Thesis for doctoral degree (Ph.D.) 2009

Molecular Aspects of Parathyroid Tumorigenesis - with Focus on Parafibromin and the Wnt Pathway

Christofer Juhlin

Karolinska Institutet

Thesis for doctoral degree (Ph.D.)
2009

Molecular Aspects of Parathyroid Tumorigenesis

- with Focus on Parafibromin and the Wnt Pathway

Christofer Juhlin

Karolinska Institutet

From the Departments of
Molecular Medicine and Surgery / Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

MOLECULAR ASPECTS OF PARATHYROID TUMORIGENESIS

- WITH FOCUS ON PARAFIBROMIN AND THE WNT PATHWAY

Christofer Juhlin

Stockholm 2009

Supervisors

Catharina Larsson, M.D, Ph.D, Professor
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Anders Höög, M.D, Ph.D
Department of Oncology-Pathology
Karolinska Institutet, Stockholm, Sweden

Andrea Villablanca, B.Sc, Ph.D
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Faculty Opponent

Peter Stålberg, M.D, Ph.D, Associate Professor
Department of Surgery
Uppsala University Hospital, Uppsala, Sweden

Examination Board

Peter Zaphiropoulos, M.Sc, Ph.D, Professor
Department of Biosciences and Nutrition
Karolinska Institutet, Stockholm, Sweden

Ylva Pernow, M.D, Ph.D, Associate Professor
Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

Johan Botling, M.D, Ph.D
Department of Genetics and Pathology
Uppsala University Hospital, Uppsala, Sweden

Front cover: Parathyroid tumour cells engaging the capsule. Picture taken by the author.

Published and printed by Larserics Digital Print AB
Landsvägen 65, 172 65 Sundbyberg

ISBN 978-91-7409-682-8

”Nature is never governed by a single solution”

Anders Höög, Endocrine Pathologist

ABSTRACT

Primary hyperparathyroidism (PHPT) denotes the tumorous enlargement of one or several parathyroid glands and constitutes a common disorder, particularly pronounced among postmenopausal women. PHPT patients display hypercalcemia as a consequence of parathyroid hormone hypersecretion, and the symptomatology is habitually based on this metabolic aberrancy. Parathyroid adenomas constitute the vast preponderance of PHPT (80-85%), multiple gland disease/hyperplasia comprises 15% and the malignant parathyroid carcinomas represent about 1% of all cases. Although recent genetic advances have improved our understanding regarding the development of these tumours, a great deal regarding the molecular backdrop to parathyroid tumorigenesis remains obscure.

In **paper I**, we investigated the frequency of allelic loss at the known parathyroid tumour suppressor *MEN1* locus at 11q13 as well as in chromosome regions 1p and 6q in two different patient groups, namely screening-detected (all asymptomatic) and routine clinical practice cases (a large fraction with symptomatic disease). Loss at 1p, 6q and 11q were commonly found in similar frequencies suggesting the presence of putative tumour suppressor genes at 1p and 6q. Allelic loss of 1p and 11q was predominantly demonstrated in screening detected patients and appear to confer a more mild-mannered disease, whereas loss at 6q was primarily established among patients recruited from routine clinical practice exhibiting a more clinically explicit hyperparathyroidism.

In **paper II**, we turned to parafibromin, a protein derived from the *HRPT2* tumour suppressor gene recently implicated in the hereditary hyperparathyroidism-jaw tumour (HPT-JT) syndrome as well as in parathyroid malignant disease. We demonstrated that the *HRPT2* gene and parafibromin is expressed in various human tissues, and showed that parafibromin is a predominantly nuclear protein. Moreover, three parathyroid adenomas carrying *HRPT2* gene mutations were devoid of parafibromin expression, and two cases with wildtype *HRPT2* genotype exhibited aberrantly sized parafibromin. All remaining tumours exhibited parafibromin expression, suggesting that loss of parafibromin is a rare event among parathyroid adenomas. In addition, our results support the notion that *HRPT2* exhibits tumour suppressor properties in the parathyroid glands.

Parathyroid carcinomas can only be diagnosed if exhibiting an invasive growth pattern or metastases. Since the *HRPT2* gene is mutated in the majority of parathyroid malignant tumours, parafibromin has subsequently been proposed as a distinguishing marker for the detection of parathyroid cancer. In **paper III**, we examined a large group of parathyroid tumours by immunohistochemistry (IHC) using four parafibromin antibodies. The majority of unequivocal parathyroid carcinomas demonstrated reduced parafibromin expression as opposed to retained expression in all benign tumours examined. We conclude that parafibromin IHC can be used as an adjunct, but not a solitary marker when assessing parathyroid tumours, as a positive finding is indicative of benign disease while reduced or negative expression could either signify a parathyroid carcinoma or an *HRPT2*-mutated parathyroid adenoma. Cases with reduced or absent parafibromin expression should be subjects to *HRPT2* gene sequencing to detect possible familial disease.

Following the discovery that parafibromin is associated to the wingless (Wnt) signaling pathway, we assessed a number of Wnt pathway members **in paper IV** by IHC to investigate whether any of these proteins could be of additional value in separating malignant and benign parathyroid disease. We discovered that adenomatous polyposis coli (APC) expression was completely absent in the majority of carcinomas but retained in all adenomas studied, and propose that APC immunoscreening is a promising tool for the detection of parathyroid malignant tumours. Thus, in **paper V** we examined a group of atypical parathyroid tumours with unknown malignant potential for parafibromin and APC expression using IHC and found that a subset of atypical tumours displayed concurrent reduced levels of parafibromin as well as absence of APC expression, suggesting that a fraction of atypical tumours display a molecular phenotype similar to malignant tumours which may necessitate intensified follow-up. In addition, as a marker APC confer a higher specificity that parafibromin, however parafibromin might detect familial cases suitable for *HRPT2* gene screening, thus making both markers helpful when evaluating parathyroid tumours. Finally, in **paper VI** we discovered that the *APC* and *RASSF1A* promoters exhibited hypermethylation in the vast majority of parathyroid adenomas using bisulphite pyrosequencing. Moreover, parathyroid tumours generally display a genome-wide hypermethylation as compared to normal tissues indicated by *LINE-1* pyrosequencing. Hypermethylation of the *APC* promoter and global hypermethylation was associated to *MEN1* and *HRPT2* mutational status respectively, indicating that the corresponding proteins menin and parafibromin may exhibit epigenetic regulation properties in parathyroid cells.

LIST OF PAPERS

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

I. Correa P, **Juhlin C**, Rastad J, Åkerström G, Westin G, Carling T.
Allelic loss in clinically and screening-detected primary hyperparathyroidism.
Clinical Endocrinology (Oxf). 2002 Jan;56 (1):113-117.

II. **Juhlin C**, Larsson C, Yakoleva T, Leibiger I, Leibiger B, Alimov A, Weber G, Höög A, Villablanca A.
Loss of parafibromin expression in a subset of sporadic parathyroid adenomas.
Endocrine Related Cancer 2006 Jun;13 (2):509-523.

III. **Juhlin CC**, Villablanca A, Sandelin K, Haglund F, Nordenström J, Forsberg L, Bränström R, Obara T, Arnold A, Larsson C, Höög A.
Parafibromin immunoreactivity – its use as an additional diagnostic marker for parathyroid tumor classification.
Endocrine Related Cancer 2007 Jun;14 (2):501-512.

IV. **Juhlin CC**, Haglund F, Villablanca A, Forsberg L, Sandelin K, Bränström R, Larsson C, Höög A.
Loss of expression for the Wnt components adenomatous polyposis coli and glycogen synthase kinase 3-β in parathyroid carcinomas.
International Journal of Oncology 2009 Feb;34 (2):481-492.

V. **Juhlin CC**, Nilsson I-L, Johansson K, Haglund F, Villablanca A, Höög A, Larsson C.
Parafibromin and APC as screening markers for malignant potential in atypical parathyroid tumours.
Submitted manuscript

VI. **Juhlin CC**, Kiss N, Villablanca A, Haglund F, Nordenström J, Höög A, Larsson C.
Frequent promoter hypermethylation of the *APC* and *RASSF1A* tumour suppressors and *LINE-1* repeats in parathyroid tumours.
Submitted manuscript

RELATED PUBLICATIONS

1. Lu M, Forsberg L, Höög A, **Juhlin CC**, Vukojevic V, Larsson C, Conigrave AD, Delbridge L, Gill A, Bark C, Farnebo L-O, Bränström R. Heterogeneous expression of SNARE proteins SNAP-23, SNAP-25, Syntaxin1 and VAMP in human parathyroid tissue.
   Molecular and Cellular Endocrinology 2008 Jun;287 (1-2):72-80.

2. Geli J, Kogner P, Lanner F, Natalishvili N, **Juhlin CC**, Kiss N, Clark GJ, Ekström TJ, Farnebo F, Larsson C. Assessment of *NORE1A* as a putative tumor suppressor in human neuroblastoma.
   International Journal of Cancer 2008 Jul;123 (2):389-394.

3. Laurell C*, Velazquez-Fernandez D*, Lindsten K, **Juhlin C**, Enberg U, Geli J, Höög A, Kjellman M, Lundeberg J, Hamberger B, Larsson C, Nilsson P, Bäckdahl M. Transcriptional profiling enables molecular classification of adrenocortical tumours. *European Journal of Endocrinology* 2009 Jul;161 (1):141-152.

4. Lee JJ*, **Juhlin CC***, Foukakis T, Robinson BG, Zedenius J, Larsson C, Höög A. *HRPT2* gene screening and parafibromin expression analyses in thyroid carcinomas.
   Manuscript

5. Kiss NB, Muhr A, **Juhlin CC**, Geli J, Bäckdahl M, Höög A, Wängberg B, Nilsson O, Ahlman H, Larsson C. Acquired hypermethylation of the $p16^{INK4A}$ promoter in abdominal paraganglioma: relation to adverse tumor phenotype and predisposing mutation.
   Manuscript

6. Sofiadis A, Orre L, Dinets A, **Juhlin CC**, Foukakis T, Wallin G, Höög A, Zedenius J, Larsson C, Lehtiö J. Proteomic study of thyroid tumors reveals frequent up-regulation of the Ca^{2+}-binding protein S100A6 in papillary carcinoma.
   Manuscript

* Both authors contributed equally

Contents

LIST OF ABBREVIATIONS

aa	Amino acid
ABC	Avidin biotin complex
APC	Adenomatous polyposis coli
bp	Base pair
BRCA2	Breast cancer gene 2
Ca^{2+}	Calcium ion
CaSR	Calcium sensing receptor
CDKN2A	Cyclin-dependent kinase inhibitor 2A gene
CCND1	Cyclin D1 gene
Cdc73	Cell division cycle protein 73 homolog
Cdk	Cyclin dependent kinase
CTNNB1	Beta-catenin gene
hPAF	Human polymerase associated factor
HPT-JT	Hyperparathyroidism-jaw tumour
HRPT2	Hyperparathyroidism 2 gene
hTERT	Human telomerase reverse transcriptase
DNA	Deoxyribonucleic acid
DAB	Diaminobenzedine
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
FHH	Familial hypocalciuric hypercalcemia
FIHP	Familial isolated hyperparathyroidism
GFP	Green fluorescent protein
GSK3-β	Glycogen synthase kinase 3 beta
IHC	Immunohistochemistry
kDa	kilo-Dalton
LINE-1	Long interspersed nuclear elements-1

LOH	Loss of heterozygosity
LRP5	Low density lipoprotein receptor-related protein 5
Mdm2	Mouse double minute 2 homolog
MEN	Multiple endocrine neoplasia
MDR1	Multi-drug resistance protein 1
MPF	Maturation promoting factor
MS-PCR	Methylation-specific polymerase chain reaction
NLS	Nuclear localization signal
$p16^{INK4A}$	Protein 16 inhibitor of Cdk 4
PCR	Polymerase chain reaction
PHPT	Primary hyperparathyroidism
PTCH1	Patched 1
PTH	Parathyroid hormone
RAR-β	Retinoic acid receptor beta
RASSF1A	Ras association domain family 1 isoform A
Rb	Retinoblastoma
RET	Rearranged during transfection proto-oncogene
RIZ1	Retinoblastoma protein-binding zinc finger 1
RNAi	RNA interference
Shh	Sonic hedgehog
SHPT	Secondary hyperparathyroidism
SMO	Smoothened
THPT	Tertiary hyperparathyroidism
VEGF	Vascular endothelial growth factor
VDR	Vitamin D receptor
Wnt	Wingless type

INTRODUCTION

Fundamental genetics of tumour development

The tumour concept

The word *tumour* stems from the Latin word *tumor*, meaning “swelling” or “lump”. In the clinical context however, the term *tumour* is preserved for neoplastic tissues, i.e. tissues with an uncontrolled proliferation rate stemming from genetic alterations. A tumour in turn could be benign, premalignant or malign. In contrast to benign tumours, malignant tumours are characterized by their abilities to grow in a completely uncontrolled manner, spread into adjacent tissues and possess the ability to metastasize to distant sites. The term *carcinoma* refers exclusively to a malignant tumour. On the other hand, the term *benign* implies a placid and harmless disease; however, a benign tumour can sometimes be hazardous to the patient through local symptoms termed *mass effect* resulting from the compression of vital organs such as laryngeal or thyroid tumours congesting air supply to the lungs and benign brain tumours compressing vital cerebral centers et.c. In addition, certain benign, endocrine tumours with hormone overproduction can cause aversive health effects, for example adenomas of the adrenal and parathyroid glands to name a few.

Basics of tumour development

The living cell is a dynamic blend of signaling pathways and interacting proteins, providing precise regulation of diverse phenomenon such as proliferation, apoptosis, migration, cell to cell communication, to name a few. These processes are all tightly controlled by the normal cell, as faulty mechanisms in this machinery could prove disastrous for the organism as a whole. As one of the fundamentals of eukaryotic evolution, a cell is bound to delete itself when needed and replicate when considered necessary. Any disturbances in these carefully supervised systems, whether caused by intrinsic (sporadic genetic alterations) or extrinsic factors (for example UV irradiation damaging the DNA) might propel increased proliferation and decreased self-destruction by apoptosis. This event, termed *initiation*, leads to excessive

cell divisions in the parental (original) cell which produces clones of a cell with the same genetic aberration. Hence, the cell with the initial genetic change will; if the alteration gives rise to a proliferative advantage, outgrow its normal cell neighbor. This is called *promotion*. As the clone continues to divide, multiple genetic aberrations are acquired along the way, further propelling the division rate or diminishing the rate of senescence/apoptosis in the clone. This process, termed *progression*, will thus lead to expansion of the fittest clone and the formation of a true neoplasm. Genetic and epigenetic changes together with environmental factors (blood supply, immunological responses etc) will determine whether the growing tumour develops benign features or transform into a full-blown metastatic carcinoma. Tumour progression is schematically represented in Figure 1.

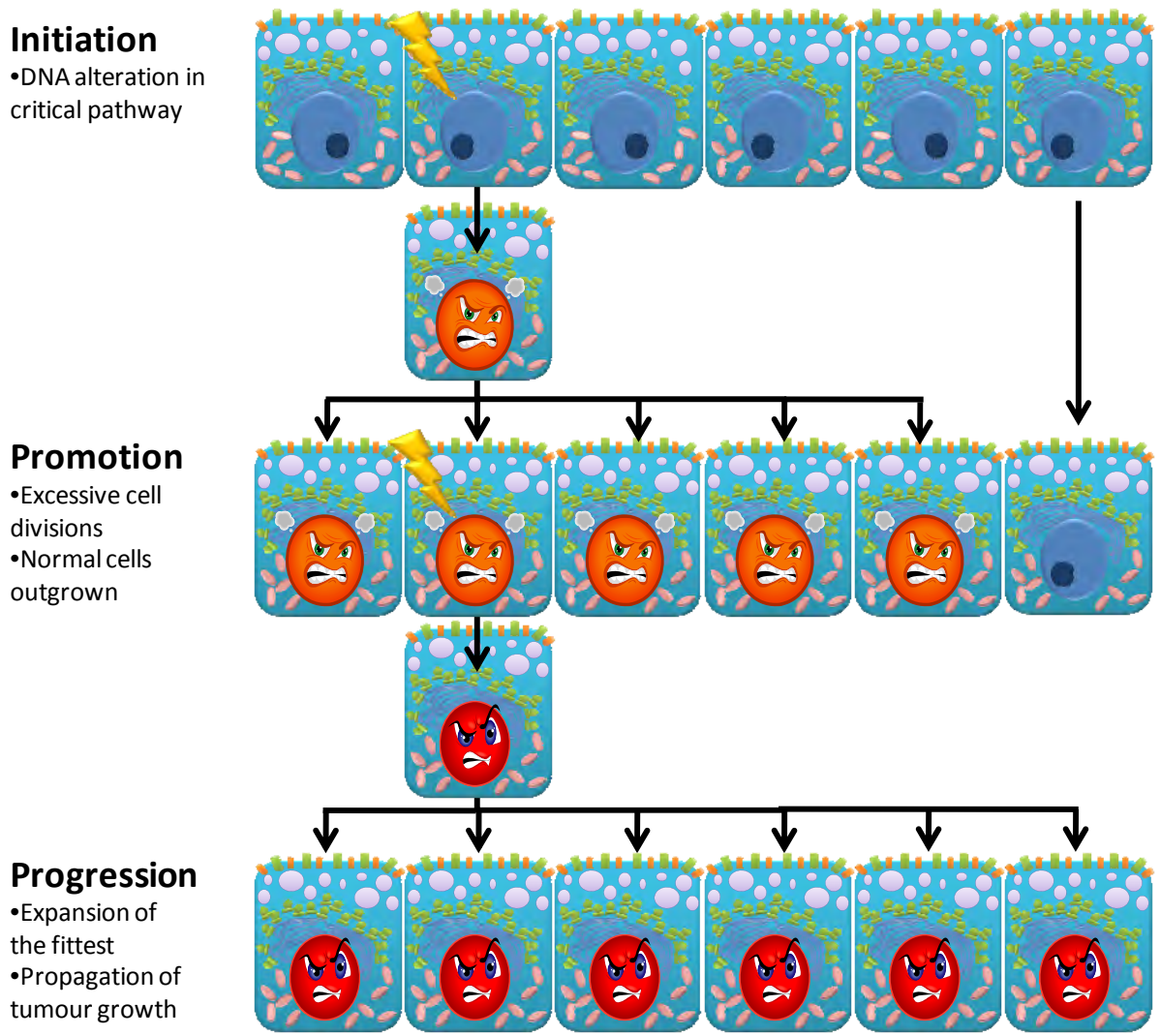

Figure 1 Schematic overview of tumour progression.

Underlying genetic aberrations in human tumorigenesis

Tumour growth, whether benign or malignant, is basically a genetically caused imbalance between the rate of cell proliferation and cell death. Normally, this balance is maintained in each cell by a tightly regulated assembly of proteins. Certain proteins are prone to inhibit cellular growth and elicit apoptosis; hence they are termed tumour suppressor proteins based on their anti-proliferative functions. On the other side we have the oncoproteins, proteins which promote cellular growth through stimulation of proliferation and inhibition of apoptosis. All human neoplasias are believed to exhibit inhibition of one or several tumour suppressor proteins and/or abnormal activity of oncoproteins in order to progress as a tumour. This faulty regulation of the proliferative homeostasis is thus the basis for tumour development in man, and is in turn caused by different types of genetic or epigenetic aberrations. These abnormalities might origin from different types of events such as activating or inactivating gene mutations, epigenetic modifications, abnormal posttranscriptional or posttranslational regulation and chromosomal imbalances. These events are commonly caused by intrinsic factors (errors during replication, faulty chromosomal segregation, hormonal profile etc) as well as extrinsic factors (the usage of tobacco and alcohol, dietary products, irradiation, viruses as well as various chemical compounds) in a complex manner which is only partially understood.

In theory, all human neoplasias need to acquire a critical number of genetic changes in order to progress as a tumour, afflicting critical cellular processes and giving the tumour cell advantages over their wildtype neighbors. Examples might include autonomy for growth stimulating signals and resistance to anti-growth signals which propagate proliferation through changes in gene expression and cell cycle progression (Figure 2). Other examples include methods to escape apoptosis, infinite replication potential and continuous angiogenesis. In addition to these rules applicable to all tumours, true malignant tumours also develop features for tissue invasiveness and metastasis that are hallmarks of carcinomas (Hanahan and Weinberg 2000). In the following sections, some of these fundamental genetic anomalies in tumour development will be presented.

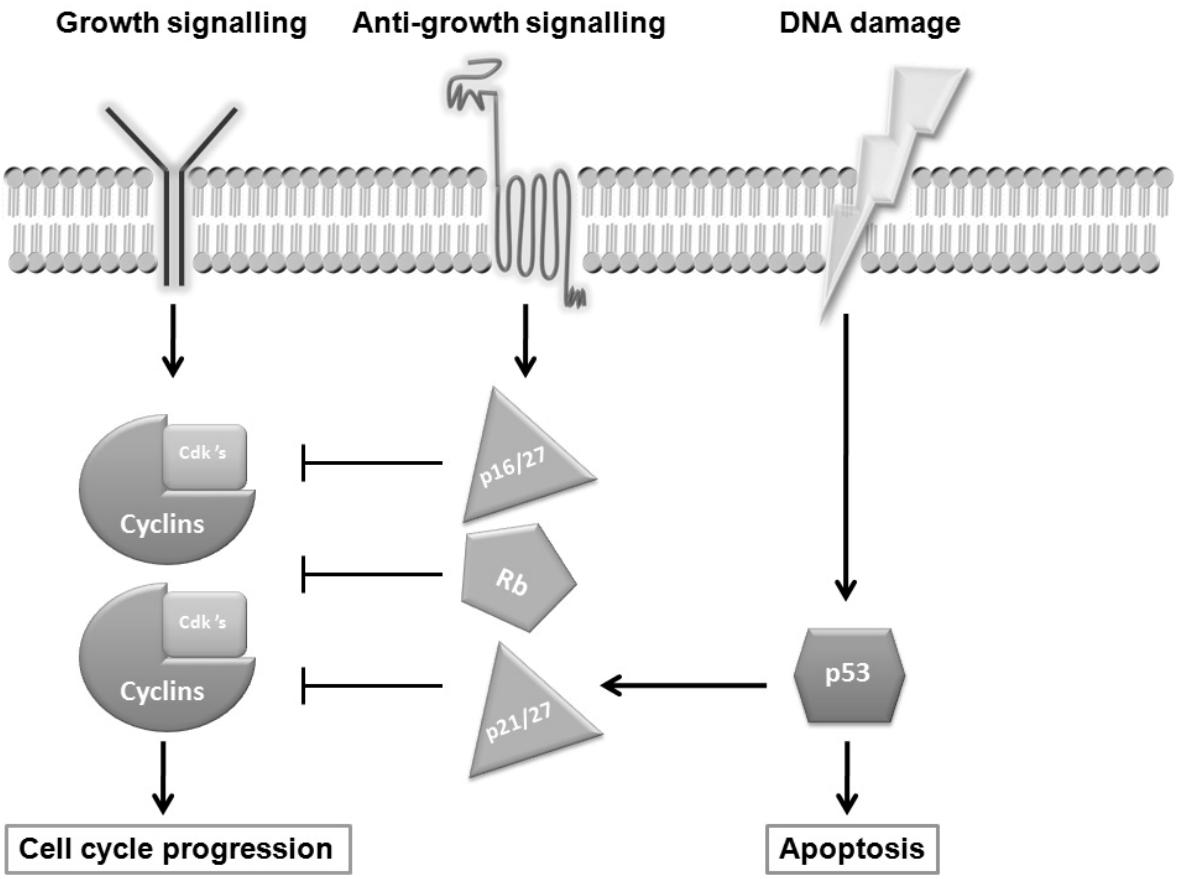

Figure 2 Major signaling pathway systems of importance in tumour development. Tumours frequently display decreased sensitivity to anti-growth signaling, augmentation of growth stimulatory pathways and aberrations in p53 expression leading to inhibition of apoptosis.

The cell cycle

The notion that every cell has sprung from a previous cell is best summarized in a phrase by the great German pathologist Rudolf Virchow: "*omnis cellula e cellula*", meaning "every cell from a cell". This process, which secures the intransience of life, is due to cell division and is managed through a complex series of regulated events known as the cell cycle.

The cell cycle consists of two main phases, the short M phase (mitotic) and the much longer interphase, the latter in turn divided into the subphases G_1 phase (gap 1), S phase (synthesis) and G_2 (gap 2) phase. At any given time, most fully differentiated cells throughout the human body are not proliferating. Hence, they are not functionally residing in any of the cell cycle phases but instead rest quiescent in the so called G_0 phase where no replication occurs. However, upon stimulation through external signaling and from growth factors and decreased

cell-cell mediated density-dependent inhibition, the cell will leave its non-dividing state and enter the cell cycle to initiate cell division. The first phase upon leaving the G_0 phase is the highly anabolic G_1 phase, where the cells grow in size and synthesize new organelles. After the G_1 phase, the cell is now ready to fully replicate its DNA (S phase). During this stage, the cell will go from a diploid genome to a quadrupled genome. Following this episode, the cell enters the short G_2 phase where the cell once again prepares for an energy-consuming process, namely the M phase. The M phase contains two distinct events, mitosis (separation of daughter chromatides) and cytokinesis (division of cytoplasm to form two separate cells). The result is two identical daughter cells with diploid genomes.

As stated previously, the cell cycle is a stringently regulated process. The cell must provide extreme care so that the DNA is correctly replicated and transferred to the daughter cells without alterations, since an incorporated error in the genomic information could prove deleterious to the individual cell. The cell cycle is driven forward by kinase proteins which uses phosphorylation as a method of activating/deactivating other target proteins within the cell cycle machinery. These kinases in turn require proteins termed *cyclins* to function properly; hence they are termed cyclin-dependent kinases (Cdks) (Satyanarayana and Kaldis 2009). The cell cycle has three major cell cycle checkpoints which all involve Cdks. The first checkpoint resides within the G_1 phase, and is also known as the restriction point since it is crucial to the entire cell cycle process. The checkpoint is regulated by the tumour suppressor proteins p15, $p16^{ink4A}$, p18 and p19, well characterized Cdk inhibitors which restrain the interaction between Cdk 4/6 and cyclin D1 (Sherr and Roberts 1999). The Cdk 4/6-cyclin D1 complex normally phosphorylates the tumour suppressor protein retinoblastoma (Rb), which will lead to increased expression of cyclin E through the release of the transcription factor E2F from the inhibited Rb. Cyclin E in turn binds Cdk2 and initiates the G_1 to S phase transition. Knowing this, one would generally expect the cyclin D1 and Rb proteins as well as the cdk inhibitors (for example $p16^{ink4A}$) to be altered in tumourigenesis, since aberrations in this checkpoint could drive the cell into S phase and hence initiate cellular proliferation. It is therefore not surprising that $p16^{ink4A}$ is found inactivated in multiple tumours such as cancers of the pancreas, esophagus and skin, cyclin D1 over-expression in tumours of the parathyroids, breast, prostate and colon as well as Rb mutations in the hereditary retinoblastoma syndrome and down-regulation in parathyroid carcinomas to name a few (Cryns *et al.* 1994, Kamb 1995, Vasef *et al.* 1999, Knudson 2005, Kim and Diehl 2009). The G_1 to S restriction point is further detailed in Figure 3.

