CLINICAL AND MOLECULAR STUDIES OF PAPILLARY THYROID CARCINOMA

- WITH AN EMPHASIS ON PROGNOSTIC FACTORS

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- with an Emphasis on Prognostic Factors

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Fast vi förefaller sörja över livets korthet, önskar vi dock få ett slut på varje enskild period av det. Den omyndige önskar bli vuxen, därpå att bli affärsman, samla en förmögenhet, uppnå hedersbevisningar och slutligen dra sig tillbaka från affärerna. Om således var och en medger att livet som helhet är kort, förefaller dess enskilda avsnitt långa och tråkiga.

Addison

Abstract

Thyroid cancer accounts for approximately 1% of all cancers reported. In Sweden 4-5/100, 000 inhabitants annually present with thyroid carcinoma. There are four different variants of thyroid carcinoma: papillary thyroid carcinoma (PTC), follicular thyroid carcinoma, medullary thyroid carcinoma and anaplastic thyroid carcinoma. PTC comprises 60-80% of thyroid cancers. The PTC prognosis is usually excellent with a long-term survival rate exceeding 90%. However, some patients develop distant metastases or die from the disease, and these are classified as having aggressive PTC. With the aim to predict the prognosis at the time for surgery, several prognostic factors have been established. Still, the natural course of the disease cannot always be predicted from the initial clinical presentation. Hence, there is a need for additional markers for the identification of patients who are at risk of developing aggressive PTC.

To evaluate associations between clinical and histopathological features and the patients´ outcome, 220 patients who underwent surgery for PTC at the Karolinska University Hospital, Solna between 1980 -1999 were reviewed retrospectively. Of these, 19 patients (9%) developed aggressive PTC. Due to invasive tumor growth radical surgery could not be performed in 58% of these patients as compared to in 2% of the patients with non-aggressive PTC. Non-radical surgery proved the most powerful predictor for development of aggressive PTC. The reported excellent prognosis for patients < 45 years of age could not be confirmed; three of the patients who eventually died from PTC were below the age of 45 (Paper I).

The antibody MIB-1 detects the Ki-67 antigen in proliferating cells. To investigate if assessment of the percentage of MIB-1 positive cells (MIB-1 index) can add prognostic information in PTC, MIB-1 immunoreactivity was analyzed in 30 PTCs. Thirteen patients were classified as having aggressive PTC, and tumors from these patients had a significantly higher MIB-1 index (median 5.4%) as compared to those from patients with non-aggressive PTC (median 1.1%). MIB-1 index ≥ 1.85% was found to be an independently significant risk factor for aggressive PTC (Paper II).

The *RET* proto-oncogene encodes a receptor tyrosine kinase normally not expressed in thyroid follicular cells. *RET* can be activated through rearrangements, generating the fusion oncogene *RET/PTC*. Today several variants have been described, but their association to clinical outcome is debated. To search for expression of *RET* or the oncogenes *RET/PTC*1-4, 61 PTCs were analyzed using RT-PCR. *RET*-TK expression was detected in 48%. This proved to be due to a *RET/PTC* rearrangement in three cases only, and expression of wild-type *RET* in 12 cases. The remaining 14 tumors expressed *RET*-TK only, indicating presence of yet unidentified rearrangements. Expression of wild-type *RET* was detected significantly more often in aggressive PTCs and in poorly differentiated PTCs (Paper III).

In an attempt to identify chromosomal regions harboring potential oncogenes and tumor suppressor genes involved in PTC initiation and progression, 25 PTCs were screened for chromosomal imbalances using comparative genomic hybridization (CGH). Gain of 9q was the most common change, detected in close to 30% of the tumors. The total number of alterations was higher in tumors from patients with aggressive PTC. Gain of 1q and loss of 9q were exclusively seen in tumors from patients with aggressive disease, suggesting the location of genes important for PTC progression in these regions (Paper IV).

In summary, complete removal of all tumor cells appears to be most important and postoperative I^{131} treatment probably cannot compensate for an incomplete tumor resection. MIB-1 index is a valuable prognostic marker, which can add prognostic information to established prognostic parameters. *RET/PTC*1-4 oncogenes are rare in Swedish tumors, suggesting other mechanisms involved in PTC development. Still, expression of *RET*-TK may be of prognostic importance, and may point towards new treatment modalities. Although PTC is generally a genetically stable tumor, aggressive PTC tumors exhibit signs of chromosomal instability. At least three chromosomal regions have been defined as potential locations for tumor suppressor genes or oncogenes involved in PTC. Gain of 9q may be an early event, while loss of 1q and 9q may be involved in progression toward aggressive variants of PTC.

Key words: Papillary thyroid carcinoma, MIB-1, *RET*-proto-oncogene, *RET/PTC*, CGH, prognosis

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Contents

1. Abbreviations

AGES	age, grade, extent and size of the tumor
AMES	age, metastases, extent and size of the tumor
ATC	anaplastic thyroid carcinoma
CDK	cyclin dependent kinase
cDNA	complementary DNA
CGH	comparative genomic hybridization
CIN	chromosomal instability
DNA	deoxyribonucleic acid
DTC	differentiated non-medullary thyroid carcinoma
ECM	extracellular matrix
FISH	fluorescent *in situ* hybridization
FTA	follicular thyroid adenoma
FTC	follicular thyroid carcinoma
FGF	fibroblast growth factor
IHC	immunohistochemistry
MACIS	metastases, age, completeness of resection, invasion and size of the tumor
MAPK	the mitogen-activated protein kinase pathway
Mb	megabase
MEN2A	multiple endocrine neoplasia type 2A
MEN2B	multiple endocrine neoplasia type 2B
MMP	matrix metalloproteinase
MIN	microsatellite instability
MMR	mismatch repair system
MTC	medullary thyroid carcinoma
NER	nucleotide excision repair system
p53	the protein encoded by the *TP53* gene
PCR	polymerase chain recation
PTC	papillary thyroid carcinoma
pTNM	tumor, node, metastases classification system
RB1	the retinoblastoma gene
RET	the *RET* proto-oncogene
***RET*-TK**	the tyrosine kinase domain of *RET*
RET/PTC	rearranged form of *RET*
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
T3	triiodothyronine
T4	thyroxine
Tg	thyroglobulin
TSH	thyroid stimulating hormone
TSG	tumor suppressor gene
VEGF	vascular endothelial growth factor
WT-*RET*	wild-type (not mutated) *RET*

2. List of papers included in this thesis

Kjellman P, Zedenius J, Lundell G, Bäckdahl M, Farnebo L-O, Hamberger B, Larsson C and Wallin G. Which factors best predict the outcome in patients with papillary thyroid carcinoma? – A comparison of three different prognostic scoring systems.
Submitted

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Thyroid, 13: 371-380, 2003

Kjellman P, Learoyd DL, Messina M, Weber G, Höög A, Wallin G, Larsson C, Robinson BG and Zedenius J. Expression of the *RET* proto-oncogene in papillary thyroid carcinoma and its correlation with clinical outcome.
British Journal of Surgery, 88: 557-563, 2001

Kjellman P, Lagercrantz S, Höög A, Wallin G, Larsson C and Zedenius J. Gain of 1q and loss of 9q21.3-q32 are associated with a less favorable prognosis in papillary thyroid carcinoma.
Genes Chromosomes and Cancer, 32: 43-49, 2001

3. Introduction

Thyroid carcinoma accounts for approximately 1% of all cancers reported worldwide (Parkin 1997). In Sweden 4-5/100,000 inhabitants annually present with clinically detectable thyroid carcinomas (The National Swedish Board of Health and Welfare, Swedish Cancer Registry).

In spite of thyroid carcinoma being a comparatively uncommon type of tumor, increasing attention has over the past two decades been directed towards this malignancy. After the Chernobyl nuclear accident in 1986 the incidence of thyroid cancer increased dramatically among children who lived in the regions most contaminated with radioactive isotopes at the time of the disaster. This attracted the attention of medical experts from all over the world, resulting in increased awareness of the disease. In addition, as the thyroid covers a broad spectrum of malignancies, ranging from well differentiated carcinomas carrying a very good prognosis to undifferentiated tumors with close to 100% mortality, thyroid tumors provide an ideal model for studying tumorigenesis in epithelial tissue. According to the National Cancer Institute, the rate of increase in the incidence of thyroid cancer among women in the United States is today more rapid than for all other types of tumors.

In patients with differentiated thyroid carcinoma the outcome is usually excellent with a ten-year-survival exceeding 90% (Schlumberger 1998, Lundgren 2003). However, some patients will develop recurrences and even die from the disease. Not surprisingly, hundreds of publications focusing on clinical and pathological data, and more recently molecular and genetic investigations, have been devoted to the identification of prognostic markers. Several of the studies have reached conflicting results and these variations are generally attributed to differences in patient selection and treatment modalities as well as different definitions of the prognostic factors considered. In addition, the disease may show considerable variation due to geographical and demographical differences.

The present thesis is focused on papillary thyroid carcinoma (PTC), which comprises 60-80% of all thyroid carcinomas (Shlumberger 1998, Lundgren 2003). It begins with an overview of general mechanisms for tumor development. Clinical and genetic aspects of thyroid pathology, with special emphasis on PTC, are described as a background for the experimental studies, and the principles of the methods used in this thesis are explained. The separate

studies focuses on genetic aberrations involved in PTC development and the evaluation of clinical, biological and genetic factors as prognostic markers for PTC.

3.1 A genetic basis for cancer

Cancer arises as a result of deregulated cell growth. Basically, a stepwise accumulation of mutations in genes regulating cell proliferation is one proposed mechanism behind the increased proliferation rate, which is characteristic of cancer. The genetic alterations in a particular cell are passed on to the next generation of cells, and in this sense cancer is considered a genetic disease. The majority of alterations are somatically acquired, however, some are also present in the germline, giving rise to familial disease.

A heritable basis has been hypothesized for most cancer types for more than a century. In 1866, Broca described a family with many members affected by breast and liver cancer. He suggested that some heritable aberration present within the affected tissue allowed the tumors to form. This and other similar studies (Haaland 1911, Warthin 1913) supported the idea that a genetic basis for cancer might account for the familial clustering. Still, other explanations such as clustered environmental exposure were possible, and it was for long argued that all cancer occur in sporadic or isolated cases. Today we know that at least 5-10% of all cancers are caused by hereditary genetic defects.

The hypothesis that cancer can arise from somatic alterations in the genetic material was first proposed by Boveri in 1914. He had previously noted abnormal mitotic divisions and atypical cell masses in sea urchin eggs fertilized by two sperms, and those abnormal masses appeared very similar to tumors. Unfortunately, these studies did not gain favor at that time due to lack of experimental support and uncertainty whether the chromosomal changes had caused tumors or resulted from the malignant transformation. However, during the last 30 years significant experimental support for this hypothesis has been obtained from different fields including virology, epidemiology, molecular biology and genetics. Today we know that somatic or germline mutations in three classes of genes, i.e. oncogenes, tumor suppressor genes and DNA damage recognition and repair genes, play a primary role in tumorigenesis.

3.1.1 Cell division

Any existing cell is derived from a cell division from another cell. The existence of almost 5 billion human beings today is the result of an unbroken chain of cell divisions, leading back to the very first cell that may have arisen 3.5 billion years ago. Thus, cell division is crucial for the development and maintenance of life. Since a well balanced cellular turn-over is essential for the proper function of all living organisms, the cell cycle has evolved to a tightly regulated process.

Various types of tissues are classified according to their mitotic potential and are referred to as being either renewing or permanent. Basically, in renewing tissues such as epidermis, blood cells and gut mucosal cells, the continuous cell loss is balanced by new cells, which are provided by a germinative zone composed of relatively undifferentiated cells (stem cells) in a constant state of proliferation. Some of these cells differentiate, which leads to the gain of more specialized functions but precludes further mitosis. Renewal can also take place through simple duplication, which is often recognized in various glands, the liver and the kidneys. In this case the cells multiply during maturation but become mitotically stable in the adult. Although these differentiated cells never lose their mitotic potential, they only proliferate in response to certain stimuli. Permanent tissues, such as neurons and striated muscle consist of differentiated cells, which are incapable of division beyond early stages of development and live as long as the organism as a whole survives.

Thus, cellular life span is highly variable between different types of cells. At any given time the absolute majority of the cells are not dividing, but exists in a resting and metabolically active state called G_0. In response to internal or external stimuli a cell can leave the resting state and enter the cell cycle, during which the DNA is duplicated followed by the division of the cell. This process is supervised by checkpoint controls, which act to ensure that identical chromosome copies are transferred to the two daughter cells.

3.1.2 The cell cycle

The cell cycle comprises a short stage of cell division, the mitosis phase (M), and a long interphase including the DNA synthesis phase (S) and the gaps before and after the S phase, which are termed G1 (gap 1) and G2 (gap 2), respectively (Fig 1). After mitosis the cells enter

the G1 phase, in which they respond to extracellular signals that ultimately determine whether they will progress towards a new division or exit the cell cycle into the resting G_0 state. Alternatively, cells can permanently exit the cell cycle to enter postmitotic states associated with differentiation and cell senescence. Once the cells make the decision to begin DNA replication, they are irreversibly committed to completion of the cell cycle and dividing. The time in late G1 phase at which this decision is made is designated the "restriction point" and when cells have left the G_0 resting state upon growth factor stimulation, they generally need continuous mitogenic stimulation to be driven to the restriction point. On the contrary, antiproliferative compounds can only arrest proliferation of cells proceeding through the G1 phase, but which have not yet reached the restriction point (Sherr 2000).

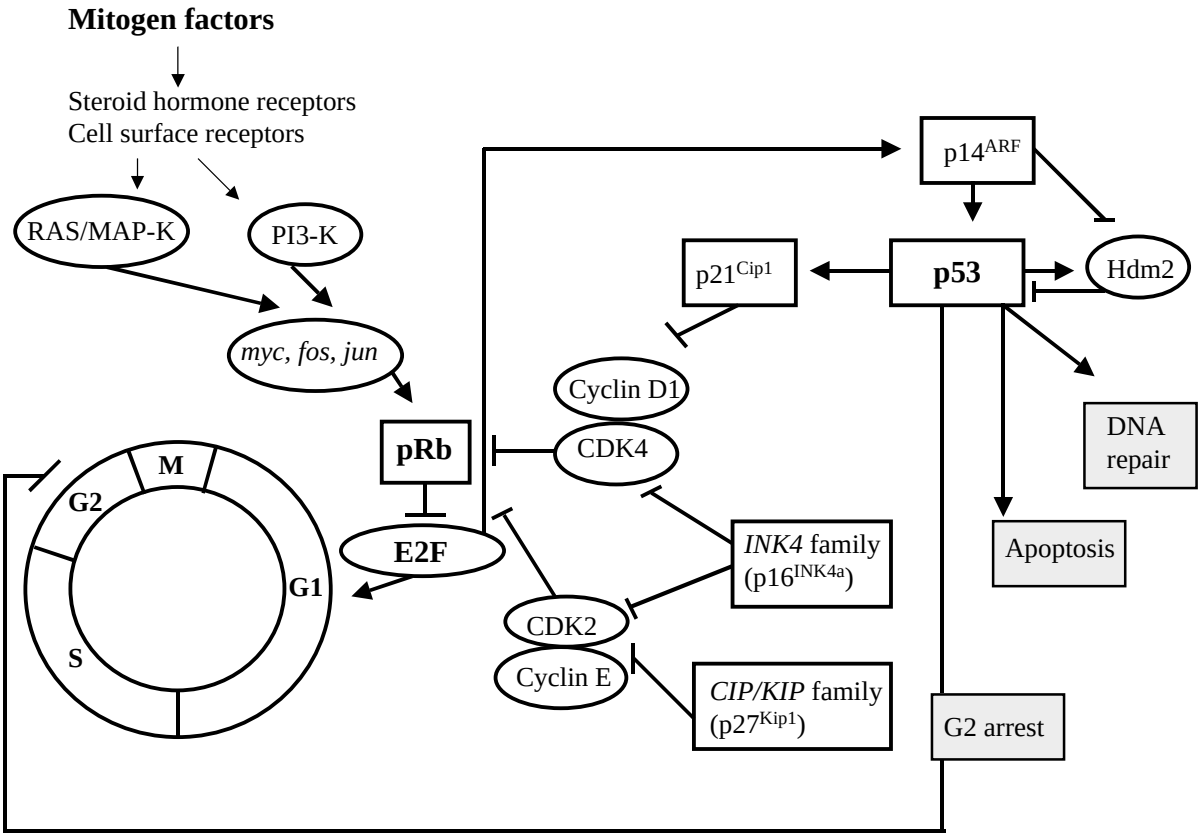

Figure 1. Schematic illustration of gene products involved in cell cycle regulation. Growth-promoting proteins are marked by circles and growth-inhibiting proteins by squares.

3.1.3 Restriction point regulation – the RB pathway

Members of the transcription factor family E2F regulate genes governing DNA replication, thus E2F plays a crucial role in restriction point control and is required for G1/S transition. In non-dividing cells E2F activity is repressed by the binding of pRB, the *RB1* gene product, which has been activated by dephosphorylation. In response to mitogenic stimulation, genes encoding cyclin-dependent kinases (CDKs) and D-type cyclins are transcribed and the gene products assemble into enzyme complexes. The cyclin D1-CDK4 complex appears to be of greatest significance in the G1 phase as it acts together with cyclin E-CDK2 to phosphorylate pRb, canceling its growth inhibitory function and facilitating S phase entry (Fig 1). The phosphorylating activity of CDKs is inhibited by proteins from the INK4 family ($p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$) and the CIP/KIP family of protein inhibitors ($p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$). Disruption of the RB pathway through alterations of $p16^{INK4a}$, cyclin D1, CDK4 or pRb is detected in almost all cancer types analyzed. For example, cyclin D1 is overexpressed in >50% of breast carcinomas and $p16^{INK4a}$ is lost in 80% of pancreatic carcinomas (Sherr 2000, 2002).

3.1.4 G1 check point and apoptosis – the p53 pathway

The *INK4a* locus encodes a second gene product, $p14^{ARF}$, from an alternative reading frame, the coding sequence of which is designated *ARF*. The *ARF* gene is induced by abnormal mitogenic signals, for example from overexpression of oncoproteins such as Myc, Ras or -catenin. The $p14^{ARF}$ protein activates p53, which in turn induces the CDK inhibitor $p21^{Cip1}$, resulting in cell cycle arrest. In this way the RB and p53 pathways are interconnected (Fig 1). Another linkage between the p53 and RB pathways is the ability of deregulated E2F to induce *ARF* transcription (Sherr 2002).

Thus, p53 plays a critical role as “the guardian of the genome”. In addition to the p53-mediated growth arrest in G1, other “cellular programs” such as DNA repair, G2 arrest or apoptosis (programmed cell death) may be triggered through an increase of p53 levels in response to various cell stress stimuli, for example DNA damage, hypoxia, overexpression of oncogenic proteins or the absence of pRb (Levine 1997, 2001).

Similar to the RB pathway, disruption of the $p14^{ARF}$-Hdm2-p53 pathway appears to be part of the life history of almost all cancer cells, regardless of tumor type. *TP53* is the single most commonly mutated gene, altered in >50% of all human cancers. Deletions of *ARF* or Hdm2 overexpression occur in high frequencies in the remaining cases (Sherr 2000).

3.1.5 Proto-oncogenes and oncogenes

Oncogenes are illegitimately activated proto-oncogenes. Initially, oncogenes were described as genes, carried by viruses, which could cause neoplastic transformation of target cells. This happens because the viral oncogenes (*v-onc*) have cellular counterparts, the proto-oncogenes (*c-onc*), which they are highly similar to and from which they derive.

Proto-oncogenes cover a broad spectrum of genes, having in common that they encode proteins which promote cell proliferation and differentiation. Some of these proteins function as growth factors or cell surface receptors. Others are components of the intracellular signal transduction pathways or the network of cyclins and CDKs that positively regulate progression through the cell cycle. Another group acts as transcription factors.

Transformation of proto-oncogenes into oncogenes (generally termed oncogene activation) involves a quantitative or qualitative gain of function. The activation can result from various genetic mechanisms including point mutations or amplifications of the proto-oncogene, or through translocations resulting in fusion genes and chimeric proteins. Activated oncogenes usually act dominantly, meaning that only one allele of the gene needs to be activated. As a consequence, mutations leading to oncogene activation were expected to be somatic events, while constitutional mutations would be lethal. However, constitutionally occurring activating mutations have been linked to different hereditary tumor forms.

