillary thyroid carcinomas by comparative genomic hybridization and microsatellite instability analysis. Cancer genetics and cytogenetics 2002;135(2):182-6.
119. Wreesmann VB, Ghossein RA, Hezel M*, et al.* Follicular variant of papillary thyroid carcinoma: genome-wide appraisal of a controversial entity. Genes, chromosomes & cancer 2004;40(4):355-64.
120. Wreesmann VB, Sieczka EM, Socci ND*, et al.* Genome-wide profiling of papillary thyroid cancer identifies MUC1 as an independent prognostic marker. Cancer research

2004;64(11):3780-9.
121. Richter H, Braselmann H, Hieber L*, et al.* Chromosomal imbalances in post-chernobyl thyroid tumors. Thyroid 2004;14(12):1061-4.
122. Rodrigues RF, Roque L, Rosa-Santos J, Cid O, Soares J. Chromosomal imbalances associated with anaplastic transformation of follicular thyroid carcinomas. British journal of cancer 2004;90(2):492-6.
123. Wilkens L, Benten D, Tchinda J*, et al.* Aberrations of chromosomes 5 and 8 as recurrent cytogenetic events in anaplastic carcinoma of the thyroid as detected by fluorescence in situ hybridisation and comparative genomic hybridisation. Virchows Arch 2000;436(4):312-8.
124. Miura D, Wada N, Chin K*, et al.* Anaplastic thyroid cancer: cytogenetic patterns by comparative genomic hybridization. Thyroid 2003;13(3):283-90.
125. Ward LS, Brenta G, Medvedovic M, Fagin JA. Studies of allelic loss in thyroid tumors reveal major differences in chromosomal instability between papillary and follicular carcinomas. The Journal of clinical endocrinology and metabolism 1998;83(2):525-30.
126. Gillespie JW, Nasir A, Kaiser HE. Loss of heterozygosity in papillary and follicular thyroid carcinoma: a mini review. In vivo (Athens, Greece) 2000;14(1):139-40.
127. Huang Y, de la Chapelle A, Pellegata NS. Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. International journal of cancer 2003;104(6):735-44.
128. Califano JA, Johns MM, 3rd, Westra WH*, et al.* An allelotype of papillary thyroid cancer. International journal of cancer 1996;69(6):442-4.
129. Antonini P, Levy N, Caillou B*, et al.* Numerical aberrations, including trisomy 22 as the sole anomaly, are recurrent in follicular thyroid adenomas. Genes, chromosomes & cancer 1993;8(1):63-6.
130. Belge G, Thode B, Rippe V, Bartnitzke S, Bullerdiek J. A characteristic sequence of trisomies starting with trisomy 7 in benign thyroid tumors. Human genetics 1994;94(2):198-202.
131. Bondeson L, Bengtsson A, Bondeson AG*, et al.* Chromosome studies in thyroid neoplasia. Cancer 1989;64(3):680-5.
132. Roque L, Castedo S, Clode A, Soares J. Deletion of 3p25-->pter in a primary follicular thyroid carcinoma and its metastasis. Genes, chromosomes & cancer 1993;8(3):199-203.
133. Teyssier JR, Liautaud-Roger F, Ferre D, Patey M, Dufer J. Chromosomal changes in thyroid tumors. Relation with DNA content, karyotypic features, and clinical data. Cancer genetics and cytogenetics 1990;50(2):249-63.
134. Kitamura Y, Shimizu K, Ito K, Tanaka S, Emi M. Allelotyping of follicular thyroid carcinoma: frequent allelic losses in chromosome arms 7q, 11p, and 22q. The Journal of clinical endocrinology and metabolism 2001;86(9):4268-72.
135. Trovato M, Fraggetta F, Villari D*, et al.* Loss of heterozygosity of the long arm of chromosome 7 in follicular and anaplastic thyroid cancer, but not in papillary thyroid cancer. The Journal of clinical endocrinology and metabolism 1999;84(9):3235-40.
136. Zhang JS, Nelson M, McIver B*, et al.* Differential loss of heterozygosity at 7q31.2 in follicular and papillary thyroid tumors. Oncogene 1998;17(6):789-93.
137. Zedenius J, Wallin G, Svensson A*, et al.* Allelotyping of follicular thyroid tumors. Human genetics 1995;96(1):27-32.
138. Zedenius J, Wallin G, Svensson A*, et al.* Deletions of the long arm of chromosome 10 in progression of follicular thyroid tumors. Human genetics 1996;97(3):299-303.
139. Grebe SK, McIver B, Hay ID*, et al.* Frequent loss of heterozygosity on chromosomes 3p and 17p without VHL or p53 mutations suggests involvement of unidentified tumor suppressor genes in follicular thyroid carcinoma. The Journal of clinical endocrinology and metabolism 1997;82(11):3684-91.