The second checkpoint of the cell cycle occurs during the G_2 phase, and controls the start of the subsequent M phase. The main player at this control point is called "maturation promoting factor", MPF, a complex consisting of Cdk 1 and cyclin B_1. Cyclin B_1 levels rise throughout the G_2 phase, and when sufficiently abundant it associates to Cdk1 and initiates phosphorylation of target proteins, for instance histones (H1/H3) and nuclear lamins for facilitating the access to DNA prior to mitosis as well as various microtubule-associated proteins for initiation of the mitotic spindle formation.

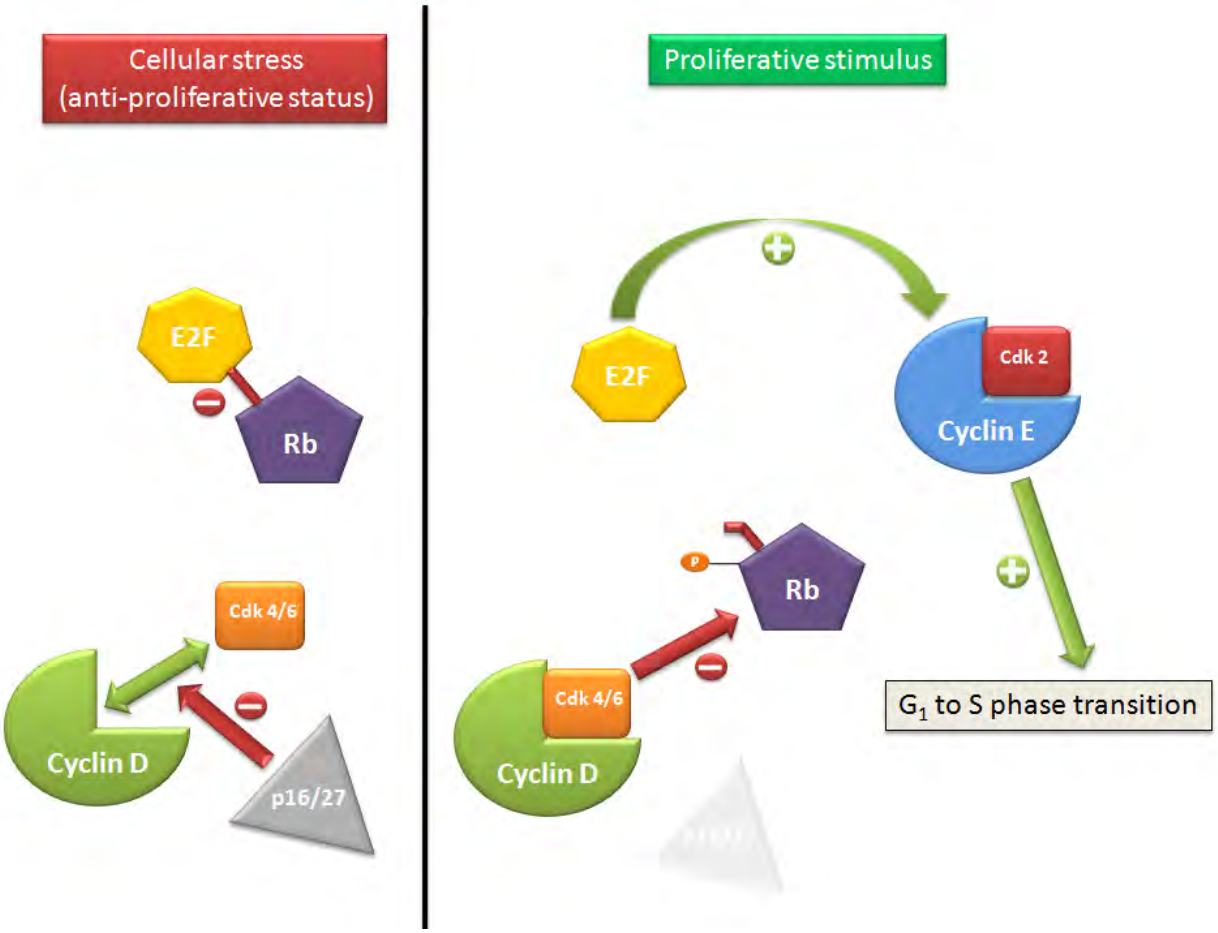

Figure 3. Major players involved in the regulation of G_1 to S phase transition of the cell cycle. High levels of p16 and p27 normally inhibit the interaction between cyclin D1 and Cdk 4/6. A proliferative stimulus (for instance increased signaling from a growth signaling pathway) will result in diminished levels of p16/p27, thereby allowing the cyclin D1-Cdk 4/6 complex to inhibit Rb, resulting in cyclin E accumulation and subsequent G_1 to S phase transition.

The third checkpoint during mitosis occurs in the metaphase when all chromatides are aligned at the mitotic plate. The MPF inhibits the *anaphase promoting complex*, a cluster of proteins which initiates the anaphase that marks the separation of sister chromatides and the subsequent telophase and cytokinesis. When all chromatides are brought into line, the cyclin B_1 component of the MPF is degraded and the inhibition of the anaphase promoting complex is lifted. Hence, cyclin B_1 appears as a central component in later stages of the cell cycle. Cyclin B_1 over-expression is common in several tumour types, for example in tumours of the esophagus and lung (Murakami *et al.* 1999, Soria *et al.* 2000). Cyclin B_1 has also been shown to be aberrantly expressed during the G_1 phase which is thought to cause uncontrolled cell-cycle progression (Shen *et al.* 2004).

Apoptosis

"*This is the way the world ends, not with a bang - but a whimper*". The words derive from the poem "The Hollow Men" where T.S Eliot describes the insincerity of modern man, but could just as well serve as an introduction to the phenomenon of programmed cell death, termed apoptosis. Apoptosis, as opposed to the uncontrolled, traumatic *necrosis* during acute cellular injury, represents a controlled and carefully regulated process which includes cell shrinkage, packing of organelles, condensation of chromatin, fragmentation of DNA and the formation of vesicles containing cellular debris (apoptotic bodies). These vesicles are then phagocytosed by macrophages in the surroundings without eliciting an inflammatory response. Apoptosis as a phenomenon plays important roles in different biological processes, for example as a cell termination process after irreversible DNA damage, as a mode of sculpting the emergent individual during embryonic development as well as a mode of antiviral response as circulating lymphocytes may terminate a infected cell through activation of apoptosis. The process is initiated through cellular stress such as DNA damage, heat, deprivation of nutrients/oxygen and irradiation to name a few. Apoptosis is firmly regulated in part through internal mechanisms including several intracellular pathways and in part through extracellular signaling by cytokines and direct extracellular receptor binding by extravasated lymphocytes. One of the most fundamental internal mechanisms controlling apoptosis is regulated by the well characterized tumour suppressor gene p53, encoded by the *TP53* gene located on the short arm of chromosome 17 (17p13.1). The p53 protein is normally inhibited by mdm2, a protein functioning as an E3 ubiquitin ligase marking p53 for proteosomal degradation as well

as inhibiting *TP53* gene transcription. Upon cellular stress, the INK4a/ARF encoding gene *CDKN2A* is activated, leading to transcription of two products with alternate reading frames, namely $p16^{ink4A}$ and $p14^{ARF}$ (Sherr and Roberts 1999). The $p16^{ink4A}$ protein (as mentioned earlier) exerts its function through inhibition of the Cdk 4/6-cyclin D1 interaction required to propel the cell cycle from the G_1 to the S phase. The $p14^{ARF}$ protein however, directly inhibits mdm2, releasing and activating p53. p53 is then free to exercise its functions upon the cell through its role as a transcription factor, including up-regulation of p21 and Bax. The protein p21 inhibits cdk2-cyclin E mediated actions and the Bax protein induces apoptosis through the activation of the mitochondrial cytochrome C pathway.

p53 is one of the best characterized proteins in human tumour development. Germ-line *TP53* mutations predispose for the Li-Fraumeni syndrome, where the afflicted develop multiple tumours a relatively young age, for example soft tissue sarcomas, breast and adrenocortical carcinomas as well as different types of brain tumours (Malkin *et al.* 1990). Sporadic tumours also display *TP53* alterations to a large extent, and somatic mutations have been found in a huge variety of human neoplasias, in addition to *TP53* promoter hypermethylation and allelic loss of the 17p13 region (Hollstein *et al.* 1990). Moreover, loss of p53 protein expression has been reported in a variety of tumours, for example in parathyroid and thyroid carcinomas to name a few (Cryns *et al.* 1994, La Perle *et al.* 2000). In the same manner, patients with Li-Fraumeni syndrome carrying certain single nucleotide polymorphisms (SNP's) at the *mdm2* gene locus display augmented levels of *mdm2* RNA and protein, further attenuating the p53 activity and enhancing tumorigenesis in these patients (Bond *et al.* 2004). Moreover, $p14^{ARF}$ expression is altered in the development of acute myeloid leukemia and a certain mutations in the *CDKN2A* gene encoding $p14^{ARF}$ and $p16^{ink4A}$ has been associated with increased risk for the development of malignant melanomas of the skin (Hayward 2003, Müller-Tidow *et al.* 2004)

Angiogenesis

Unlike certain prokaryotic life forms, tumours are eukaryotic and hence more or less reliant to aerobic metabolism. As oxygen-dependent, a tumour must therefore as a precondition nurture its excessive growth by concurrent blood vessel expansion. Angiogenesis denotes endothelial

cell proliferation and the formation of new vessels, and this phenomenon is repeatedly observed in early to mid stage tumour development of various tissues.

One way of conceiving this cross-talk between tumour tissue and normal stroma is through the release of soluble growth factors which associate to membrane bound receptors on the endothelial cell surface, such as the vascular endothelial growth factor (VEGF) and the fibroblast growth factors 1 and 2 (FGF1/2) to name a few. Numerous tumour types display augmented expression of VEGF and/or FGFs as compared to the normal tissue equivalents. In other neoplasias, expression of angiogenic inhibitors such as thrombospondin-1 or β-interferon is downregulated (Hanahan and Weinberg 2000). For instance, VEGF will upon binding its tyrosine kinase receptor mediate intracellular signaling leading to caspase inhibition. This will cause prolonged survival, Ras pathway activation as well as numerous changes in the cytoskeleton organization leading to filopodial extension and an overall increase in migration capacity. The cross-talk between tumour tissue and normal stroma is further propagated through cell adhesion molecule signaling, mostly through integrins, in which the endothelium express certain types of these molecules during the angiogenic process. Inhibition of integrin signaling *in vitro* can block angiogenesis altogether, underlining the importance of these molecules in vascular formation. The significance of proper vascularization for tumour growth has propelled the development of angiogenesis inhibitors, for example bevacizumab (a VEGF monoclonal antibody) which is now used as a therapeutic agent in patients with advanced colorectal cancer.

Infinite replication potential

Even though a tumour might reach the stages of promotion and progression through the acquirement of genetic aberrancies which alter the selected clone's proliferatory status, the tumour itself cannot grow for an infinite period of time without alterations of pathways controlling cellular senescence. The ability to multiply without confines is referred to as cellular immortalization, and is probably a phenotype which all tumours are expected to acquire in order to sustain proliferation. Normal cells *in vivo* as well as *in vitro* die after a certain number of divisions, and one elucidation put forward explaining this phenomenon is the shortening of telomeres. Telomeres are situated at the termini of linear chromosomes of most eukaryotic organisms, and are made up of multiple repeats of a short sequence constituent. Thus, evolution has arranged these repetitive, non-coding sequences at the ends of

chromosomes as a mode of protecting vital DNA from end-to-end chromosomal fusions and imbalances. However, the problem with this solution is that DNA polymerases progress from 5' to the 3' end of the DNA strand during replication. While this works fine for the leading DNA strand, the replication of the lagging DNA strand require RNA primer formation and the formation of so called tiny Okazaki fragments. This means that the 3' end of the lagging strand cannot be fully replicated as the last 3' end sequence will be occupied by an RNA primer which is subsequently degraded. This will result in a net loss of telomeric DNA at the lagging strand's end of each chromosome. This continuous attrition of telomeres through consecutive cycles of replication ultimately gives rise to diminished capacity to protect chromosomal DNA, leading to chromosomal imbalances which cause cellular senescence and triggers apoptosis.

To counter this problem, tumours frequently gain access to a system which is strictly forbidden ground for normal cells, namely the up-regulation of telomerase, an enzyme which prolong the telomere sequences by addition of TTAGGG repeats to the chromosomal ends. In humans, telomerase expression results from transcriptional activation of the *hTERT* gene (human telomerase reverse transcriptase) which encodes the critical catalytic subunit of telomerase. As of this, *hTERT* overexpression is evident in many different tumours, including breast and cervical carcinomas to name a few (Cerni 2000). Interestingly, from a parathyroid perspective, *hTERT* has been shown to be a modulator of cyclin D1 expression, and in addition has found to be partly regulated through menin, a protein encoded by the *MEN1* gene underlying a clinical syndrome predominantly presenting with primary hyperparathyroidism (Jagadeesh and Banerjee 2006, Hashimoto *et al.* 2008).

Oncogenes and tumour suppressor genes

Several aberrant genetic mechanisms can pilot cellular proliferation as discussed previously; and two well-characterized processes which are profusely present in human tumours are activation of oncogenes as well as inactivation of tumour suppressor genes.

The word "oncogene" stems from the gene's ability to promote tumour development when overly expressed. Usually these are genes promoting cellular growth and typically comprise regulators of proliferatory pathways. Oncogenes can be overly activated through various intrinsic mechanisms; including stabilizing point mutations of regulatory regions which make

them insensitive to degradation (β–catenin in hepatocellular carcinoma) or functionally overactive (*RET* oncogene in the MEN 2 syndromes), gene copy number amplifications (*HER2* in breast carcinomas) or chromosomal rearrangements (*CCND1*/*PTH* chromosome 11q inversion in subsets of parathyroid tumours) to name a few examples (Slamon *et al.* 1987, Arnold 1995, Miyoshi *et al.* 1998, Lodish and Stratakis 2008). Furthermore, oncogenes can also be altered through promoter hypomethylation (*Wnt5a* in prostate carcinoma) or through reduced RNA interference (let-7 repression up-regulates the oncogene *HMGA2* in uterine leiomyosarcomas), both processes leading to oncogene overexpression (Wang *et al.* 2007, Shi *et al.* 2008). Oncogenes are also prone to extrinsic modulation, for example *RET* activation due to external radiation as well as *T-cell leukaemia 1* (*TCL-1*) upregulation through Epstein-Barr viral antigens in lymphomas (Bell and Rickinson 2003, Volpato *et al.* 2008).

Tumour suppressor genes are genes which normally inhibit cellular proliferation and promote apoptosis. As a rule, inactivation of tumour suppressor genes complies with Knudson's famous two hit theory, in which both alleles must be silenced to obtain the expected loss-of-function properties. These inactivating processes might be due to missense or nonsense point gene mutations, giving rise to altered amino acid composition in a single location or a prematurely truncated protein respectively. Other mechanisms include promoter hypermethylation or increased RNA interference, both means leading to reduced or absent gene expression. In addition, tumour suppressor genes can also be altered through chromosomal loss or entire deletions.

The parathyroid glands

Anatomy and histology

The parathyroid glands, usually four to the number, are most commonly localized adjacent to the dorsal surface of the thyroid capsule (Figure 4A). The parathyroid glands stem from the third and fourth branchial pouches of a human embryo, and migrate towards the thyroid at gestational week 7. The parathyroid glands derived from the fourth branchial pouch usually stop at the superior surface of the thyroid; hence they are termed superior parathyroid glands.

However, the parathyroid glands which stem from the third branchial pouch are pulled down by the co-migrating thymus tissue created from the same pouch; hence these parathyroid glands migrate further caudally than the superior glands and are therefore termed inferior parathyroid glands. Supernumerary glands can be found in approximately 5% of cases with various locations along the migratory route from the branchial pouches (Åkerström *et al.* 1984). To complicate the picture, failure of the inferior parathyroid glands to separate from the descending thymus tissue might give rise to intrathoracical residing parathyroid tissue, and co-formation of the superior parathyroids along with the thyroid gland might result in intrathyroidal parathyroid glands. The colour of the normal parathyroid gland is brown to yellowish, and the weight is usually less than 70 milligrams. The glands are supplied with blood through branches of the inferior thyroid artery, stemming from the thyrocervical trunk.

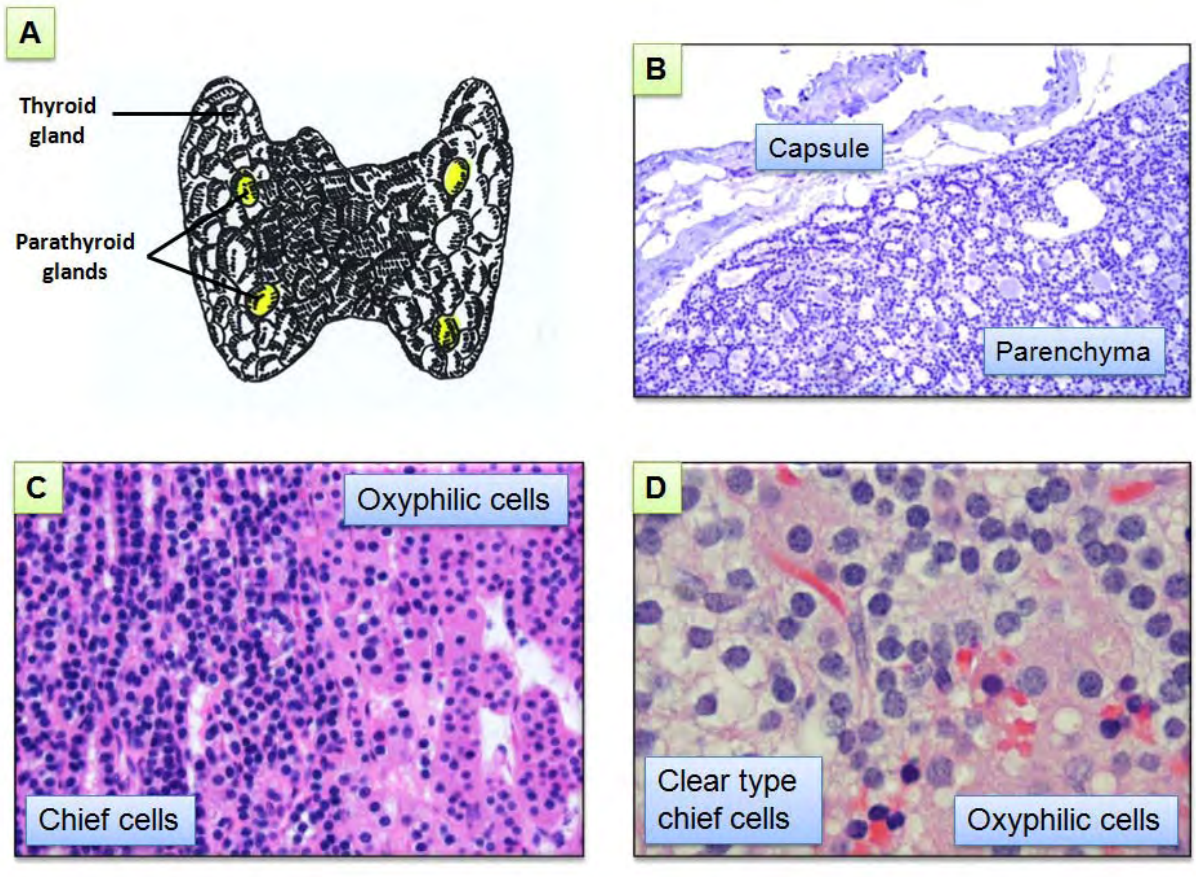

Figure 4 Overview of parathyroid anatomy and histology. **A;** The parathyroid glands are situated adjacent to the dorsal surface of the thyroid. **B;** A fibrous capsule surrounds the parathyroid parenchyma. **C;** Chief cells with condensed nuclei and larger oxyphilic cells with eosinophilic cytoplasm. **D;** Clear type (or "water-clear cells") chief cells with characteristic cytoplasm.

Under the microscope, the parathyroid tissue is enclosed by a fibrous capsule (Figure 4B). The histology revolves mainly around two parenchymal cell types, chief cells and oxyphilic cells. The chief cells is the most abundant cell type of the parathyroid gland, and is recognized as a small polyhedral cell with a centrally located, chromatin-dense nucleus and pale cytoplasm. This cell type produces and releases parathyroid hormone (PTH) into the blood. The oxyphilic cell is a relatively larger cell with a dense nucleus and an eosinophilic and mitochondria-rich cytoplasm. The function of this cell type is largely unknown (Figure 4C). In addition, clear type chief cells (sometimes referred to as water-clear cells) are also present within the parathyroid glands, so-called because of their glycogen-rich deposits in the cytoplasm which remain unstained in the usual preparation of tissue sections (Figure 4D).

Physiology

The main function of the parathyroid glands is to regulate the serum levels of calcium. Calcium ions (Ca^{2+}) are essential for multicellular, eukaryotic organisms, and play substantial roles in diverse fields such as signal transduction, signal transmitter substance release, contraction in smooth, heart and skeletal muscle, apoptotic signaling and bone mass formation. Therefore, it comes to no surprise that the serum levels of Ca^{2+} need to be firmly regulated. The level of serum Ca^{2+} is maintained due to an intricate collaboration between the small intestine, the kidneys, the skeletal system and the parathyroid glands. The key hormones which regulate this process are activated vitamin D3 and PTH. Vitamin D3 is formed in the dermis through UV light exposure, as well as ingested in certain dietary products. To form active vitamin D3, the vitamin needs to be hydroxylated in two sequential steps; the first reaction takes place in the liver, the second in the kidney. The second hydroxylation is PTH-dependent, meaning that the parathyroids regulate active vitamin D3 levels directly through PTH stimulation of the tubule cells of the kidneys.

When the serum levels of Ca^{2+} drops, PTH is secreted into the blood via the parathyroid chief cells. This is due to the normally inhibitory effect of the trans-membranous calcium-sensing receptor (CaSR) on PTH secretion. The CaSR is a 120 kDa 7-transmembrane G-protein coupled receptor which is rapidly activated by an increase in extracellular Ca^{2+} (Brown *et al.* 1993). Binding of Ca^{2+} to the extracellular domain of the CaSR confers a conformational change which activates phospholipase C (PLC). PLC in turn increases the levels of inositoltriphosphate (IP_3) through the cleavage of phosphatidylinositol 3-phosphate (PI(3)P).

IP_3 acts on the IP_3 receptor located on the surface of the endoplasmic reticulum (ER), in turn opening a calcium channel resulting in the release of Ca^{2+} into the cytoplasm. High intracellular Ca^{2+} levels inhibit the exocytosis of vesicles containing PTH, leading to reduced serum levels of PTH and hence a drop in extracellular Ca^{2+} levels (Nemeth and Scarpa 1986). When the serum levels of Ca^{2+} falls, the inhibitory effect from the CaSR will cede in a typical regulatory feedback manner. The net result is immediate PTH secretion.

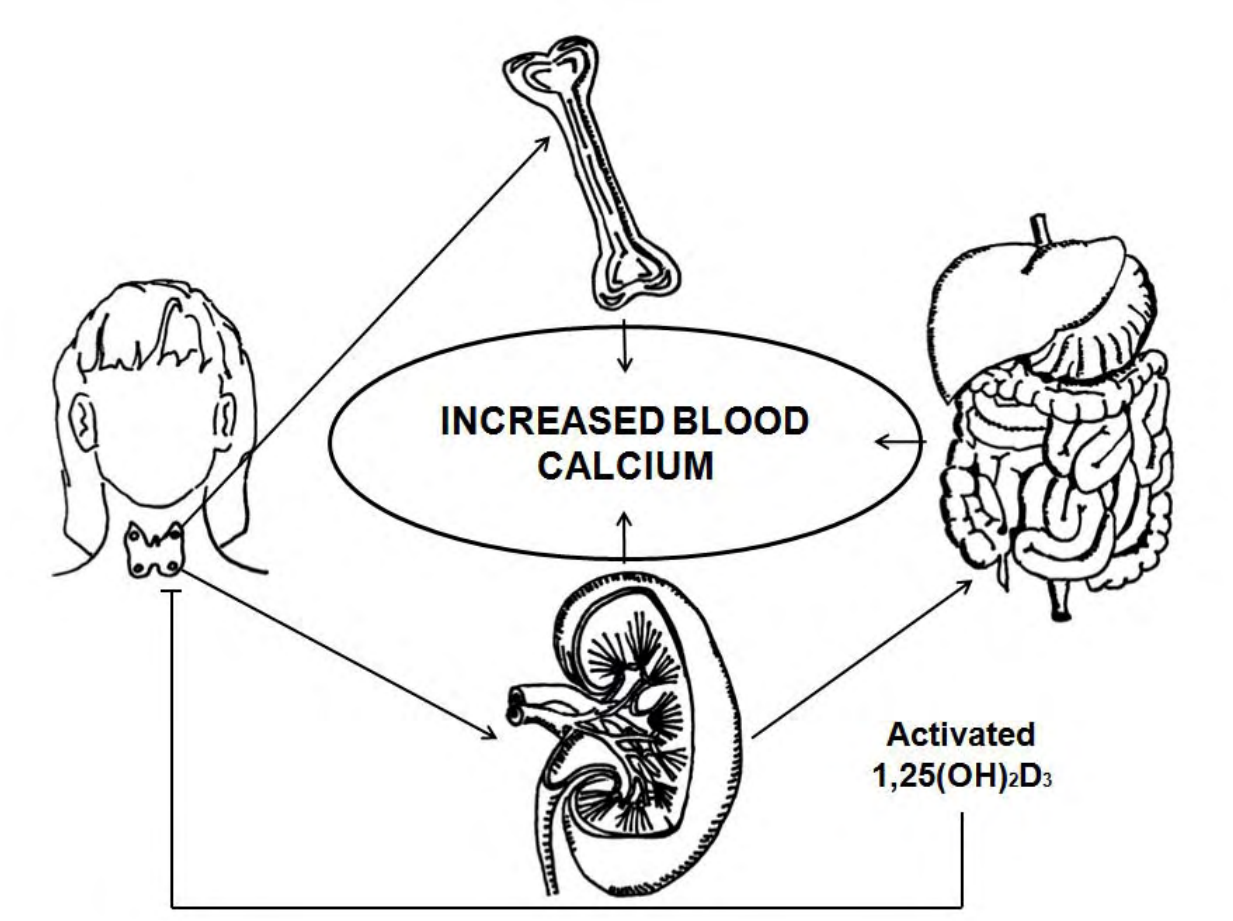

Figure 5. Physiologic regulation of calcium homeostasis in man. Arrows indicate stimulatory signals and the stop line represent a negative feedback loop. Note that increased blood calcium levels also inhibit PTH secretion (stop line not included).