The *RAS* and *RAF* genes, encoding proteins involved in intracellular signaling, are proto-oncogenes frequently mutated in human malignancies (Bos 1989, Davies 2002). Another example is the *MYC* family of nuclear proto-oncogenes, which have been found overexpressed in many types of human carcinomas (Nesbit 1999). Amplification of *MYC* is often detected in breast carcinomas and appears to have a negative influence on the prognosis (Liao 2000). Germline activating mutations in the *RET* proto-oncogene, associated with

multiple endocrine neoplasia types 2A and 2B and familial medullary thyroid carcinoma, is one rare example of a disease triggered by dominant inheritance of an activated proto-oncogene (Eng 1999). Another example is constitutional *MET* mutations, predisposing to familial papillary renal carcinoma.

3.1.6 Tumor suppressor genes

The observation that the tumor phenotype could be suppressed by fusing malignant cells with either normal cells or certain chromosomes, led to the hypothesis that loss of genetic material is a critical event in tumorigenesis in addition to oncogene activation (Harris 1988). Subsequently, tumor suppressor genes (TSG) were identified and shown to contribute to cancer when both alleles are inactivated. Many identified TSGs encode proteins which function as negative regulators of the cell cycle, therefore termed “gatekeepers”, for example the *RB* and *TP53* genes. Others encode proteins that promote apoptosis or are involved in differentiation, cell signaling or DNA repair. Since a defect in one allele may be compensated by the remaining normal allele, both copies of a TSG need to be inactivated to promote tumorigenesis, thus TSG mutations are recessive on the cellular level. The requirement of two mutations to turn a normal cell into a cancer cell was proposed by Knudson in 1971, based on epidemiological studies of retinoblastoma, a rare eye tumor (Knudson 1971). This is referred to as the “two-hit theory”. In most cases tumorigenesis due to TSG inactivation involves two somatic mutations. However, in hereditary cancers the first mutation is present constitutionally in all cells in the body, thus only one somatic “hit” is needed for tumor initiation.

Different types of genetic alterations can result in TSG inactivation. In hereditary forms the first “hit” is usually a discrete change of the coding sequence, such as base substitution, deletion or insertion of a few nucleotides. The second hit commonly involves loss or deletion of the wild-type non-mutated allele. Alternatively, biallelic inactivation of a TSG results from mitotic recombination with segregation of both alleles into one cell, or hypermethylation of CpG dinucleotides within promoter regions resulting in gene silencing (Robertson 1999).

3.1.7 DNA repair genes and subtle sequence instability

During an average human lifetime an estimated 10^{17} cell divisions will take place, and in each of these 3×10^9 nucleotides need to be correctly duplicated and incorporated. Most mutations arise due to copying errors but the DNA is also subjected to the damaging effect of numerous chemical and physical agents, giving rise to mutations which may compromise DNA function. To protect the genome, an intricate network of DNA repair systems has developed early in the evolution. Genes encoding such proteins constitute a subclass of TSGs termed caretaking genes, and loss of function mutations in those genes are a severe threat to the integrity of the genome.

There are two major repair systems: nucleotide excision repair (NER) and mismatch repair (MMR). NER is mainly responsible for repairing single- or double strand breaks or crosslinks caused by exogenous mutagens, for example UV-light, and the system is defective in patients with the autosomal recessive disease xeroderma pigmentosum. The main role of MMR is to immediately after replication check the genome for mismatched base pairs, and subsequently replace the incorrect nucleotides. In cells with defect MMR the mutation rate is approximately 100-1000 times higher than normally, and usually those cells also show characteristic microsatellite instability (MIN). Microsatellites are repetitive genetic elements dispersed throughout the genome, in which the repeating units usually consist of 1-4 bases. The number of repeats can be highly varying between the alleles, giving rise to alleles of different lengths, so called polymorphisms. Within an individual the allele sizes at a given locus should be kept constant between normal cells. Because of the repetitive nature of microsatellites they are particularly prone to DNA polymerase slippage, which is efficiently repaired provided the MMR system is intact. However, defects in MMR result in increasing length and number of microsatellites, termed MIN. Most patients with hereditary non-polyposis colorectal cancer (HNPCC) exhibit MIN as a result of constitutional mutations in the *hMSH2* or *hMLH1* genes, encoding proteins involved in MMR (Lengauer 1998).

3.1.8 Chromosomal instability – mutations in mitotic “checkpoint” genes

Subtle sequence instabilities are comparatively rare events. Conversely, chromosomal instability (CIN) is observed in the majority of human cancers, and involves gains or losses of entire chromosomes or other gross chromosomal abnormalities, such as deletions, inversions,

translocations or amplifications. It has long been debated whether these genetic changes contribute to tumor formation and progression, or is a consequence of a general genetic instability in the tumor cells. Several genes involved in chromosome segregation during mitosis have been found mutated in CIN cancers, including genes important for the spindle-checkpoint (prevents S phase entry in case of spindle damage) and the DNA damage checkpoint (prevents M phase entry in case of DNA damage). This leads to incorrect chromosome segregation causing chromosome number abnormalities with an imbalance in gene dosage, which is likely to induce cancer. Several genes are involved in mitotic checkpoint control, of which the best characterized are *ATM, ATR, BRCA1* and *BRCA2*. Loss of *TP53* was earlier suggested to be responsible for CIN, but *TP53* mutations are presently regarded as late events as gross chromosomal abnormalities can be observed in small colon and breast tumors in combination with wild type *TP53*. Thus, *TP53* mutations are unlikely to be the primary cause of CIN but may have an exacerbating effect (Lengauer 1998, Sen 2000).

3.1.9 Telomere erosion and telomerase activity

Human chromosomes have nucleoprotein structures at each end, consisting of stretches of repetitive TTAGGG sequences and specific proteins, designated telomeres. Their primary functions are to protect the ends of the chromosomes from degradation and to mark the ends of chromosomes as distinct from broken DNA ends. In addition, telomeres are believed to function as determinants of cellular lifespan: telomeric length decline with each cell division and when the telomeres have reached a specific length, p53 and pRb dependent checkpoints are activated and the cells irreversibly cease to proliferate. This state is recognized as replicative senescence. If the checkpoint arrest is restrained, cells continue to proliferate although lacking the protective telomeric function, resulting in massive chromosomal instability, a stage termed cellular crisis.

In “immortal” cells, such as germ cells and tissue stem cells, the continuous telomere erosion is compensated for by the action of telomerase, a specialized reverse transcriptase, adding nucleotides to the telomeric ends. In the majority of the differentiated human cells telomerase is normally not expressed. However, tumor cells can reactivate telomerase or activate another mechanism to maintain stable telomere lengths, called alternative lengthening of telomeres. As a result, cells are provided with the capability to escape senescence or survive crisis, thus

they have been immortalized, a feature crucial for cancer development (Sherr 2000, Masutomi 2003).

3.1.10 Stepwise development of cancer

As stated before, cancer arises as a result of deregulated cell proliferation together with suppressed cell death. The mutations that drive cancer development are usually stochastic somatic mutations in multiple genes regulating cell growth, illustrated by the fact that in most cancers several key pathways involved in the control of cell proliferation are perturbed. One single mutation is generally not enough to convert a normal cell into a tumor cell; instead accumulation of a number of successive mutations is needed. The estimated mutation rate is about one in 2×10^7 per gene and cell division, which makes it unlikely that the mutations required for tumor formation will take place in one single cell.

Already in the 1950s, tumorigenesis was believed to be a multistep process, but it was first in the late 1980s that molecular mechanisms underlying initiation and progression of human tumors began to be elucidated. Basically, an initiating event provides the cell with a growth advantage, and the subsequent clonal expansion of the affected cell results in the propagation of the initial mutation, as well as an increased number of cells available for a second mutation. This process, which usually evolves during decades, results in a progressive accumulation of mutations, providing a platform for neoplastic formation. Alternatively, mutations occur in genes responsible for the integrity of the genome, increasing the overall mutation rate (Fig 2).

In 1990 Fearon and Vogelstein proposed a genetic model for the development of colorectal tumors, which illustrates a stepwise accumulation of particular mutations, along with a gradual progress from adenoma to carcinoma and metastases. This model serves as an excellent tool for understanding how human epithelial tumors develop, although the sequence of mutations is not invariant, and analogous schemes have been constructed for other types of human cancers (Fearon and Vogelstein 1990).

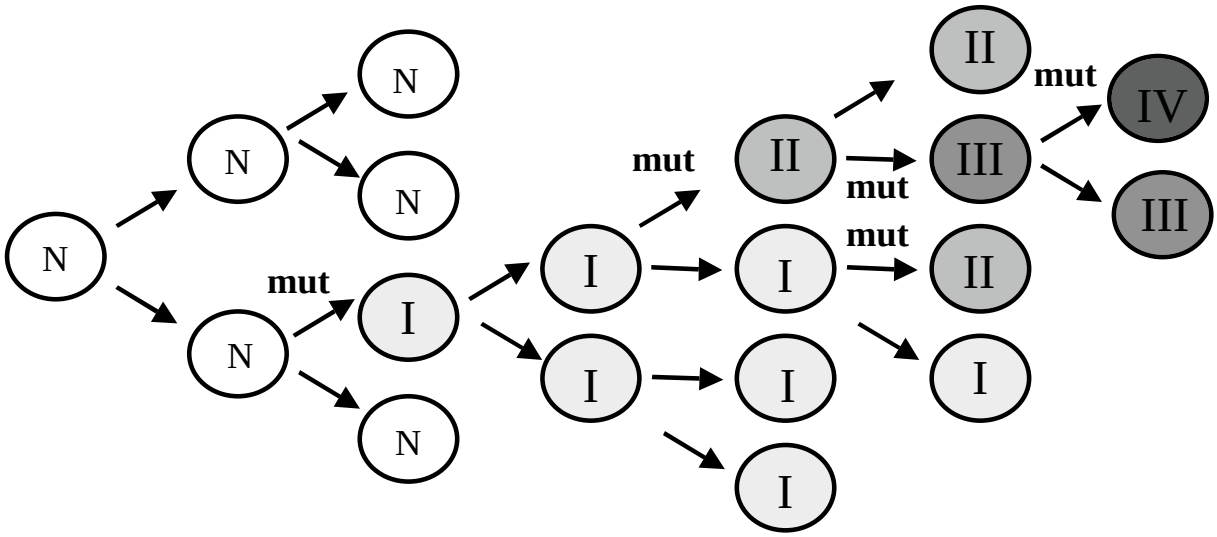

Figure 2. Multistep progression toward cancer. A normal cell (N) acquires one (I) mutation (mut), which provides the cell with a growth advantage and constitutes a platform for accumulation of additional mutations resulting in clones with increased proliferative capacity (I-IV).

3.1.11 Tumor progression and metastases

In order to restrict clonal cell expansion, which may lead to neoplastic formation, potentially oncogenic proliferative signals trigger anti-tumorigenic mechanisms such as apoptosis, senescence or differentiation. Cancer arises only when these mechanisms have failed. Somatic cells which have obtained the capacity to proliferate autonomously still face major difficulties to their continued expansion. All cells require oxygen for their survival and when tumors reach 1 cm^3 the oxygen supply is depleted. A prerequisite for further tumor growth is the recruitment of new blood vessels by angiogenesis. Briefly, tumor cells produce and secrete various angiogenic substances of which the most potent are the vascular endothelial growth factors (VEGF) and fibroblast growth factors (FGF), which leads to the formation of new vessels from already existing vessels by sprouting. It was initially suggested that hypoxia triggered the production of angiogenetic factors but the process is today considered more complex, involving interactions with the extra-cellular matrix (ECM) as well as with different cell types chemotactically recruited by the tumor (Hanahan and Folkmann 1996, Verheul 2004). Although most human solid tumors are angiogenesis dependent, this is not the only factor that determines tumor growth, which may be one explanation why the therapeutic effect of angiogenesis inhibitors on tumors has so far been modest.

Cell-cell interactions through adhesion molecules as well as interactions between cells and the ECM are important for determining cell polarity and maintaining normal tissue architecture. Normal ECM serves as a border that can delay or prevent tumor progression. On the other hand, abnormal ECM components may induce tumors in epithelial cells, or indirectly promote tumor progression as the altered ECM may release the growth suppression on pre-existing context-inhibited malignant cells. The most important system of cell adhesion molecules is the cadherins, which cross the cell membrane to associate with cadherins on adjacent cells. Inside the cell each cadherin molecule is attached to □-catenin, □-catenin, and actin filaments in the cytoskeleton. Impairment of cadherins causes loss of polarity, which induces cell proliferation and subsequent tumor formation (Fig 3). E-cadherin, the most prevalent type of cadherin in epithelial tissue, is lost in many tumor types and restoration of E-cadherin is able to suppress further cellular transformation (Bissell 2001). In addition E-cadherin sequesters □-catenin, which apart from its anchoring function also acts as a transcriptional coactivator stimulating cell-cycle entry (Behrens 1996). Loss of E-cadherin in tumor cells causes detachment of cells from the primary tumor mass. A cell membrane glycoprotein, dysadherin, was recently reported to down-regulate E-cadherin and promote metastases (Ino 2002). Loss of polarity can also occur through loss of genes required for proper localization of apical proteins (Bilder 2000). The attachment of cells to the extracellular matrix is largely mediated by integrins (Fig 3).

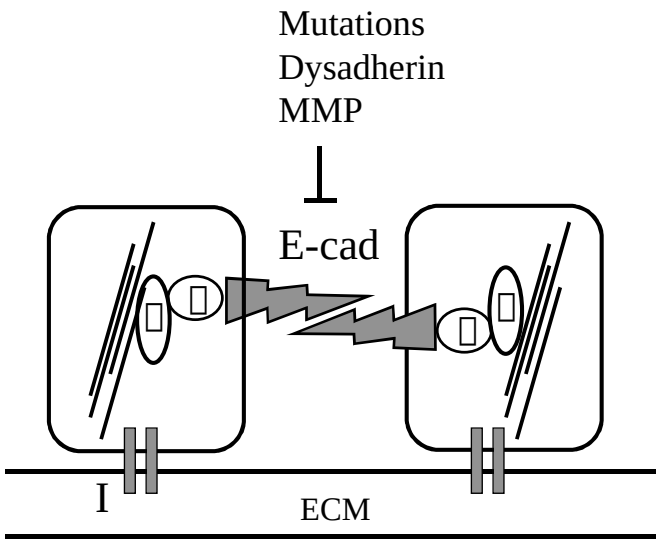

Figure 3. Illustration of adhesion molecules, linking adjacent cells to each other and to the extra cellular matrix (ECM). E-cadherin (E-cad) is linked to intracellular □ -catenin (□) and □-catenin (□). Integrins (I) anchors the cells to the ECM. Mutations, dysadherin and matrixmetalloproteinases (MMP) abrogate E-cadherin function, causing cellular detachment.

After the detachment of cells from the primary tumor mass, degradation of the ECM is required for cell migration and penetration into the vascular lumen, which are the first steps in the metastasizing process. Once in the circulation cells must survive, adhere to distal vascular endothelium and penetrate into a new host tissue microenvironment to finally establish a new tumor colony. A group of proteases, matrix metalloproteinases (MMPs), are involved in most steps in this process. MMPs can cleave E-cadherin causing cellular detachment (Fig 3). The cleavage fragments appear to promote cellular migration (Noe 2001). The proteolytic cleavage of various components of the ECM is carried out by MMPs secreted from the tumor cells as well as from stromal cells, which are induced by tumor cell infiltration. A large number of MMPs involved in different processes have been recognized. For example MMP-9 plays a central role in the intravasation process. Several of the mechanisms for tumor cell evasion of immune surveillance in the circulation are MMP-dependent, as many proteins required for immune cell survival and action are impaired by MMP cleavage. Extravasation may not require MMPs. Instead, it results from mechanical disruption of vascular endothelium following proliferation of adhering tumor cells. The establishment of new tumor colonies is a result of an intricate interplay between the tumor cells and the new host tissue stroma. MMPs are also believed to play a role in tumor initiation by activating growth factors, structurally alter ECM components and stimulate cell survival. In several human cancers MMP expression correlates with poor prognosis and MMP inhibitors have been shown to abrogate tumor development (Stamenkovic 2003). There is also strong support that MMPs participate in the promotion of angiogenesis (Heissig 2003).

3.2 The thyroid gland

3.2.1 Embryology and Anatomy

The thyroid gland was named so because of its close relationship to the shield-shaped thyroid cartilage (greek: *thyreos* = shield). The gland consists of two lobes connected with the isthmus (Fig 4). In a healthy adult the thyroid gland weights about 15-35 g, but can increase considerably in size and weight due to pathological conditions, for example goiter or tumors. During embryogenesis the thyroid follicular cells, originating from epithelial cells located in the base of the tongue, fuse with the neuroendocrine C-cells while descending to the final location in the neck. In many individuals thyroid remnants along this migration pathway constitute a third lobe, the pyramidal lobe.

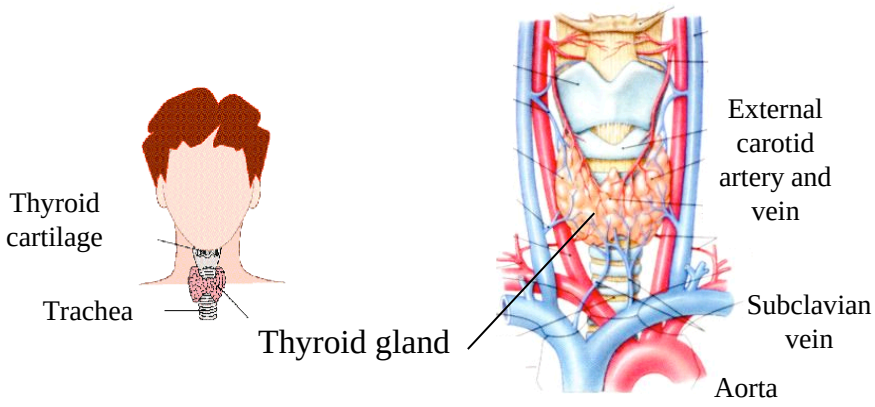

Figure 4. Anatomical localization of the thyroid gland.

The thyroid gland receives its blood supply mainly from the superior and inferior thyroid arteries, and the venous return empties into the brachiocephalic vein. Lymphatic vessels drain to para- and pretracheal lymph nodes as well as to lymph nodes in the supraclavicular fossa and along the internal jugular vein. The drainage is usually ipsilateral and each lobe can be regarded as a separate entity. The recurrent laryngeal nerves and the parathyroid glands are of great practical importance during surgery due to their close topografic relation to the thyroid gland.

3.2.2 Physiology

The thyroid gland consists of a large number of round structures, follicles, which are made up of follicular epithelial cells surrounding a central lumen filled with colloid. The thyroid also contains parafollicular cells, which are called C-cells because they produce calcitonin, a hormone involved in the calcium homeostasis. The synthesis of the thyroid hormones, triiodothyronine (T3) and thyroxine (T4), takes place in the follicular cells and requires enzymatic activity provided by thyroxine peroxidase (TPO). The hormones are secreted directly into the blood stream or stored in the colloid, bound to the thyroglobulin protein (Tg), until needed. Of the circulating T3 and T4, 80% is bound to thyroxine-binding globulin (TBG), 10% to prealbumin and 10% to albumin.

The activity of the thyroid gland is basically regulated by the secretion of thyroid stimulating hormone (TSH) from the pituitary gland, which in turn is under the influence of thyrotropin releasing hormone (TRH) from the hypothalamus (Fig 5). TSH stimulates growth of the follicular epithelium and the synthesis of thyroid hormones. The system is subjected to feed back control: increased T3 inhibits the release of TSH.

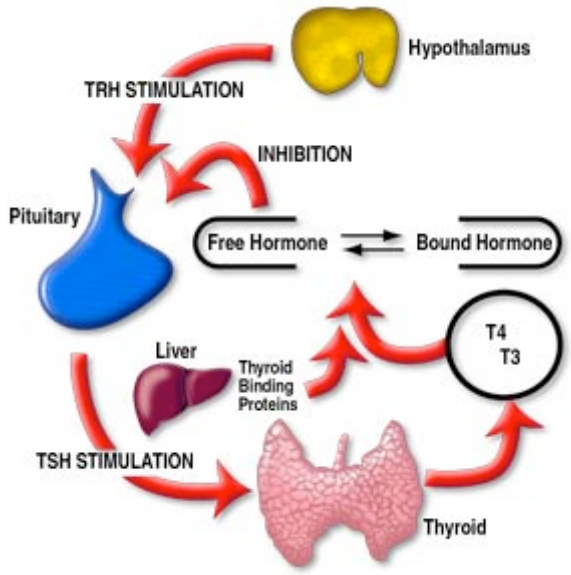

Figure 5. Regulation of thyroid hormone secretion. Increased amounts of T3 suppress TRH and TSH release in a feed-back system.