140. Hu S, Liu D, Tufano RP*, et al.* Association of aberrant methylation of tumor suppressor genes with tumor aggressiveness and BRAF mutation in papillary thyroid cancer. International journal of cancer 2006;119(10):2322-9.
141. Hoque MO, Begum S, Topaloglu O*, et al.* Quantitative detection of promoter hypermethylation of multiple genes in the tumor, urine, and serum DNA of patients with renal cancer. Cancer research 2004;64(15):5511-7.
142. Joseph B, Ji M, Liu D, Hou P, Xing M. Lack of Mutations in the Thyroid Hormone Receptor

{alpha} and {beta} Genes, but Frequent Hypermethylation of the Thyroid Hormone Receptor {beta} Gene in Differentiated Thyroid Tumors. The Journal of clinical endocrinology and metabolism 2007.
143. Kondo T, Zheng L, Liu W, Kurebayashi J, Asa SL, Ezzat S. Epigenetically controlled fibroblast growth factor receptor 2 signaling imposes on the RAS/BRAF/mitogen-activated protein kinase pathway to modulate thyroid cancer progression. Cancer research 2007;67(11):5461-70.
144. Galusca B, Dumollard JM, Lassandre S*, et al.* Global DNA methylation evaluation: potential complementary marker in differential diagnosis of thyroid neoplasia. Virchows Arch 2005;447(1):18-23.
145. Aldred MA, Huang Y, Liyanarachchi S*, et al.* Papillary and follicular thyroid carcinomas show distinctly different microarray expression profiles and can be distinguished by a minimum of five genes. J Clin Oncol 2004;22(17):3531-9.
146. Huang Y, Prasad M, Lemon WJ*, et al.* Gene expression in papillary thyroid carcinoma reveals highly consistent profiles. Proceedings of the National Academy of Sciences of the United States of America 2001;98(26):15044-9.
147. Lubitz CC, Ugras SK, Kazam JJ*, et al.* Microarray analysis of thyroid nodule fine-needle aspirates accurately classifies benign and malignant lesions. J Mol Diagn 2006;8(4):490-8; quiz 528.
148. Puskas LG, Juhasz F, Zarva A, Hackler L, Jr., Farid NR. Gene profiling identifies genes specific for well-differentiated epithelial thyroid tumors. Cellular and molecular biology (Noisy-le-Grand, France) 2005;51(2):177-86.
149. Jarzab B, Wiench M, Fujarewicz K*, et al.* Gene expression profile of papillary thyroid cancer: sources of variability and diagnostic implications. Cancer research 2005;65(4):1587-97.
150. Finley DJ, Arora N, Zhu B, Gallagher L, Fahey TJ, 3rd. Molecular profiling distinguishes papillary carcinoma from benign thyroid nodules. The Journal of clinical endocrinology and metabolism 2004;89(7):3214-23.
151. Onda M, Emi M, Yoshida A*, et al.* Comprehensive gene expression profiling of anaplastic thyroid cancers with cDNA microarray of 25 344 genes. Endocr Relat Cancer 2004;11(4):843-54.
152. Montero-Conde C, Martin-Campos JM, Lerma E*, et al.* Molecular profiling related to poor prognosis in thyroid carcinoma. Combining gene expression data and biological information. Oncogene 2007.
153. Salvatore G, Nappi TC, Salerno P*, et al.* A cell proliferation and chromosomal instability signature in anaplastic thyroid carcinoma. Cancer research 2007;67(21):10148-58.
154. Saggiorato E, De Pompa R, Volante M*, et al.* Characterization of thyroid 'follicular neoplasms' in fine-needle aspiration cytological specimens using a panel of immunohistochemical markers: a proposal for clinical application. Endocr Relat Cancer 2005;12(2):305-17.
155. Cheung CC, Ezzat S, Freeman JL, Rosen IB, Asa SL. Immunohistochemical diagnosis of papillary thyroid carcinoma. Mod Pathol 2001;14(4):338-42.
156. Fryknas M, Wickenberg-Bolin U, Goransson H*, et al.* Molecular markers for discrimination of benign and malignant follicular thyroid tumors. Tumour Biol 2006;27(4):211-20.
157. Wallin G, Backdahl M, Tallroth-Ekman E, Lundell G, Auer G, Lowhagen T. Co-existent anaplastic and well differentiated thyroid carcinomas: a nuclear DNA study. Eur J Surg Oncol 1989;15(1):43-8.
158. Takano T. Fetal cell carcinogenesis of the thyroid: theory and practice. Seminars in cancer biology 2007;17(3):233-40.
159. Wallin G, Lundell G, Tennvall J. Anaplastic giant cell thyroid carcinoma. Scand J Surg 2004;93(4):272-7.
160. Salman M, Jhanwar SC, Ostrer H. Will the new cytogenetics replace the old cytogenetics? Clinical genetics 2004;66(4):265-75.