If the hypocalcemia is prolonged (for example in chronic renal failure), the rate of degradation of intracellular PTH is reduced, followed by an increase in *PTH* gene transcription. Long term hypocalcemia finally induces proliferation of the parathyroid cells. Furthermore, an additional

potential calcium regulating receptor from the LDL receptor family (termed LRP2 or megalin) has been identified as present on the parathyroid cells but the suggested mode of action is largely unrevealed (Juhlin *et al.* 1987). The PTH-mediated regulation of serum calcium is outlined in Figure 5.

When excreted into the blood stream as a response to hypocalcemia, PTH stimulates the osteoclasts to break down the hydroxyl apatite, the calcium containing substance that gives the skeletal bone its hardness, leading to a Ca^{2+} efflux into the blood. PTH will also stimulate tubule cells of the kidney to reabsorb calcium from the primary filtered urine as well as induce the conversion of vitamin D3 to its active form. The delivery of dietary calcium to the extracellular volume (ECV) is regulated via the small intestine and its specific Ca^{2+} transporters on the luminal side of the intestinal epithelium. Activated vitamin D3 stimulates the synthesis of these calcium transporter proteins, in turn increasing the uptake of dietary calcium to the ECV.

As described above, both vitamin D3 and PTH works together towards elevating the Ca^{2+} plasma levels. In many other systems monitoring homeostasis in physiological systems there is also an established self-regulatory process. In our case, a Ca^{2+} elevation will stimulate the CaSR, leading to reduced PTH secretion and down-regulation of *PTH* gene transcription. In addition, active vitamin D3 will act on the parathyroid glands, inhibiting *PTH* gene transcription and parathyroid cellular proliferation, thus counteracting a proliferative stimulus from an eventual long term hypocalcemia.

Development of parathyroid tumours

Overview of the proliferative diseases of the parathyroid glands

The pathological basis of abnormal parathyroid growth can roughly be divided in to three subgroups, namely primary hyperparathyroidism (PHPT), secondary hyperparathyroidism (SHPT) and tertiary hyperparathyroidism (THPT).

PHPT is a common endocrine disorder and denotes the tumorous enlargement of one or several parathyroid glands, leading to excessive PTH release and consequently hypercalcemia. The usual finding is a benign adenoma (~80%), as opposed to the less

commonly observed multiple gland disease or “hyperplasia” (~20%) or the malignant parathyroid carcinoma (~1%). Overall, PHPT is the most common form of hyperparathyroidism. The symptoms are derived from the increase in PTH blood levels, and include polydipsia, fatigue, muscular weakness, psychic disorders, osteoporosis and gall/kidney stones to name a few. However, most individuals with PHPT have very mild symptoms or lack manifestations altogether, although the hypercalcemia itself might be pronounced. The frequent absence of symptoms thus indicates that PHPT might be under-diagnosed as a whole. Furthermore, recent work indicates an association between PHPT and cardiovascular disease as well as various neoplasias such as cancers of the breast, kidney, colon and skin (Palmer *et al*. 1988, Nilsson *et al*. 2007, Walker and Silverberg 2008). The treatment is surgical, and the removal of the diseased gland/s usually is curative and requires no further medical handling.

Parathyroid adenoma

A single parathyroid adenoma is as previously mentioned the most common finding in PHPT. As the name implies, it is a benign tumour consisting of an enlarged parenchymal mass. Macroscopically, the tumour is dark red to brownish and often enlarged to allow distinction from the other three (or more) regular glands. Two different histological subtypes of parathyroid adenoma are recognized based on the predominant cell type visible at histopathological examination; the most common chief cell adenoma and the more unusual oxyphilic adenoma. Most adenomas are believed to be monoclonal lesions. The histological criteria for a parathyroid adenoma is based on the finding of a predominant cell type usually presenting with nuclear pleomorphism, reduced or absent levels of adipocytes and intracytoplasmatic fat content (assessed using an Oil Red staining technique), the presence of a remnant with normal histological phenotype (the so called “normal rim”) as well as a visible fibrous capsule which may or may not surround the parenchyma (DeLellis *et al.* 2004). However, since primary hyperplasia also can present with all or some of these phenotypes and an adenoma can lack some of the above mentioned criteria, the precise distinction between these two tumour types lies in the retrieval of an adjacent gland biopsy. A normal histological finding from the biopsy strongly suggests adenoma, whereas aberrant findings in the biopsy imply hyperplasia.

Primary hyperplasia/multiple gland disease

Primary hyperplasia is a variant of primary hyperparathyroidism which denotes the abnormal growth of all four glands. It can be divided into two histological subgroups, chief cell hyperplasia and water clear cell hyperplasia, the former much more common than the latter. In the microscope, primary hyperplasia consists of abundant number of cells with little or no adipocytes or intracytoplasmatic fat as well as an absence of a normal rim. The growth pattern can be diffuse or nodular (DeLellis *et al*. 2004). A true primary hyperplasia of all four glands is treated with subtotal parathyroidectomy (leaving some 25-80 mg of parathyroid tissue in the patient) or a total parathyroidectomy with re-implantation of minced tissue in the patient's forearm.

The underlying molecular events regarding this entity is rather obscure, however an association to the hereditary MEN 1 syndrome is reported, in which patients with inactivating *MEN1* gene mutations often display multiglandular involvement. Primary hyperplasia is believed to be monoclonal in origin in most cases; this has also been demonstrated for MEN 1 related cases (Arnold *et al*. 1995, Miedlich *et al*. 2000). There has been a substantial debate whether primary hyperplasia represents a synchronous, physiological growth mechanism of all four glands due to hormonal/immunological deregulation, or whether these lesions are true neoplastic tumours with sporadic gene aberrations emerging independently in several glands. Arguing for the latter, mutations in tumour suppressor genes known to play significant parts in the aberrant growth of parathyroid adenomas and carcinomas are sometimes observed in sporadic cases with primary hyperplasia. In contrast, studies have found indirect evidence of circulating auto-antibodies in primary hyperparathyroidism, possibly suggesting an autoimmune pathogenesis much like the mechanisms observed in Grave's disease of the thyroid gland (Bjerneroth *et al*. 1998). To further complicate the picture, the phenomenon "multiple adenoma" is an acknowledged entity consisting of two separate, monoclonal tumours presenting simultaneously and where the pathologist excludes hyperplasia based on a normal appearing biopsy from a third gland. Hence the term "multiglandular disease" should be preferred instead of "hyperplasia", the latter which denotes a physiological response and by definition would exclude true clonal origin.

Parathyroid carcinoma

Parathyroid carcinoma is the malignant manifestation of primary hyperparathyroidism. It is an unusual but potentially lethal condition, and the prognosis depends on early-on detection and surgical removal of all tumorous tissues including eventual spread of metastatic tissue (Shane 2001, Rodgers and Perrier 2006). If the diagnosis is suspected prior to surgery, the preferred technique is an *en bloc* resection where the entire tumour is removed together with the ipsilateral thyroid lobe.

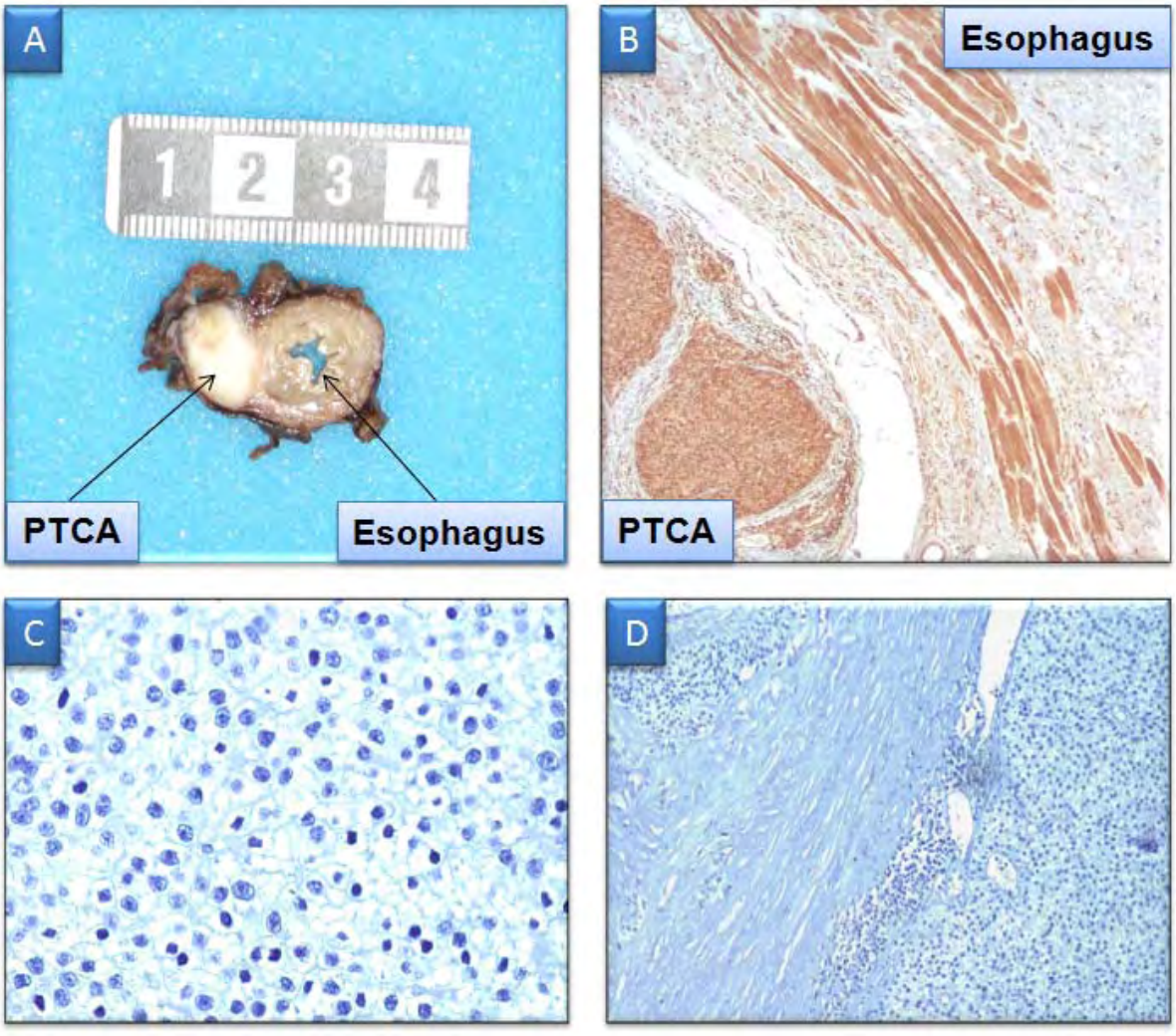

Figure 6 Examples of a parathyroid carcinoma (A-B) and atypical adenomas (C-D). **A;** Gross specimen of a parathyroid carcinoma adherent to the esophagus, thus displaying indisputable (unequivocal) signs of malignancy. **B;** The same tumour at histopathological examination, stained for parafibromin expression. **C;** Nuclear atypia and macronucleoli in a case of atypical parathyroid adenoma. **D;** Fibrous band splitting the parenchyma in a parathyroid atypical adenoma.

Suspicion of parathyroid carcinoma is raised if the patient exhibit profound hypercalcemia, an eventual palpable neck mass as well as positive family history regarding the HPT-JT syndrome (reviewed later). The definite diagnosis of parathyroid carcinoma is based on histopathology, and requires direct evidence of tumour overgrowth onto adjacent organs or blood vessel/perineural infiltration alternatively metastatic spread to distant sites (DeLellis *et al*. 2004, Figure 6).

Parathyroid atypical adenoma

Apart from these unequivocal criteria, there are also a number of minor histological features that may indicate a malignant tumour with varying sensitivity and specificity although the unequivocal characteristics are absent. Examples of these attributes include elevated number of mitoses, marked proliferation count using Ki-67 immunostaining, fibrous bands or distinct macronucleoli to mention a few (Figure 6). If several of these features are present when reviewing the sample although there are no unequivocal signs of malignancy, the tumour is termed “atypical adenoma” and the patient should be monitored closely (DeLellis *et al*. 2004). The occasionally used expression “equivocal carcinoma” is synonymous with atypical adenoma. Clinical characteristics such as profound hypercalcemia, large tumours and palpable neck masses indicate malignant disease, but are also established phenomenon in subsets of benign tumours with or without atypical histological findings. The current dilemma of diagnosing malignant potential in a parathyroid tumour is discussed in detail in subsequent chapters.

Secondary and tertiary hyperparathyroidism (SHPT/THPT)

In SHPT, all four parathyroid glands are synchronously enlarged due to chronic hypocalcemia. Thus, SHPT denotes “true” hyperplasia as a physiological response and is not considered a tumour. Most commonly the cause is chronic kidney failure, where the impaired renal parenchyma is unable to activate vitamin D3 although sufficient PTH levels. The drop in active vitamin D3 levels will subsequently stimulate parathyroid cell proliferation since the vitamin D3 dependable negative feedback onto parathyroid cellular growth is lifted. Furthermore, parathyroid proliferation is further propagated through a fall in calcium levels due to the drop in vitamin D3 concentration. Other, unusual causes of SHPT are

malabsorbtion due to inflammatory bowel disease and short bowel syndrome resulting from excessive surgery, leading to intestinal loss of calcium (Fraser 2009). The serum levels of calcium in SHPT are usually low. As SHPT represents a physiological phenomenon rather than a defined neoplastic event, the treatment consists of correction of the underlying cause. Vitamin D3 analogs and calcium substitution are regularly used in trying to correct the serum levels of calcium. Parathyroidectomy is sometimes also performed to reduce symptomatology prior to kidney transplantation.

The end-result of long-standing SHPT is THPT, in which one or several of the hyperplastic parathyroid glands reach an autonomous proliferation state, i.e. develop into an adenoma. Usually these patients have long-standing SHPT due to severe, chronic renal failure which necessitates dialysis treatment or kidney transplantation. The treatment of THPT is surgical (Fraser 2009).

Hereditary syndromes predisposing to primary hyperparathyroidism

PHPT is mostly a sporadic disease, however up to 5% of cases might comprise a heritable form due to constitutional predisposing mutations that can be passed on to the following generations. Table 1 summarizes the tumour genes with recurrent constitutional or somatic mutations present in PHPT patients. Below the different hereditary syndromes as well as the genes responsible will be discussed in detail.

The multiple endocrine neoplasia type 1 (MEN 1) syndrome

Multiple endocrine neoplasia type 1 (MEN 1) is caused by an inactivating, germ-line mutation in the *MEN1* tumour suppressor gene located at chromosome 11q13. MEN 1 constitute a high-penetrance, autosomal dominant disorder with an estimated prevalence of 1 in 20,000-40,000 individuals. The disorder is distinguished by several endocrine lesions of varying origin, most prominently including tumours of the parathyroid glands (>90% of patients), the

endocrine pancreas (30-75%) and the anterior pituitary gland (20-60%) (Figure 7). In addition, tumours of the adrenal cortex, skin, thymus, bronchi and soft tissues can also be demonstrated (DeLellis *et al*. 2004). The afflicted patients often present with tumour(s) in one of the endocrine tissues discussed above and subsequently may develop a full-blown syndrome with multiple tumours from different tissues. Most MEN 1 patients (90%) display previous familial history with involvement of endocrine tumours, whereas only 10% of the patients have *de novo* mutations without preceding accounts of familial disease (Bassett *et al.* 1998). Therefore, the diagnosis is often based on genetic screening of individuals with single endocrine lesions and familial history indicative of the MEN 1 syndrome. In addition, about 5% of patients with seemingly sporadic PHPT have been shown to harbor constitutional *MEN1* mutations, although no evidence of ancestral MEN 1 related disease (Uchino *et al*. 2000). Consequently, the prevalence of the MEN 1 syndrome might be higher than indicated as patients with apparent sporadic disease not often are subjects to germline *MEN1* sequencing. Taken together, the MEN 1 syndrome should be suspected in patients presenting with one or several endocrine tumour/s associated to the MEN 1 syndrome, especially if the patient is relatively young (<50 years of age) and display positive familial history and or multiple endocrine organ involvement. The diagnosis is based upon the identification of endocrine tumours in at least two of the tissues associated to the MEN 1 syndrome; alternatively a single tumour type is sufficient for the MEN 1 diagnosis if the patient also has a first-degree relative affected with MEN 1 (DeLellis *et al*. 2004).

The etiology of MEN 1 was initially mapped to chromosome 11q13 based on tumour deletion mapping and subsequent linkage analyses in a kindred with the MEN 1 phenotype (Larsson *et al.* 1988). The *MEN1* gene was subsequently identified as the culprit gene in 1997 based on frequent constitutional mutations in probands from MEN 1 families, all presumed to be inactivating mutations (Chandrasekharappa *et al.* 1997, Lemmens *et al.* 1997). Presently, inactivating germline mutations in all 9 coding exons of the *MEN1* gene has been demonstrated in MEN 1 families, and most MEN 1 related tumours display somatic loss of the wildtype allele by LOH analysis. Thus, *MEN1* seems to be a *bona fide* tumour suppressor gene which requires bi-allelic inactivation in accordance with Knudson's two hit theory. To date, more than one thousand somatic and germ-line *MEN1* mutations have been reported in MEN 1 families, with no apparent mutational clustering regions ("hot spots") identified.

Table 1. Overview of reported mutations in PHPT related genes in hyperparathyroid disease.

	MEN1 (11q13)	*RET (10q11)*	*HRPT2 (1q25)*	*CaSR (3q13)*	*p27 (12p13)*	*CTNNB1 (3p21)*	*TP53 (17p13)*
Familial disease							
MEN1	Mut (G) 1	-	-	-	-	-	-
MEN2A	-	Mut (G) 1	-	-	-	-	-
HPT-JT	-	-	Mut (G) 1	-	-	-	-
FHH	-	-	-	Mut (G) 1	-	-	-
MEN1 variant	-	-	-	-	Mut (G) 4	-	-
FIHP	Mut (G) 2	-	Mut (G) 3	Mut (G) 3	-	-	-
Sporadic disease							
PTAD	Mut (S) 3	-	Mut (S) 4	-	-	Mut (S) 4	-
MGD/HYP	Mut (S) 3	-	-	-	-	-	-
PTCA	Mut (S) 4	-	Mut (S) 2	-	-	-	Mut (S) 4
SHPT	Mut (S) 4	-	-	-	-	-	-

Mut (G): germline mutation, **Mut (S)**: somatic mutation, **-**: not reported/not known

1: almost always mutated (81-100%), **2**: often mutated (41-80%)

3: recurrently mutated (11-40%), **4**: infrequently mutated (≤10%)

PTAD: parathyroid adenoma, **MGD/HYP:** multiglandular disease/hyperplasia

PTCA: parathyroid carcinoma, **SHPT:** secondary hyperparathyroidism

PHPT is, as stated above, the most common feature of the MEN 1 syndrome. The MEN 1 syndrome display an exceedingly high penetrance for parathyroid disease, and one study has shown that >90% of all *MEN1* mutational carriers develop PHPT prior to reaching 50 years of age (Trump *et al*. 1996). PHPT in the MEN 1 syndrome is often characterized by multiglandular involvement, and the histological findings are identical to the observations in sporadic primary chief cell hyperplasia. Since *MEN1* gene inactivation follows the Knudson two hit model, a second hit occurring on the wildtype allele is probably required to propel tumorigenesis in the hereditary setting. As a consequence, the parathyroid glands are asymmetrically enlarged and the size can vary considerably with one or several glands presenting with near-normal masses. The diagnosis of hyperplasia in the MEN 1 setting does not differ from the diagnosis criteria for sporadic hyperplasia, and hence require histological investigations of multiple glands in which the pathologist identifies copious cell numbers with scarce adipocyte counts or intracytoplasmatic fat, as well as a lack of a normal rim (DeLellis *et al*. 2004). The treatment consists of subtotal parathyroidectomy or total parathyroidectomy with re-implantation, as the majority of MEN 1 patients develop recurrent PHPT. As a consequence, uniglandular parathyroidectomy should not be routinely performed if the MEN 1 syndrome is suspected or verified, and the treating physician should be quite liberal with germline *MEN1* gene mutational screenings in young patients with positive family history as well as if primary hyperplasia is suspected preoperatively. Since a rather large subset of MEN 1 patients might exhibit supernumerary glands, they should undergo cervical thymectomy to reduce the risk for recurrent PHPT and thymic carcinoid tumours (Marx *et al*. 2002). The risk of developing parathyroid carcinoma in the MEN 1 setting is believed to be extremely low, although a few cases of malignant parathyroid tumours have been reported to exhibit somatic *MEN1* mutations (Haven *et al*. 2007).

Certain families have an observably congregation of tumours of the parathyroid and pituitary glands, however lack germline *MEN1* mutations. These families are said to exhibit a MEN 1 syndromic variant, and in a single case in one particular study the authors detected a germline inactivating mutation of the *CDKN1B* gene encoding $p27^{Kip1}$, a cyclin dependent kinase (Cdk) inhibitor which negatively regulates cell cycle progression and cellular proliferation (Pellegata *et al*. 2006). Various studies have since failed to detect germline *CDKN1B* gene mutations in large cohorts of families with MEN 1/MEN 1 variant but without demonstrable *MEN1* gene alterations (Owens *et al*. 2009, Igreja *et al*. 2009). However, others have subsequently detected *CDKN1B* mutations as well as mutations in other cyclin dependent

kinase inhibitors (p15, p18, p21) in small subsets of families without observable *MEN1* gene mutations, suggesting that the inactivation of menin and certain Cdk inhibitors could give rise to similar tumorigenic phenotypes (Agarwal *et al*. 2009).

Menin structure and functions

The *MEN1* gene encompasses 10 exons, of which 9 are protein-coding. Two major *MEN1* transcripts have been demonstrated; the ubiquitously expressed *MEN1* transcript of 2.9 kb which encodes menin, as well as a larger 4.2 kb transcript present in the pancreas and thymus (Lemmens *et al*. 1997). The menin protein is 610 amino acids long (68 kDa) and exhibits two C-terminal nuclear localizations signals (NLS) as well as two leucine zipper motifs, both of which are features necessary for controlling nuclear gene expression (Guru *et al*. 1998). Not surprisingly, menin is a mainly nuclear protein but also display cytoplasmic localization depending on the contemporary cell cycle phase (Kaji *et al*. 1999). Given the frequent *MEN1* gene alterations in PHPT predisposition, menin was early on hypothesized to be associated with the regulation of cellular proliferation. Indeed, several roles for menin as a regulator of proliferatory pathways have since been put forward in the literature. For example, menin has been shown to interact with the JunD proto-oncogene, thereby repressing JunD mediated gene activation (Agarwal *et al*. 1999). JunD constitute a transcription factor thought to shield cells from senescence or apoptosis mediated by p53 activation (Weitzman *et al.* 2000). Menin also regulates the Ras signaling pathway through inhibition of Jun N-terminal kinase and is implicated in the TGF-β signaling meshwork through SMAD protein interaction (DeLellis *et al*. 2004).

Extremely interesting from a parathyroid research perspective, menin was recently associated to the Wingless type (Wnt) signaling pathway. In rodent pancreatic islet cells, menin was shown to directly bind the central component β-catenin, which in turn binds to the known parathyroid tumour suppressor parafibromin in the nucleus. Furthermore, menin over-expression increased the levels of the Wnt protein axin2 by histone methylation of the axin2 gene promoter area. Finally, over-expression of menin led to the up-regulation of Wnt target genes, suggesting a positive role for menin in Wnt signaling (Chen *et al*. 2008).

The MEN1 gene in sporadic parathyroid tumorigenesis

Not surprisingly given its role as a parathyroid tumour suppressor, somatic loss of one *MEN1* allele is found in approximately 25-40% of all sporadic parathyroid adenomas and in half of these cases an inactivating mutation of the second allele is also demonstrated (Heppner *et al*. 1997, Carling *et al*. 1998, Farnebo *et al*. 1998a, Miedlich *et al*. 2000). Interestingly, given the fact that subsets of small parathyroid adenomas derived from patients with modest hypercalcemia harbour *MEN1* mutations might indicate that these aberrations denote early-stage events in parathyroid tumorigenesis (Carling *et al*. 1998). *MEN1* mutations are very infrequent findings in parathyroid carcinomas, suggesting that *MEN1* gene mutations are not required for evoking a full malignant potential in parathyroid tumours. However, modest amounts of expression data covering the menin protein are available in the current literature, both regarding parathyroid adenomas and carcinomas. In one study, the authors investigated a small number of parathyroid tumours for menin expression by Western blot analysis and found retained expression in all normal tissues (n=3), adenomas (n=9) and cases with secondary HPT (n=3). The only sample devoid of menin expression was an MEN 1 associated hyperplasia sample carrying a truncating *MEN1* germline mutation (Corbetta *et al*. 2005). Surprisingly, Bhuiyan et al. investigated cases of PHPT and SHPT for menin expression using RT-PCR and Western blot analysis and observed strong menin expression of ~67 kDa in all sporadic PHPT and SHPT cases investigated, including three MEN 1 related cases carrying either missense or nonsense *MEN1* germline mutations. Two additional cases endowed with *MEN1* germline mutations displayed reduced but not absent levels of menin. Unexpectedly, the cases with the lowest levels of menin expression were cases of non-specified thyroid tumours used as controls (Bhuiyan *et al*. 2000). Given the observations that approximately 10-20% of all parathyroid adenomas display *MEN1* mutations, further studies covering menin expression in parathyroid tumours are clearly warranted.