T3 is the biologically active hormone and T4 can be converted to T3, either in the thyroid gland or in periferal tissue cells. T3 increases the cellular metabolism by binding to the T3 receptors, which are members of the nuclear receptor family. Increased level of thyroid hormone (hyperthyroidism) results in increased oxygen demand and heat production, which in turn leads to a variety of symptoms such as heat intolerance, weight loss and tachycardia.

3.3 Human thyroid tumors – general aspects

3.3.1 Benign thyroid tumors

Thyroid nodules are common. In non-iodine deficient areas clinically detectable thyroid nodules occur in 4-7% of the general population, the absolute majority of which are benign (Mazzaferri 1993). Thyroid nodules do not reflect a single disease. Instead they represent clinical manifestations of a variety of thyroid disease ranging from non-neoplastic conditions, such as goiter or thyroiditis, to neoplastic nodules, either benign (adenomas) or malignant (carcinomas). Only the neoplastic lesions will be described here.

Follicular thyroid adenomas (FTA) are benign, encapsulated and commonly solitary tumors. A follicular lesion is defined as benign in the absence of vascular and capsular invasion, parameters that are not possible to evaluate on routine fine needle aspiration and cytology. Consequently, virtually all patients with follicular tumors are subjected to surgery and the resected tumor specimen is carefully examined for signs of malignancy. A variety of markers that can assist in the preoperative separation of FTA from its malignant counterpart have been evaluated but yet none has proved reliable. The question whether an adenoma can progress into a carcinoma or not is still debated.

3.3.2 Malignant thyroid tumors

Thyroid carcinoma accounts for 1% of all cancers reported worldwide, but the annual incidence varies in different parts of the world from 0.5-10 cases per 100 000 individuals. Differentiated thyroid carcinomas of follicular cell origin (DTC) are classified as follicular thyroid carcinoma (FTC) or papillary thyroid carcinoma (PTC). In Sweden FTC comprises approximately 35% of all DTC, and if age is adjusted for, FTC carries the same low mortality

as PTC (Lundgren 2003). The diagnosis of FTC is based on the presence of follicular differentiation, but the normal thyroid tissue architecture with cyst-like structures covered by single layers of follicular cells is disrupted. Vascular and capsular invasion is present, while histopathological features typical for PTC (see below) are missing (Hedinger 1988).

Medullary thyroid carcinoma (MTC) arises from the calcitonin producing C-cells and accounts for 5-10% of all thyroid carcinomas. Most MTC cases occur sporadically. In addition, 20-30% have a familial form of the disease. Hereditary MTC can present as a single entity called familial MTC (FMTC), or it can be a part of the multiple endocrine neoplasia syndrome 2A or 2B (MEN 2A, and MEN 2B). Hereditary forms are transmitted with an autosomal dominant pattern with a high penetrance (>90%). The 10-year survival rate in patients with clinically apparent MTC is approximately 65% (Wells Jr 2000).

Undifferentiated or anaplastic thyroid carcinoma (ATC) constitutes less than 10% of all thyroid cancers and is one of the most aggressive cancers encountered in man. Only few patients survive more than 12 months. The tumor is typically made up of grotesque giant cells that do not produce Tg or trap iodine. Most investigators are convinced that ATC represent a terminal stage in the dedifferentiation of a FTC or PTC, for example triggered by the loss of p53 function. Others believe it is a separate clinical and molecular entity possibly arising from an immature cell type. However, in many ATCs a well differentiated component is present supporting the progression theory (Giuffrida 2000).

3.4 Papillary thyroid carcinoma (PTC)

3.4.1.Epidemiology and etiology

PTC is the most common type of thyroid carcinoma, and constitutes 60-80% of all cases. PTC is rare in children and adolescents, occurring only exceptionally before the age of 10. In adults the incidence increases with age. Females are affected 2-4 times more often than males and the mean age at diagnosis is 45-50 years (Franceschi 1993, Clark & Duh 1997). According to a recent study where all patients (n = 5, 554) in Sweden diagnosed with DTC between 1958 and 1987 were evaluated, 65% were classified as PTC (Lundgren 2003). The majority of the PTC patients were females (76%) and the mean age at diagnosis was 50 years. The PTC

incidence started to increase already before the age of 20, increased steadily until the age of 65 and decreased thereafter. This pattern was similar in men, although less pronounced. In addition, the PTC incidence (per 100, 000 person-years) increased over time from 1.5 between 1958-67 to almost 3 during 1978-87, which is in line with other studies (Franceschi 1993).

The only clearly demonstrated etiological factor in the development of PTC is a previous history of radiation exposure. For example thyroid carcinoma was the first solid malignant tumor found to be increased in Japanese atomic bomb survivors (Socolow 1963). In 1986 the nuclear accident in Chernobyl released large amounts of radioactive particles into the atmosphere, including iodine isotopes. A remarkable increase in the number of thyroid carcinomas was observed already four years after the accident. The increase was especially pronounced in those under the age of 15 years, most of whom was under the age of 10 at the time of the accident (Kazakov 1992). More than 90% of the identified thyroid carcinomas were classified as PTC.

Other suggested risk factors for PTC are pre-existing benign thyroid disease (Franceschi 1993) or a family history of PTC, which will be discussed in section 3.5.4. In geographical areas where iodine intake is adequate or elevated the PTC incidence is higher than in areas with iodine deficiency, where, on the other hand, FTC and ATC are relatively more frequent. Other yet unknown environmental or dietary factors appear to influence the development of PTC. In immigrants the PTC incidence is similar to the incidence in the general population in the country where they reside, but different from the country of origin (Spitz 1988). The striking gender differences in PTC incidence, especially during the reproductive part of female life, suggest a possible influence of sex hormone related factors (Lundgren 2003).

3.4.2 PTC diagnosis and histopathological variants

Virtually all thyroid nodules are investigated using fine needle aspiration and cytology. Aspiration smear from PTC may reveal papillary structures, but the preoperative diagnosis is mainly based on the recognition of typical nuclear characteristics, such as intranuclear pseudoinclusions (due to cytoplasmic invaginations) and nuclear grooves (folds in the nuclear membrane). In addition psammoma bodies (calcium salt deposits) may be present (Fig 6). The

diagnosis of PTC is confirmed by postoperative microscopic examination of the surgical specimen, which typically reveals branching papillae, composed of a central fibrovascular core covered with a single layer of neoplastic columnar epithelial cells. The crucial diagnostic feature is the atypical nuclei, which are large and clear (due to uncondensed chromatin) and referred to as “ground-glass nuclei”, and if these are present the tumor is classified as PTC regardless of the other features of the tumor. PTC frequently invades lymphatic vessels, but vascular invasion is fairly uncommon (Rubin & Farber 1994).

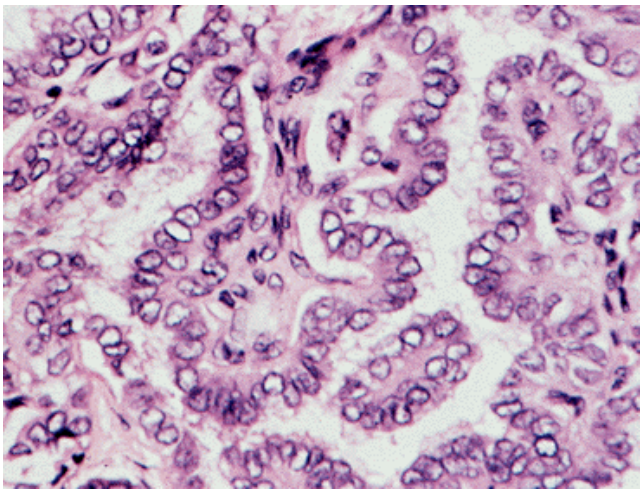

Figure 6. Photomicrograph of PTC showing typical branching papillae consisting of fibrovascular core structures covered by crowded overlapping optically clear nuclei so called ground-glass nuclei.

Most PTCs are classified as “classical” or “common” PTCs. These always exhibit papillae as described above, although neoplastic follicles are commonly present. Others are classified as histopathological variants of PTC, some of which have clinical relevance. One well known variant is the microcarcinoma, or occult carcinoma, which according to the WHO includes PTC measuring 1 cm or less. These tumors are present in 4-35% of thyroids investigated at autopsy (Rosai 1992) and are common incidental findings in thyroids removed for other abnormalities, such as goiter and hyperthyroidism. Microcarcinomas are usually considered harmless, and no further treatment is indicated upon their fiding. Still, a few cases have been described where patients with microcarcinomas have developed metastases and died from the disease (Harach 1984).

The follicular variant of PTC represents a pathological paradox as the diagnosis of PTC is made without visible papillae. Instead, it is entirely composed of follicles containing eosinophilic colloid, but the clear nuclei characteristic for classical PTC are present. The follicular variant of PTC appears to behave similarly to classical PTC.

Tall cell and columnar cell variants are thought to have worse prognosis as compared to classical PTC. The tall cell variant (described by Hawk and Hazard 1976) is characterized by cells which are twice as high as they are wide. The columnar cell variant differs from the tall cell variant only by the nuclei, which are stratified and hyperchromatic in the columnar cell variant.

3.4.3 Treatment and follow-up

The initial treatment of choice in PTC patients is indisputably surgery. However, the extent of surgery is highly controversial, although most clinicians presently perform total thyroidectomy for clinically detected PTC tumors (Sherman 2001). Some investigators report no differences in survival between patients treated with lobectomy as compared to those who have had a total thyroidecomy, but the number of locoregional and distant recurrences appears higher in those who have had a lobectomy only (Hay 1998, Taylor 1998). Others report a reduced PTC specific mortality in patients treated with total thyroidectomy (DeGroot 1994, Mazzaferri 2001).

The rationals for total thyroidectomy when treating PTC are usually: 1) many PTCs are multifocal; 2) I^{131} treatment requires as little remnant thyroid tissue as possible; and 3) postoperative Tg assessment is more reliable. The rationals for lobectomy would then be: 1) lower complication rates i.e. nerve palsy and hypoparathyroidism; 2) survival is possible without thyroxine replacement therapy; and 3) most patients do well regardless of surgical strategy, at least when classified as being at low risk for PTC related mortality.

After the initial thyroidectomy, I^{131} ablation usually follows. Prior to the ablation I^{131} uptake measurement can be performed in order to scan for residual thyroid tissue or metastases, but many patients are treated with I^{131} without a prior diagnostic procedure, thus the diagnostic procedure is considered optional (Sherman 2001). There are three reasons to perform I^{131}

adjuvant therapy: 1) to destroy microscopic residual tumor or metastatic disease, 2) to eliminate uptake by residual normal thyroid tissue, thus increasing the specificity of I^{131} uptake measurements which facilitates early detection of recurrent or metastatic disease, and 3) to improve the value of serum Tg measurements as detectable Tg is then expected to derive from malignant cells (see below). However, the beneficial effect of I^{131} ablation on recurrences and mortality is a matter of debate. It is generally thought to have no impact on intrathyroidal tumors measuring <1.5 cm (Baudin 1998). In patients with tumors that are large (≥1.5 cm), multicentric or exhibit extrathyroidal extension, a decrease in recurrence rate and PTC-specific mortality following I^{131} ablation is seen (Mazzaferri 1994, Taylor 1998). In cases of gross extrathyroidal tumor extension or presumed residual disease after the initial surgical procedure, external radiation to the thyroid bed is usually recommended (Sherman 2001).

To prevent hypothyroidism and to keep a minimum stimulation of tumor growth by TSH, all patients who have been thyroidectomized will need lifelong thyroid hormone replacement. With this therapy disease-free survival is reported to increase two to three-fold, especially in high-risk patients (Cooper 1998).

Long-term monitoring basically consists of clinical examination, I^{131} scanning and serum Tg measurements. A post-treatment I^{131} scanning is recommended 6-12 months after the initial ablation, and if this shows no abnormalities additional scanning procedures are recommended only for patients in whom recurrence is suspected (Sherman 2001). Tg is a glycoprotein exclusively produced by thyroid follicular cells. After thyroidectomy and successful remnant ablation Tg should be undetectable, thus an increase in Tg level provides indirect evidence of recurrent disease (Spencer 1999).

3.4.4 Prognostic factors in PTC

Although PTC generally carries a good prognosis, some patients are at high risk of recurrences or PTC related death. Hundreds of publications evaluating clinical and histopathological data, as well as genetic and molecular features of PTC, have been devoted to defining prognostic markers. The fact that PTC has a comparatively low incidence and a generally excellent long-term prognosis makes prospective studies regarding prognostic

factors impractical. Instead, the identification of suggested prognostic factors (Table 1) have usually been based on retrospective analyses. However, as many of the patients included in these analyses have been collected during decades when surgical techniques improved and progresses were made in PTC diagnosis and treatment the results are frequently conflicting. As no single prognostic factor successfully identifies patients who are at risk of recurrences or death from PTC, different prognostic scoring systems have been developed, each taking a selection of the prognostic factors into account. In this way, each patient is given a particular score and from this the patient´s long-term outcome is predicted with reasonable accuracy.

Table 1: Factors associated with a less favorable outcome of PTC

Patient characteristics
Age > 45 years
Male sex
Tumor features
Histopathology
Tall cell or columnar cell variant
Poor differentiation
Tumor extent
Large tumor size (> 4 cm)
Extrathyroidal tumor extension
Distant metastases
Others
Multifocality
Lymph node metastases
Aneuploidy
Family history
Treatment
Incomplete resection
No administration of ablative radioiodine postoperatively
Lack of TSH supression
Follow-up
Elevated Tg > 3 months postoperatively

The pTNM staging system was introduced in 1987 and revised in 1992 (Hermanek and Sobin 1992). This system takes age at diagnosis, size and extension of the primary tumor, and the presence or absence of lymph node and distant metastases into account (Table 2). Patients are classified into four risk groups with varying cancer specific mortality rate (Loh 1997).

Table 2: TNM classification of PTC

Stage			Death rate *
	< 45 years	≥ 45 years	
I	M0	T1	1.7%
II	M1	T2-T3	15.8%
III		T4 or N1	30%
IV		M1	60.9%

T1= tumor size ≤ 1 cm
T2= tumor size > 1-4 cm
T3= tumor size > 4cm
T4= extension beyond the thyroid capsule
N1= presence of lymph node metastases
M1= presence of distant metastases
* PTC specific death rate according to a study including 700 patients (Loh 1997)

The AMES system was created in 1988 and is based on **A**ge at diagnosis, presence of distant **M**etastases, **E**xtent and **S**ize of the primary tumor (Cady 1988). According to this system 89% of the patients belong to a subgroup with very low mortality rate (1.8%), while the remaining patients have a 46% mortality rate (Table 3). In 1992 the information provided by assessment of DNA ploidy in the tumor was added to the AMES system, recognized as the DAMES system, which increased its predictive value (Pasieka 1992).

Table 3: The AMES scoring system

High-risk group (mortality rate 46 %)

Presence of distant metastases regardless of age.

Men > 40 and women > 50 years of age at diagnosis with:
Primary tumor extending beyond the thyroid capsule
or
Tumor size > 5cm (primary tumor)

Low-risk group (mortality rate 1.8 %)

Men ≤ 40 or women ≤ 50 years of age at diagnosis regardless of extent and size of the primary tumor.
Older patients with primary tumors < 5 cm confined to the thyroid.

In 1987 the AGES scoring system was developed at the Mayo Clinic, taking **A**ge at diagnosis, tumor **G**rade, tumor **E**xtension and **S**ize of the primary tumor into account (Hay 1987). As tumor grade is not used at most instances this parameter was excluded and the system revised in 1993. The new system was designated MACIS and included distant **M**etastases, **A**ge at diagnosis, **C**ompleteness of surgery, **I**nvasion of extrathyroidal tissue and **S**ize of the primary tumor (Hay 1993). This system defines four risk groups with decreasing 20-year PTC-specific survival rate (Table 4).

Table 4: MACIS prognostic scoring system

Calculated as
3.1 if age ≤ 39 years *or* 0.08 x age if ≥ 40 years + 0.3 x tumor size (cm) + 1 if incompletely resected + 1 if locally invasive + 3 if distant metastases
20-year survival rate for a score of: < 6 : 99%, 6 - 6.99: 89%, 7 - 7.99: 56%, > 8: 24%

3.5 Molecular genetic alterations in papillary thyroid carcinoma

3.5.1 Screening for genetic alterations in PTC

During the past decade there has been an increasing number of publications regarding genetic alterations in thyroid tumors, but the results are frequently conflicting. In the following sections the search for DNA alterations in PTC will be briefly described and the most important genetic lesions found in PTC to date will be discussed.

DNA content

Earlier investigations focused on variations in the total DNA content in cancer cells. A cell carrying complete chromosome sets (2n, 3n, 4n etc., where n = 23 chromosomes) is regarded as euploid. Normal human somatic cells are diploid (2n) during G_0-G_1, and tetraploid (4n) when passing through G_1 and S phase, while cancer cells often show an extreme aneuploidy (one or more chromosomes extra or missing from an euploid set). When presence of aneuploidy was compared with the patients´ outcome, DNA content was found to be associated with decreased survival in PTC patients. On the contrary, in patients with other types of thyroid cancer the DNA content did not affect the prognosis (Cohn & Bäckdahl 1984). Furthermore, subtypes of FTA (atypical) exhibited aneuploidy in up to 40% of the cases (Zedenius 1992). Subsequent DNA analyses were focused on genetic changes of the individual chromosomes.

Karyotyping using classical cytogenetics

Although chromosomes were described already in the 1880s it was not until in the 1950s that proper chromosome spreads could be produced and the correct number determined (Tijo & Levan 1956). The advances relied on the findings that colchicine could arrest the cell cycle in metaphase and that hypertonic saline improved the chromosome spreading. Before 1970 groups of chromosomes were identified on the basis of size and centromere position, but the introduction of banding techniques (Caspersson 1970) made it possible to identify separate chromosomes on the basis of their unique banding pattern. Chromosome banding (usually G-banding) allows for the identification of translocations as well as subchromosomal gains or deletions with a minimum size of 4-5 Mb. A disadvantage with traditional karyotyping is the requirement for good quality metaphases from cultured cells and unfortunately PTC has proved relatively difficult to culture.

Early cytogenetic studies on PTC identified chromosome 10q rearrangements as the most frequent aberration, consistent with the *RET/PTC* oncogene activation (see section 3.5.3). To date approximately 100 karyotypes of PTC have been published, in which additional clonal numerical or structural genetic alterations are described, although generally in isolated cases only (Antonini 1992, Pierotti 1996, Roque 2001). Based on the low frequency of alterations detected, PTC is considered a genetically stable tumor. However, in a cohort including 56 radiation-induced PTC, multiple structural chromosome aberrations giving rise to complex karyotypes were more frequently detected than in spontaneous PTC (Zitzelberger 1999).

Loss of heterozygosity (LOH)

The invention of the polymerase chain reaction method (PCR) revolutionized molecular genetics by permitting rapid amplification of DNA fragments (Mullis 1987). PCR has numerous applications in mutation analysis, the most direct being nucleotide sequence determination of the PCR product which enables analysis of small mutations or sequence polymorphisms. One of the most general approaches is the use of gel electrophoresis allowing for identification of differences in size between the amplified sequences. LOH is a method in which tumors are screened for deleted DNA regions. Here, highly polymorphic microsatellite markers, which are able to distinguish between the alleles of homologous chromosomes, are spaced across the whole genome. Paired blood and tumor samples are analyzed using PCR for allelic losses in the tumor DNA indicating a putative TSG at this locus. However, screening for LOH is rather laborious and deleted regions cannot be readily distinguished from amplifications.

Apart from one study, where LOH was detected in 50% of cases frequently involving 4q, 5p and 7p (Califano 1996), the frequency of LOH in PTC is generally low with no prevalence for LOH at any particular locus (Ward 1998, Oriola 2001). Instead, hypermethylation of promoter regions appears to be a more common mechanism for gene silencing in PTC (Huang 2003).