161. Albertson DG, Pinkel D. Genomic microarrays in human genetic disease and cancer. Human molecular genetics 2003;12 Spec No 2:R145-52.
162. Schrock E, du Manoir S, Veldman T*, et al.* Multicolor spectral karyotyping of human chromosomes. Science (New York, NY 1996;273(5274):494-7.
163. Kallioniemi A, Kallioniemi OP, Sudar D*, et al.* Comparative genomic hybridization for

molecular cytogenetic analysis of solid tumors. Science (New York, NY 1992;258(5083):818-21.
164. Thiagalingam S, Foy RL, Cheng KH, Lee HJ, Thiagalingam A, Ponte JF. Loss of heterozygosity as a predictor to map tumor suppressor genes in cancer: molecular basis of its occurrence. Current opinion in oncology 2002;14(1):65-72.
165. Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R. Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 1992;12(2):241-53.
166. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic acids research 2002;30(12):e57.
167. Ronaghi M, Uhlen M, Nyren P. A sequencing method based on real-time pyrophosphate. Science (New York, NY 1998;281(5375):363, 5.
168. Karimi M, Johansson S, Stach D*, et al.* LUMA (LUminometric Methylation Assay)--a high throughput method to the analysis of genomic DNA methylation. Experimental cell research 2006;312(11):1989-95.
169. Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer communications 1991;3(7):207-12.
170. Santoro M, Carlomagno F, Romano A*, et al.* Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science (New York, NY 1995;267(5196):381-3.
171. Fritz A, Walch A, Piotrowska K*, et al.* Recessive transmission of a multiple endocrine neoplasia syndrome in the rat. Cancer research 2002;62(11):3048-51.
172. Heldin NE, Cvejic D, Smeds S, Westermark B. Coexpression of functionally active receptors for thyrotropin and platelet-derived growth factor in human thyroid carcinoma cells. Endocrinology 1991;129(4):2187-93.
173. Dahlman T, Lammerts E, Wik M*, et al.* Fibrosis in undifferentiated (anaplastic) thyroid carcinomas: evidence for a dual action of tumour cells in collagen type I synthesis. The Journal of pathology 2000;191(4):376-86.
174. Ain KB, Taylor KD, Tofiq S, Venkataraman G. Somatostatin receptor subtype expression in human thyroid and thyroid carcinoma cell lines. The Journal of clinical endocrinology and metabolism 1997;82(6):1857-62.
175. Fabien N, Fusco A, Santoro M, Barbier Y, Dubois PM, Paulin C. Description of a human papillary thyroid carcinoma cell line. Morphologic study and expression of tumoral markers. Cancer 1994;73(8):2206-12.
176. Belge G, Roque L, Soares J*, et al.* Cytogenetic investigations of 340 thyroid hyperplasias and adenomas revealing correlations between cytogenetic findings and histology. Cancer genetics and cytogenetics 1998;101(1):42-8.
177. Lui WO, Kytola S, Anfalk L, Larsson C, Farnebo LO. Balanced translocation (3;7)(p25;q34): another mechanism of tumorigenesis in follicular thyroid carcinoma? Cancer genetics and cytogenetics 2000;119(2):109-12.
178. Rodrigues-Serpa A, Catarino A, Soares J. Loss of heterozygosity in follicular and papillary thyroid carcinomas. Cancer genetics and cytogenetics 2003;141(1):26-31.
179. Bol S, Belge G, Thode B, Bonk U, Bartnitzke S, Bullerdiek J. Cytogenetic tetraclonality in a rare spindle cell variant of an anaplastic carcinoma of the thyroid. Cancer genetics and cytogenetics 2001;125(2):163-6.
180. Pallante P, Berlingieri MT, Troncone G*, et al.* UbcH10 overexpression may represent a marker of anaplastic thyroid carcinomas. British journal of cancer 2005;93(4):464-71.
181. Arnold A, Papanikolaou A. Cyclin D1 in breast cancer pathogenesis. J Clin Oncol 2005;23(18):4215-24.
182. Khoo ML, Ezzat S, Freeman JL, Asa SL. Cyclin D1 protein expression predicts metastatic behavior in thyroid papillary microcarcinomas but is not associated with gene amplification. The Journal of clinical endocrinology and metabolism 2002;87(4):1810-3.
183. Ewen ME, Lamb J. The activities of cyclin D1 that drive tumorigenesis. Trends in molecular medicine 2004;10(4):158-62.
184. Ball E, Bond J, Franc B, Demicco C, Wynford-Thomas D. An immunohistochemical study of p16(INK4a) expression in multistep thyroid tumourigenesis. Eur J Cancer 2007;43(1):194-201.

185. Ferru A, Fromont G, Gibelin H*, et al.* The status of CDKN2A alpha (p16INK4A) and beta (p14ARF) transcripts in thyroid tumour progression. British journal of cancer 2006;95(12):1670-7.