The multiple endocrine neoplasia 2 (MEN 2) syndrome

Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominant tumour syndrome caused by activating germline mutations of the *rearranged during transfection* (*RET*) proto-oncogene located at chromosomal region 10q11 (Eng *et al*. 1996). The *RET* gene encodes a tyrosine kinase type membrane-bound receptor which conveys signalling for proliferation and

differentiation. Gain-of-function mutations within the *RET* coding exons thus lead to subsequent augmentation of associated proliferatory pathways. For example, upon activation the RET receptor directly binds and phosphorylates the Wnt pathway oncoprotein β-catenin and the latter then escapes APC/Axin/GSK3-β complex degradation and accumulates in the nucleus where it can initiate transcription of Wnt target genes (Gujral *et al*. 2008). Furthermore, subsets of *RET* gene mutations have been demonstrated to activate the PI3K/AKT cascade, a signalling pathway heavily implicated in human tumorigenesis (Segouffin-Cariou and Billaud 2000). Germline activating *RET* mutations are demonstrated in the three variants of the MEN 2 syndrome, MEN 2A, MEN 2B and in the familial medullary thyroid carcinoma (FMTC) syndrome. MEN 2A comprises medullary thyroid carcinomas of the parafollicular C-cells (MTC), pheochromocytomas and parathyroid adenomas, whereas MEN 2B include pheochromocytomas as well as a mixture of associated features such as neuromas of the tounge and/or intestinal ganglioneuromatosis to name a few (DeLellis *et al*. 2004). Unlike *MEN1* gene mutations, *RET* gene alterations seem to demonstrate a more distinct genotype to phenotype correlation, as mutations in the extracellular domains of specified codons of exons 8, 10 and 11 correlate strongly to the development of MEN 2A, although subsets of mutations in the intracellular domain of exons 13-16 have also been reported (Lodish and Stratakis 2008). In MEN 2B, the mutations are most frequently located in intracellular domains involving the tyrosine kinase domains or the carboxyl terminus, the most common aberration constitutes a mutation in exon 16 that affects codon 918 and alters the structure of the substrate recognition pocket. The same mutational spectrum as observed in MEN 2A is commonly observed on the somatic level in MTC's (Lodish and Stratakis 2008). However mutations of the *RET* gene has not yet been demonstrated in sporadic parathyroid tumours, although the authors limited their search to MEN 2A mutation-prone exons (Pausova *et al*. 1996, Willeke *et al*. 1998).

The hyperparathyroidism-jaw tumour (HPT-JT) syndrome

The hyperparathyroidism-jaw tumour (HPT-JT) syndrome is a hereditary and autosomal dominant condition which involves primary hyperparathyroidism, ossifying fibromas of the mandible and maxilla, renal lesions such as cysts and Wilm's tumour as well as uterine manifestations, for example endometrial hyperplasia, adenofibromas and leoimyomas (Figure 7). The cause is an inactivating germline mutation in the *Hyperparathyroidism 2* (*HRPT2*)

gene located at 1q25-q31, which was identified by positional cloning after linkage analyses in kindreds with hereditary PHPT coupled to the 1q region (Carpten *et al*. 2002). *HRPT2* encodes parafibromin, a 531 amino acid protein with putative tumour suppressor properties. *HRPT2* gene mutations are mostly of frameshift or nonsense type, yielding premature stop codons and protein truncations. Generally, germline mutations are most commonly located to exon 1, but are also reported in exons 2 and 7 and sometimes in exons 3, 4, 5 and 14 (Carpten *et al*. 2002, Howell *et al*. 2003, Cetani *et al*. 2004b, Simonds et al. 2004, Villablanca *et al*. 2004). There is no obvious genotype-phenotype correlation, as hereditary mutations across the gene seem to result in the same type of syndrome. Subsets of tumours from HPT-JT patients have been shown to display LOH involving the 1q25-q31 region, suggesting that *HRPT2* is a classical tumour suppressor gene which obeys the Knudson two hit theory.

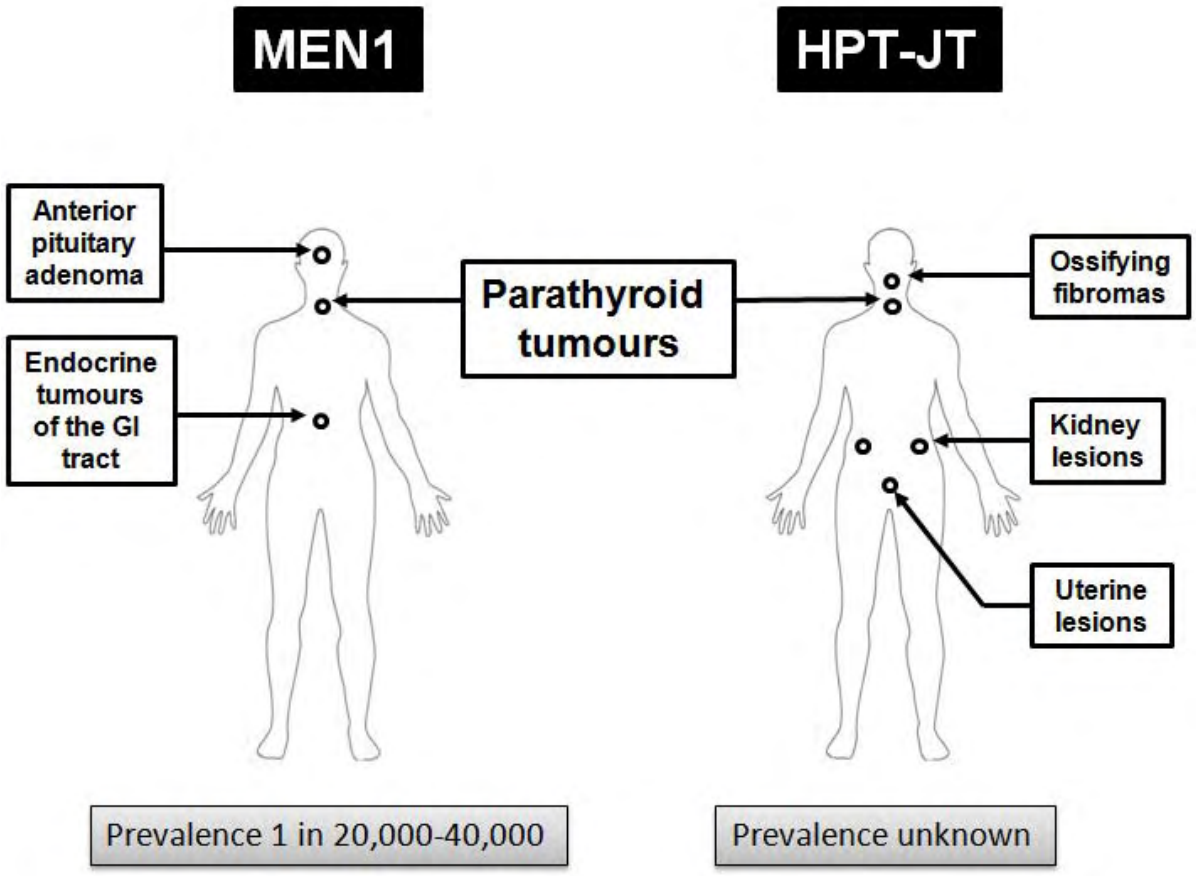

Figure 7 The MEN1 and HPT-JT syndromes with predominant manifestations.

The most common manifestation of the HPT-JT syndrome is PHPT, which afflict approximately 80% of the mutational carriers. A single parathyroid adenoma is the most frequent finding (85%), often with a cystic phenotype; however parathyroid carcinoma constitutes a fairly frequent entity in the HPT-JT syndrome (DeLellis *et al.* 2004). The reported risk is approximately 15% as compared to the much lower incidence rate in the general populace (Carpten *et al*. 2002). Indeed, *HRPT2* gene mutations are also frequently found in the sporadic setting, as studies have found this alteration in the majority of malignant cases examined (Howell *et al*. 2003, Shattuck *et al*. 2003a, Cetani et al. 2004b). The authors of these studies also detected *HRPT2* germline mutations in subsets of patients with apparent sporadic disease; the patients surprisingly lacked family history suggestive of familial PHPT. As a consequence, patients outwardly displaying sporadic parathyroid carcinoma should probably be subjects to germline *HRPT2* screening, regardless of family history for the disease or not.

Recent reports furthermore suggest that patients with parathyroid carcinomas should be investigated by immunohistochemical analysis of parafibromin expression, as reduced expression of this protein might indicate *HRPT2* gene alterations and may well detect cases which require further attention (paper III).

The HRPT2 gene in sporadic parathyroid tumorigenesis

In 2002, Carpten *et al.* identified inactivating germline mutations of the *HRPT2* gene in families with the HPT-JT syndrome, and interestingly *HRPT2* gene mutations were also demonstrated in a small fraction of sporadic parathyroid adenomas with cystic features (Carpten *et al*. 2002). Subsequent studies have shown that these sporadic adenomas also displayed loss of parafibromin expression (paper II). Given the fact that parathyroid carcinomas are over-represented in the HPT-JT syndrome, sporadic parathyroid carcinomas in turn became the focus of several groups. Howell *et al.* demonstrated *HRPT2* mutations in 4 out of 4 sporadic parathyroid carcinomas, and in two of the cases a second hit (second *HRPT2* mutation and LOH respectively) was evident (Howell *et al*. 2003). Shattuck *et al.* detected one or more *HRPT2* inactivating mutations in 10 out of 15 samples of sporadic parathyroid carcinoma and a surprisingly high frequency of germline mutations (Shattuck *et al*. 2003a). Subsequent studies demonstrate similar results, with *HRPT2* gene mutations discovered in the majority of parathyroid carcinomas but very rarely encountered in parathyroid adenomas

(Cetani *et al*. 2004b). In addition, Hewitt and colleagues recently demonstrated *HRPT2* promoter methylation in two of 11 parathyroid carcinomas (18%) and in a single HPT-JT associated tumour by methylation-specific PCR (MS-PCR). By contrast, all 37 sporadic adenomas investigated exhibited absence of methylation (Hewitt *et al*. 2007).

As a whole, the findings strongly advocate that *HRPT2*/parafibromin exhibit tumour suppressive properties in parathyroid cells. Furthermore, *HRPT2* gene alterations are found in the majority of sporadic parathyroid carcinomas as well as in small fractions of sporadic parathyroid adenomas, preferentially in those with cystic features.

Familial hypocalciuric hypercalcemia and the calcium-sensing receptor (CaSR)

The calcium-sensing receptor, as previously discussed, is responsible for the PLC-IP_3 mediated decrease in PTH secretion in the parathyroid cells, thus functioning as a negative feedback brake which regulates calcium homeostasis. Faulty expression of this receptor therefore has implications for parathyroid PTH hypersecretion and is also proposed to be associated to parathyroid tumourigenesis. The *CaSR* gene maps to 3q13-q21, and germ-line mutations in this gene cause several different syndromes, for example familial hypocalciuric hypercalcemia (FHH) and autosomal dominant mild hypercalcemia (ADMH) (both exhibiting heterozygous loss-of-function *CaSR* mutation), neonatal severe hyperparathyroidism (exhibiting homozygous/bi-allelic loss-of-function *CaSR* mutations), familial hypoparathyroidism as well as the Bartter syndrome (both displaying activating *CaSR* mutations) (Egbuna and Brown 2008). FHH is a syndrome characterized by hypercalcemia and concurrent low urinary calcium excretion. PTH levels are usually slightly elevated due to failure of the CaSR to inhibit PTH emission from vesicles, and the result is an increase in serum levels of calcium. The hypocalciuric status results from the failure of the renal system to reduce reabsorption of Ca^{2+} to the plasma as a response of hypercalcemia, since this event is mediated through the CaSR. Patients with FHH are usually asymptomatic and respond poorly to subtotal parathyroidectomy as hypercalcemia often recur (Marx *et al*. 1980). Furthermore, the present diagnostic routines do not always permit dependable discrimination between FHH and milder cases of neoplastic parathyroid disease; consequently it is imperative to recognize patients with FHH prior to surgical intervention to prevent unnecessary parathyroidectomy (Pearce *et al*. 1995). ADMH probably denotes an FHH

variant in which a *CaSR* mutation in the C-terminal domain caused familial disease with similar symptoms as FHH, but with a marked absence of hypocalciuria (Carling *et al*. 2000). Neonatal severe hyperparathyroidism denotes an FHH variant with homozygous *CaSR* mutations, causing severe hypercalcemic symptoms in the newborn. Early-on diagnosis and subtotal parathyroidectomy is essential to survival. Familial hypoparathyroidism represents a hereditary syndrome in which the inherited *CaSR* mutation is activating, meaning that the PLC-IP_3 cascade is augmented in the parathyroid cells. The result is an inhibition of PTH release. The disorder is distinguished by low levels of PTH and serum calcium as well as raised serum phosphorus in the presence of normal kidney function. Finally, activating *CaSR* mutations are also present in subsets of kindreds with the Bartter syndrome, a syndrome characterized by defective reabsorption of sodium and chloride in the tubules of the kidney among other electrolyte disturbances. A few patients displaying the Bartter syndrome phenotype have been found to be hypocalcemic with low levels of serum PTH, and the subsequent genetic analysis confirmed activating mutations of the *CaSR* gene (Watanabe *et al*. 2002). In sporadic parathyroid tumours, down-regulation of *CaSR* mRNA levels has been demonstrated (Farnebo *et al*. 1997a), however no *CaSR* mutations have been identified and only a few cases of parathyroid adenomas displaying LOH of the 3q13-21 locus have been shown (Thompson *et al*. 1995).

Familial isolated hyperparathyroidism (FIHP)

Familial isolated hyperparathyroidism (FIHP) is an uncommon disease in which the afflicted presents with PHPT as the sole manifestation, and the cause is often a single parathyroid adenoma or multiple gland disease. The diagnosis is based on the identification of inheritable PHPT where all other syndromes have been excluded. Linkage analyses of FIHP families have demonstrated coupling to the 1q21-q32 and 11q13 chromosomal regions, and subsequent direct DNA sequencing has identified *HRPT2* and *MEN1* germline mutations in the same cases (Huang *et al*. 1997, Teh *et al*. 1998, Kassem *et al*. 2000, Villablanca *et al*. 2002, Villablanca *et al*. 2004). In addition, subsets of FIHP families display inactivating *CaSR* gene mutations. No clear genotype to phenotype correlation has been proposed for these three genes for the development of FIHP versus the three regular syndromes MEN 1, HPT-JT and FHH. In clear writing, there is no apparent association between the germline mutation type and/or exon number and the development of either FIHP or the full-blown respective

syndrome. Interestingly, a large proportion of FIHP kindreds fail to display mutations in the *MEN1*, *HRPT2* or *CASR* genes, and subsets of FIHP families have been linked to chromosomal region 2p13.3-14, suggesting the presence of an unknown parathyroid tumour suppressor or protooncogene at this locus (Warner *et al*. 2006).

Parafibromin – a tumour suppressor of the parathyroid glands

Parafibromin is a member of the human PAF1 complex

The name parafibromin derives from an integration of the two most prominent tumour types of the HPT-JT syndrome, namely tumours of the parathyroid glands as well as ossifying fibromas of the mandible and maxilla (Carpten *et al*. 2002). Initially regarded as a classical tumour suppressor protein based on the initial findings regarding germline *HRPT2* gene mutations and loss of parafibromin expression in HPT-JT related tumours, more recent data imply that parafibromin is a rather complex, multifaceted protein with assorted roles and functions in the human cell – including growth stimulating properties in addition to its known growth inhibitory abilities.

Parafibromin bear similarities with the yeast protein Cdc73, in that the 200 most C-terminal amino acids shares 27% sequence identity between the two proteins. Cdc73 is a component of the yeast polymerase-associated factor 1 (yPAF1) complex, a key transcriptional regulatory complex that interacts directly with RNA polymerase II. In both yeast and mammalian systems, studies have demonstrated that Cdc73/parafibromin is a key mediator of transcriptional events such as histone modifications and chromatin remodeling as well as transcription initiation and mRNA elongation (Carpten *et al*. 2002). Parafibromin has been shown to bind directly to members of the human PAF1 complex (hPAF1), including PD2/hPaf1, hLeo1, and hCtr9, as well as to the large subunit of RNA polymerase II (RNAP II) (Rozenblatt-Rosen *et al*. 2005, Yart *et al*. 2005). Indeed, parafibromin expression itself was required for proper hPaf1 and RNAP II binding. hPAF1 is comprised of five subunits that include PD2/hPaf1, parafibromin, hLeo1, hCtr9 and hSki8, and a key role of the hPAF1 complex includes the histone 2B (H2B) monoubiquitination and H3 methylation, which

generate efficient transcription. In short, H2B ubiquitination is necessary for the RNAP II protein to access the entire chromatin, which is normally bundled up around histone proteins. The hPAF1 complex modifies the histone structure by associating to FACT (FAcilitates Chromatin Transcription), a protein which orchestrates the subsequent histone modifications. The dissociated nucleosomes are then traversed by RNAP II, and thus transcription is maintained (Chaudhary *et al*. 2007). The hPAF1 complex is also involved in the recruitment of transcriptional elongation factors required for transcriptional elongation as well as posttranscriptional roles including regulation of poly(A) tail length which in part control mRNA stability. Furthermore, the hPAF complex has been suggested to have growth stimulatory growth properties, as its core subunit PD2/hPaf1 is amplified and over-expressed in many cancers (Chaudhary *et al*. 2007).

Tumour-suppressor properties of parafibromin

Parafibromin is a protein with diverse functional properties, and is physically dispersed throughout the cell with evidence of nuclear, cytoplasmic and nucleolar accumulation (Figure 8). Based on the initial findings of *HRPT2* gene mutations in HPT-JT cases, FIHP and sporadic parathyroid tumours, parafibromin was assumed to bear tumour suppressor like properties. This assumption was further fortified when a monoclonal antibody raised against the N-terminal part of parafibromin was employed to demonstrate complete loss of parafibromin expression in the majority of tumour types commonly associated with *HRPT2* alterations, namely parathyroid carcinomas and HPT-JT-associated parathyroid tumours (Tan *et al*. 2004). In one of the first functional studies of parafibromin, Woodard and colleagues noticed that the transfection of plasmids containing *HRPT2* into cell lines inhibited cellular proliferation. Furthermore, the transfection led to up-regulation of the oncoprotein cyclin D1, whereas the introduction of a plasmid carrying known *HRPT2* missense mutation (L64P) resulted in lower levels of proliferation and absence of cyclin D1 up-regulation. Based on these observations, parafibromin was proposed to regulate cyclin D1 levels and act as an inhibitor of proliferation, the latter a hallmark for a tumour suppressor protein (Woodard *et al*. 2005). Subsequent studies using *HRPT2* inhibition by RNAi and FACS analysis showed that parafibromin inhibits cell cycle progression from the G_1 to the S phase (Yart *et al*. 2005). However, these studies did not assess whether or not this effect was a direct result of up-regulated cyclin D1 levels.

Further studies have since confirmed the role of parafibromin as a tumour suppressor protein. Zhang *et al.* showed that *HRPT2* overexpression *in vitro* led to reduced colony formation, and interestingly truncated parafibromin generated by site-directed mutagenesis could stimulate proliferation even in the presence of wild-type parafibromin. These observations are in agreement with a dominant negative effect, possibly implying that parafibromin suppression of cell growth is a dose-dependent mechanism or that mutant parafibromin directly or indirectly inhibits the functions of wild-type parafibromin (Zhang et al. 2006). Parafibromin has also been shown to be a pro-apoptotic protein, as the inhibition of endogenous parafibromin expression through RNAi led to diminished basal rates of apoptosis and apoptosis inflicted by the application of a topoisomerase inhibitor (Lin *et al*. 2007).

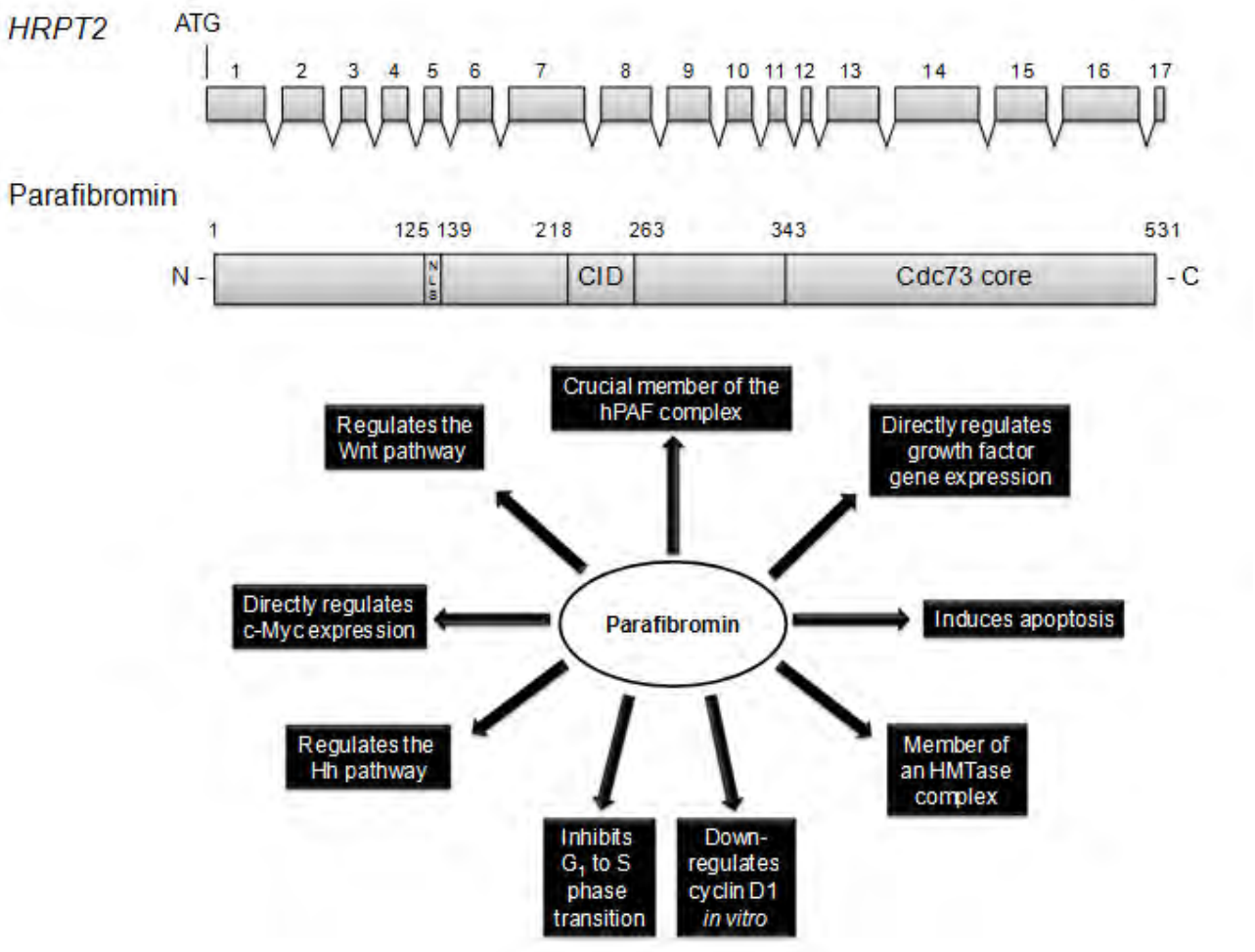

Figure 8 Schematic representation of the 17 exon long *HRPT2* gene, wildtype parafibromin structure pinpointed with important domains as well as an overview of parafibromin functions in the human cell. ATG: start codon, NLS: nuclear localization signal, CID: β-catenin interaction domain, Cdc73 core: hPAF1 complex binding domain.

Parafibromin regulates the Wingless type (Wnt) and Hedgehog (Hh) pathways

The Wingless type (Wnt) signaling pathway constitutes a multifaceted network of proteins regulating highly complex mechanisms such as embryogenesis, tissue development and cancer (McDonald *et al*. 2009). Roughly, the Wnt pathway is divided into two fractions, the canonical and the non-canonical Wnt pathway. Both pathway fractions make use of membrane-bound Frizzled type receptors, but when the canonical fraction employs LRP5/6 co-receptors for its activation, non-canonical signaling is dependent of Ror1/2 co-receptors. While canonical Wnt signaling revolves around the key oncoprotein β-catenin, non-canonical Wnt pathway acts independently of β-catenin and mediates its signaling through various signaling cascades such as the protein kinase C/calcium signaling system and the c-Jun kinase pathway to name a few. Canonical Wnt has been shown to be responsible for cell proliferation and differentiation of human cells, and aberrations in the central components of this signaling cascade have been identified as tumorigenic.

The canonical Wnt pathway is depicted in Figure 9. In its off-state, the Wnt pathway oncoprotein β-catenin is marked for degradation through a cytoplasmic, tri-molecular complex consisting of glycogen-synthase kinase 3-β (GSK3-β), adenomatous polyposis coli (APC) and axin. GSK3-β phosphorylates certain serine-threonine phosphorylation consensus motifs at aa 33, 37 and/or 41 of β-catenin, which triggers beta-transducing repeat-containing protein (β-TRCP) to mediate β-catenin destruction through the ubiquitin-proteasome pathway. When soluble Wnt ligands bind the extra-cellular domain of the Frizzled type receptors, it triggers a conformational change in the receptor's structure. This causes the recruitment of the membranous LRP5/6 co-receptor, which in turn activates Dishevelled (Dsh). Dsh subsequently inhibits β-catenin degradation by recruiting Frat-1, which displaces GSK3-β from axin. This causes stabilization of β-catenin, which will enter the nucleus and connect with LEF/TCF transcription factors, leading to the up-regulation of certain Wnt target genes, such as the *c-Myc* and *CCND1* (cyclin D1) oncogenes to name a few (McDonald *et al*. 2009).

Parafibromin was coupled to the canonical Wnt pathway in 2006, when Mosimann and coworkers discovered that RNAi against Hyx, the Drosophila melanogaster homolog of parafibromin, gave a significant reduction in Wnt pathway signaling. Further overexpression of *HRPT2* in a human cell line resulted in Wnt pathway activation, and parafibromin was also shown to directly bind β-catenin. The authors conclude that parafibromin, somewhat surprisingly given its tumour suppressor functions, has a positive role in Wnt signaling

(Mosimann *et al*. 2006). Given the fact that the Wnt pathway activates numerous target genes through β-catenin association to transcriptional activators in the nucleus, the transcriptional targets of β-catenin in the presence of parafibromin are assumed to encode several proteins which inhibits cellular growth. Interestingly, the parathyroid tumour suppressor protein menin was as discussed previously also shown to positively influence canonical Wnt signaling output, suggesting that the current view on canonical Wnt solely as a growth stimulating pathway is somewhat inadequate (Chen *et al*. 2008). At least in theory, the positive influence of two major parathyroid tumour suppressors on this pathway suggest that a subset of canonical Wnt target genes in fact inhibit as opposed to stimulate cellular growth.