CGH, M-FISH and SKY

Over the past two decades cytogenetic methodologies have improved and new techniques facilitating DNA studies have been developed, making it possible to analyze faster, more accurately and with higher resolution. In the early 1990s comparative genomic hybridization (CGH) was described (Kallionemi 1992). CGH is a cytogenetic method which compares the ratio between tumor DNA and normal reference DNA by competitive fluorescent *in situ*

hybridization (FISH). This allows screening of the entire genome for gains or losses of DNA material in one single experiment (see section 5.2.3).

The frequency of PTC tumors in which chromosomal imbalances have been detected using CGH varies between 12 and 84% (Chen 1998, Hemmer 1999, Singh 2000, Kjellman 2001, Wreesmann 2002, Bauer 2002). Although the studies show inconsistent results regarding the chromosome regions displaying DNA imbalances, the results point toward that less well differentiated PTC exhibit significantly more abnormalities as compared to their well differentiated counterparts (Kjellman 2001, Bauer 2002, Wreesmann 2002). This suggests that the development of CIN may underlie a progression to more aggressive PTC phenotypes. MIN, on the other hand, is uncommon in PTC (Lazzereschi 1999, Bauer 2002).

With the development of multicolor FISH (M-FISH) and spectral karyotyping (SKY) assays, in which whole chromosome painting probes in 24 different colors are used, the analysis of complex karyotypes involving translocations or other structural or numerical chromosomal abnormalities is substantially facilitated. However, as for classical karyotyping the need for metaphases obtained from the culturing of PTC tumors is a limiting factor. Recently the investigation of PTC tumors using SKY revealed a new balanced translocation between chromosomes 3 and 15 in one tumor and a complex karyotype with translocations involving several chromosomes in another metastasizing PTC tumor (Foukakis, abstract ETA 2003)

Global gene expression profiling using microarrays

Although a variety of cells with different functions exists, every somatic cell carries identical genetic information. Basically, it is the differential expression of the genes and subsequent protein production in each cell that determine the properties of the cell. In mid 1990s a technique that allowed for the simultaneous measurement of gene expression levels of thousands of genes in one single experiment was described (Schena 1995). The most common variant is the use of commercially available Affymetrix GeneChips, which contains oligonucleotides corresponding to the genes of interest. Total RNA from the tissue sample is labeled and hybridized to the array and the amount of RNA bound to each probe set is quantified. When gene expression was studied in PTC using microarray, concordant over-expression of many genes, for example *FN1*, *MET* and *TIMP-1*, was detected (Huang 2001, Wasenius 2003). The role for these genes in thyroid pathogenesis needs to be further elucidated.

3.5.2 Signaling pathways in thyroid follicular cells

All cells in a multicellular organism contain an elaborate system of proteins which enables the cells to respond to signals from other cells. The signaling molecules bind to cell-surface or intracellular receptor proteins in the target cell. The cell surface receptor proteins act as signal transducers: signals received at the surface are, via second messengers, relayed to the nucleus where they alter the expression of specific genes and subsequently change the behavior of the cell. The majority of cell surface receptor proteins are either linked to GTP-binding regulatory proteins (G-proteins) or are enzyme-linked (kinases).

Follicular cell proliferation is basically regulated by extracellular growth factors that, upon binding to membrane receptors, mediate proliferative signals to the nucleus via three key signaling pathways: the TSH receptor/adenylate cyclase (AC)/protein kinase A (PKA) pathway, the tyrosine kinase receptor (RTK)/ras/mitogen-activated protein kinase (MAPK) pathway and the receptor/phospholipase C (PLC)/protein kinase C (PKC) pathway (Fig 7).

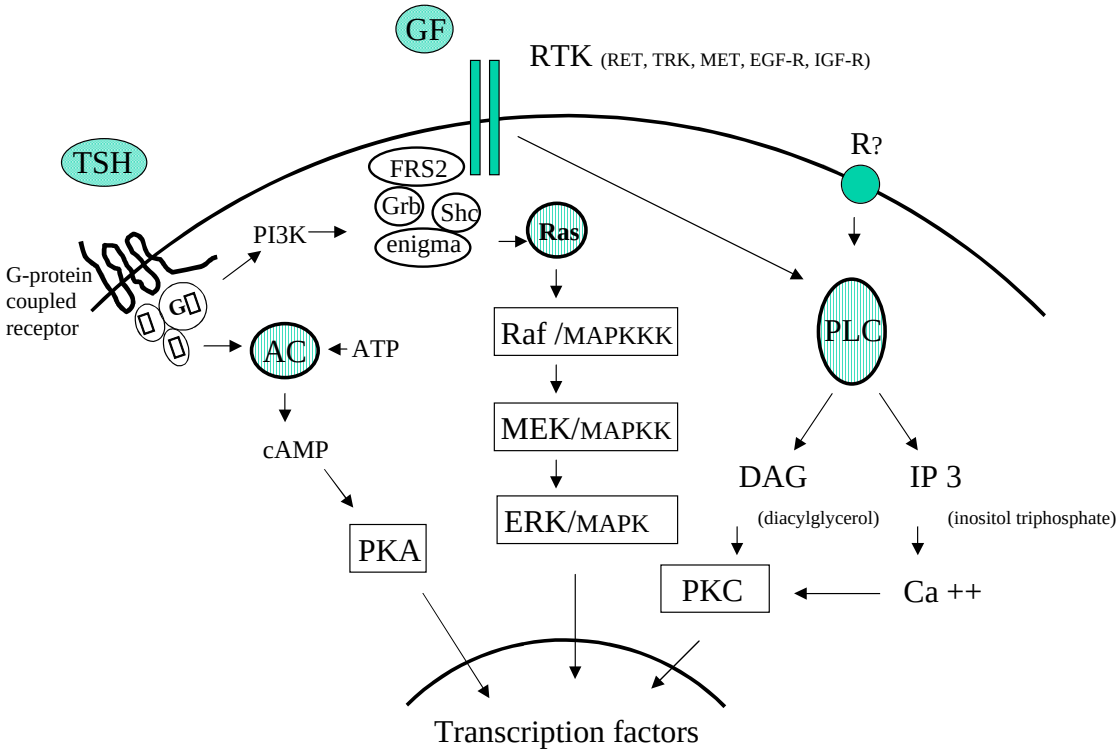

Figure 7. The three main signal transduction pathways activated through growth stimulation in follicular thyroid cells.

3.5.3 Oncogenes and PTC

The RET/PTC oncogene

The *RET* proto-oncogene is located on chromosome 10q11.2 and encodes a transmembrane receptor of the tyrosine kinase family. The RET protein consists of a ligand-binding extracellular domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity (Itoh 1989, Pasini 1995). RET is the receptor for growth factors belonging to the glial cell line-derived neurotrophic factor (GDNF) family, comprising GDNF, neurturin (NTN), persephin (PSP) and artemin (ART), which all have trophic influences on various neuronal populations (Sariola 2003). In adults RET expression is normally restricted to cells of neural crest origin (Nakamura 1994), but during embryogenesis RET plays an important role in kidney development, urethral branching and innervation of the hindgut (Sariola 2003). To activate RET, the different RET ligands form multicomponent receptor complexes with ligand-binding subunits known as GDNF family receptor-(GFR-), and the RET receptor (Fig 8).

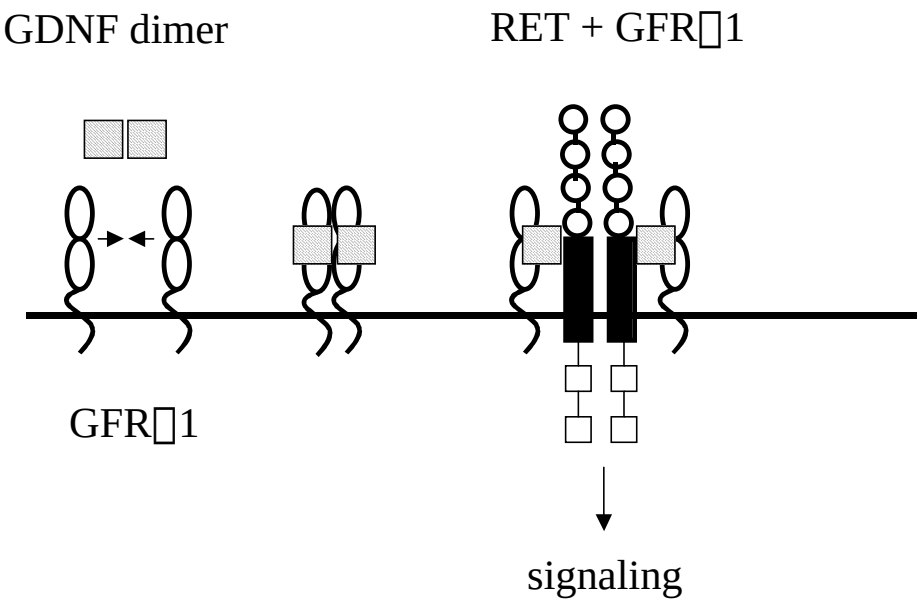

Figure 8. GDNF family of ligands interacting with the RET receptor. GDNF, NTN, ART and PSP specifically bind to GFR-1, GFR-2, GFR-, and GFR-4 respectively. A dimer of GDNF brings together two molecules of GFR-1. The complex dimerizes two molecules of RET leading to transphosphorylation of their tyrosine kinase domains.

The *RET* proto-oncogene was originally discovered in a transfection assay (Takahashi 1985). A DNA fragment with transforming activity was identified in a PTC and after characterization it was shown to consist of sequences from two different unrelated genes, producing a fusion gene. As no rearrangements were detected in the original tumor, the gene fusion was supposed to be an artefact that had occurred during transfection. Therefore, the first gene was named *RET* (*RE*arranged during *T*ransfection), and the second *RFP* (*R*et *F*used *P*rotein).

The finding that DNA extracted from PTC could transform NIH-3T3 cells indicated the presence of an activated oncogene in PTC (Fusco 1987). The oncogene was initially designated *PTC* (*P*apillary *T*hyroid *C*arcinoma) and assigned to chromosome 10q11-q12 (Donghi 1989). Further studies revealed that the *PTC* oncogene resulted from the rearrangement of an unknown amino-terminal sequence to the DNA sequence encoding the tyrosine kinase domain of *RET* (*RET*-TK), thus generating a chimeric oncogene, which was designated *RET/PTC* (Grieco 1990). The unknown part of *RET/PTC*, called *H4*, was mapped to 10q (Sozzi 1991), and the mechanism behind the rearrangement was soon revealed to be an inversion of 10q (Fig 9), resulting in the fusion of the *H4* promoter to the TK part of *RET* (Pierotti 1992). In addition to *H4*, several new *RET*-TK fusion partners have been detected including *Ri* as in *RET/PTC2* (Bongarzone 1993) and *ELE1* as in *RET/PTC3* and *4* (Santoro 1994, Klugbauer 1996). Similar to *RET/PTC1*, *RET/PTC 3* and *4* arise as a result of chromosome inversions, while *RET/PTC 2*, *RET/PTC5* (Klugbauer 1998), *RET/PTC6* and *7* (Klugbauer 1999), *RET/PTC8* (Salassidis 2000), *RET/ELKS* (Nakata 1999) and *RET/PCM* (Corvi 2000) are generated by balanced translocations (Fig 10).

In thyroid follicular cells harboring *RET/PTC* rearrangements, the normally transcriptionally silent *RET* promoter is substituted with those of the constitutively activated *RET* fusion partners, allowing for *RET* expression. In addition, *RET/PTC* rearrangements generate ligand-independent constitutively active oncoproteins (Santoro 2002). RET activation results in autophosphorylation of intracellular RET tyrosine residues, generating docking sites for adaptor proteins involved in downstream signaling. Several pathways activated by RET and RET/PTC have been identified. The RAS/MAPK pathway (Fig 7) is activated as a result of the phosphorylation (adding a phosphate group) of adaptor proteins such as Shc, Enigma, Grb and FRS2, which all have docking sites on activated tyrosine residues of RET (Durick 1998, Melillo 2001, Knauf 2003). Activated RET can also interact with and activate PLC (Fig 7), which in turn leads to downstream signaling via the activation of various PKCs (Borrello

1996, Andreozzi 2003). Phosphorylation of PIK3, resulting in the activation of PIK3/Akt signaling pathway, provides an additional oncogenic mechanism for activated RET. Akt promotes survival through phosphorylation of members of the Bcl-2 family which results in suppressed apoptosis (Datta 1997, Besset 2000).

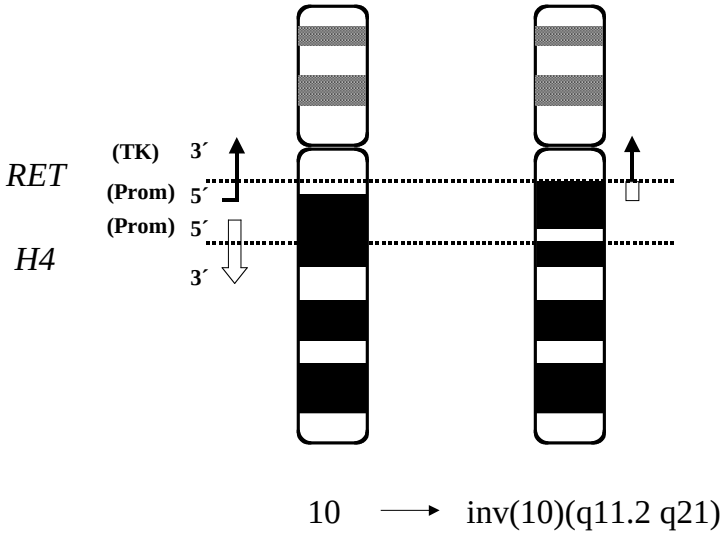

Figure 9. Schematic view of the inversion on chromosome 10q involving the *RET* and *H4* genes, generating the *RET/PTC1* fusion gene. The gene order and the direction of gene transcription are indicated. The 5´promoter region (Prom) and the 3´ part of the *RET* gene which encodes the tyrosine kinase domain (TK) of the RET protein is marked.

The frequency of *RET/PTC* rearrangements in patients without a history of radiation towards the thyroid gland varies between 2.5% and 40%. As *RET/PTC* activation is induced by irradiation of thyroid cells (Mizuno 2000), it is not surprising that a higher prevalence (50-70%) of rearrangements is reported in PTC associated with radiation exposure from the Chernobyl reactor accident (Rabes 2001). *RET/PTC1* and *3* are by far the most frequent rearrangements, while the more recently described ones are mainly obtained from PTCs from the radioactively contaminated Chernobyl area. The clinical significance of the rearrangements is controversial. Some report an association between *RET/PTC* and aggressive disease, while others report *RET/PTC* to be more frequent in small, slow growing and less aggressive PTC (Jhiang 2000). Recently, *RET/PTC* activation was reported *not* to influence clinical and pathological features of PTC and to *not* be more frequent in radiation-induced PTC compared with those spontaneously occurring (Elisei 2001, Puxeddu 2003). In addition, *RET/PTC* was detected in 14-52% of benign thyroid nodules (Elisei 2001), which is in

contrast to most other reports advocating that *RET/PTC* is restricted to PTC (Santoro 1992, 2002). Although the role for *RET/PTC* in tumorigenesis is not completely elucidated, the fact that expression of *RET/PTC* in transgenic mice results in tumors resembling PTC (Powell 1998) implicates that *RET/PTC* rearrangements are initiating events in the development of PTC.

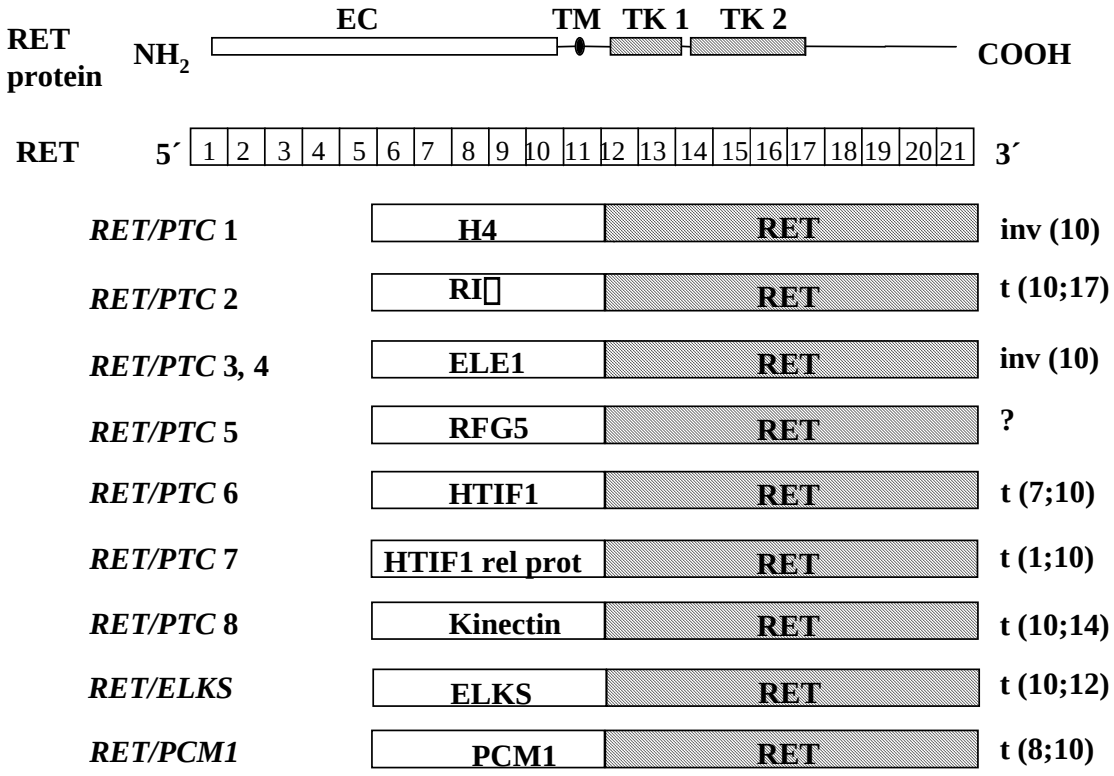

Figure 10. The 21 exons of the RET gene and the different parts of the corresponding RET protein (top). EC, extracellular part; TM, transmembrane domain; TK, tyrosine kinase domain. Below are the different *RET/PTC* rearrangements shown. The particular fusion gene is depicted for each of the rearrangements, together with the underlying genetic alteration generating the fusion genes (right).

The *RET* gene was in 1993 shown to be the disease gene for the familial forms of MTC observed in MEN 2A, MEN 2B and FMTC. In more than 95% of the patients with these syndromes germline missense or single point mutations affecting selected codons are detected. Similar mutations are also detected as *de novo* mutations in 7-25% of apparently

sporadic MTC (Zedenius 1994, Eng 1999). Moreover, a few patients with *RET* germline mutations and PTC has been reported (Brauckhoff 2002).

Inactivating *RET* mutations have been detected in a subset of patients with Hirschsprung disease, a condition with congenital absence of enteric innervation leading to intestinal obstruction (Pasini 1995).

NTRK1

NTRK1 (neurotrophic tyrosine kinase receptor 1) on chromosome 1q21-22 encodes the receptor for nerve growth factor (NGF). Similar to *RET, NTRK1* has been found activated through rearrangements in PTC. The *NTRK1* gene joins to *TPM3, TPR* or *TFG* generating fusion genes designated *TRK, TRK-TI/TRK-T2* and *TRK-T3* respectively (Alberti 2003) The frequency of *NTRK1* rearrangements in PTC is low, ranging between 3-12 %, also in radiation induced PTC (Beimfohr 1999, Rabes 2000, Musholt 2000).

The RAS and RAF oncogenes

The small *RAS* encoded proteins ($p21^{ras}$) participate in signal transmission from cell surface receptors of the tyrosine kinase family to the nucleus via the Ras/Raf/MEK/ERK pathway (Fig 7). The Ras proteins act as G-proteins and exist in a resting state, bound to guanosine diphosphate (GDP) and an active state in which Ras is bound to guanosine triphospate (GTP). Inactivation of Ras occurs through hydrolysis of GTP to GDP. As a result of point mutations in certain *RAS* codons, impairing the inactivating mechanism, Ras is constitutively activated. Activated Ras phosphorylates Raf, which in turn activates a series of kinases along the signaling pathway (Peyssonnaux 2001). Activating *RAS* mutations have been detected in both benign and malignant thyroid tumors (Bos 1989), and some report an association between *RAS* mutations and poor prognosis in various types of thyroid cancer (Garcia-Rostan 2003). In PTC *RAS* mutations are infrequently detected (Soares 2003), but when present have been suggested to be associated with a poorer prognosis (Hara 1994, Basolo 1994).

The Raf proteins are serine-threonine kinases acting down-stream of Ras. In humans three genes encoding isoforms of Raf have been detected: *ARAF, BRAF* and *CRAF-1*. Point mutations in *BRAF* have recently been reported to be a frequent finding in melanomas (>60%) and to a lesser degree in lung, colon and ovarian carcinoma (Davies 2002). A nucleotide change at codon 599, substituting valine with glutamic acid (V599E), has been reported to

result in a protein with elevated kinase activity that can transform NIH3T3 cells. This hot spot accounts for more than 80% of *BRAF* mutations (Davies 2002). Three recent studies on thyroid tumors showed consistently that *BRAF* ^{V599E} mutations were unique to PTC and a frequent finding in PTC, detected in 36-53% of the tumors, thus representing the most common genetic change in PTC to date. A small number of *RAS* mutations and *RET/PTC* rearrangements were also detected, but in no PTC tumor *RET/PTC*, *BRAF* or *RAS* mutations coexisted. Accordingly, in the majority of PTCs thyroid cell transformation appears to occur through activation of effectors along the RET/Ras/Raf pathway (Kimura 2003, Soares 2003, Fukushima 2003).

cAMP pathway

Stimulation of thyroid growth and function classically depends on TSH, which binds to the TSH-receptor (TSHR), belonging to the family of G-protein coupled receptors. The G-protein, composed of an □ (G□), □ and □subunit, exists under basal condition as an inactivated heterotrimer with GDP bound to the G□. Upon receptor activation GDP is replaced with GTP, and the activated GTP- G□ dissociates from the □□subunits to interact with effectors and propagate the signal. The most well known second messenger is the adenylate cyclase, converting ATP to cAMP, which leads to transduction of the signal to the nucleus via protein kinase A (Fig 7). Mutations in the gene encoding G□ (*gsp*) or in the *TSHR* gene, resulting in constitutive activation of the cAMP pathway, are frequently detected in hyperfunctioning (toxic) nodules of the thyroid, implicating a role in hyperproliferative disturbances. However, such mutations are rare in thyroid carcinomas (Goretzki 2000). When studying G-protein coupled receptors, Ras dependent MAPK-activation mediated by the □□ subunits through phosphoinositide 3-kinase (PI3K) was detected (Lopez-Ilasaca 1997). *TSHR* mutations in codon 623 and 632 have been detected in thyroid carcinomas (Russo 1995) and have proved to have transforming capacity on mouse 3T3 cells (Du Villard 2000). It has been speculated that *TSHR* gene mutations, depending on the location, may stimulate either cAMP or Ras or both, and that activation of the cAMP pathway alone is not sufficient for malignant transformation.

MET

The *c-MET* proto-oncogene encodes the Met protein, a receptor tyrosine kinase binding the hepatocyte growth factor (HGF). In PTC Met has been reported markedly overexpressed (Di

Renzo 1992). No amplifications or rearrangements of *c-MET* have yet been detected in PTC, neither does the protein show any structural alterations. Instead, Met overexpression is considered a result of mutations in other genes such as *RAS* and *RET* (Ruco 2001). In most studies Met expression does not correlate to tumor features or prognosis. However, two studies report that negative or low Met expression is a sensitive predictor for distant metastases (Belfiore 1997, Ruco 2001).

TP53

The *TP53* gene product, p53, plays a key role in arresting the cell cycle (Fig l). Inactivating mutations are infrequent in differentiated thyroid carcinomas, including PTC, but are detected in 25-71% of poorly differentiated and anaplastic thyroid carcinomas (Donghi 1993). Inactivated p53 has been suggested to be involved in the de-differentiating process, which is supported by the fact that PTC tumors developed in *RET/PTC1* mice progress to invasive and less differentiated tumors upon p53 loss (La Perle 2000).

PTEN

The *PTEN* tumor suppressor gene on 10q23.3 encodes a protein belonging to the family of protein serine phosphatases. PTEN induces apoptosis and cell cycle arrest by reducing the activity of antiapoptotic Akt. Germline mutation of *PTEN* is the genetic background of Cowden syndrome, an autosomal dominant syndrome characterized by multiple hamartoma and an increased risk of breast and thyroid neoplasia. In individuals with Cowden syndrome the lifetime risk of developing thyroid carcinoma is approximately 10%, with predominance for FTC although PTCs have also been observed (Eng 2002).

Adhesion molecules

Decreased levels of E-cadherin was reported as an independent adverse prognostic factor in DTC (von Wasielewski 1997). It is more prevalent in PTC as compared to FTC and correlates with lymph node metastases. E-cadherin gene alterations are rare. Instead the decreased expression of E-cadherin appears associated with CpG island hypermethylation (Soares 1997, Graff 1998, Naito 2001). In addition, overexpression of dysadherin, which down-regulates E-cadherin, is significantly associated with adverse clinical outcome (Ino 2002).

Reduced IHC staining of β-catenin at the cell membrane has been associated with tumor recurrence and cancer-related mortality in DTC (Böhm 2000). However, mutations in the β-

catenin gene result in over-expression and cytoplasmic or nuclear localization of β-catenin, which in turn activates the Wnt-signaling pathway resulting in activation of downstream *myc* and *cyclin D1* (Garcia-Rostan 2001, Ishigaki 2002).

Angiogenetic factors and matrix metalloproteinases

Both over-expression and increased serum levels of VEGF and bFGF have been documented in DTC and are associated with local and distant metastases (Klein 2001, Komorowski 2002, Tuttle 2002). In contrast to VEGF which stimulates angiogenesis, VEGF-C is believed to promote lymphangiogenesis. VEGF-C expression is increased as compared to normal tissue in PTCs (Hung 2003).

The production of different MMPs is increased in PTC with lymph node metastases as compared to those without metastases (Nakamura 1999). This is in concordance with a recent microarray study demonstrating up-regulation of MMPs (Wasenius 2003).

3.5.4 Familial PTC

Although most PTC is sporadic, familial aggregation of PTC occurs. Several studies have shown an increased risk for PTC in first-degree relatives of patients with PTC (Hemminki 1999). Familial PTC is estimated to account for 5-10% of all PTC and may occur as a distinct entity, or be a component of an inherited cancer syndrome (Table 5). For example, patients with Cowden syndrome frequently develop FTA and FTC, and to a lesser extent PTC. In patients with familial adenomatous polyposis (FAP), caused by germline mutations in the *APC* gene on 5q21, about 2% also develop PTC (Giardiello 1993). However, *APC* has been ruled out as the disease gene for familial PTC without FAP association (Malchoff 1999). Other genes or loci have through linkage studies been proposed to predispose for familial PTC. The *TCO* (*T*hyroid tumors with *C*ell *O*xyphilia) locus on 19p13.2, was proposed to be responsible for thyroid tumors showing cell oxyphilia (Canzian 1998). A locus on 14q, harboring a putative *MNG1* (*M*ulti*N*odular *G*oiter) gene, was identified in a family with multinodular goiter and low prevalence of PTC (Bignell 1997). A *PRN1* (*P*apillary *R*enal *N*eoplasia) locus on 1q21 was identified in a large family affected with PTC and papillary renal neoplasia (Malchoff 2000). However, neither of these loci account for a significant fraction of the familial PTC (Lesueur 1999, McKay 2001). Instead, linkage analysis in a set of

80 pedigrees with isolated PTC identified a new locus mapped to 2q21 (McKay 2001). Although *RET* rearrangements may be found in familial PTC, the low incidence (<5%) excludes *RET* as a major cause (Corvi 2001). Familial PTC, including familial papillary microcarcinomas, are thought to be clinically more aggressive than the sporadic forms, with high rate of recurrences and reduced disease-free survival (Lupoli 1999, Uchino 2002).

Table 5: Familial disorders predisposing to PTC

Disorder	Locus	Gene/locus	Characteristics
Familial adenomatous polyposis	5q21	*APC*	Colon cancer, PTC
Cowden syndrome	10q23.3	*PTEN*	Hamartoma, breast cancer, FA, FTC, PTC
-	19p13.2	*"TCO"*	Thyroid tumors with cell oxyphilia
-	14q	*"MNG1"*	Multinodular goiter and PTC
-	1q21	*"PRN1"*	Papillary renal neoplasia and PTC
-	2q21	?	Familial PTC

4. Aims of the thesis

Patients with PTC usually have an excellent prognosis but some patients are at increased risk of recurrences and death. The following thesis was undertaken to:

▲ Evaluate patient and tumor characteristics and their impact on the outcome.

▲ Investigate if Ki-67 immunoreactivity in tumor cells (MIB-1 index) can add prognostic information.

▲ Estimate the frequency of rearrangements involving the *RET* gene in Swedish patients.

▲ Search for genetic alterations with the intention to find chromosomal regions harboring genes involved in the development or progression of PTC tumors.

▲ Analyze whether or not *RET/PTC* rearrangements or other genetic alterations can be used for diagnostic or prognostic purposes.

5. Material and methods

5.1 Patient and tumor material

Since 1986, fresh frozen tissue samples have been consecutively collected from all patients operated on for endocrine tumors at the Department of Surgery, Karolinska University Hospital, Solna. Immediately after the surgical removal, parts of the macroscopically identified tumorous tissue were separated and snap frozen in liquid nitrogen and then stored in –70 °C until use. In parallel the majority of the tumor tissue was embedded in paraffin and subjected to routine histopathological examination to establish the diagnosis. Additional corresponding patient peripheral blood cells were collected in connection with the surgical procedure to ensure access to matched pairs of constitutional and tumor samples.

The diagnosis of PTC and the histopathological classifications of the thyroid tumors included in the studies were carried out according to the standards of the World Health Organisation (Hedinger 1988), and poor differentiation was defined in accordance with the criteria of the Armed Forces Institute of Pathology (Rosai 1992). Furthermore, the suitability of the frozen samples for genetic and molecular analyses was confirmed by histopathological evaluation of the proportion of tumor cells on representative sections. This showed that the tumor specimens included in the studies contained a majority of tumor cells (70% or more). An advantage of the Swedish health care system is the accessibility to clinical information, thus information about the follow-up was retrieved from the patients´ medical records either at the Karolinska University Hospital, Solna or elsewhere. The studies of the tumor material were approved by the ethics committee of the Karolinska University Hospital, Solna.

The patients studied in this thesis basically consist of all 220 patients operated on for primary PTC at the Karolinska University Hospital, Solna between 1980 and 1999 (Fig 11). From these, clinical information was collected from medical records from all relevant hospital departments and follow-up institutions, and reviewed retrospectively in **Paper I**. From this group of patients, closely followed for a mean period of 8 years (range 1-20), tumors were selected and subjected to molecular genetic studies in **Papers II-IV**.

In **Paper II,** paraffin-embedded material from 30 primary PTC tumors (28 of which were included in Paper I and two who were operated in 1977 and 1979) was used for

immunohistochemical analysis of MIB-1 immunoreactivity. For comparison 10 FTC, 8 ATC, 96 FTA and 11 locally recurrent tumors from the above mentioned primary PTC tumors were similarly analyzed.

In **Paper III,** RNA extracted from 61 PTC (59 of which were included in Paper I, and two who were operated in 1972 and 1979) were analyzed using RT-PCR for expression of *RET* and the fusion oncogenes *RET/PTC* 1-4. In addition, 25 FTA and 5 FTC were included as a reference group and similarly analyzed.

In **Paper IV,** DNA was extracted from 25 primary PTC tumors (also included in Paper I) and analyzed with comparative genomic hybridization (CGH).

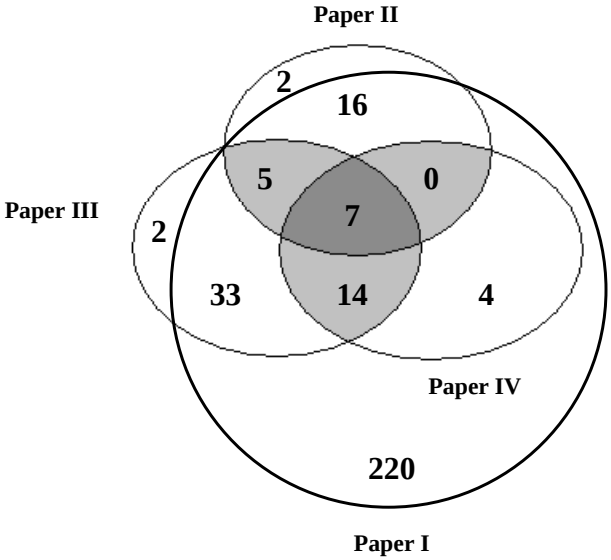

Figure 11. Illustration of patient overlapping in Papers I to IV. The 220 patients operated during the period of 1980-1999 and reviewed in Paper I was further subjected to molecular genetic studied (Papers II-IV). An additional 4 patients operated before 1980 were included in paper II and III.

5.2 Methods

5.2.1 Immunohistichemistry (Paper II)

In 1941 the immunoglobulin fraction of an antiserum was for the first time labeled with fluorescein to visualize the corresponding antigen in tissue sections by fluorescence microscopy (Coons 1941). The discipline of immunohistochemistry (IHC) was founded.

Since then the technique has improved and today a variety of methods are applied involving different types of antibodies, labeling and detection. In addition to fluorescent antibodies, enzyme labeled antibodies and their chromogens (as in paper II), and hapten labeled antibodies are also used. The various methods all have in common that they allow for the precise identification of individual cells containing a certain protein, as well as where in the cell the protein is located. In Paper II the proliferative activity in tumors was examined using the monoclonal antibody MIB-1, which recognizes the Ki-67 antigen expressed in the nucleus of all proliferating cells. A monoclonal antibody refers to a solution of identical antibodies (originating from one single B-cell) with a single specificity, produced *in vitro*. Those are distinct from polyclonal antibodies, which are raised by immunization, giving rise to antibodies, where each recognize different epitopes (from numerous B-cells).

In practice, slides with 4 μm thick tissue sections were prepared from formalin-fixed and paraffin-embedded specimens. The formalin fixation allows storage of tissue material for a long time, but carries the risk that antigenic sites are masked, and that antibody binding is inhibited. One can overcome this situation by microwave treatment of the tissue sections in sodium citrate prior to the incubation. The tissue sections were then exposed to MIB-1 antibodies. After washing, biotinylated anti-mouse antibodies were applied as secondary antibodies or bridge antibodies, followed by incubation with the avidin-biotin-peroxidase-complex (Fig 12). This procedure is usually referred to as the ABC method (Hsu 1981). The glycoprotein avidin has an extraordinarily high affinity for the small coenzyme biotin, and one molecule of avidin strongly binds four molecules of biotin. The substrate used for the peroxidase enzyme was diaminobenzidine, which polymerizes upon oxidation and produces a brown color. In the sample used herein, endogenous peroxidase activity was blocked by hydrogen peroxide treatment to avoid unspecific staining. In addition, areas with necrosis and/or lymphocyte infiltration were excluded to rule out an incorrectly high number of positively stained cells.

The proportion of MIB-1 positive cells was evaluated in light microscopy. With the aid of a 10 x 10 squares ocular grid, a minimum of 3,500 cells were classified as having a positive or negative immunoreaction in each tumor sample. The MIB-1 index, given as the percentage of positively stained tumor cell nuclei, was calculated in the areas with the highest number of immunoreactive cells.

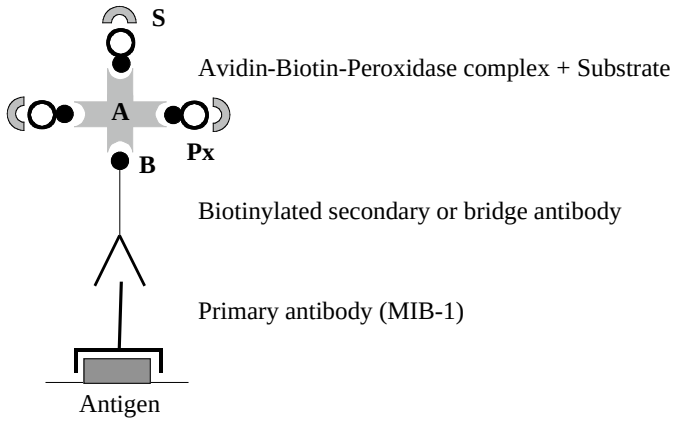

Figure 12. Three step immunoenzyme method using avidin (A), biotin (B), peroxidase (Px) and a substrate (S).

5.2.2 Reverse transcriptase – polymerase chain reaction (Paper III)

Gene expression can be studied at the RNA level using several different methods. When compared to protein studies of the corresponding gene product, the results are often inconsistent. These discrepancies may have many different causes such as alternative splicing, translation efficiency, posttranscriptional modification and protein export. Reverse transcriptase – polymerase chain reaction (RT-PCR) is frequently used for expression studies. The method is relatively fast, sensitive and simple and gives information about expression of a specific transcript in a cell population or tissue. However, this method cannot provide spatial patterns of expression, which instead is possible using RNA *in situ* hybridization. Similarly, information about transcript sizes and their relative intensities are best determined by Northern analysis.

PCR is a method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers that each hybridizes to opposite strands and flanks the region of interest in the target DNA (or cDNA). Basically, a PCR reaction involves a repetitive series of cycles with template denaturation at 93-95 ˚C, cooling down to 50-70 ˚C to allow for primer annealing, and extension of the annealed primers by a heat stable DNA polymerase at 70-75 ˚C. The result is an exponential accumulation of the specific fragment, limited by the 5´end of the primers. The annealed primers are incorporated into the newly synthesized strand. The 3´

ends are variable in length during the first cycle but become fixed in the next turn since synthesis cannot proceed past the terminus of the template strand (Fig 13). Consequently, after a few cycles the desired fixed length product will begin to predominate. After 25 cycles there are about 10^5 copies of the target sequence, an amount clearly detectable at agarose gel electrophoresis.

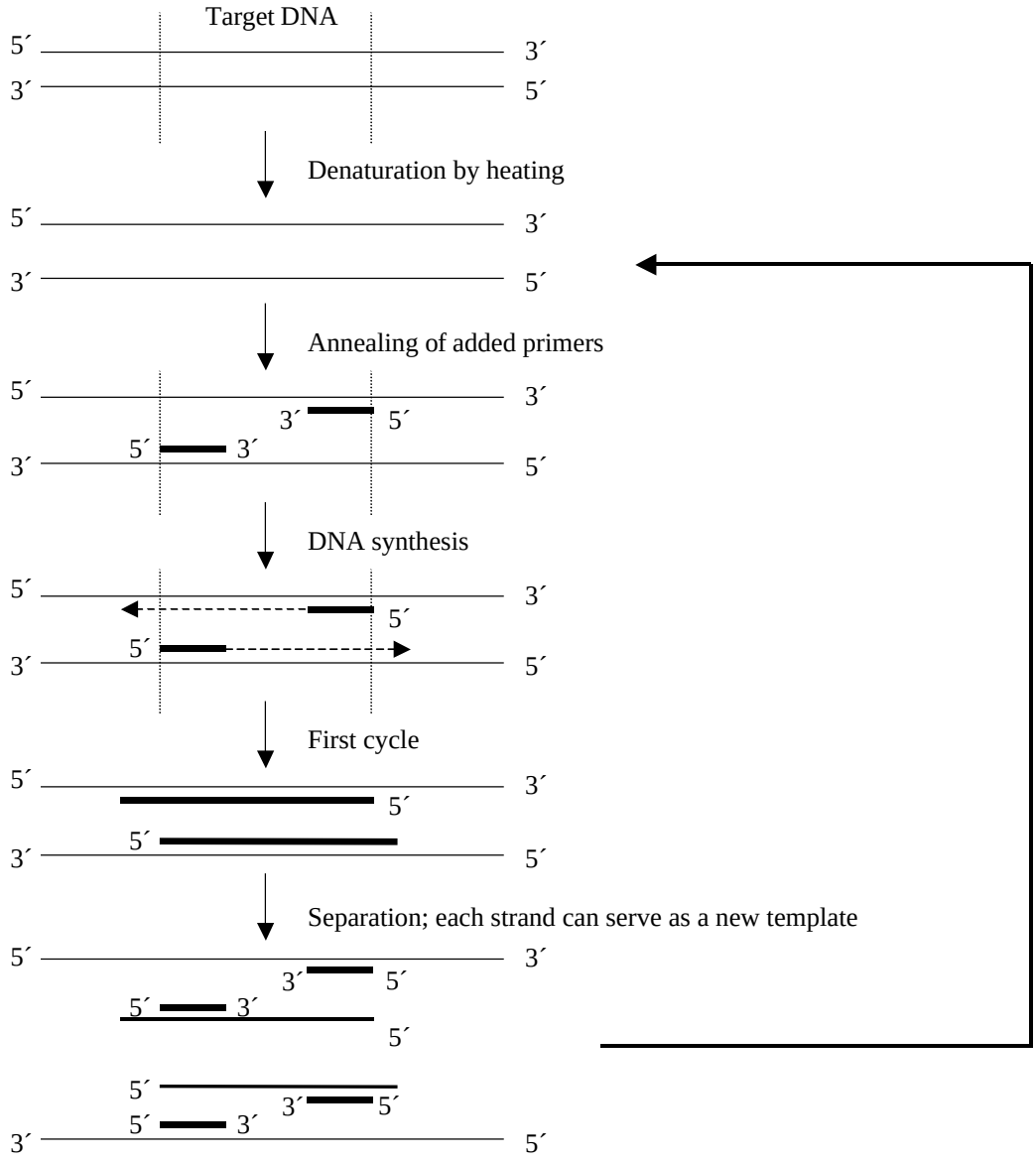

Figure 13. Principles for the PCR method, allowing for amplification of DNA sequences using oligonucleotide primers. Bars in bold indicate the initial localization of the primers which are incorporated into the newly synthesized strand. After completion of each cycle, the strands are separated and can serve as new templates, to which new primers will bind.