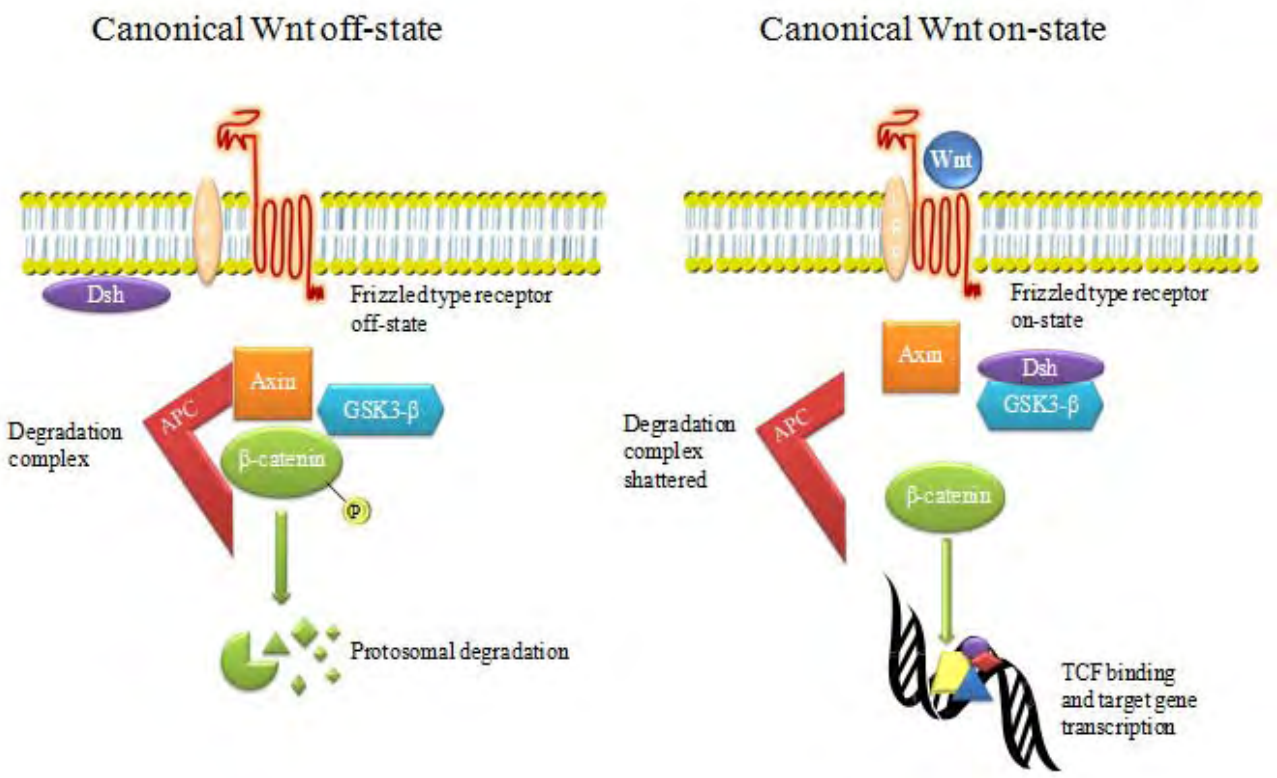

Figure 9 Canonical Wnt in its off-state (left) and on-state (right).

Interestingly, recent data also suggest a role for parafibromin in the hedgehog signaling pathway (Hh) through its direct communication with the nuclear transcriptions factors Gli (Mosimann *et al*. 2009). The Hh signaling pathway plays significant roles in a variety of

embryonic and tissue renewal events, for example in orchestrating the formation of limbs. One of the best studied Hh ligands is the sonic hedgehog protein (Shh). Shh binds to the transmembranous Patched-1 (PTCH1) receptor, thereby lifting the constitutional inhibition of Smoothened (SMO), a downstream pathway protein which then triggers the Hh pathway through the activation of the Gli transcription factors, consisting of Gli1, Gli2 and Gli3. Activated Gli molecules then enter the nucleus to control the transcription of various target genes (Lum and Beachy 2004). Interestingly, the Hh pathway also promotes stem cell proliferation and has been implicated in various tumours, for example brain-, lung- and breast cancer (Gialmanidis *et al*. 2009, Thomas *et al*. 2009, Zardawi *et al*. 2009). For example, activating mutations in SMO as well as deleterious mutations in PTCH1 have been demonstrated in patients with basal cell nevus syndrome as well as in sporadic basal cell carcinomas of the skin (Johnson *et al*. 1996, Xie *et al*. 1998). Intriguingly, parafibromin has been shown to act as a Gli binding partner and might be essential for expression of particular Hh target genes, thus parafibromin exhibit functions as a key Hh pathway controller in addition to its previously known role as a regulator of the canonical Wnt pathway. Future studies regarding SMO, PTCH1 and Gli gene and protein expression in parathyroid tumours could consequently be of great interest as the genetic mechanisms underlying the development of parathyroid tumours are further dissected.

Parafibromin as a gene regulator

In 2005, parafibromin was shown to associate with a histone methyltransferase (HMTase) complex in addition to its properties as a member of the hPAF complex (Rozenblatt-Rosen *et al*. 2005). HMTase complexes are required for histone methylation, an epigenetic phenomenon used to regulate the accessibility of certain chromatin sequences and thereby indirectly the rate of target gene transcription. Interestingly, parathyroid adenomas have been shown to display higher levels of global DNA methylation as compared to normal parathyroid cells, and this phenomenon correlated to the presence of an *HRPT2* mutation (paper VI). In fact, the two tumours with the highest levels of global methylation densities in the entire series were both carried germ-line *HRPT2* gene mutations, indicating that parafibromin might play a principal role in the epigenetic regulation of parathyroid gene expression in addition to histone modifications.

In addition to the role of parafibromin as a member of a noted epigenetic apparatus, studies have shown that parafibromin directly binds to the promoter regions of various genes and controls their expression. Wang and colleagues used chromatin immunoprecipitation and showed that parafibromin directly occupies promoter and/or coding regions of genes encoding cellular growth factors, including *IGF1*, *IGF2*, *IGFbp 4*, *Hmga 1*, *Hmga 2*, *H19* and *Hmgcs2* (Wang *et al*. 2008). The insulin-like growth factors (IGF) 1 and 2 are both growth hormones, stimulating cellular proliferation by endocrine, paracrine and autocrine mechanisms. Interestingly, IGF2 over-expression is a regular phenomenon in Wilm's tumour (nephroblastoma) which is also an observed component of the HPT-JT syndrome. As a part of the pathogenesis of this tumour type, the *Wilm's tumour 1* gene (WT1) has been found to be mutated in a substantial subset of nephroblastomas. WT1 has also been shown to directly bind the promoter region of the *IGF2* gene and down-regulate IGF2 protein expression (Drummond *et al*. 1992). Further IGF2 protein and *WT1* gene analyses in parathyroid tumours devoid of parafibromin expression could therefore be of future value, as well as parafibromin analyses in sporadic nephroblastomas.

In 2008, Lin et al. discovered that parafibromin directly binds to the *c-Myc* oncogene promoter region and down-regulates *c-Myc* expression (Lin *et al*. 2008). *c-Myc* is a well-established oncogene which encodes a DNA-binding protein that regulates gene transcription of various genes in charge of proliferation and cell cycle progression. Interestingly, c-Myc also constitutes a Wnt pathway target gene, and is over-expressed in many different cancers (Nesbit *et al*. 1999). Regarding parathyroid tumours, Björklund and coworkers have demonstrated c-Myc over-expression in a considerable fraction of parathyroid adenomas using quantitative real time-PCR and immunohistochemistry, suggesting that parathyroid tumours in part are driven by this oncoprotein. The status of parafibromin and GSK3-β, both known to negatively regulate *c-Myc*, was not assessed in these tumours (Björklund *et al*. 2007).

Parafibromin – subcellular localization

With few exceptions, most tumour suppressor proteins exert their functions in the nucleus. In 2004, Tan and colleagues published data regarding parafibromin expression in parathyroid tumours, and for this purpose the authors produced a monoclonal parafibromin antibody termed 2H1 directed at aa 87-100 of human parafibromin, corresponding to exon 3 of *HRPT2*

(Tan *et al*. 2004). Employing 2H1, the authors demonstrated exclusively nuclear expression of parafibromin in parathyroid tumours using immunohistochemistry (IHC), and the results were validated using immunofluorescence with an *HRPT2*-transfected cell line.

Woodard *et al.* constructed a polyclonal parafibromin antibody targeting an epitope within residues 2-135 of human parafibromin, and by analyzing subcellular fractionated protein extracts from cell lines transfected with *HRPT2* plasmids the authors concluded that parafibromin was expressed in both the nucleus and cytoplasm (Woodard *et al*. 2005). A third study detected parafibromin solely in the nucleus by immunofluorescence employing a polyclonal parafibromin antibody directed at a 24 aa sequence within residues 250-300 of parafibromin (Rozenblatt-Rosen *et al*. 2005). Parafibromin has since been shown to carry a dominant, bipartite nuclear localization signal (NLS) at residues 125-139, and *HRPT2* gene mutations within this region was found to totally abolish parafibromin nuclear expression (Hahn and Marsh 2005). Furthermore, a second NLS at residues 76-92 was later identified by an independent group (Lin *et al*. 2007). Conversely, the authors still observed nuclear accumulation of parafibromin although mutations within the dominant NLS was introduced in cell lines, however the nuclear expression was totally eradicated if the secondary NLS also was disrupted by an *HRPT2* gene mutation (Lin *et al*. 2007).

A subsequent study in which a C-terminal polyclonal parafibromin antibody towards aa 509-531 was employed revealed predominant parafibromin nuclear expression with a weak cytoplasmic component in parathyroid tumours by IHC. The results were verified using live cell imaging of cell lines with transfected *HRPT2* constructs tagged with green fluorescent protein (GFP) (paper II). In 2008, Agarwal and coworkers cemented the notion that parafibromin could locate to the cytoplasm by demonstrating direct parafibromin interaction with muscle alpha-actinins but not with non-muscle actinins (Agarwal *et al*. 2008). In addition, parafibromin was shown to cross-link actin filaments. The authors speculate that actin and actinin could -by their binding properties- play a role as regulators of parafibromin nuclear levels by keeping parafibromin bound in the cytoplasm. However, given the different roles of Wnt pathway members (for example β-catenin as a membranous anchor in addition to its oncogenic properties in the nucleus et.c) it is not entirely impossible that parafibromin also exhibits auxiliary roles in the cell in addition to its proliferation regulating properties.

To summarize, parafibromin is a predominantly nuclear protein with concurrent cytoplasmic localization that exhibits functional roles in both compartments. In addition to this,

parafibromin has been endowed with three nucleolar localization signals (NoLS) at residues 76-92, 192-194 and 393-409 of human parafibromin (Hahn & Marsh 2007). However, the role of nucleolar parafibromin is currently unknown.

Parafibromin – the only child?

The current notion of *HRPT2* as a parathyroid tumour suppressor gene stems from its inactivation in the HPT-JT syndrome, in subsets of families with FIHP as well as in sporadic parathyroid tumours. In turn, much focus has been put into analyzing parafibromin, the ~60 kDa protein encoded by the 2.7 kb long *HRPT2* transcript originating from exons 1 to 17. However, current studies of parafibromin expression in parathyroid tumours are somewhat contradictory regarding subcellular localization, parafibromin expression levels (100% positive nuclei versus mixed patterns) and the overall intensity of the staining reactions. The finding of parafibromin expression in subsets of parathyroid nuclei only could indicate a cell cycle dependent expression pattern; alternatively parafibromin variants could be expressed in subsets of tumour cells with functions other than suppression of proliferation, but still imperative to tumour growth. These observations have led to speculations whether or not parafibromin is the only *HRPT2* product. Indeed, Northern blot studies early on indicated that *HRPT2* harbored an additional transcript of approximately double the size of regular parafibromin (Carpten *et al*. 2002), but more profound studies regarding this transcript have yet to be published.

In recent times, the online genome browser Ensembl has published data regarding two additional *HRPT2* transcripts; CDC73-201 and CDC73-202, both suggested to be protein coding. CDC73-201 corresponds to exons 3-17 plus a previously unknown exon (termed “exon 0”) upstream of the regular *HRPT2* ATG start site. Exons 1 and 2 are excluded from of the transcript. The Ensembl database predicts a corresponding 257 aa protein of 29 kDa. The lack of an apparent ATG site in exon 0 of CDC73-201 suggests that further upstream elements are involved in the regulation or alternatively CDC73-201 could represent a non-ATG start site transcript, the latter very unusual in eukaryotic systems. Protein similarity analyses by gene ontology predict that a putative CDC73-201 protein exhibits serine/threonine kinase properties, thus making the CDC73-201 protein a possible pathway intervener and an interesting topic in further studies. Of further interest, the putative CDC73-201 protein retains NLS sequences, the β-catenin interacting domain in exon 7-8 and the

hPAF1 interacting domain in the C-terminus of regular parafibromin, suggesting that CDC73-201 could translocate to the nucleus for interaction with the Wnt signaling pathway and the hPAF1/RNAP II machinery. Of great interest to the parathyroid field is the fact that the vast majority of inactivating *HRPT2* mutations within sporadic and hereditary parathyroid disease reside within exons 1 and 2. The exclusion of exons 1 and 2 in CDC73-201 may indicate that a parathyroid cell with an acquired *HRPT2* mutation in exon 1 or 2 still propels the production of a protein with retained β-catenin and hPAF1 interaction properties.

The second novel transcript CDC73-202 corresponds to 14 of the 17 *HRPT2* exons with skipping of exons 10 to 12. The predicted protein of 316 amino acids and 36 kDa would correspond to wildtype exons 1-8 followed by a novel sequence based on a frameshift from exon 9 downwards to exon 13 where a novel stop codon is present. Gene ontology analyses suggest that CDC73-202 could be involved in cell differentiation and responses to ionizing irradiation. According to the Ensembl database, the CDC73-202 protein would retain the NLS sequence and the β-catenin interacting domain, but lack the C-terminal hPAF1 interacting domain. Theoretically, the elusive CDC73-202 protein would be able to interact with β-catenin in the nuclear compartment, however without involvement of the hPAF1 complex. Clearly, both CDC73-201 and CDC73-202 are potentially parafibromin-related products which, if expressed in the parathyroid gland, would constitute an interesting starting point for future studies.

Gene expression profiling in parathyroid tumours

The advent of microarray techniques for mass investigations of gene expression in tissue samples have been of great assistance in evaluating how a specific tissue or tumour takes advantage of the genome. Today's technology allows cancer researchers to correlate the expression of thousands of genes in tumour samples and normal tissues, and the amount of available information is overwhelming. The tumour and reference samples are commonly hybridized onto microarray slides containing oligonucleotides or cDNA clones representing thousands of unique genes, yielding an expression profile for each sample investigated. This kind of molecular classification has allowed many tumour types to be categorized into clinical or pathogenetical subgroups of diagnostic or prognostic value.

MEN1 and HRPT2 – two separate molecular paths

Using microarray analysis, Haven and colleagues conducted expression profiling analysis of 53 parathyroid lesions of hereditary (HPT-JT, MEN 1, MEN 2A and FIHP cases) and sporadic origin (Haven *et al*. 2004). All histological subtypes of parathyroid lesions were employed, including adenomas, primary hyperplasias, carcinomas, SHPT and THPT. In addition, pooled samples of normal parathyroid tissues were included in the study. Utilizing microarray slides spotted with approximately 20,000 cDNA clones, the authors identified three distinct tumour groups according to the clustering of respective gene expression profiles. Cluster 1 denoted hyperplastic specimens and the pooled normal sample. Cluster 2 contained sporadic carcinomas and benign and malignant tumours with *HRPT2* associated familial disease. The third cluster constituted the vast majority of all sporadic adenomas, all MEN 1 cases as well as two FIHP cases of unknown genetic background. In addition, this cluster harbored the majority of THPT cases as well as one SHPT case. These data suggest that parathyroid tumours carrying *HRPT2* gene mutations, regardless of histological subgroup or hereditary status, have a similar molecular profile. This profile clearly differs from parathyroid adenomas and MEN 1 related cases, the latter two groups which exhibit a similar molecular expression pattern. These data further delineate the association between the *MEN1* gene and the development of benign parathyroid tumours. These results are in line with previous work based on expression profiling in parathyroid lesions, in which the authors demonstrate that tumours displaying chromosomal loss at 11q13 separate from tumours without this alteration (Forsberg *et al*. 2005).

To conclude, based on their gene expression parathyroid tumours segregate into at least four different groups; one *HRPT2* related group, one *MEN1* related group, one group unrelated to *HRPT2* or *MEN1* and one hyperplastic group displaying an expression phenotype which is also present in normal parathyroid tissues. Interestingly, HPT-JT-associated adenomas clustered tightly with parathyroid carcinomas. Based on these observations, it could be speculated that the former tumour type have the capability to progress into malignant tumours. Theoretically, these tumours might represent early stages of malignant tumours in the progression to full blown malignancy, possibly lacking one or several genetic alterations required for the ultimate transformation.

Other molecular aberrancies in sporadic PHPT

Cyclin D1

Cyclin D1 is a key regulator of the G_1 to S transition of the cell cycle, as its association with Cdk 4/6 initiates inhibition by phosphorylation of the Rb protein. Cyclin D1 thus functions as a proto-oncogene, and its over-expression has been demonstrated in a wide variety of tumour types (Arnold 1995). In PHPT, the cyclin D1 protein has brought out particular attention, partly since it was first identified and acknowledged as an oncogene in parathyroid tumours and partly because small subsets of parathyroid tumours display a defined chromosomal aberration involving the cyclin D1 gene *CCND1*. Basically, the mechanism denotes the chromosomal inversion of the regulatory sequence of the *PTH* gene, placing it in proximity to the *CCND1* oncogene. Thus the *CCND1* gene is placed under the control of the regulatory region of the abundantly expressed *PTH* gene, causing its over-expression by augmented transcription (Arnold 1995). However, this mechanism has been demonstrated in a small number of parathyroid tumours only. By contrast cyclin D1 over-expression in parathyroid tumours occur in a substantial fraction of the cases based on immunohistochemistry and/or Western blot analysis (Hsi *et al*. 1996, Vasef *et al*. 1999). Subsequent genetic analyses of the known cyclin D1-Cdk 4/6 inhibitors p15, $p16^{ink4A}$ and p18 failed to demonstrate mutations in sporadic parathyroid tumours (Tahara *et al*. 1996b, Tahara *et al*. 1997). Thus, additional unknown mechanisms propelling the up-regulation of *CCND1* are probably present in parathyroid tumours.

The retinoblastoma protein (Rb)

As previously discussed, the Rb protein prevents the E2F transcription factor from entering the nucleus and activating gene expression of cyclin E. Thus Rb acts as a tumour suppressor protein, obstructing G_1 to S phase transition of the cell cycle. Loss of Rb expression is implicated in various tumour types, and the parathyroid glands are no exception. Indeed, in 1994 Cryns and coworkers postulated that loss of Rb expression determined by immunohistochemistry was diagnostic for parathyroid carcinoma. A total of 16 carcinoma

specimens from 9 patients were studied of which 88% revealed total or near-total loss of nuclear Rb expression. Furthermore, the group also demonstrated frequent loss of heterozygosity (LOH) of the *RB* locus at 13q14 in the same malignant tumours using intragenic markers, in which all 11 specimens investigated from 5 patients with parathyroid carcinoma lacked an *RB* allele. Controls consisted of 19 parathyroid adenomas, which all retained nuclear Rb expression and only one case showed allelic loss at the *RB* locus (Cryns *et al*. 1994b). The findings are supported by Cetani and colleagues who discovered LOH of the *RB* allele and loss of RB expression in six out of six parathyroid carcinomas studied (Cetani et al. 2004a). Despite these encouraging findings, other studies have displayed much lower frequencies regarding loss of Rb expression in malignant parathyroid tumours, thereby diminishing the diagnostic value of the Rb protein as a discriminating marker between malignant and benign disease. For example, Farnebo *et al*. showed that adenomas could lack Rb expression altogether, whereas carcinomas could display full immunoreactivity, thereby greatly lowering both sensitivity and specificity for the method. This was also supported by LOH data, in which some malignant tumours retained both *RB* alleles while some benign tumours did not (Farnebo *et al*. 1999). Interestingly, the 13q14 locus contains at least one additional tumour suppressor gene besides *RB*, namely *BRCA2*. Turning to the gene level, a subsequent study demonstrated a total absence of *RB* and *BRCA2* mutations in the same parathyroid carcinomas previously identified to lack Rb protein expression altogether (Shattuck *et al*. 2003b). Thus; although Rb protein down-regulation is associated with parathyroid carcinomas, an additional putative tumour suppressor gene or important miRNA sequence within the same locus might be responsible for the intriguing findings of LOH at 13q14 in malignant PHPT.

Retinoblastoma protein-binding zinc finger 1 (RIZ1) protein

The *RIZ1* gene located at 1p36 encodes a 1,719 aa protein with retinoblastoma protein-binding properties that is implicated in transcriptional regulation. *RIZ1* inactivating mutations have been demonstrated in subsets of colorectal cancer and malignant melanomas (Chadwick *et al*. 2000, Poetsch *et al*. 2002). So forth, all recognized mutations are restricted to the RIZ1-specific PR domain, which is believed to exhibit a regulatory protein methyltransferase activity. In 2003, Carling and coworkers investigated parathyroid tumours and pheochromocytomas for *RIZ1* promoter methylation, mutations and LOH at the *RIZ1* locus.

For the 47 parathyroid tumours studied, LOH within the *RIZ1* locus was demonstrated for 13 cases (28%). Using MS-PCR, *RIZ1* promoter hypermethylation was evident in 17 of the parathyroid tumours (36%) and interestingly correlated to the existence of 1p36 LOH as well as to *MEN1* wildtype sequence, suggesting that *MEN1* mutations are superfluous for parathyroid tumour growth if *RIZ1* already carries aberrations (Carling *et al*. 2003).

Megalin

The multi-ligand receptor megalin is mainly expressed in the proximal tubule cells of the kidney where it functions as an endocytic receptor for a wide range of ligands filtered by the glomeruli apparatus. Examples of ligands include PTH, 25-hydroxyvitamin D3 and vitamin D3-binding proteins. The megalin receptor is responsible for the uptake of 25-hydroxyvitamin D3 into the tubule cells for subsequent activation to 1,25-dihydroxyvitamin D3, as well as for the uptake of PTH which triggers internalization and degradation. Hence, megalin have functional properties which might influence calcium homeostasis in man. As mentioned earlier, megalin has also been proposed as a second calcium-sensing receptor of the parathyroid glands; although the evidence that has been put forward to demonstrate such a function is limited to one study (Juhlin *et al*. 1987). A few studies have shown down-regulation of the megalin mRNA and protein in sporadic parathyroid adenomas and SHPT as compared to normal parathyroid tissues (Juhlin *et al*. 1989, Farnebo *et al*. 1998b) while others have failed to find LOH in the 2q21-q22 region covering the megalin gene or CpG hypermethylation of the megalin gene promoter (Farnebo *et al*. 1997b, Knutson *et al*. 1998). Germ-line mutations of the megalin gene give rise to the Donnai-Barrow syndrome characterized by several developmental anomalies, however no reported relation to parathyroid disease. Hence, the bulk of evidence argues against direct involvement of the megalin protein in parathyroid tumourigenesis.

Interestingly, as previously discussed there is now emerging evidence that parafibromin acts as an Hh pathway interacting protein. It is also known that the Hh pathway ligand Shh binds to the parathyroid cell receptor megalin with high affinity, followed by internalization of the Shh protein through endocytosis, conceivably regulating the levels of Shh available to PTCH1 (McCarthy *et al*. 2002). The megalin receptor is thus associated to a signaling pathway for a

known parathyroid tumour suppressor protein, a circumstance which may require further attention in the future.

The vitamin D3 receptor (VDR)

The vitamin D3 receptor (VDR) is a nuclear receptor that exclusively binds the active form of vitamin D3 (1,25-dihydroxyvitamin D3). The binding between vitamin D3 and VDR induces the formation of a hetero-dimer between the VDR and the retinoid X receptor (RXR). The VDR-RXR complex subsequently binds to specific DNA hormone response elements which induces expression of a wide variety of target genes. The main function of active vitamin D3 in the parathyroid glands is to negatively regulate *PTH* gene transcription and to inhibit proliferation of the parathyroid cells. Therefore, the VDR could be seen as a potential tumour suppressor of the parathyroid glands, as its inhibition would theoretically induce parathyroid cell proliferation. Indeed, polymorphisms of the *VDR* gene have been shown to be overrepresented among postmenopausal women with PHPT (Carling *et al*. 1995). If *VDR* polymorphisms would affect VDR expression levels, this could in turn alter the inhibitory effect of vitamin D3 upon parathyroid cell proliferation.

Multi-drug resistance protein 1 (MDR1)

Technetium-99m-sestamibi scans have gained ground as a preoperative imaging technique in patients with PHPT, as a focused uptake in a pathological parathyroid gland could convey a minimally invasive surgical approach. Studies regarding this phenomenon have shown that parathyroid adenomas usually exhibit reduced expression of the multi-drug resistance protein 1 (MDR1) and the multidrug resistance-associated protein 1 (MRP1), both members of the ATP-binding cassette family. These proteins cause resistance to various drugs by functioning as drug-transport pumps in the cell membrane. Parathyroid normal tissue exhibits strong MDR1 expression, and some authors argue that the difference between MDR1 expression in parathyroid tumours as opposed to the findings in normal parathyroid explains the fact that parathyroid adenoma cells often accumulate technetium-99m-sestamibi while normal cells do not. In 2007, Takeuchi and coworkers showed that the *MDR1* gene promoter was methylated in 19 out of 27 parathyroid adenomas (70%) by MS-PCR, whereas methylation of the *MRP1* gene was uncommon (10% of cases). The authors furthermore found a significant correlation

between hypermethylation for *MDR1* and sestamibi-positive scans, suggesting that the inactivation of this gene might propel sestamibi accumulation (Takeuchi *et al*. 2007). However, the methylation of the *MDR1* gene was exclusively found in parathyroid tumours, suggesting that the downregulation of this gene could confer proliferatory advantages as well. Interestingly, *MDR1* mutations have been demonstrated in subsets of colorectal cancers with high microsatellite instability (Potocnik *et al*. 2001).