RT-PCR was used to detect expression of the tyrosine kinase domain of *RET* (*RET*-TK), wild type *RET* (WT-*RET*) and the fusion oncogenes *RET/PTC* 1-4 in a panel of PTC tumors. Since the chromosomal breakpoint for the rearrangements is located within intron 11, RT-PCR was a suitable method for analysis. Total RNA was extracted from the tumors using commercially available kits. A reverse transcriptase enzyme and non-specific hexanucleotide primers were then used to synthesize the first strand, which is complementary to the RNA template. In the resulting cDNA, the regions of interest were amplified by PCR using different sets of primers (Fig 14). Additional primer sets were used for exons 15-17 and exons 6-8. The specificity of the PCR products was confirmed by restriction enzyme cleavage and gel electrophoresis. In addition, PCR products indicative of a *RET/PTC* rearrangement were all sequenced. The integrity of the cDNA was confirmed using primers for *GAPDH* or β_2 *– microglobulin.* Screening for calcitonin, to exclude *RET* expression from C-cells present in the specimens, was negative in all samples.

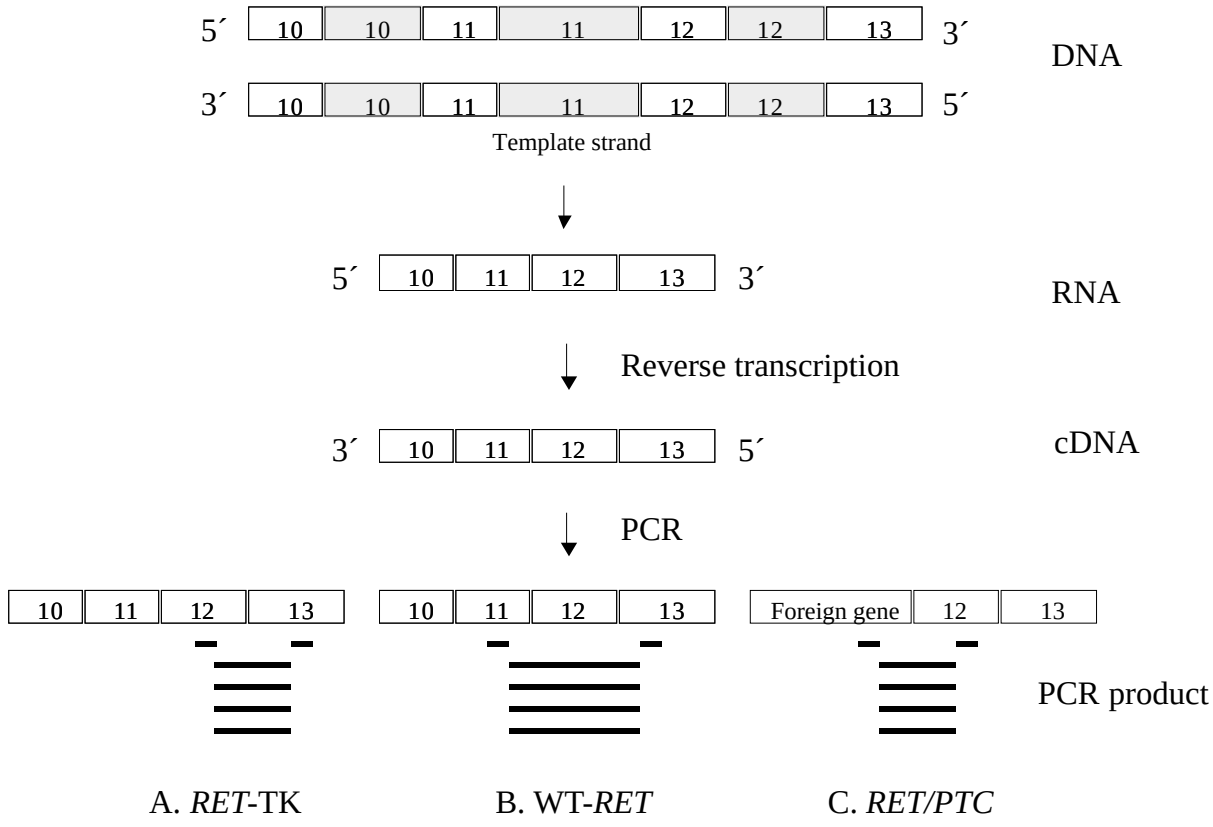

Figure 14. The part of the *RET* gene spanning the regular breakpoint for the *RET/PTC* rearrangements. cDNA was prepared from total RNA followed by PCR using primers in exons 12 and 13 to detect *RET*-TK expression (A), in exons 11 and 13 for WT- *RET* expression (B), and primers specific for the rearrangements *RET/PTC* 1-4 (C).

5.2.3 Comparative genomic hybridization (Paper IV)

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique, which permits screening of the entire genome for DNA sequence copy number changes in a DNA sample, e.g. from a tumor specimen (Fig 15). Chromosomal regions with increased copies of a given DNA sequence (gains or amplifications) or where one or both copies are lost (losses) can thus be detected, and are interpreted as potential sites for oncogenes and tumor suppressor genes, respectively (Kallioniemi 1992).

In this study CGH was performed on high molecular weight DNA isolated from samples of fresh frozen PTC tissue. The extracted DNA was labeled with a green fluorochrome (FITC-dUTP) by nick translation and mixed together with normal sex-matched reference DNA, labeled with a red fluorochrome (Texas red-dUTP), and unlabelled Cot-1 DNA which prevents binding of repetitive sequences present in both the test and reference genomes. The mixture was then denatured and hybridized onto commercial slides with denatured normal chromosomes in metaphase, the phase of cell division during which the chromosomes are fully condensed and visible in light microscope. The slides were then washed and stained with DAPI, which gives rise to a Q-like banding pattern and facilitates the recognition of the individual chromosomes. During the hybridization process, tumor and normal DNA will bind to their complementary sequences in the target metaphase chromosomes in a concentration dependent manner. Consequently, gains of DNA sequences can be seen as green regions on the target chromosome and losses are visualized as red parts. The differences may be visualized in the microscope but the detection of imbalances was made by digital image analysis of hybridization intensities along the length of all chromosomes, and ratio profiles were determined by computer analysis. Data from several metaphase preparations were combined to improve the signal to noise ratio. In Paper IV, a minimum of 12 ratio profiles from six separate metaphases was analyzed for each chromosome. Green-to-red ratios exceeding 1.20 were scored as gains, ratios less than 0.80 as deletions, and regional aberrations with a ratio >2 were regarded as high-level amplifications. Heterochromatic regions around the centromeres and the paracentromeric parts of some chromosomes (1, 9, 16 and 19), as well as the short arms of the acrocentric chromosomes (13-15, 21 and 22) and the Y chromosome, were excluded from the evaluation. CGH is not able to detect balanced chromosomal aberrations such as translocations and inversions where there is no change in the DNA sequence copy number. Under optimal conditions, the limit for the detectable size of

an aberration is approximately 2-4 Mb (Piper 1995, Kirchhoff 1999). Alternatively, for a small region to be detectable by CGH the increase in copy numbers must be many fold. For example, a 50-fold increase of a 300 kb region around the *myc* oncogene was detectable with CGH in a colon cancer cell line (Kallioniemi 1992).

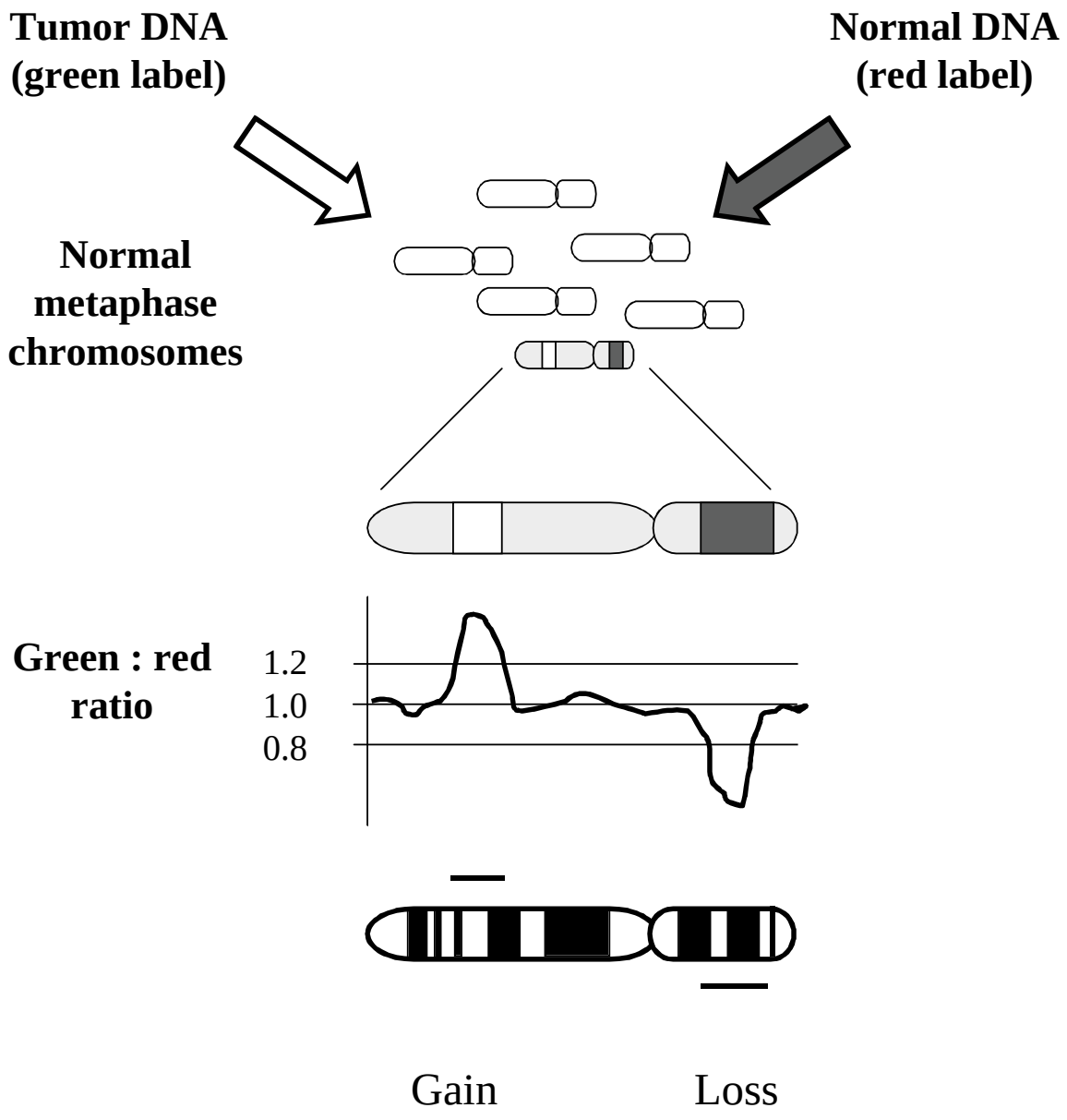

Figure 15. Principles for the detection of chromosomal imbalances by CGH. Differently labeled tumor and reference DNA are simultaneously hybridized to normal metaphase chromosomes. Location of sequence copy number changes are determined from the relative ratio between red and green fluorescence.

5.2.4 Statistical analysis

The statistical analyses were performed using either the Statistica, SAS or SPSS for Windows, or StatsView software. The methods applied basically consisted of Chi^2 or Fisher´s exact tests or logistic regression analyses, to test for significant associations between dichotomized variables such as patient and tumor characteristics on the one hand, and clinical outcome on the other. The significant variables thus identified were subsequently examined by stepwise logistic regression in order to identify independently significant variables. To compare levels of a continuous variable in two groups, the Mann-Whitney-U test was applied (Paper IV). Probabilities of less than 0.05 were regarded as significant.

6. Results

6.1 PTC in Stockholm – evaluation of prognostic factors (Paper I)

In this study, 220 patients operated on for PTC between 1980 and 1999 at the Karolinska University Hospital, Solna were reviewed retrospectively. Patient and tumor characteristics at the time of surgery were compared to the outcomes. In addition, patients were classified according to the pTNM, AMES and MACIS prognostic scoring systems (see section 3.4.4) in order to evaluate the validity of these systems when applied to the present PTC series. Analyses employing univariate and multiple logistic regression models were then used to identify independently significant prognostic factors with respect to the outcome in PTC patients from the Stockholm region.

PTC incidence

The average number of patients operated on for PTC increased gradually during the study period from 12 during 1980-1984, to 99 between 1995 and 1999 (Fig 16). This 10-fold increase cannot entirely be explained by an increase in the number of patients referred to our clinic. In fact, the incidence of PTC in Sweden has steadily increased during the period of 1957-1987, especially in women (Lundgren 2003). According to the National Cancer Institute, the rate of increase in the incidence of thyroid cancer among women in the United States is presently more rapid than for any other type of cancer. An increase in the incidence of thyroid cancer in France was also recently reported (Leenhardt 2004). Improved diagnostic methods have been suggested as one explanation for the increased incidence. However, if this was the reason, other types of thyroid cancer would show a similar increase in incidence, which appears not to be the case.

According to the WHO standard, tumors measuring 1 cm or less were classified as microcarcinomas (Hedinger 1989). Of the 220 samples included in the study, 58 PTC (26%) were microcarcinomas. Thirty-nine of these were diagnosed preoperatively and the remaining 19 were incidental findings at thyroid surgery for other reasons. Microcarcinomas constituted about 10% of the total number of PTCs diagnosed during 1980-1989, which increased to 31% during 1995-1999 (Fig 16). Increased health awareness in the general population and better diagnostic tools, facilitating earlier detection, may explain the increased fraction of microcarcinomas. Still, the reason for the over-all increase in PTC incidence is yet unknown.

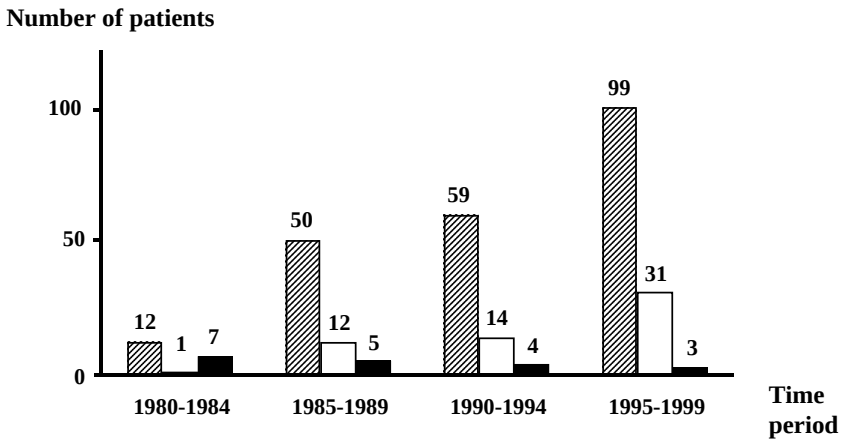

Figure 16. The total number of patients operated (hatched column), the total number of these patients who died from PTC (black column) and the total number of microcarcinomas (white column) during each 5-year period between 1980 and 1999.

Outcome

The surgical procedure was considered to be curative in 97% of the patients. The remaining six patients suffered from persisting disease. By the end of the study period 16 patients (7.5%) had died with or from PTC and three patients were alive with generalized disease, and those were all classified as having a poor clinical outcome. Of the remaining 201 patients, 180 were alive with no signs of PTC and 21 had died from other reasons but without signs of PTC (Fig 17). Recurrences were detected in 30 (14%) of the 214 patients initially considered as being cured.

Treatment and outcome

The standard treatment was total or near-total thyroidectomy, which was applied for 79% of the patients. Lobectomy or resection only was carried out in the remaining 21% of the patients. No difference in outcome was noticed between patients in these two groups.

In 16 patients (7%) the surgeon could not achieve macroscopical radicality. In another 15 patients (7%), judged as radically operated by the surgeon, the histopathological examination revealed an incomplete excision. Only five of the 16 patients (31%) in whom macroscopic radicality could not be achieved are today alive without disease, as compared to 91% of the radically operated (Fig 17). Indeed, macroscopic non-radical surgery was the most powerful

predictor for non-curable disease. In addition, failure to achieve radicality (both macroscopic and microscopic) was the strongest independent predictor for both local and distant recurrences. In connection with surgery, lymph node metastases were detected in 43% of the patients, the presence or absence of which appeared to have no influence on the outcome.

Postoperative measurement of I^{131} uptake was performed in 84% of the patients undergoing total thyroidectomy. Of these, 73% exhibited an increased uptake indicating the presence of remnant thyroid tissue (normal or tumorous). There was no difference in outcome between patients with increased uptake (≥ 1%) as compared to those with low or no uptake. In patients with elevated uptake, an ablative dose of I^{131} was administered.

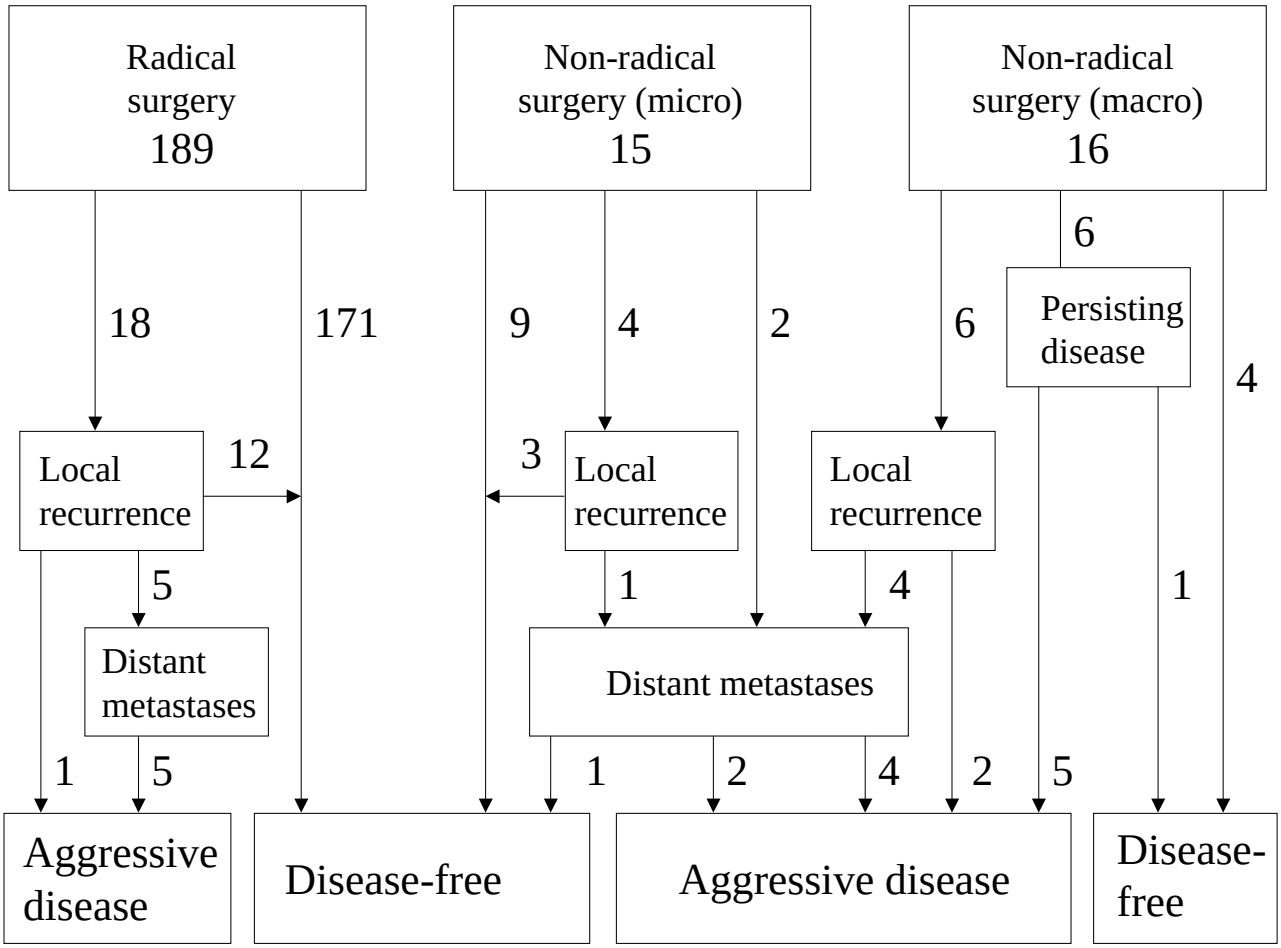

Figure 17. The outcome for patients treated with radical surgery, microscopic (micro) or macroscopic (macro) non-radical surgery.

Age and outcome

Three of the 14 patients (21%) who died were 33, 35 and 42 years old at diagnosis, which is not in line with the general idea that patients aged < 45 at diagnosis are at almost no risk of dying from PTC. Still, older patients developed local recurrences and distant metastases more frequently (≥ 55 and ≥ 60, respectively) than the younger patients, which in turn was associated with poor outcome. It should be mentioned that surgical radicality could not be achieved in any of the three young patients who died.

Prognostic scoring systems

The pTNM system (Table 2) classifies only patients ≥ 45 years of age who have distant metastases at diagnosis into stage IV (highest risk group). Accordingly, only one of the 220 patients in our study belonged to stage IV. In addition, patients aged < 45 are considered as having an excellent prognosis in spite of the presence of distant metastases and extensive local tumor growth, and patients with lymph node metastases are assigned to stage III if aged ≥ 45. When our patients were staged according to pTNM, the low mortality rate for stage I patients (2%) was confirmed. From the single patient in stage IV conclusions could not be drawn. Furthermore, patients in stages II and III had similar outcomes with 86.5% and 83% of the patients being disease-free at the end of follow-up.

When the AMES system is used, all patients with distant metastases are classified into the high-risk group, but younger patients are still regarded as having a low (2%) risk for PTC related mortality, even if the tumor is not resectable due to extensive local growth. This is not in line with our results as young age apparently does not guarantee disease-free survival, at least not if radical surgery cannot be performed. Of the patients assigned to the low-risk group, 97% were disease-free at the end of the study period, as compared to 73% of the patients in the high-risk group.

The MACIS system takes completeness of resection into account. Using the MACIS system, 99% of the patients classified into Group I (lowest risk group) were still disease-free at the end of follow-up, as compared to only 60% of the patients in Group IV (the highest risk group). Thus, the MACIS system appeared to most accurately predict the outcome in the present patient series.

6.2 Assessment of the proliferative activity (Paper II)

Although several risk factors for tumor recurrence and PTC related mortality have been established (Table 1), some of the individuals who are classified as low risk patients will still experience an unfavorable outcome. Assessment of the proliferative activity using the MIB-1 antibody has been useful in predicting survival in a variety of human neoplasms, for example breast carcinomas. To investigate if MIB-1 immunoreactivity could add prognostic information to the conventional prognostic variables, 30 PTC tumors were subjected to immunohistochemical staining procedures (see section 5.2) using the MIB-1 antibody. For comparison, 10 FTC, 8 ATC and 96 FTA together with recurrent tumors from eight of the primary tumors were similarly analyzed.

MIB-1 index in different types of thyroid tumors

The median MIB-1 index was lowest in the FTAs and highest in the ATCs. In the FTCs the MIB-1 index was higher than in the PTCs although this difference was not significant (Fig 18). The difference in MIB-1 index between FTAs and FTCs was significant, but as a substantial overlap of single values occurs, MIB-1 index appears not to be useful for the discrimination between FTA and FTC.

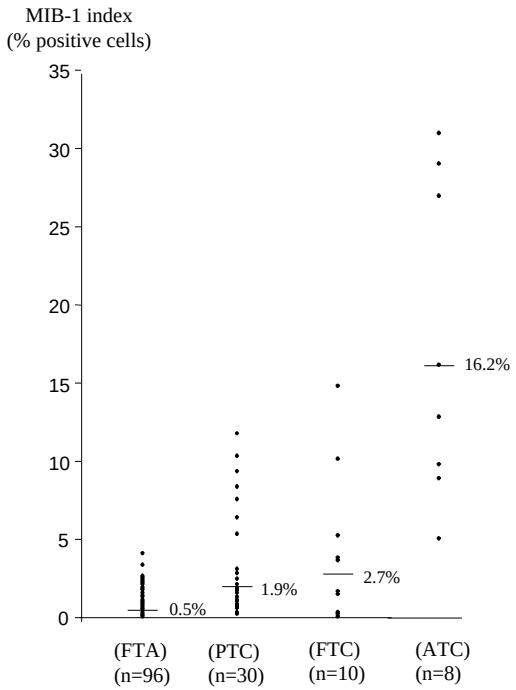

Figure 18. MIB-1 index in different types of thyroid neoplasm. Each tumor is represented by a dot and the median MIB-1 index for each subgroup is marked with a horizontal line.

MIB-1 index in PTC

The 13 patients who were regarded as having aggressive PTC included those who died from the disease, those with persisting PTC, together with those exhibiting distant metastases. The remaining 17 had an excellent clinical outcome with no evidence of PTC at the end of follow-up. Patients with aggressive PTC had significantly higher MIB-1 index (median: 5.4%, range 0.6-11.8), as compared to those with non-aggressive PTC (median: 1.1%, range 0.3-1.9), (Fig 19). A MIB-1 index of 1.85% or more was defined as the optimal cut-off value for separating aggressive tumors from their non-aggressive counterparts. Predictive values for this cut-off level are summarized in Table 6. MIB-1 index ≥ 1.85 was found to be an independently significant predictor of aggressive PTC, and so was large tumor size. The disease-free survival in patients with tumors with MIB-1 index ≥ 1.85 and < 1.85 is compared in Fig 20.

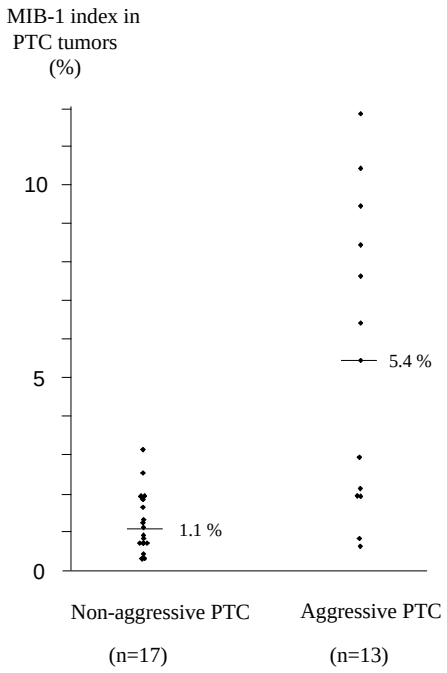

Figure 19. Comparison of MIB-1 index in tumors from patients with aggressive PTC (left) and non-aggressive PTC (right). Each tumor is represented by a dot and the median MIB-1 index for each subgroup is marked with a horizontal line.

However, the usefulness of MIB-1 index in separating tumors likely to be or develop to aggressive ones from the rest is somewhat limited as overlap of single values occurs. Still, a MIB-1 index well above or below the defined cut-off value appears to add prognostic information. For example, all tumors with MIB-1 index > 3.1% were diagnosed or developed

into aggressive tumors. Similarly, all patients with MIB-1 < 0.6% experienced an excellent clinical course giving positive and negative predictive values of 100%, respectively.

Table 6. Predictive values of MIB-1 index in patients with aggressive or non-aggressive PTC

	MIB-1 index ≥1.85%
Sensitivity	85%
Specificity	76%
Positive predictive value (PPV)	73%
Negative predictive value (NPV)	87%
Diagnostic accuracy	77%

Sensitivity = (aggressive PTC with MIB-1 index ≥ 1.85 / total number of aggressive PTC),
specificity = (non-aggressive PTC with MIB-1 index <1.85 / total number of non-aggressive PTC),
PPV = (aggressive PTC with MIB-1 index ≥ 1.85 / total number of PTC with MIB-1 index ≥ 1.85),
NPV = (non-aggressive PTC with MIB-1 index <1.85 / total number of PTC with MIB-1 index <1.85)
Diagnostic accuracy = (the number of correctly predicted PTC/the total number of PTC analyzed)

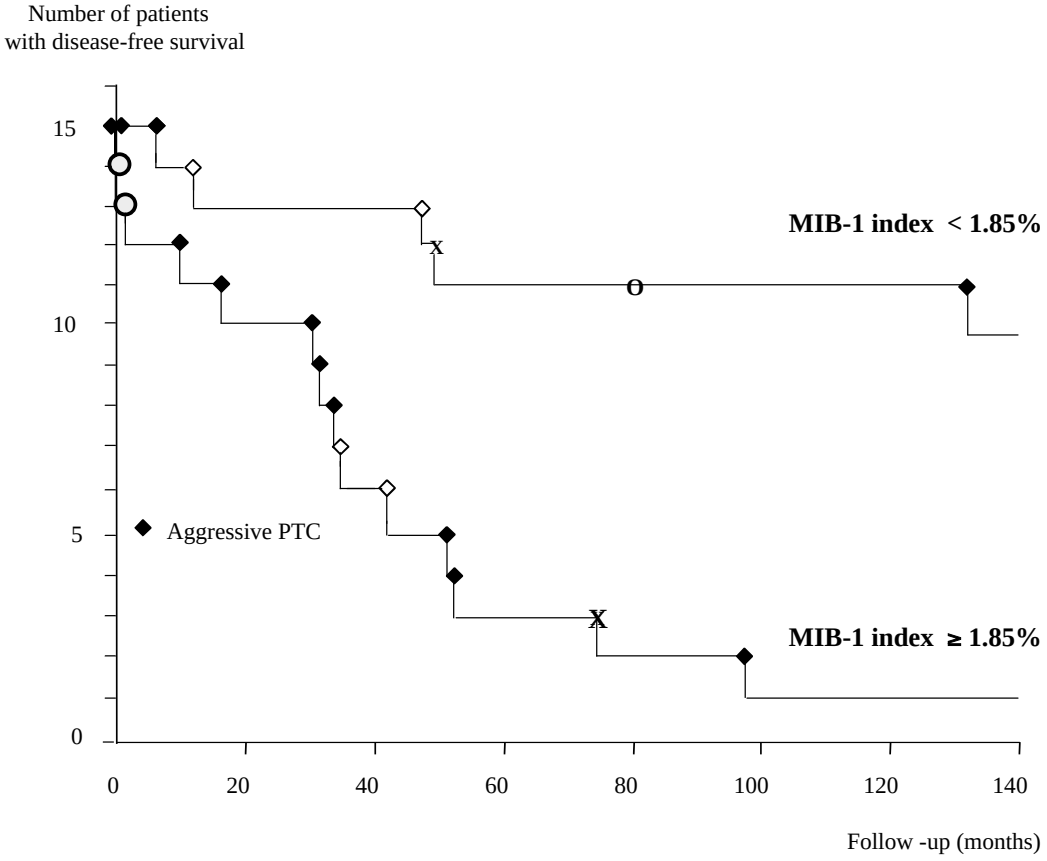

Figure 20. Disease-free survival in PTC patients with MIB-1 index < 1.85% and ≥ 1.85%. A solid rhomboid = occurrence of recurrence leading to persisting PTC or death from PTC. Open rhomboid = dead without signs of PTC; X = occurrence of a local recurrence which was successfully treated, grey circles, occurrence of distant metastases which was successfully treated.

MIB-1 index in recurrent PTC tumors

The recurrent PTC tumors all had higher MIB-1 index than their corresponding primary tumor. In case of recurrences, the MIB-1 index in the primary tumor tended to influence the time to recurrence (Fig 21).

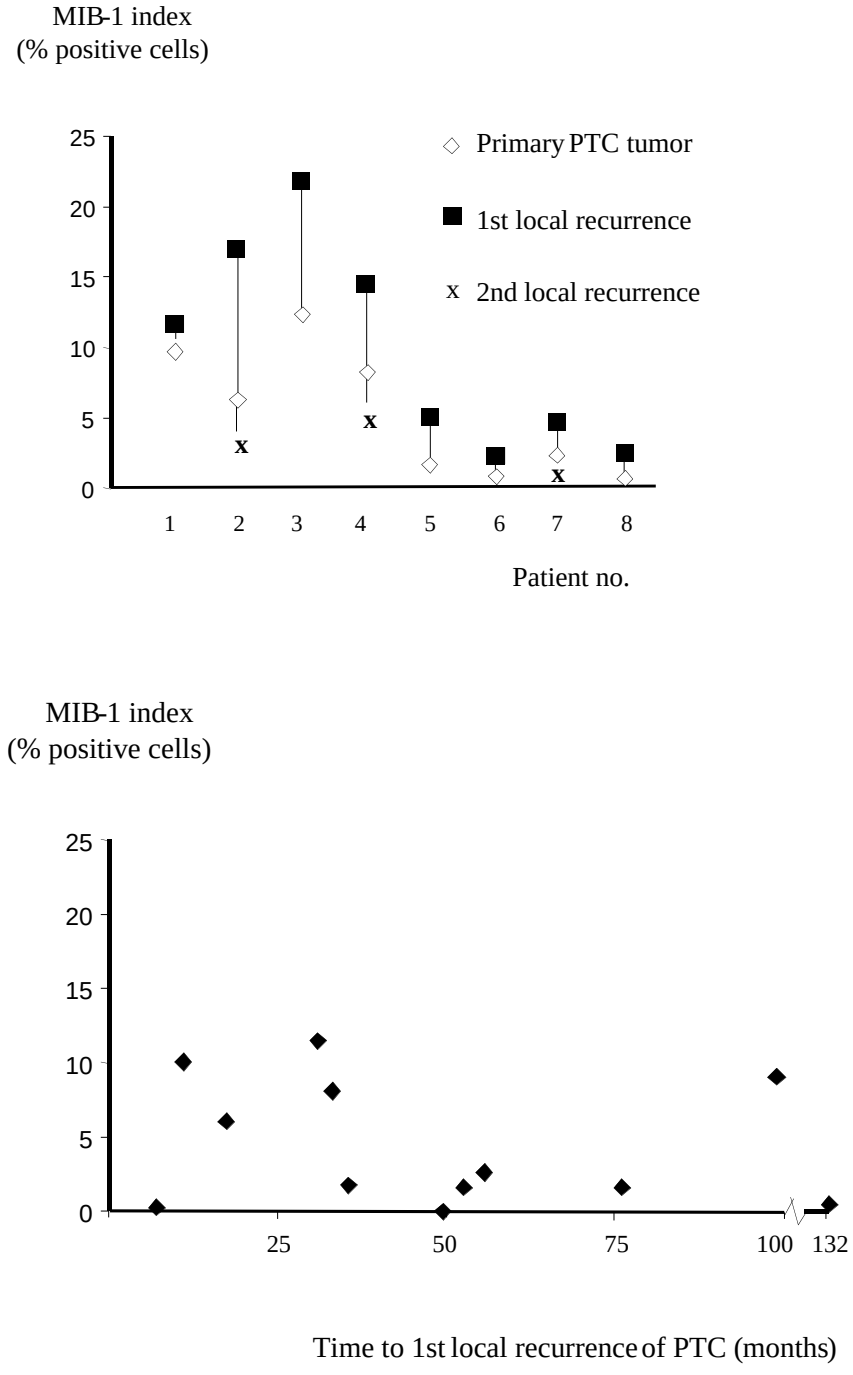

Figure 21. MIB-1 index in matched pairs of primary and recurrent PTC (top), and in relation to time to occurrence of the 1st local recurrence (bottom).

6.3 Expression of *RET* and *RET/PTC* (Paper III)

The reported prevalence of tumors harboring *RET/PTC* oncogenes varies considerably between different groups of patients with PTC (see section 3.5.3). However, in a subset of tumors, expression of the tyrosine kinase domain of *RET* (*RET*-TK) has been detected although presence of any of the *RET/PTC* oncogenes cannot be found. In the present study, 61 PTC were analyzed regarding expression of *RET*-TK, *RET/PTC* 1-4 or wild-type *RET* (WT-*RET*) using RT-PCR (see section 5.2.2). In addition, the experimental results were compared with the patient and tumor characteristics, as well as with the outcomes.

RET expression in different subgroups of patients

The patients were divided into four groups according to their clinical outcome (Table 7). Fourteen patients with aggressive PTC, including those who died from the disease, those with persisting PTC and those with distant metastases, were assigned to Group 1. Thirty-eight patients did not exhibit recurrence or distant spread during the follow-up period. Of these, 24 presented with lymph node metastases at the time of surgery (Group 2), while 14 never showed any signs of lymph node involvement (Group 3). The remaining nine patients experienced one local recurrence during follow-up, which was successfully treated (Group 4).

Altogether, *RET*-TK mRNA was detected in tumors from 29 of the patients investigated (48%). In 13 of these expression of exons encoding the extracellular part of *RET*, indicative of WT-*RET* expression, was also detected. These tumors all expressed *RET* exons 11-13, while *RET* exons 6-8 were only detected in eight of the tumors (Table 8). The discordance is most likely due to alternative splicing of *RET* mRNA (Fluge 2001). Whether or not expression of WT-*RET* or any of the splice variants play a role in PTC carcinogenesis is not entirely clear.

The *RET/PTC* oncogenes were found in three tumors only, which is less frequent than in most other reports. The rearrangements consisted of one *RET/PTC* 1, one *RET/PTC* 3 and one rare variant of *RET/PTC* 3, previously only described in a few patients from Belarus. In the *RET/PTC* 1 positive tumor, *RET* mRNA implying expression of WT-*RET* was concomitantly detected. In tumors from the remaining 14 patients expression of *RET*-TK only was noted. In these tumors yet unidentified *RET/PTC* rearrangements may be present. The prevalence of the various types of *RET* expression in the different groups of patients is outlined in Table 8.

Table 7: Criterias for inclusion of the patients with PTC into group 1-4

Characteristic	Group 1	Group 2	Group 3	Group 4
Dead from PTC	Yes	No	No	No
Persisting PTC	Yes	No	No	No
Distant metastases	Yes	No	No	No
Lymph node metastases	Yes	Yes	No	Yes
Recurrence	Yes	No	No	Yes
No of patients included in each group	14	24	14	9

WT-*RET expression correlates with aggressive PTC*

WT-*RET* expression was detected significantly more frequent in patients with aggressive PTC (7 of 14) than in those with non-aggressive PTC (6 of 47, $p<0.01$). WT-*RET* expression correlated well with staging of patients according to MACIS (see section 3.4.4), where those with MACIS score ≥ 7 expressed WT-*RET* significantly more often (7 of 18) than patients with MACIS <7 (6 of 43). Expression of WT-*RET* was found to be an independently significant risk factor for aggressive disease together with poor differentiation and tumor size > 40 mm. However, it is still not clear if the WT-*RET* expression is a secondary effect of tumor progression or an event initiating aggressive PTC development.