β-catenin

β-catenin is the main effector of the canonical Wnt pathway, and its functions and modes of regulation are thoroughly discussed above. Over-expression of β-catenin has recently been proposed as a mechanism underlying parathyroid tumorigenesis, as the authors demonstrated frequent activation of the Wnt pathway through excessive β-catenin signaling (Björklund *et al*. 2007a). The study reveals stabilization and accumulation of non-phosphorylated β-catenin in parathyroid lesions from patients with PHPT and SHPT, and in a small subset of the cases (9 out of a total of 124 cases; 7.3%) the activation was associated with homozygous (i.e. bi-allelic) mutations in the third exon of the *CTNNB1* gene. The mutation consisted of a single nucleotide substitution at position c.109 (T>G) in exon 3, giving rise to an amino acid change at position 37, from the polar serine to the unpolar alanine. This region comprises the GSK3-β phosphorylation sites of β-catenin, and the substitution will render β-catenin un-phosphorylated at position 37. As of this, the levels of β-catenin subsequently rise and stabilized β-catenin can enter the nucleus, initiating transcription of certain Wnt target genes. Stabilizing β-catenin mutations affecting exon 3 of the *CTNNB1* gene are also observed in ovarian cancer, hepatoblastomas and Wilm's kidney tumours to name a few (Polakis *et al*. 2007). Contrasting results have been reported in studies from other geographical regions (Japan, United States and Italy) that showed lack of *CTNNB1* exon 3 mutations and/or absence of β-catenin activation (Ikeda *et al*. 2002, Costa-Guda and Arnold 2007, Cetani *et al*. 2009, paper IV). Thus, it remains to be determined whether the positive findings of *CTNNB1* exon 3 mutations in a small subset of parathyroid adenomas represent a geographical divergence, random variation or an association to an undefined subtype of PHPT.

LRP5

By analyzing the upstream membrane-bound receptor LRP5 in the Wnt signaling cascade, Björklund et al. reported aberrant splicing of this Wnt co-receptor in 32 out of 37 cases of PHPT and in 20 out of 20 cases of SHPT (Björklund *et al*. 2007b). The abnormal splicing creates an in-frame deletion of LRP5 between amino acids 666 and 809, and as a consequence β-catenin driven transcription in parathyroid tumour cells is increased. The authors specifically knocked down this aberrantly spliced variant of LRP5 in a novel human parathyroid cell line, and as a result the rate of cell growth as well as the levels of active β-catenin diminished. The effect of the LRP5 variant on Wnt signaling was augmented by application of the WNT3 soluble ligand in the culture medium. Furthermore, upon injection of parathyroid tumour cells derived from the cell line into severe combined immunodeficiency (SCID) mice, a significant reduction in tumour growth was observed if the cells were pre-treated with siRNA against the LRP5 splicing variant.

Mitochondrial DNA mutations

Mitochondrial DNA (mtDNA) mutations in genes encoding respiratory chain components have been demonstrated for various tumour types, but their exact contribution to tumorigenesis remains to be established. In 2007, Costa-Guda and colleagues investigated 30 parathyroid adenomas for mtDNA mutations by direct sequencing of the complete mtDNA genome, and found a grand total of 27 mutations (Costa-Guda *et al*. 2007). Eleven mutations were detected in 6 out of 18 chief cell adenomas and 16 mutations were demonstrated in 9 of 12 oxyphilic adenomas. Mutations affecting complex I genes of the respiratory chain comprised 56% of all mutations, including inactivating mutations of the *NADH dehydrogenase subunit 1*, *4* and *5* genes. Complex I is the initial electron acceptor from NADH which initiate the respiratory chain reaction leading to the formation of the high-energy compound ATP. Interestingly, a significantly higher proportion of mutations were found among oxyphilic adenomas than in the chief cell population. Given the fact that oxyphilic adenomas display high quantities of mitochondria in the cytoplasm, these findings indicate that the mitochondrial accumulation observed might be due to mtDNA mutations in

genes encoding respiratory chain members. Indeed, similar observations have been made in oncocytic tumours of the thyroid (Gasparre *et al*. 2007).

Recurrent DNA copy number alterations

Apart from single gene aberrations in PHPT, analyses of chromosomal gains or losses by loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) techniques have confirmed frequent deletions of chromosome regions at 1p, 6q, 9p, 11q, 13q, 15q, and X as well as gain of chromosomes 7, 16p and 19p in parathyroid tumours. The frequency of these alterations in PHPT suggests that these regions harbor several tumour suppressor genes and proto-oncogenes respectively of importance for developing parathyroid tumours (Tahara *et al*. 1996a, Agarwal *et al*. 1998, Palanisamy *et al*. 1998, Kytöla *et al*. 2000, Villablanca *et al*. 2002, paper I).

Molecular markers for parathyroid carcinoma diagnosis

The pathologist's dilemma

The current criteria for the proper identification of a parathyroid carcinoma require histopathological evidence of vascular or perineural invasion, local tissue invasion or distant metastasis (DeLellis *et al*. 2004). Hence, the classification is dependent on the visualization of ongoing malignant properties and is clearly not satisfactorily since the tumour might have spread before malignancy can be diagnosed. Optimally, a definite diagnosis for malignancy is made prior to the onset of full-blown malignant properties, and hence a great number of research studies have tried to identify a suitable molecular marker for early detection of parathyroid cancer. The concept revolves around the perception that malignant potential is best detected through associated molecular events rather than through morphological findings observed in routine stained sections, and most studies have assessed this question by using IHC analyses. The reasons for employing IHC are many; it denotes a golden standard technique for rapid detection of various proteins from frozen or paraffin embedded tissues; it is a generally applied method within pathology institutions; it is relatively inexpensive as

compared to more advanced molecular analysis methods and it exhibits both high sensitivity and specificity if carried out during stringent conditions. Previous attempts to identify parathyroid malignant tumours based on histopathological characteristics (such as nuclear atypia, fibrous bands, trabecular growth patterns et.c) alone have not been sensitive or specific enough, and hence the attention has turned to the molecular level (Sandelin *et al*. 1992, Bondeson *et al*. 1993).

One of the first IHC markers that were assessed for the proper identification of parathyroid carcinoma was Ki-67, a widely used proliferation marker. Although initial studies demonstrated high sensitivity for the method (i.e. a high number of parathyroid carcinomas with high Ki-67 counts), subsequent studies have shown a significant overlap between adenomas and carcinomas (Lloyd *et al*. 1995, Farnebo *et al*. 1999, Stojadinovic *et al*. 2003). Still, the World Health Organization Classification of Tumours advocate that Ki-67 counts above 5% in PHPT should require a more prolonged follow-up since this aberration is frequently found in parathyroid malignant tumours.

In 1994, lack of immunohistochemical expression of the retinoblastoma protein (Rb) was evaluated as a distinguishing marker for parathyroid malignant disease (Cryns *et al*. 1994). Subsequent studies were not able to reproduce these promising results (Lloyd *et al*. 1995, Subramaniam *et al*. 1995, Farnebo *et al*. 1998a). However, in 2004 Cetani and coworkers reported six parathyroid carcinomas that stained completely negative for Rb, while 30 adenomas all stained positive with varying intensity/distribution. Hence, the group postulates a “reappraisal” of the Rb protein in carcinoma diagnostics (Cetani *et al*. 2004a).

In subsequent years, an IHC microarray study from Stojadinovic et al. suggested a profile with high specificity towards parathyroid benign tumours, namely positive expression for p27, bcl-2 and mdm2, as well as low Ki-67 counts (Stojadinovic *et al*. 2003). This profile was not found in a single carcinoma studied but seen in the majority of adenomas (76%). Interestingly, bcl-2 and mdm2 are members of the p53 pathway, indicating that other p53 pathway members than p53 itself might be dysfunctional and play a role in benign tumour development of the parathyroid glands. Although promising, the downside of employing 4 different markers in the general screening process revolves around the meticulous work of the required methodology as well as in the interpretation of these data. Furthermore, given the reduced sensitivity in identifying benign tumours, a considerable amount of adenomas will be misclassified as potentially malignant. Furthermore, succeeding studies have demonstrated

significant overlap in both bcl-2 and Ki-67 expression when comparing benign and malignant parathyroid tumours (Naccarato *et al*. 1998).

Cyclin D1 is a well-established parathyroid oncoprotein as discussed previously. Initial IHC analyses found cyclin D1 to be useful for distinguishing parathyroid carcinomas from adenomas, since the malignant group displayed strong and diffuse cyclin D1 staining whereas the benign group in general exhibited a lower proportion of positively stained cells (Hsi *et al*. 1996). More recent studies however demonstrate an overlap between carcinomas and adenomas regarding cyclin D1 immunoreactivity, thus it is plausible that cyclin D1 up-regulation is a common molecular event for both malignant and benign parathyroid tumours and hence has reduced value as a separating marker (Vasef *et al*. 1999, paper IV).

In a similar fashion, p53 was initially found to be of additional value to the pathologist since allelic loss of the p53 gene was evident in a subset of parathyroid carcinomas, although mutations were not observed (Cryns *et al*. 1994a). However, the greater part of the parathyroid carcinomas examined exhibited negative p53 immunoreactivity as opposed to the adenoma control group in which the vast majority was p53 positive. Controversially, Wang et al. discovered p53 expression in only 15% of all adenomas investigated, and Kayath and coworkers found 36% of adenomas and 40% of carcinomas to be p53 positive respectively, diminishing the usefulness for p53 as a marker of malignancy in the parathyroid glands (Wang *et al*. 1996, Kayath *et al*. 1998). In both studies, p53 expression was not revealed in the normal parathyroid tissues examined.

To summarize, these data suggest that overexpression of cyclin D1, loss of Rb and increased Ki-67 counts are associated with malignant forms of primary hyperparathyroidism, but with a considerable overlap towards benign tumours. In addition, p27 overexpression is mostly found in parathyroid adenomas, thus suppressing the Rb pathway through interaction with cyclin D1. Meanwhile, aberrations in the p53 pathway might be coupled to the development of benign parathyroid tumours. However, no marker has yet demonstrated the sensitivity and specificity needed to efficiently distinguish parathyroid malignant tumours from adenomas.

Prevalence and predictive values

One of the greatest challenges in developing a useful molecular marker for the identification of malignant-prone parathyroid tumours is based on the fact that parathyroid adenomas outrank carcinomas by 99 to 1, measured in prevalence of PHPT. This known attribute puts high demands particularly on the specificity of the marker, and explains the need to incorporate negative and positive predictive values (NPV and PPV respectively) into the evaluation. The NPV and PPV take into account the prevalence of a given feature, as oppose to the terms sensitivity and specificity which focus solely on number of positives and negatives among healthy and affected in a particular study group. As of this, the NPV is defined as the prevalence-adjusted proportion of all true negative tests divided by the total number of negative tests (including false) and the PPV is defined as all true positive tests divided by the total number of positive tests (including false). As an example, if a hypothetical IHC study demonstrates that 9 out of 10 parathyroid carcinomas investigated are positive for the marker X, the sensitivity is 9/10 = 90%, i.e. the proportion of the malignant samples that are correctly identified by positive X expression. Similarly, if the researchers discovered that 7 out of 10 parathyroid benign tumours were negative for the same marker, the specificity would be 7/10 = 70%, i.e. the proportion of the benign lesions which were correctly identified by an absence of marker X. These values seem reasonably promising, but if we now introduce the true prevalence of parathyroid carcinomas into this scheme, things will differ.

Let's assume that the relative prevalence of parathyroid carcinoma in this imaginary example is 2% of all cases with PHPT, whereas the rest (98%) constitute benign lesions. This would indicate that out of 100 PHPT tumours, 2 would be malignant and 98 would be benign. By employing our marker X with a sensitivity of 90% and specificity of 70%, 0.9x2 = 1.8 out of the two malignant tumours would stain positive (true positive) and 0.7x98 = 68.6 benign tumours would stain negative (true negative). 2-1.8 = 0.2 for every 2 carcinomas would stain negative (false negative) and 98-68.6 = 29.4 benign tumours would stain positive (false positive). The ensuing NPV is as discussed above calculated as the number of true negative samples divided by the number of true negatives plus the number of false negatives. In our example, the NPV is calculated as 68.6 / (68.6+0.2) ≈ 99.7%. The PPV would in a similar fashion be calculated as the number of true positive cases divided by the number of true

positives plus the number of false positives; 1.8 / (1.8+29.4) ≈ 5.8%. The interpretation of the high NPV suggests that almost all negative tests for the marker X will be true, i.e. identify benign tumours more or less exclusively. However, a PPV of only 5.8% implies that only a fraction of cases with positive X expression will be correctly identified as malignant tumours. This is rather disheartening given the initial sensitivity of 90%, and can be attributed to the introduction of prevalence into our calculations. Since benign lesions are so much more frequently encountered in the clinical setting than carcinomas and given the rather low specificity of 70%, 30% of all adenomas will be classified as false positives, which widely outrank the true positive cases in the infrequent carcinoma group. As a result, absent expression of our marker X would be great in identifying benign tumours, but a positive staining would not provide any information regarding the tumour type, as it could be either an adenoma or carcinoma. If our initial specificity for marker X instead was reported as 99%, the corresponding NPV and PPV values would end up as 99.8% and 65% respectively, clearly improving the utility for this hypothetical marker. In turn, a specificity of 100% would render the NPV and PPV 99.8% and 100% respectively; demonstrating that near-perfect or perfect specificity is required for proper usage of this marker in the clinical context. To conclude, a marker for the proper identification of a parathyroid carcinoma demands exceedingly high specificity, probably around 100%, as a consequence of the very low prevalence of malignant PHPT.

Parafibromin – a possible discriminator?

Shortly after the identification of the *HRPT2* gene, Shattuck and colleagues demonstrated *HRPT2* gene mutations in 10 out of 15 samples of sporadic parathyroid carcinoma (Shattuck *et al*. 2003). As a consequence, these data inspired the production of a parafibromin antibody and subsequent investigation of parafibromin expression in a large cohort of parathyroid tumours. The first study of parafibromin expression employed tissue arrays and regular sections, including 52 cases of parathyroid carcinoma (Tan *et al*. 2004). Using a citrate based antigen retrieval technique and the avidin-biotin complex (ABC) methodology; the authors reported complete loss or reduced expression (focal loss) of parafibromin nuclear staining in the vast majority of all parathyroid carcinomas and HPT-JT related adenomas investigated as opposed to the findings in sporadic parathyroid adenomas which retained parafibromin expression. The outcome resulted in an overall 96% sensitivity and 99% specificity in

diagnosing definite carcinoma by focal or total loss of parafibromin immunoreactivity, yielding an NPV of 100% and a PPV of 49% if assuming a carcinoma prevalence of 1% in PHPT. These numbers indicate that all parathyroid adenomas would be identified by a positive staining, which denotes a "negative" result when using absence of immunoreactivity as a malignant marker, hence we are consulting the NPV. In contrary, only half the amount of parathyroid carcinomas would be similarly detected by negative parafibromin staining, where absence of parafibromin equals a "positive" result, hence referring to the PPV. As a conclusion, Tan *et al.* states that parafibromin immunostaining is a promising marker for the diagnosis of parathyroid carcinoma.

The findings were affirmed in a second study in which the authors employed the same monoclonal antibody, although the sensitivity was somewhat lower; 76% as compared to 96% by Tan and coworkers (Gill *et al*. 2006). This is due to the fact that the authors only considered complete lack of parafibromin immunoreactivity as diagnostic; therefore approximately 1/3 of the carcinomas examined which displayed focal loss rather than complete absence of parafibromin were classified as positive. The reason for this classification was to avoid inter-observer error and less reproducibility that could occur for cases with partial but not total loss of parafibromin. It is worth mentioning that the authors used a different antigen retrieval technique than Tan, and the monoclonal parafibromin antibody used was employed in a ten times higher dilution than in Tan's paper (1:200 versus 1:20). Furthermore, the detection system was based on a polymer system rather than the ABC methodology. The authors conclude, given the reasons discussed above, that complete absence of parafibromin is diagnostic of parathyroid carcinoma or an HPT-JT-related tumour (adenoma or carcinoma).

However, subsequent studies have displayed somewhat reduced sensitivity and/or specificity for parafibromin IHC, yielding suboptimal NPV and PPV values (papers II and III, Cetani *et al*. 2007, Tominaga *et al*. 2008, Fernandez-Ranvier *et al.* 2009). In addition, based on the methodology different expression patterns of parafibromin is observed in parathyroid carcinomas, ranging from total absence of parafibromin, through a mixture of positive and negative cells in the same section, to 100% positive nuclei using the 2H1 antibody and/or various parafibromin polyclonal antibodies.

The high prevalence of parathyroid carcinoma among HPT-JT kindreds together with the fact that most sporadic parathyroid carcinomas display somatic *HRPT2* gene mutations strongly

suggests that loss of parafibromin propel malignant transformation. However, the exact mechanisms remain largely elusive. Interestingly, cyclin D1 constitutes a target gene of the Wnt pathway, and in addition parafibromin has been shown to regulate expression levels of cyclin D1 *in vitro*, providing a speculative explanation for the proliferative advantage in tumours with parafibromin deletions (Woodard *et al*. 2005). However, studies have shown that cyclin D1 might be regulated in part from a parafibromin-independent mechanism since both high and low levels of cyclin D1 has been found in parafibromin deleted parathyroid tumours (paper II, Cetani *et al*. 2007). One should also bear in mind that the majority of HPT-JT patients develop parathyroid adenomas instead of carcinomas. Therefore, since small subsets of parathyroid adenomas also exhibit inactivating *HRPT2* gene mutations, parafibromin inactivation is not expected to be the only event in malignant transformation. Additional aberrations are likely to be required for the ultimate conversion into a full blown malignant tumour of the parathyroid glands.

AIMS OF THE STUDY

The overall aims of this thesis were to elucidate the molecular background of parathyroid tumorigenesis by molecular genetics and protein expression approaches, to evaluate currently proposed markers for the identification of parathyroid malignancy and to assess the involvement of previously unanalyzed molecules linked to the parathyroid proliferatory pathways. More specifically, the aim of each of the individual papers is given below:

I To investigate and compare the frequency of loss of heterozygosity (LOH) at certain chromosomal hot spot regions in parathyroid tumours derived from population-based screening as well as from routine clinical practice.

II To characterize the expression levels and subcellular localization of the tumour suppressor protein parafibromin in cell lines and in parathyroid adenomas by the generation of a polyclonal parafibromin antibody.

III To assess the value of parafibromin as an immunohistochemical screening marker for parathyroid carcinoma by employing a set of regional-specific antibodies.

IV To explore Wnt pathway proteins by immunohistochemistry and Western blot analyses, and to evaluate the potential role of the tumour suppressor protein APC as a discriminating marker between malignant and benign parathyroid tumours.

V To assess if parafibromin and/or APC could detect malignant potential prior to necessary histopathological requirements in cases of atypical parathyroid adenomas with available follow-up data, and to investigate the specificity of APC in an independent series of parathyroid adenomas.

VI To investigate whether promoter hypermethylation of the well-known tumour suppressor genes *APC*, *RASSF1A*, $p16^{INK4a}$ and *RAR-β* is present in parathyroid tumours, as well as to assess the levels of global methylation by *LINE-1* pyrosequencing.

MATERIALS AND METHODS

This section briefly describes the methodology used in papers I-VI. More precise and detailed information concerning the practical implementation of each method is found in the respective original paper.

Human material and antibody production

Human tissue samples

This thesis includes molecular analyses of human tissue samples (papers I-VI) and established human cell lines (papers II-III). Classifications of parathyroid tumours were according to the World Health Organization (WHO) criteria, and parts of the material studied have been previously assessed in preceding publications. Tissue samples were snap frozen in -70 ºC liquid nitrogen tanks in conjunction to surgery and subsequently stored in -70 ºC freezers until used. Paraffin embedded samples from formalin fixated specimen were cut at 4μm, mounted on slides and stored in 4 ºC until use. All parathyroid tumour samples were collected with informed verbal consent and ethical approval from patients undergoing surgery for PHPT.

Established cell lines

Six established human cell lines were analyzed for parafibromin expression in paper II; including HEK-293 (human embryonic kidney), HeLa (cervical squamous carcinoma), U2020 (small cell lung cancer), HepG2 (hepatoblastoma), SH-SY5Y (neuroblastoma), COS-1 and COS-7 (SV40 transformed monkey kidney) cells. In paper III, a HeLa cell line transfected with *HRPT2* was used for control experiments using Western blot and immunohistochemistry analyses.

Antibody production

Altogether four parafibromin antibodies were used: the 2H1 monoclonal antibody targeting aa 87-100, the BL648 polyclonal antibody directed at an epitope within aa 250-300 and two polyclonal parafibromin antibodies termed APVF and TNYV. The latter two were generated by immunizing rabbits with C- and N-terminal fragments of human parafibromin respectively (papers II and III). APFV targets an epitope within aa 509-531 of parafibromin, while TNYV is directed against aa 39-58 (Figure 10). The antibodies were commercially raised by Agrisera Co, Umeå, Sweden and ethical approval was given by the animal experiment ethical committee in Umeå. In short, rabbits were repetitively immunized with 200 µg peptide in Freund's incomplete and complete adjuvant. The incomplete adjuvant denotes a mineral oil in which the antigen is emulsified in to enhance the immune response, the complete variant also contains inactivated mycobacteria, which elicit an even more pronounced immune reaction. Raw serum was drawn from each rabbit and further affinity purified against the immunizing peptide through a column. Pre-immunization serum was collected and used as negative controls in subsequent immunohistochemical and Western blot analyses.

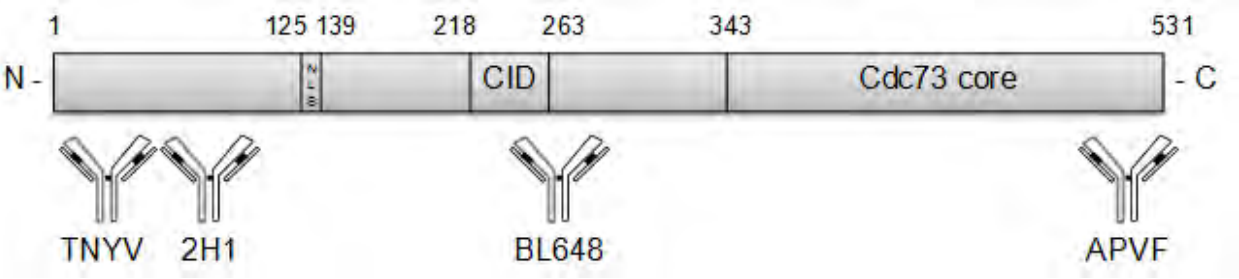

Figure 10 Overview of parafibromin and the four different parafibromin antibodies used with respect to their site of binding. NLS: nuclear localization signal, CID: β-catenin interaction domain, Cdc73 core: hPAF1 complex binding domain.

Protein expression studies

Western blot analysis

Edwin Southern, developer of the Southern blot method for DNA fragment analysis, is the man indirectly responsible for the baptizing of two subsequent methods for analyzing RNA and protein contents in a similar fashion as his own invention. Consequently the subsequent RNA method was named Northern blot and the protein analysis variant was referred to as the Western blot technique. Western blot analysis is a fairly straightforward technique for the antibody mediated identification of a certain protein of interest, and the analysis primarily requires a gel-based separation of proteins. The protein sample is associated to a highly negatively charged molecule termed sodium dodecyl sulphate (SDS) either present in the gel or applied to the sample in advance. SDS will give the proteins a negative net charge independently of previous net charge values. The samples are then separated by size only, in which smaller proteins travel faster through the gel than larger proteins. After transferring the separated proteins to a nitrocellulose or polyvinylidene fluoride (PVDF) membrane, the membrane is incubated with a primary antibody targeting the protein of interest. Different detection methods are available, however in this thesis the development was based solely upon horse radish peroxidase coupled secondary antibodies and the application of a peroxidase sensitive developing solution. Staining with Ponceau solution was used as a control of equal loading and successful protein transfer and membrane incubation with tubulin (paper II), α-prohibitin (paper III) α-actinin (papers IV and V) served as controls for equal protein loading. Western blot analysis was used to analyze the expression levels of parafibromin, cyclin D1, APC, GSK3-β well as total and active levels of β-catenin (papers II-V).

Immunohistochemistry (IHC)

Immunohistochemistry (IHC) denotes the *in situ* identification of a protein by the application of an antibody directed at an epitope within the target protein sequence and visualization by a chromogen reaction. IHC provides information whether or not a protein is present in a certain

tissue type, and also visualize its subcellular distribution. In addition, if the target protein is expressed in subsets of cells or in specific cell types only, this can be properly visualized. This is in contrast to Western blot analysis, which cannot retrieve such data (with the exception of subcellular localization if the protein sample is pre-fractionated). However, Western blot provides exact information regarding the size of the target protein, something which cannot be estimated by IHC alone. As a consequence, the combination of the two methods constitutes a potent tool in assessing proteins with unknown expression levels for a certain tissue type.

IHC is performed on tissue sections of frozen or paraffin embedded cases, in which the initial sample handling differs. Frozen sections retain more of the antigenicity, but the storage and handling of frozen tissues is complicated. Paraffin sections display superior morphology and are easier to handle, however the tissue might lose antigenicity over time. This thesis will focus on methodology revolving paraffin sections and the avidin biotin complex (ABC) method.

All samples were first fixated in 4% formaldehyde in a neutrally buffered solution prior to paraffin embedment in blocks. After 4 μm thin sectioning the samples are applied onto glass slides and stored best in 4 ºC prior to usage. To gain access to the protein of interest, all paraffin wax has to be properly removed by xylene treatment, a non-polar solvent. After paraffin removal, the sections are carefully dehydrated in ethanol followed by water. Usually, the sections are now succumbed to various modes of so called antigen retrieval techniques (AR), in which the tissue is treated with for example heated citrate or EDTA solutions. The reason for this treatment is supposed to be due to the calcium ion binding properties of the citrate/EDTA systems, which removes otherwise interfering calcium ions which might distract the following binding between the primary antibody and the protein of interest. In addition, it has been suggested that the heating induces cross-linkage breakage provoked by the previous formalin fixation, making the target protein more accessible for the antibody (Shi *et al*. 2001). The introduction of AR in research as well as in clinical pathology has led to an augmentation of IHC immunoreactivity on paraffin-embedded tissue sections for a huge assortment of antibodies in terms of increased sensitivity, such as for the Ki-67 proliferation marker, the androgen receptor, and many cluster of differentiation (CD's) markers, which are otherwise negative in standard IHC devoid of AR (Shi *et al*. 2001). It is however important to mention that not all antibodies or tissues demand the same AR technique. Therefore all experiments in which either the antibody or the tissue type or both have not yet been validated

in concert should be subjects to preceding careful control experiments. For example, some proteins are not properly identified without heated citrate treatment whereas other proteins lose antigenicity when exposed to high temperatures. Therefore, different AR heating times could be of great value when assessing new antibodies for IHC usage (paper III, discussion).