Table 8: Expression of *RET* variants in patients grouped according to their clinical outcome

Gene sequence	Primers in exons	Group 1	Group 2	Group 3	Group 4	Total
RET -TK	12-13, 15-17	10	14	3	2	29
WT-*RET*	11-13, 12-13, 15-17	7	5*	1	0	13*
	6-8	7	1	0	0	8
RET/PTC	fusion gene - 12	1	2	0	0	3
RET -TK only	12-13, 15-17	2	8	2	2	14

*one patient exhibited both *RET/PTC1* and WT-*RET*

RET expression and clinical and hisopathological variables

A significant difference in WT-*RET* expression was also noted between poorly differentiated (4 of 7) and well differentiated (9 of 54, $p<0.05$) PTC. *RET* expression of any kind could not be associated with sex, age at diagnosis, tumor size, extrathyroidal growth, tall cell PTC, lymph node involvement, distant metastases or single local recurrences.

RET/PTC expression has been proposed to occur in patients with small, slow-growing and clinically indolent tumors. However, one of our patients harboring the *RET/PTC3* variant was diagnosed with PTC infiltrating the surrounding soft tissue, and with pulmonary metastases.

6.4 Searching for genomic alterations (Paper IV)

To approach genetic mechanisms behind initiation and progression of PTC tumorigenesis, 25 primary PTC tumors were investigated regarding chromosomal imbalances using CGH (see section 5.4). The results were compared with the outcomes as well as to clinical and histopathological features at the time of surgery.

Overall, 21 of the 25 tumors investigated (84%) exhibited chromosomal imbalances with an approximately equal frequency of gains and losses. Recurrent alterations (found in ≥ 3 tumors) involved chromosomes 1, 9, 17, 22 and X, the distribution and subchromosomal regions of which are illustrated in Fig 23 and listed in Table 9. The number of alterations per tumor ranged from 0 to 13 (mean 2.8). The most frequent aberration, gain of 9q33-qter, was detected as the sole alteration in three tumors and equally distributed between aggressive and non-aggressive PTC, suggesting that this region may contain oncogenes involved in early initiating events.

Table 9: Recurrent CGH alterations in relation to the clinical outcome

	No of tumors with alteration			
CGH alteration	Total	AD (7 patients)	NAD (18 patients)	p-value
Gain 9q33-qter	7 (28%)	2/7	5/18	n.s
Gain X	5 (20%)	1/7	4/18	n.s
Gain 1q	4 (16%)	4/7	0/18	0.003
Gain 17q	4 (16%)	3/7	1/18	n.s
Gain 22q	3 (12%)	0/7	3/18	n.s
Loss 9q21.3-q32	3 (12%)	3/7	0/18	0.015
Loss 22q	3 (12%)	1/7	2/18	n.s
Mean number of genetic alterations	2.8	5.9	1.7	0.009

AD; aggressive disease
NAD; non aggressive disease

CGH alterations in relation to clinical outcome

Seven patients were classified as having an aggressive form of PTC, which included those who died from the disease, those with persisting PTC and those with distant metastases. PTC is generally thought to be a genetically stable tumor, which is confirmed by the low number of alterations detected in most PTC. However, the total number of genetic alterations was significantly higher in patients with aggressive disease as compared to those with non-aggressive disease (Fig 22, Table 9), thus aggressive phenotypes appear associated with increased genomic instability.

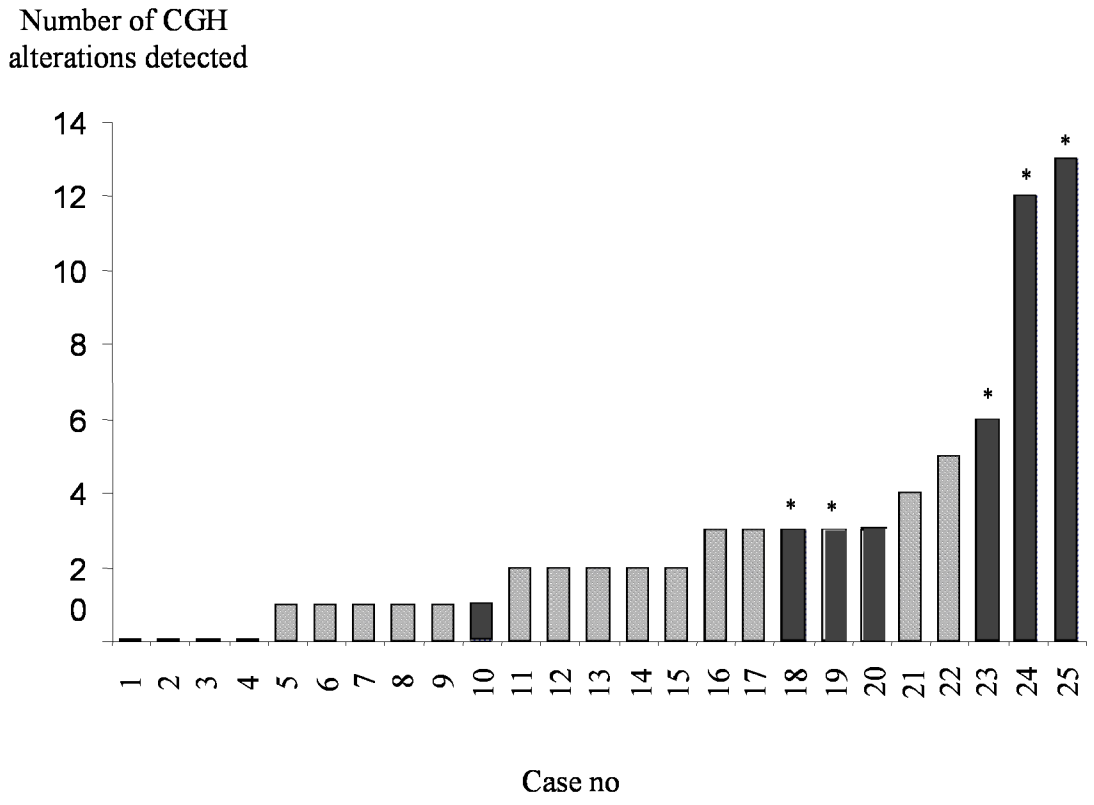

Figure 22. The number of CGH alterations detected in each tumor. Hatched bars indicates tumors from patients with aggressive PTC and filled bars tumors from patients with non-aggressive PTC. Those exhibiting distant metastases are marked with an asterisk.

Gain of 1q and loss of 9q21.3-q32 were seen exclusively in tumors from patients with aggressive disease (Table 9), suggesting the localization of genes involved in the progression towards aggressive PTC variants in these regions. For further understanding, identification of genes in these regions would be helpful.

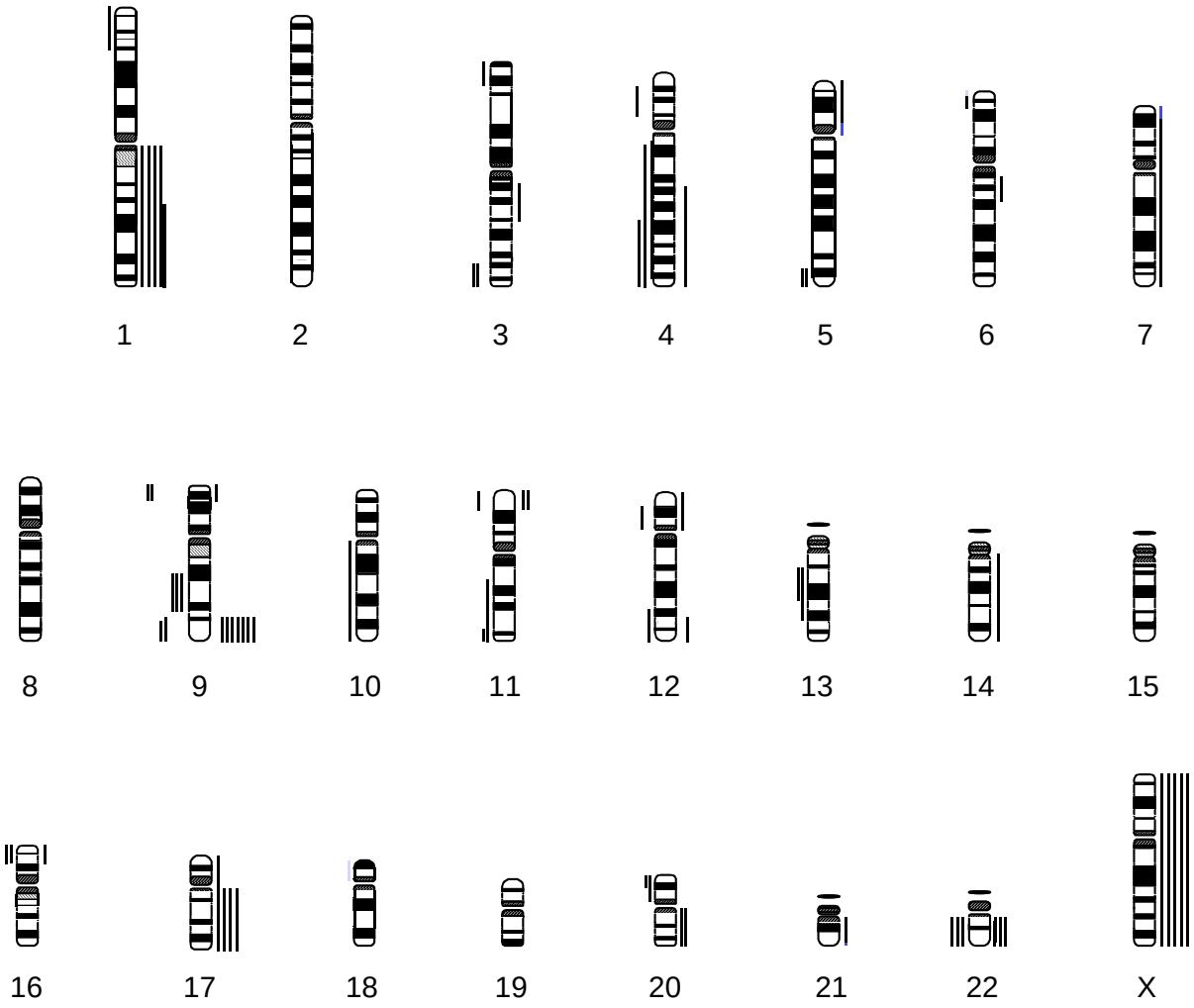

Figure 23. Illustration of DNA copy number changes detected by CGH. Each line represents one alteration detected in one tumor, with losses to the left and gains to the right. High level amplifications are shown by bold lines.

7. Discussion

Although the vast majority of patients with PTC carries an excellent prognosis, some patients will die from the disease. Most patients who are at increased risk of dying from the disease can be identified at the time of surgery when established prognostic variables or prognostic scoring systems are taken into account. However, identification of variables influencing the prognosis in PTC patients has only been carried out by retrospective studies. The drawback with this type of studies is that the patient samples included are heterogenous groups of patients, collected from several decades when progress was made in diagnosis and treatment of PTC. Thus, the results are often conflicting. Since PTC is a comparatively rare malignancy and recurrences can be detected up to 30 or even 40 years after treatment of the primary tumor, it would take very long time to complete a prospective study, although this would be of great interest. Since a subset of patients regarded as being at low risk for PTC related mortality will still die from the disease, one of the aims with the present thesis has been identification of additional tools, which can aid in the recognition of these patients at the time of surgery.

Assessment of the proliferative activity using MIB-1 proved to be such a tool. Although the theoretical cut-off value defined in Paper II should not be used as a watershed, separating aggressive PTC from their non-aggressive counterparts, MIB-1 immunoreactivity adds information if a tumor lacks all features of an aggressive tumor, but displays a MIB-1 index well above the 1.85%. This patient should perhaps be subjected to a more careful follow-up than the routine.

When established risk factors for PTC related mortality were evaluated, large tumor size was confirmed to influence the outcome among patients included in the present thesis, especially if the tumor grew with infiltration of the surrounding tissues so that the tumor was impossible to radically remove. All patients in whom radical surgery (macroscopic or microscopic) could not be achieved were subjected to postoperative adjuvant treatment (I^{131}, external radiation or cytotoxic drugs). Still these patients experienced an unfavorable outcome significantly more often than radically operated patients. Thus, removal of all tumor cells is important to a favorable outcome and may not be compensated for by postoperative treatment.

Young patients are considered to be at almost no risk of dying from PTC. However, this study proves that young age does not guarantee a disease-free survival, especially if the tumor cannot be radically operated. This is the reason why the MACIS prognostic scoring system, which takes completeness of resection into account, proved to be the most accurate system with respect to predicting the prognosis in patients with PTC.

The second overall aim with this thesis was to try to identify genetic factors involved in the initiation and progression of PTC tumors. The low frequency of tumors expressing the oncogenes *RET/PTC1-4* indicates that the majority of PTC from Swedish patients develops without involvement of these rearrangements. However, the detection of *RET*-TK only in a subset of tumors suggested the presence of yet unidentified rearrangements, the nature of which could be further investigated using, for example FISH.

In one fifth of the tumors investigated expression of WT-*RET* was detected. The role of this expression is not clear, but the fact that WT-*RET* expression was associated with aggressive PTC suggests that it is not just an unspecific and random event. *RET* expression whether it occurs through a rearrangement or is a result of activation of WT-*RET* through other mechanisms, may result in the transmission of proliferative signals to the nucleus. Thus, *RET* expression may be involved in PTC formation even in the absence of *RET/PTC*. However, depending on whether it is *RET/PTC*, WT-*RET* or any of the splice variants of *RET* that are expressed, the resulting RET protein may acquire different properties, which in turn can give rise to different phenotypes.

In the future, we will hopefully be able to already preoperatively identify patients who are at risk of dying from PTC. If genetic or other markers can be applied at the time for surgery, future patients may benefit from a more individualized treatment modality. At the same time, those who prove to have a truly indolent tumor may be spared unnecessarily extensive treatment.

8. Conclusions

The clinical and molecular genetic studies herein have aimed at the identification of factors which could improve prognostic judgment and hence treatment of PTC patients. The main findings were:

▲ The number of patients treated for PTC seems to increase over time, especially microcarcinomas. The most important clinical features with respect to aggressively behaving PTC were: non-radical surgery and large primary tumor. Postoperative treatment cannot in all cases make up for remnant tumor tissue.

▲ MIB-1 index reflecting Ki-67 immunostaining may prove helpful in discriminating between indolent and aggressive PTC. Index <0.6 % and >3.1 % have predictive values of 100%.

▲ The *RET/PTC* rearrangements appear to be rare in Swedish patients with PTC. However, several show expression of the tyrosine kinase domain of *RET*, which is associated with an aggressive clinical picture.

▲ PTC is a comparably genetically stable tumor type, but aggressive variants show significantly increased number of chromosomal changes. Specifically, gain of 9q33-qter may be an early event in PTC development, and gain of 1q and loss of 9q21.3-q32 may be involved in the progression towards aggressive variants of PTC.

▲ Several of the genetic factors described here need further evaluation before they are useful in the clinical setting. However, in the future they may prove be a valuable tool in the search for better and more individual treatment strategies in patients with PTC.

9. Sammanfattning

Exklusivt för Er som har andra intresseområden än ”sköldkörtel-cancer-forskning” men ändå vill få en liten inblick i vad jag egentligen hållit på med:

Thyreoideacancer (cancer i sköldkörteln) utgör ca 1 % av all cancer. I Sverige drabbas 3-400 individer årligen. Det finns fyra varianter: papillär thyroideacancer (PTC), follikulär thyreoideacancer, medullär thyrodeacancer och anaplastisk thyreoideacancer. PTC är den vanligaste, och står för ca 60-80 % av all thyreoideacancer. De flesta patienter som drabbas av PTC har en utmärkt prognos med en 10-årssöverlevnad på över 90 %. Dessvärre kommer några patienter att få återfall (recidiv) av sin sjukdom, utveckla fjärrmetastaser (t ex i lungorna) och slutligen dö av sin PTC. Dessa patienter bedöms ha aggressiv PTC. För att på ett så tidigt stadium som möjligt (helst redan innan operation) kunna identifiera de patienter som löper ökad risk att utveckla en aggressiv PTC, så har ett stort antal parametrar utvärderats och en del av dessa har föreslagits ha betydelse för prognosen (t ex tumörens storlek, patientens ålder samt utbredd tumörväxt). Trots detta kommer en del patienter att felbedömas, dvs av de som klassificeras som tillhörande lågriskgruppen kommer några i alla fall att utveckla aggressiv PTC och dö. Ytterligare markörer som kan förbättra särskiljandet av patienter med ökad risk att utveckla aggressiv PTC vore därför önskvärt.

I den första studien (Paper I) studerades samtliga 220 patienter som opererats för PTC på Karolinska Universitetssjukhuset, Solna under åren 1980-1999. Egenskaper hos patienterna (t ex kön och ålder) och deras tumörer (t ex storlek, växt i omgivande vävnad, förekomst av metastaser i lymfkörtlar eller distalt, samt huruvida kirurgen lyckats få bort all tumörvävnad) jämfördes med hur det gått för patienterna. De faktorer som medförde en signifikant ökad risk för utvecklandet av aggressiv PTC visade sig vara stor tumör samt om all tumörvävnad ej kunnat opereras bort. Detta ska inte tolkas som att prognosen i första hand är beroende av kirurgens skicklighet, utan är snarare ett tecken på att tumören växer ut i omgivande vävnad på ett sådant sätt att det även för en erfaren kirurg är omöjligt att få bort all tumörvävnad.

I den andra studien (Paper II) utvärderades celldelningshastigheten i 30 PTC. Alla celler som befinner sig i delning uppvisar ett protein som kallas Ki-67 i cellkärnan. Med hjälp av antikroppen MIB-1 som känner igen och binder till just Ki-67-proteinet, varvid cellen färgas brun, kunde andelen celler som befinner sig i delning beräknas i tunna snitt av PTC. Det visade sig att de PTC som var eller utvecklades till aggressiva tumörer hade en betydligt

högre celldelningshastighet än ”vanliga” PTC. Hög delningshastighet utgör en oberoende riskfaktor för utvecklandet av aggressiv PTC.

RET är en gen vars protein stimulerar celldelning. Gener som stimulerar celldelning kallas ”onkogener” och gener som hämmar celldelningen kallas ”tumör-suppressor-gener”. RET tillhör alltså gruppen onkogener. Förenklat kan man säga att tumörutveckling involverar överaktivering av olika onkogener och/eller förlust av tumör-suppressor gener. I normal thyreoideavävnad är RET en inaktiv gen. RET har dock visat sig kunna aktiveras t ex genom sk translokation, vilket innebär att en kromosom bryts av och fogas ihop med en annan kromosom, vilket resulterar i en translokationsgen. Translokationen leder till att RET aktiveras, dvs dess celldelnings-stimulerande protein börjar produceras. Detta utgör ett första steg mot utveckling av PTC. Att translokationer kan stimulera celldelning och därmed leda till tumörutveckling är sedan länge känt för olika blodmaligniteter (t ex leukemi och lymfom). Det är dock relativt nyligen som man börjat misstänka att translokationer ligger bakom en hel del även av de solida tumörerna. I den tredje studien (Paper III) undersöktes huruvida RET-genen var aktiv i 61 PTC. Det visade sig att ca hälften av tumörerna hade en aktiv RET-gen. Dock hade endast tre av dessa hade en känd translokationsgen som kunde förklara varför RET var aktiv. I ett 10-tal tumörer fanns indikationer på att ytterligare translokationer än de hittills kända kunde ligga bakom RET-aktiveringen. I ytterligare ca 10 fall fanns inga tecken på translokation utan RET tycks alltså kunna aktiveras av andra mekanismer. Resterande PTC tycks ha uppstått utan inblandning av RET genen. Aktivering av RET såg signifikant oftare i de aggressiva tumörerna.

Med hjälp av den sk CGH-metoden kan man undersöka en tumörs kromosomer för att se om några delar har förlorats eller mångfaldigats. I områden som ”tappats” kan man då tänka sig att tumör-suppressor-gener kan vara lokaliserade, vars förlust har lett till tumöruppkomst. På samma sätt kan områden som mångdubblats innehålla onkogener där den ökade mängden celldelnings-stimulerande proteiner givit upphov till ökad celldelning och därmed cancer. I den fjärde studien (Paper IV) kunde två områden som mångdubblats identifieras på kromosom 1 och 9. Detta fynd gjordes endast i de aggressiva PTC. Man kan därför fundera på om onkogener som har betydelse för utvecklingen mot mer aggressiv PTC finns lokaliserade i just dessa områden.

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