Since our studies focus on a peroxidase based developing system, the slides are immersed in a hydrogen peroxide solution. This maneuver is performed to quench endogenous peroxidase still potentially present and active in the tissues examined. For example, follicular cells of the thyroid produce high quantities of thyroperoxidase and immunological cells (especially macrophages) generate the peroxidase enzyme to eradicate engulfed bacteria. Furthermore, erythrocytes contain high amounts of peroxidase, making peroxidase quenching particularly important in vessel-dense tissues. If the peroxidase enzyme would still be active during DAB development, the investigator might experience false positive staining. All cells are believed to produce peroxidase enzymes in small quantities as a protective mechanism against the development of reactive oxygen species. Consequently, peroxidase quenching is strongly recommended for any cell type if using a peroxidase-dependent detection system.

The slides should then be blocked with excessive protein solution such as bovine serum albumin (BSA) to prevent non-specific binding of the primary or secondary antibodies. When using a biotin dependent detection such as the ABC method and especially if assessing a cytoplasmic antigen, the sections should be blocked also for endogenous biotin. Parathyroid tissues are not known to express immense levels of biotin, but even small amounts of this naturally present enzyme co-factor might theoretically falsify the end results and therefore it should be included in the methodology if the researcher specifically is scrutinizing cytoplasmic proteins.

After application of primary and biotinylated secondary antibodies, the ABC solution is applied. Avidin is a tetramer protein with extremely high affinity for biotin, and the biotin in the ABC solution is in turn conjugated to peroxidase. As a consequence, the avidin molecules which are partly occupied with biotin and peroxidase enzymes can now bind to the biotin of the secondary antibodies. After application of a diaminobenzidine (DAB) substrate and hydrogen peroxide, a brownish colour substrate is formed at the site of the ABC complex where peroxidase enzyme molecules convert hydrogen peroxide to oxygen and water, consuming DAB in the process and thus generating the colour substrate. As several biotin molecules with attached peroxidase units are bound to each avidin molecule, the net result is

an amplification of the signal. The ABC method is probably the most sensitive IHC method available, but demand stringent methodology to avoid false positive results. Furthermore, the amplification process means that the sensitivity is not linear, as the amount of colour does not correlate to the amount of antigen. The ABC variant of IHC was used to assess the expression of parafibromin, Ki-67, APC, GSK3-β as well as total and active levels of β-catenin (papers II-IV) and is depicted in Figure 11.

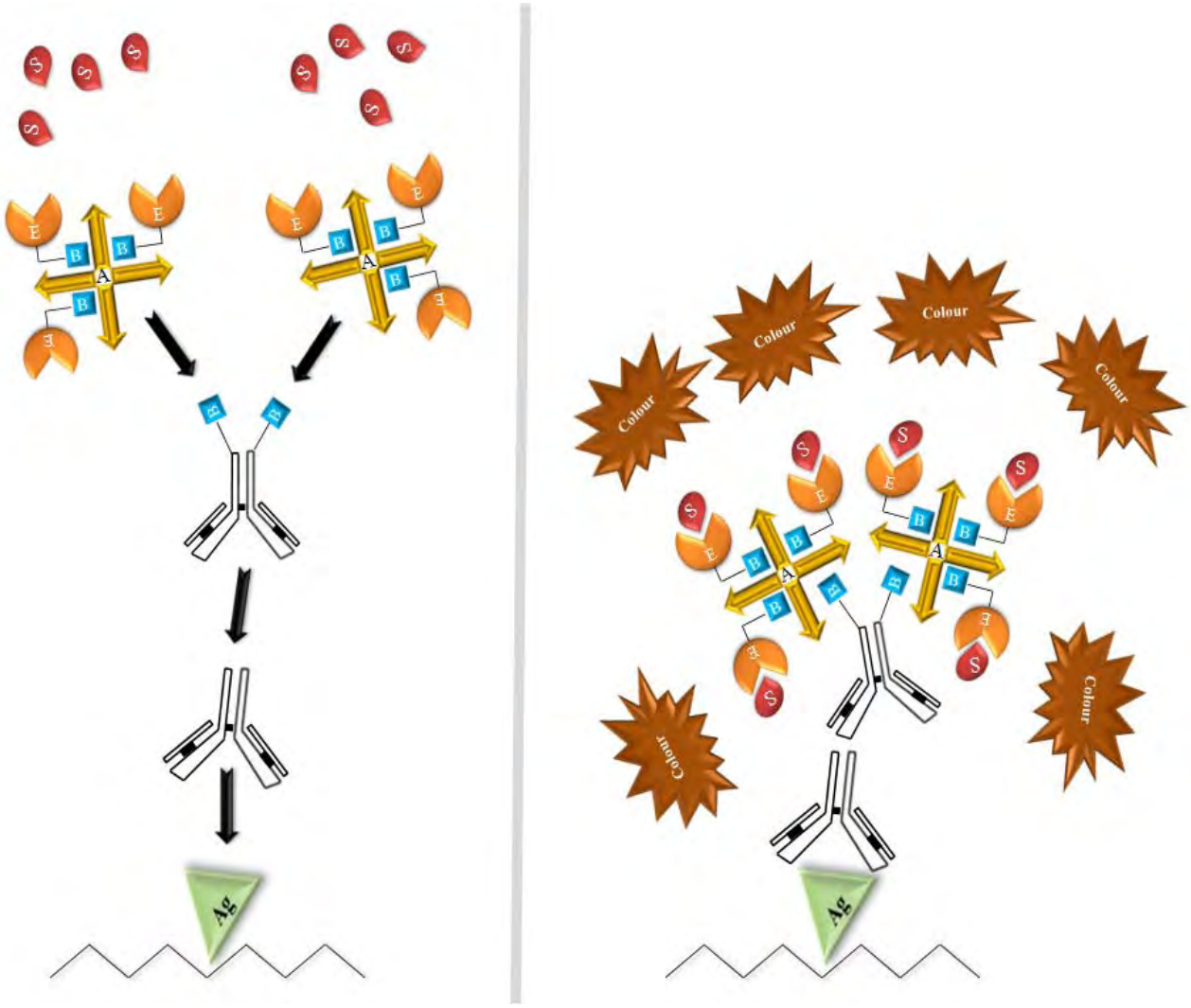

Figure 11 Basic principles of avidin-biotin complex (ABC) type immunohistochemistry. The antigen of interest is bound by the primary antibody. A secondary biotinylated antibody with high affinity towards the primary antibody is added, followed by the preformed ABC solution. The avidin tetramers will subsequently attach to the biotin molecules of the secondary antibody, and when substrate is added, the ABC-coupled peroxidase enzyme will produce a colour signal which can be detected by light microscope. Note how the signal is amplified.

Peptide neutralization tests

Positive controls for Western blot and IHC analyses may constitute of samples from tissues known to express the target protein. As negative controls, it is customary to omit the primary antibody from the reaction as a whole, although this procedure will not identify an eventual non-Fab (stemming from antibody domains other than the antigen binding part) cross-binding of the primary antibody with non-specific structures which could give rise to false positive results. An alternative is to include a tissue sample with known absence of the protein of interest, however this is not always achieved without effort as many proteins are ubiquitously expressed and a proper tissue type might be hard to find. Nevertheless, if the researcher possesses the immunizing peptide used for the production of the antibody, it can be of great value to perform a so called peptide neutralization test. In this way, the primary antibody can still be applied onto the negative control, which is more accurate *per se*. The method is based upon incubation between the primary antibody and excess amounts (20:1 or more) of the immunizing peptide, in which the antibody molecules will bind to the peptide. The pre-absorbed antibody-antigen complex is then applied instead of the primary antibody onto a sample slide with preferably known target protein expression. If the staining turns out negative, then there should be little risk for a non-Fab binding of the primary antibody giving rise to false positive staining. However, this method does not cover the aspect of molecular mimicry, and therefore a diminutive risk remains that the primary antibody could bind a similar but different protein via the Fab (antigen binding) domain and still stain negative in a peptide neutralization test which only tests non-Fab unspecific binding.

Peptide neutralization experiments could also be preceded by a dot blot assay, in which the peptide is immobilized directly onto a membrane and subsequently developed as a regular Western blot with the primary antibody targeting the peptide. By this method, the researcher can verify that the antibody binds the peptide prior to a subsequent peptide neutralization test. Peptide neutralization and dot blots were performed for all parafibromin antibodies included in papers II and III.

DNA and gene expression analyses

Loss of heterozygosity (LOH)

With the exception of the sperm and egg cells, the human genome is generally diploid. As of this, every cell exhibits two alleles of each gene locus. If the two copies are identical in terms of DNA sequence, the cell is said to be homozygous for this particular locus, as opposed to the term heterozygous which refer to the possession of two different forms of the same locus. Loss of heterozygosity (LOH) is a method which is widely employed to search for genomic deletions in tumours, and is defined as somatic (tumour DNA) homozygosity with concurrent constitutional (leukocyte DNA) heterozygosity. In other words, the tumour displays loss of a specific genomic region normally present in the constitutional DNA from the same patient. The method makes use of specific primers which anneal close to highly polymorphic loci termed microsatellites, consisting of repetitive sequences scattered throughout the genome. The probes are labeled with either radioactive phosphorus or fluorescence, and samples are subsequently amplified using PCR and separated on gel or through an automated capillary. If the length of the microsatellites differs between the two alleles, the researcher will visualize two bands/peaks at analysis. The detection of LOH in a tumour sample requires that the respective constitutional tissue is informative, i.e. heterozygous for the microsatellite marker. Likewise, if the constitutional tissue is homozygous for the microsatellite marker, it is termed non-informative. If both tumour and constitutional tissues are heterozygous with similar intensities of the two alleles, no LOH in this locus exists, termed retention of heterozygosity. LOH analysis was performed in paper I for a number of microsatellite markers covering parts of chromosomes 1p, 6q and 11q in a group of parathyroid tumours and is further detailed in Figure 12.

Transfection studies

Transfection denotes the transmission of genetic content into a cell by means other than viral transduction, for example via electroporation, liposome-mediated fusion or for the more aggressive-minded researcher; the biolistic particle “gene gun” delivery system. Transfection of plasmids carrying the DNA sequence of interest has been of exceptional value for functional studies in cell lines, in which the researcher can modify the expression of a

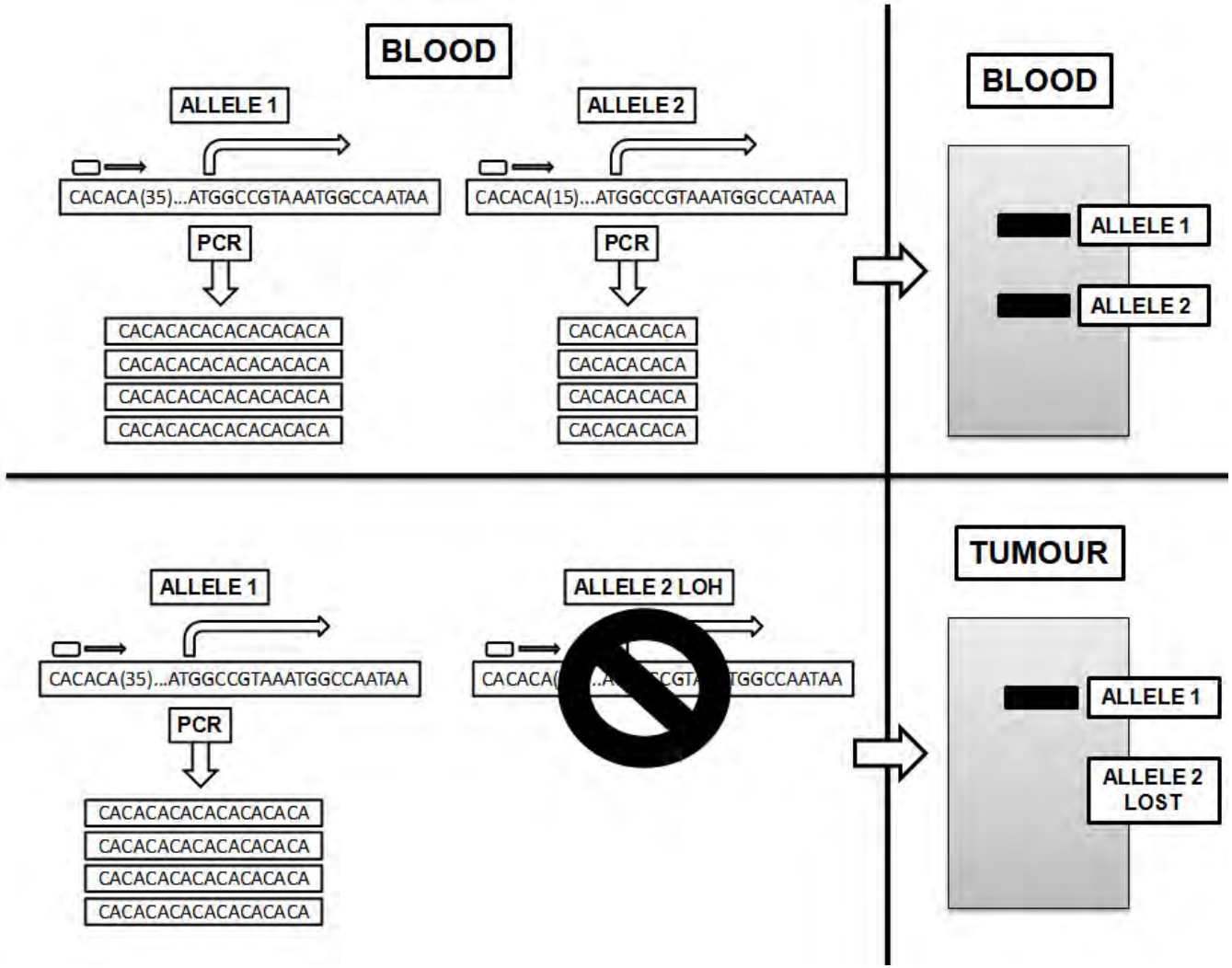

Figure 12 Principles of the loss of heterozygosity (LOH) technique. Blood: Alleles 1 and 2 exhibit microsatellite regions of different CA repeat length, and the subsequent PCR yields products of dissimilar sizes which is depicted as two separate bands at gel analysis. Tumour: Allele 2 is deleted, and hence the resulting PCR products are solely derived from allele 1 which in turn results in a single band at analysis.

particular gene and observe possible phenotypic effects on the cell, such as proliferatory actions and pure morphological changes. Transfection can be combined with site directed mutagenesis to observe how mutations or single nucleotide polymorphisms affect the gene of interest, and in turn how this alters cellular properties. Plasmids overexpressing small interfering RNA (siRNA) can be employed to silence certain genes and observe the consequences thereof. Furthermore, the gene of interest can be fused with other sequences such as the green fluorescent protein (GFP) tag which will then create a merged protein which can be easily detected by a laser-scanning microscope to scrutinize subcellular localization on a more precise plane than by standard proteomic methods alone. Transfection studies regarding the *HRPT2* gene was performed using COS and HeLA cell lines in paper II. Transfection was based on usage of Lipofectamine and FuGene agents, both lipid-based solutions which transfer the plasmid into the cells by merging with the cell membrane. These liposome mediated transfection studies are highly efficient (i.e. has a high transfection rate) and is fairly non-toxic to the cell.

DNA sequencing

DNA sequencing is a method which, like the name implies gives the researcher direct information regarding the nucleotide sequence in the selected region of the genome. The method is widely used to detect gene mutations (including substitutions, insertions and deletions) as well as polymorphisms and gene rearrangements in tumour and constitutional tissues, and is based on the chain termination scheme developed by the two-folded Nobel Prize laureate Frederick Sanger. In this method, the DNA sequence of interest is first amplified by a regular PCR with primers flanking the selected region. In a second PCR reaction, a sequencing primer is allowed to anneal to the target region and initiate DNA replication via DNA polymerase, but this time the deoxynucleotides dATP, dGTP, dCTP and dTTP are mixed with fluorescent dideoxynucleotides; ddATP, ddGTP, ddCTP and ddTTP. The dideoxynucleotides will be randomly incorporated in the replication process and hence terminate the sequence. Since the resulting PCR fragments will exhibit all possible lengths based on where in the sequence the dideoxynucleotide was incorporated, a size-dependent separation in an acrylamide gel or in an automated capillary sequencer can be established. All four ddNTP’s have different absorbance, and the result is read by an argon ion laser and visualized in a so called chromatogram (figure 3 in paper II) in which the nucleotide sequence

can be easily read. DNA sequencing was performed for *MEN1* in paper II and for *APC* in paper V.

One-step cloning

This procedure was carried out in paper II to more precisely determine the sequence of the discovered *MEN1* gene mutations. The methodology is based on the principle of allelic separation, which facilitates the visualization of the mutation in the affected allele. Briefly, PCR products of the respective exon exhibiting the mutation were ligated into a plasmid vector and directly transfected into *E. coli* bacteria. By random chance, some bacteria will receive the plasmid carrying the mutated allele exclusively while others will only be transfected with the plasmid carrying the wild type allele. After clonal growth, the bacterial clones are harvested and the DNA extracted. Direct sequencing can now be performed of single allele DNA sequences, and the resulting chromatogram will hence be free of intercalating peaks from the second allele which allows for a more precise mutational judgment of for example frameshift alterations and multiple alterations within the same exon.

Reverse transcriptase-PCR (RT-PCR)

RT-PCR is a method in which total RNA from a tissue sample is converted into complementary DNA (cDNA) by using the reverse transcriptase enzyme and random hexamer oligoprimers, and interesting genes or sequence regions within the produced cDNA can subsequently be amplified using a simple PCR scheme. The cDNA is more stable than RNA and furthermore allows amplification by DNA polymerase, the latter which can only use DNA or cDNA as a template. Importantly, the cDNA mirrors the RNA content of the tissue analyzed, and therefore the converted mRNA contains merely exons, since the introns are spliced away in mature mRNAs. Following the subsequent PCR reaction with primers flanking the region of interest, the samples are visualized on agarose gels to demonstrate either presence or absence of the specific transcript. RT-PCR was performed in paper II with a cDNA panel of human tissues to investigate *HRPT2* gene expression.

Quantitative reverse transcriptase-PCR (qRT-PCR)

RT-PCR provides information whether or not a gene transcript is present in the tissue analyzed, but confer only semi-quantitative data concerning the relative expression levels between samples. As of this, qRT-PCR has been developed as a reliable tool in measuring the total levels of a specific cDNA sequence in a sample, which in turn mirrors the RNA content of the tissue analyzed. The method was used in paper II in which 41 parathyroid adenomas and two normal parathyroid samples were assessed for *HRPT2* gene expression using primers targeting the exon 2-3 boundary and additionally a subset of cases were also analyzed using primers against the exon 10-11 overlap. The qRT-PCR was performed using the TaqMan technology from Applied Biosystems. In short, a TaqMan probe is hybridized to the cDNA sequence of interest. The probe is built up by a fluorophore covalently attached to the 5'-end of the oligonucleotide probe (termed reporter) as well as a 3' end quencher element. The fluorophore reporter displays fluorescent abilities if released from its quencher, and upon subsequent PCR reaction, the 5' to 3' nuclease activity of the Taq DNA polymerase enzyme employed will cleave the TaqMan probe as it traverses across the template strand. The result is the release of the reporter from its inhibitory quencher, which will lead to the omission of fluorescent light which is detected by a camera. The levels of fluorescence light detected are proportional to the amount of fluorophore released and therefore represent the amount of cDNA template present in the PCR.

Since not all tissue samples have the exact same rate of overall gene transcription and the same amount of cell contents, normalization against a constantly expressed housekeeping gene must be performed. A standard curve is constructed from cDNA samples diluted in sequence and subsequently assessed for target gene as well as housekeeping gene expression. These control samples must be assessed in the same TaqMan session as the samples of interest, and from this curve the levels of fluorescence from each sample can be quantified. In our case, we quantified the relative expression in all tumours as compared to two samples of normal parathyroid tissue, and the housekeeping gene selected was *36B4* which has previously been shown to be constantly expressed in parathyroid tissues.

Epigenetic analyses

Epigenetics denotes regulatory changes in gene expression resulting from other mechanisms than alteration of the primary DNA sequence. As an expanding field in molecular medicine, epigenetic modifications have been shown to play major roles in the development of human tumours. Epigenetic modifications include chromatin remodeling through histone modification and gene promoter methylation among others, the latter which is covered by this thesis. Most DNA methylation processes in human denotes a covalent bound between a methyl group ($-CH_3$) and a cytosine residue of the DNA molecule. The phenomenon is usually limited to CpG islands, genomic regions with high (>50%) cytosine-guanidine content. CpG islands are usually between 300 to 3,000 bp in length, and have been identified in the majority of the characterized gene promoter regions known to man. To date, promoter hypermethylation has been demonstrated for a wide variety of genes in assorted human tumours, and as a consequence the methodology has developed significantly over the last decade.

Bisulphite pyrosequencing

Pyrosequencing is a method for the linear sequencing of a specific genomic region of interest using a light-sensitive detection system. Pyrosequencing can be performed to assess the quantitative level of promoter methylation of a specific gene of interest. The methodology is based on preceding bisulphite treatment of the DNA sample of interest. Bisulphite treatment will convert un-methylated cytosine residues into uracil, whereas methylated cytosine residues will remain unaffected. The converted uracil residues are subsequently converted into thymidine upon the subsequent PCR of the region of interest. The PCR makes use of a biotinylated reverse primer, and the post-PCR samples are bound to streptavidine beads exhibiting very high affinity for biotin to ensure a high purity end product. The sequencing primer is then hybridized to the samples together with an assembly of enzymes, which initiate an advanced series of reactions. Firstly, the sequencing primer binds to the region of interest, and DNA polymerase initiates the sequencing elongation by adding dNTPs into the growing complementary strand just like in a normal PCR. However, when the appropriate nucleotide is inserted as DNA polymerase stroll down the template, a pyrophosphate (PP_i) molecule is cleaved from the respective nucleotide triphosphate molecule. An enzyme termed ATP

sulfurylase picks up the released PP_i and together with its substrate adenosine phosphosulphate (APS) creates a novel molecule of ATP. The newly synthesized ATP molecule will in turn be used as a substrate for a luciferase enzyme, which together with luciferin will result in the creation of oxyluciferin as well as the emission of light. The amount of light emitted is in direct proportion to the number of ATP molecules consumed, which in turn is based on the number of PP_i molecules formed during the DNA replication. Thus, if two GTPs were to be incorporated in succession at the complementary DNA strand, this would generate two PPi molecules which in turn would generate two ATP units and in the end double the intensity of the light signal as compared to the initial usage of a single GTP molecule. A camera detects the light emitted from each sample and is reflected as an individual peak in the resulting Pyrogram. The computer is fed with the sequence of interest; however CpG cytosine residues are denoted as Y in the input sequence. The computerized pyrosequencing system thus adds T and C nucleotides sequentially to the respective sample for every Y residue, and hence the proportion of cytosine versus thymidine residues can be calculated for any sample, which in turn mirrors the level of cytosine methylation. Pyrosequencing was used in paper VI to assess the density of promoter methylation for the tumour suppressor genes *APC*, *RASSF1A*, $p16^{INK4A}$ and *RAR-β* in a group of parathyroid tumours and the methodology is illustrated in Figure 13.

Assessment of global CpG methylation

A number of methods exist to assess the quantity of global (genome-wide) methylation, ranging from the IHC application of a monoclonal 5^MC antibody targeting methylated cytosine residues to more quantitative methods such as methylation sensitive restriction enzyme analysis, Pyrosequencing techniques or array-based techniques. In paper VI, *LINE-1* Pyrosequencing was used to quantitatively compare global methylation between parathyroid tumours normal parathyroid tissues. *LINE-1* is short for long interspersed nuclear elements type 1, and denotes a replicating retrotransposon element which constitutes approximately 15% of the human genome, making it the most commonly encountered sequence in the entire DNA code. *LINE-1* encodes two proteins which are both required for *LINE-1* mobilization within the genome, and one of the proteins exhibits both endonuclease and reverse transcriptase activity. As a consequence, *LINE-1* replication is based on the creation of a new *LINE-1* element trough transcription and the subsequent reverse transcriptase actions of one of its own products. Needless to say, the consequences for the cell would be deleterious if

replicating *LINE-1* sequences would be allowed to randomly insert throughout the genome. Indeed, *LINE-1* elements have for instance been found within the APC tumour suppressor gene sequence in a case of colon cancer. As a consequence, *LINE-1* repetitive sequences normally exhibit heavy CpG island methylation to inhibit its action. Given that the vast amounts of *LINE-1* sequences are scattered across the genome, assessment of *LINE-1* methylation levels has been shown to be a reliable indicator of genome-wide methylation (Yang *et al*. 2004). The measurement is performed using the standard Pyrosequencing protocol as discussed above.

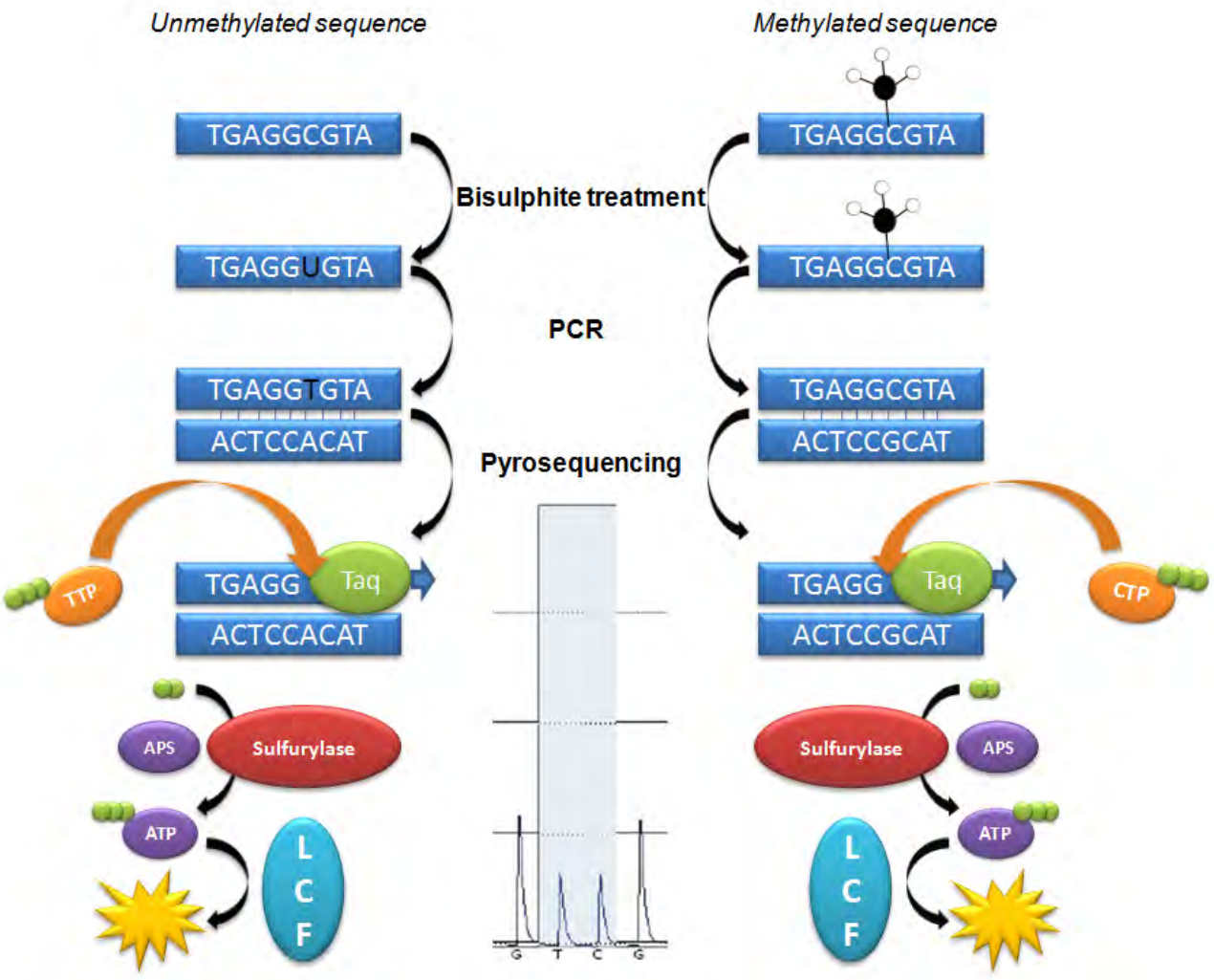

Figure 13 Schematic representation of the bisulphite pyrosequencing technique.

RESULTS AND DISCUSSION

A deletion mapping study in patients with severe vs. serene PHPT (paper I)

Allelic losses at 1p, 6q and 11q are frequent events in PHPT

As previously discussed, loss of heterozygosity (LOH) analyses is an efficient instrument to evaluate the occurrence of genomic loss at a specific chromosomal region of interest. Preceding studies have shown that parathyroid tumours frequently exhibit LOH at chromosomes 1p, 6q and 11q, and therefore we sought to determine the frequencies of LOH in an independent material presenting with dissimilar disease patterns (Tahara *et al*. 1996a). The vast majority of cases analyzed constituted parathyroid adenomas (45 cases), whereas the remainder consisted of chief cell hyperplasia (9 cases), water clear cell hyperplasia (1 case) as well as parathyroid carcinoma (1 case). Blood and tumour samples were collected from 56 patients who had undergone surgery for PHPT; 35 cases were recruited through routine clinical procedures (termed “routine group”) and the remaining 21 cases were part of a PHPT screening-detection program (termed “screened group”). When comparing the two groups with respect to clinical characteristics, the routine group exhibited higher preoperative levels of PTH (125±25 ng/l vs. 55±5.4 ng/l), a more profound preoperative hypercalcemia (2.94±0.06 mmol/l vs. 2.63±0.03) and larger tumours at surgery (765±129 mg vs. 406±85 mg). All patients from the screened group were asymptomatic, whereas 12 out of the 35 (34%) patients from the routine group exhibited hyperparathyroidism-related symptoms. These data suggest that the screened group had a more indolent hyperparathyroidism as compared to the routine group with regards to biochemical analyses and overall symptomatology.

For the LOH analyses, 18 microsatellite markers were employed (6 markers located at 1p, 9 markers located at 6q and 3 markers located at 11q) using radioactive labeling and a gel based detection system. In addition, the majority of tumours demonstrating LOH at chromosome 1p were further analyzed in detail with 7 additional fluorescent labeled markers using a capillary system. For all tumours, 27%, 23% and 23% displayed LOH within chromosomal regions 1p,

6q and 11q respectively, fully in line with previous findings which have demonstrated comparable frequencies. The high incidence of genomic deletions in these regions suggests the presence of uncharacterized tumour suppressor genes at these loci (Figure 14). At 1p, LOH at the D1S214 marker was the most prominent finding, occurring in 10 out of 56 (18%) tumours, including all tumours from the screened group with any kind of 1p involvement.

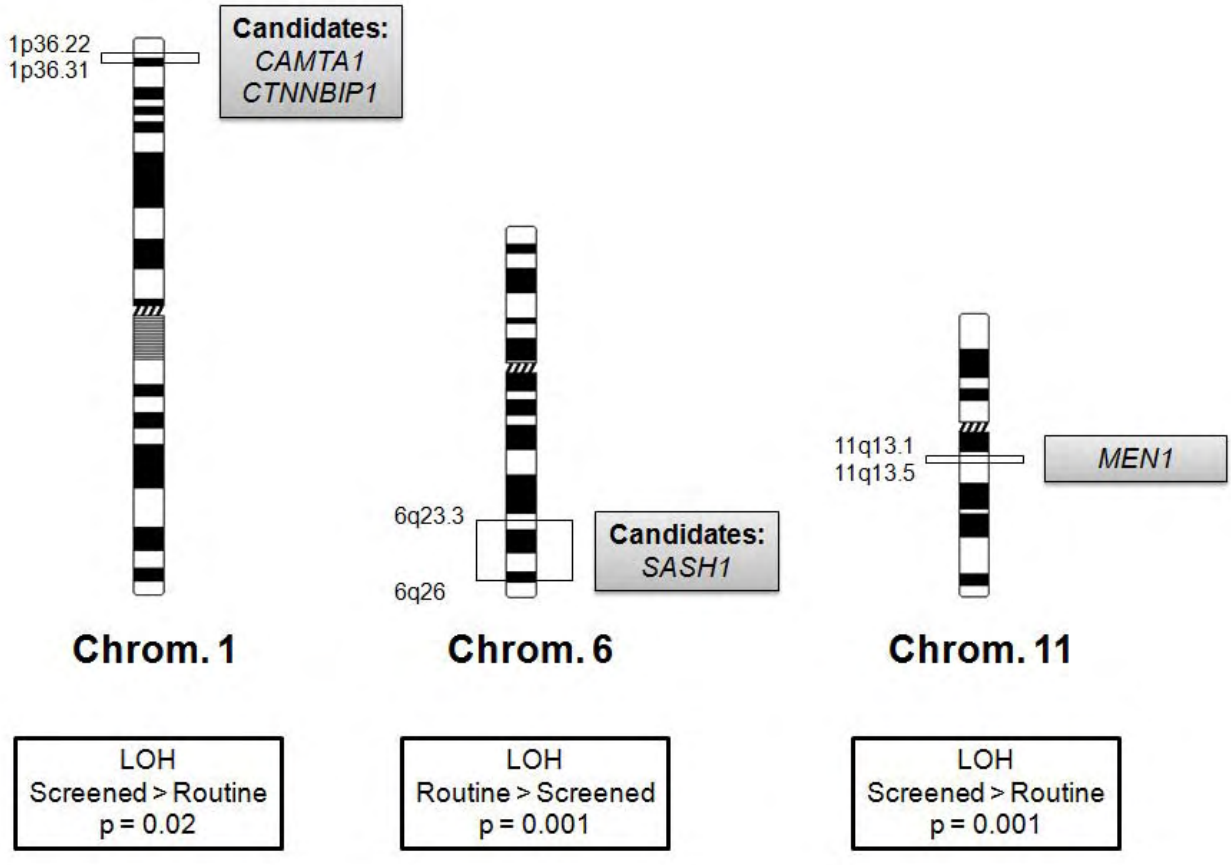

Figure 14 Schematic overview of the chromosomal regions studied in paper I. Putative candidate genes are shown for each region, and the predominant group showing LOH for the corresponding region is given below. p-values ≤0.05 were considered significant.

A more detailed mapping involving 7 additional microsatellites revealed a 6 cM region located between markers D1S214 and D1S503 (1p36.22-31) to be the most frequently deleted among the cases studied. The region encompasses a significant number of genes, for example the *calmodulin binding transcription activator 1* (*CAMTA1*) gene at 1p36.31 just telomeric of

the D1S214 marker, which constitutes a tumour suppressor candidate in neuroblastomas (Okawa *et al*. 2008).

The 1p36 region contains several interesting genes, including *RIZ1* at 1p36.21 previously shown to exhibit LOH and promoter hypermethylation in parathyroid tumours (Carling *et al*. 2003). In addition, the *CTNNBIP1* gene located at 1p36.22 encoding the inhibitor of β-catenin (ICAT) protein constitutes an interesting candidate as well. However, while *CTNNBIP1* are located in close vicinity of the D1S503 marker, RIZ1 is positioned more centromeric and should not be held responsible for the high frequency of allelic loss at the 1p region in our tumours series.

At chromosome region 6q, LOH at markers D6S292, D6S311 and D6S305 constituted the most common finding. Interestingly, this region contains the putative tumour suppressor gene *sterile alpha motifs and SH3 domain containing protein 1* (*SASH1*) gene shown to be downregulated in malignant tumours of the breast, lung, colon and thyroid (Zeller *et al*. 2003). Finally, at chromosomal region 11q encompassing the *MEN1* gene, all three markers employed displayed LOH at similar frequencies. Notably, all tumours with 11q LOH were adenomas, whereas LOH at 1p was found in 24% of adenomas, 30% of hyperplasias and in the only carcinoma investigated. Finally, loss at 6q was found with similar frequency in adenomas and hyperplasias (24% and 20% respectively). Finally, no statistically significant correlations between LOH and clinical characteristics were demonstrated.

Different genetic alterations in screening detected cases vs. the routine group

Interestingly, LOH within 1p and 11q was more commonly encountered among the screening-detected cases than in the group assembled from clinical routine practice. 38% of cases from the screened group and 20% of cases from the routine group respectively exhibited LOH at one or several 1p loci, and the difference was statistically significant (p=0.02). Regarding 11q, 43% of screened cases and 11% of routine cases displayed LOH, again with a significant disparity between the two groups (p=0.001). In contrast, LOH at 6q was a more prominent phenomenon among the cases in routine group as compared to the screened group (31% and 11% respectively, p=0.001). The findings suggest that tumours from patients with asymptomatic or mild hyperparathyroidism can develop along a specific genetic path which differs from that of patients with more severe (symptomatic) disease. The fact that LOH of the

6q region was more frequently encountered in the group recruited from clinical practice indicates that the inactivation of a putative tumour suppressor at this region results in a more prominent symptomatology. Interestingly, the 6q region covered in our study includes the *SASH1* tumour suppressor gene discussed above which could be of interest for future characterization. Furthermore, allelic loss at 1p and 11q was more prominent in the screened group, suggesting that inactivation of tumour suppressor genes at these loci might result in a more indolent growth pattern alternately lead to reduced metabolic disease, perchance through the interacting with calcium related pathways.

To conclude, allelic loss at 1p, 6q and 11q are three common events in parathyroid tumorigenesis and imply the existence of putative tumour suppressor genes at these loci. Furthermore, loss of 1p and 11q seem to confer a more placid hyperparathyroidism, as indicated by the predominance of these aberrancies in screening detected patients, whereas LOH at 6q could propel a more clinically overt disease.

Detailed characterization of *HRPT2* and parafibromin through expression analyses (paper II)

HRPT2 and parafibromin are ubiquitously expressed in human tissues

Germline inactivation of the *HRPT2* tumour suppressor gene, encoding parafibromin, causes the hyperparathyroidism–jaw tumour (HPT-JT) syndrome as well as familial isolated hyperparathyroidism (FIHP) for a subset of families. Furthermore, *HRPT2* mutations have been demonstrated in the vast majority of parathyroid carcinomas as opposed to in a very small group of parathyroid adenomas. However, since only modest amount of data regarding parafibromin expression in parathyroid tumours existed, we sought to more specifically determine the levels of *HRPT2*/parafibromin expression in human tissues, parathyroid tumours and human cell lines. For this purpose, we raised a rabbit polyclonal antibody (APVF) towards the C-terminal fragment of human parafibromin (aa 509-531) which was affinity purified and subsequently used in IHC and Western blot analyses of cell lines, normal tissues and parathyroid tumours.

To investigate the occurrence of *HRPT2* gene activity in human tissues, we assessed the levels of transcription by a multiple-tissue mRNA expression analysis using RT-PCR and primers designed towards exons 1 and 16 of *HRPT2*. A single *HRPT2* transcript of the predicted size was demonstrated for all tissues analyzed; including brain, heart, kidney, liver, lung, pancreas, placenta as well as skeletal muscle tissue. These results indicate that the *HRPT2* gene is transcribed in a wide variety of human tissues and suggests that *HRPT2*/parafibromin exhibits functions not solely limited to the parathyroid glands. Subsequent Western blot analyses using protein extracts from six human cell lines as well as normal pancreas, kidney and a SHPT gland demonstrated clear parafibromin expression of the expected ~60 kDa size. The specificity of our findings was verified through peptide neutralization experiments, in which the ~60 kDa band was completely abolished.

Parafibromin mainly localizes to the nucleus

To explore the subcellular localization of parafibromin we generated three plasmid constructs for transfection; an *HRPT2*~GFP plasmid, a GFP~*HRPT2* plasmid and a GFP plasmid without the *HRPT2* cDNA sequence as a negative control. The plasmids were validated by DNA sequencing alternatively by restriction enzyme cleavage. The plasmids were transfected into HeLa and COS cell lines using a Fugene and/or Lipofectamine based system for effective transfer of the plasmids into the cells. To verify the transfection, protein extracts from each transfected cell line were analyzed for parafibromin and GFP expression using Western blot analysis. Parafibromin expression was evident by a strong ~60 kDa band in the transfected cell extracts as compared to the much weaker signals obtained from untransfected controls. Using a GFP antibody, we observed similarly strong expression of the ~90 kDa merged parafibromin-GFP peptide in the transfected cells as opposed to the untransfected controls. By live cell imaging using a laser-scanning microscope, we observed a strong nuclear expression with concurrent weaker cytoplasmic signals after transfection with the *HRPT2*~GFP or GFP~*HRPT2* plasmids. These data indicate that parafibromin may exert its primary function in the nuclear compartment, and the coexisting cytosolic expression might represent newly synthesized parafibromin units not yet localized to the nucleus alternatively one or several cytoplasmic functions for this protein. The findings of nuclear parafibromin are in line with previous studies, for example in which the protein has been demonstrated to exhibit a functional bipartite nuclear localization signal. In addition, parafibromin has been associated

to the nuclear hPAF1 complex engaged in RNA polymerase II-mediated transcription processes and epigenetic modifications of histones, and finally previous IHC analyses using a monoclonal parafibromin antibody suggest a nuclear localization for this protein (Hahn Marsh, Rozenblatt-Rosen, Tan).

Parafibromin expression is retained in the majority of parathyroid adenomas

HRPT2 gene mutations are very uncommon events in sporadic parathyroid adenomas, nevertheless small amounts of data regarding parafibromin expression in parathyroid tumours exist. As an ever increasing amount of cancer research support epigenetic and posttranscriptional mechanisms for inactivation of tumour suppressor genes independently of mutations, we wanted to investigate whether or not parathyroid benign tumours could exhibit downregulation of parafibromin as a consequence of other mechanisms than *HRPT2* gene mutations. For this purpose we assessed a series of parathyroid tumours (n = 46) presenting with cystic phenotypes, as this histological variant has been frequently reported in tumours from HPT-JT kindreds. This tumour collection has previously been analyzed for *HRPT2* gene mutations in which 3 out of 46 cases exhibit *HRPT2* gene mutations. In this study we performed DNA sequencing of the coding *MEN1* exons for all cases, and two somatic *MEN1* gene mutations were found, 68delC in case T28 as well as 527del6 in case T39, both predicted to be inactivating. The mutated sequences were subsequently subjects to one-step cloning to allow for precise characterization.

Using qRT-PCR analyses and cDNA available from a total of 41 tumours, we targeted the *HRPT2* exon 2-3 cDNA overlap and used the housekeeping gene *36B4* as a background reference. In addition, two normal parathyroid glands were used as normal references. Samples were analyzed in quintets and a mean value was employed for analysis. Forty out of 41 (~98%) adenomas studied displayed *HRPT2* mRNA levels comparable to the mean of normal parathyroids. Using normal parathyroid *HRPT2* mRNA as a reference value (1), the relative expression in parathyroid tumours ranged from 0.35 to 1.57 for these cases. A single case (T41) exhibited strong *HRPT2* gene expression (~6 times the normal expression). Interestingly, this tumour carries a truncating *HRPT2* mutation and is derived from a 1q-linked FIHP family in which no germline *HRPT2* alteration has been demonstrated. In addition, we further characterized all adenomas with either *HRPT2* or *MEN1* alterations (n = 12), including mutations and/or allelic loss, with additional primers directed towards the

HRPT2 exon 10-11 cDNA overlap. In 11 out of 12 tumours analyzed, the levels of *HRPT2* mRNA were similar to the normal parathyroid mean. Again, case T41 exhibited increased levels of *HRPT2* mRNA expression, but interestingly these exon 10-11 mRNA levels were significantly lower than the mRNA levels detected when targeting exons 2-3.

These results indicate that *HRPT2* gene expression is unaltered in the vast majority of parathyroid adenomas as compared to normal parathyroid tissues, and the finding of normal or near-normal *HRPT2* mRNA levels in cases with *HRPT2* gene alterations could suggest the compensatory upregulation of the unaffected allele in these tumours. For case T41, the difference in mRNA levels when assessing different parts of the *HRPT2* cDNA suggest that a theoretical alternative splicing of the *HRPT2* transcripts might be of some importance in parathyroid tumours. Given the publications by the recent online browser Ensembl regarding novel *HRPT2* gene transcripts, the findings in T41 may indicate that the alternative transcripts CDC73-201 (should not be detected by the exon 2-3 primers but should be detected by the exon 10-11 primers) and CDC73-202 (should be detected by the exon 2-3 primers but not by the exon 10-11 primers) are differentially expressed in this case. Since both primers detect the regular *HRPT2* mRNA, the differences in expression with regards to these two different primer locations indicate that the levels of CDC73-202 mRNA are higher than the levels of CDC73-201 mRNA in this tumour. This observation could be of future importance.

By Western blot and IHC analyses we demonstrate three cases with absence of 60 kDa parafibromin expression and lack of parafibromin immunoreactivity on IHC. These three cases (T20, T41 and T42) all exhibit inactivating *HRPT2* gene mutations, and in addition case T42 displays 1q25 LOH, thereby following Knudson's two-hit theory. Moreover, two *HRPT2* wildtype cases (T34, T38) demonstrated an aberrantly sized parafibromin of slightly smaller size on Western analysis, and interestingly one of the cases (T34) displayed negative nuclear parafibromin expression whereas a cytosolic signal was obtained suggesting protein de-localization. All other cases (n = 41) displayed parafibromin expression of the expected 60 kDa product as well as positive nuclear immunoreactivity. In addition, a small subset of parathyroid adenomas as well as normal parathyroid controls demonstrated a weak cytoplasmic signal in addition to the nuclear staining, supporting our preceding results from live cell imaging.

Cyclin D1 levels are independent of parafibromin expression in vivo

Following the *in vitro* transfection studies by Woodard and colleagues in 2004 which indicated that parafibromin directly or indirectly regulates the levels of the parathyroid proto-oncogene cyclin D1, we sought to determine if a correlation between parafibromin and cyclin D1 expression existed *in vivo*. 44 out of the original 46 parathyroid tumours were available for Western blot analyses using a monoclonal cyclin D1 antibody, and the expression levels were classified as strong, moderate, weak or absent. The vast preponderance (n = 38) of the adenomas exhibited positive expression of diverse intensity (11 showed strong, 13 showed moderate and 14 showed weak expression respectively) whereas six adenomas 6 displayed absent cyclin D1 expression. There were no clear associations between cyclin D1 expression and parafibromin expression or *HRPT2*/*MEN1* genotype status, as cases exhibiting strong ~60 kDa parafibromin could present with both high and low/absent levels of cyclin D1 expression. These results therefore indicate that parathyroid cells can express cyclin D1 even in the presence of parafibromin. Nevertheless, the three cases with inactivating *HRPT2* gene mutations either presented with strong or moderately levels of cyclin D1 expression. Therefore, it is possible that parafibromin negatively regulates cyclin D1 expression to a certain degree, although our data point to the notion that cyclin D1 expression *in vivo* is regulated in part by parafibromin-independent mechanisms. These findings are fully in line with preceding publications in which cyclin D1 expression is found in similar levels among adenomas and carcinomas although the latter group is known to frequently harbor *HRPT2* gene mutations.

To sum up, the *HRPT2* gene is ubiquitously expressed in normal tissues and expression of its corresponding protein parafibromin is concentrated mainly to the nucleus. The majority of parathyroid adenomas display wildtype *HRPT2* gene sequence and retained parafibromin expression. Furthermore, *HRPT2* mutations are unusual findings in parathyroid benign tumours however clearly correlates to the loss of parafibromin expression. Finally, cyclin D1 expression is independent of parafibromin levels *in vivo*.

Parafibromin is an adjunct marker for parathyroid tumour classification (paper III)

Parafibromin immunoreactivity is influenced by methodological parameters

Following the initial identification of the *HRPT2* gene and the demonstration of its frequent inactivation by mutations in parathyroid carcinomas (Carpten *et al*. 2002, Shattuck *et al*. 2003), we sought to determine the usefulness of parafibromin immunohistochemistry as a marker for malignant disease by employing antibodies targeting diverse epitopes of human parafibromin. We assembled a total of 58 parathyroid tumours, including 22 unequivocal (definite) carcinomas, 11 equivocal carcinomas (currently referred to as atypical adenomas) as well as 25 sporadic parathyroid adenomas. All tumours were investigated for parafibromin immunoreactivity using IHC and four different parafibromin antibodies; 2H1, BL648, APVF and TNYV. The 2H1 monoclonal parafibromin antibody was used in previous studies and targets N-terminal parafibromin in exon 3 (Tan *et al*. 2004, Gill *et al*. 2006). BL648 is a commercially available polyclonal parafibromin antibody directed at an unknown 24 aa sequence within exons 8-9, thereby probably encompassing the β-catenin interacting domain (CID) of parafibromin. In addition, we also employed the APVF polyclonal parafibromin antibody used in paper II targeting C-terminal parafibromin (exons 16-17) as well as a novel polyclonal parafibromin antibody termed TNYV, targeting N-terminal parafibromin (exons 1-2), both antibodies raised commercially by us.

All antibodies were validated for their sensitivity and specificity towards parafibromin through Western blot analysis of a panel consisting of *HRPT2*-transfected HeLa cells, untransfected HeLa controls, subcellular fractions from a case of SHPT, two cystic adenomas (one *HRPT2* wildtype, one *HRPT2* mutated), one unequivocal carcinoma (T4) as well as one equivocal carcinoma with a germline *HRPT2* mutation (T23). For the transfected HeLa cells, all four antibodies detected a strong ~60 kDa product which was much more intense than for the untransfected control sample. These findings were validated by IHC on paraffin embedded samples of *HRPT2* transfected and untransfected HeLa cells, thereby affirming high sensitivity for the detection of parafibromin. To validate the specificity, a parathyroid adenoma carrying an *HRPT2* mutation and previously shown to lack parafibromin expression (paper II) was assessed by Western blot. All four antibodies demonstrated negative

parafibromin expression for this case. The specificity for each antibody was further validated using peptide neutralization tests for all Western blot analyzed samples.

Regarding the IHC methodology, all antibodies were assessed using different primary antibody concentrations and different modes of antigen retrieval (AR) prior to definite usage to optimize the protocols. Since different methodology for the 2H1 antibody has been used in the two preceding publications regarding parafibromin immunoreactivity, this antibody was specifically scrutinized using different antibody dilutions, different times in AR as well as different antibody incubation time (Tan *et al*. 2004, Gill *et al*. 2006). These parameters were tested on control cases including 4 adenomas and 4 carcinomas (two of the latter were available on western blot analysis, T4 and T23). We found that citrate AR for 20 minutes, a 2H1 antibody dilution of 1:20 as well as overnight incubation were all required to attain the positive signals expected in the adenomas and in the two Western blot verified carcinomas. Using shorter AR time, higher 2H1 dilution or shorter incubation time a positive signal was not obtained in the anticipated cases. These data are illustrated in Figure 15 and suggest that parafibromin immunoreactivity is highly dependent on the IHC methodology chosen, and careful assessment of the antibody is therefore crucial to obtaining dependable results at parafibromin IHC. Furthermore, our data suggest that all four antibodies exhibit similar sensitivity and specificity for the adequate detection of human parafibromin using IHC.

Parafibromin expression is frequently reduced, but rarely lost in parathyroid carcinomas

Using the ABC methodology and the parafibromin antibody panel, we demonstrate reduced parafibromin immunoreactivity in the majority of unequivocal carcinomas studied (14 out of 22, 64%). This phenomenon has been previously demonstrated for parathyroid tumours (termed “focal loss”) and in our study we denote this pattern as “partial loss”. By our definition, partial loss indicates positive parafibromin expression in subsets of nuclei only, which was observed in the range 11-89%. Less than 10% positive cells indicate a negative sample, and more than 90% denote a positive sample. Using these criteria, 7 out of 22 samples (32%) were positive for parafibromin immunoreactivity and only one case was completely devoid of parafibromin (4%). All parathyroid adenomas were positive for parafibromin expression using all four antibodies. Among the equivocal cases (atypical adenomas), 55% were positive and 45% exhibited partial loss for parafibromin.

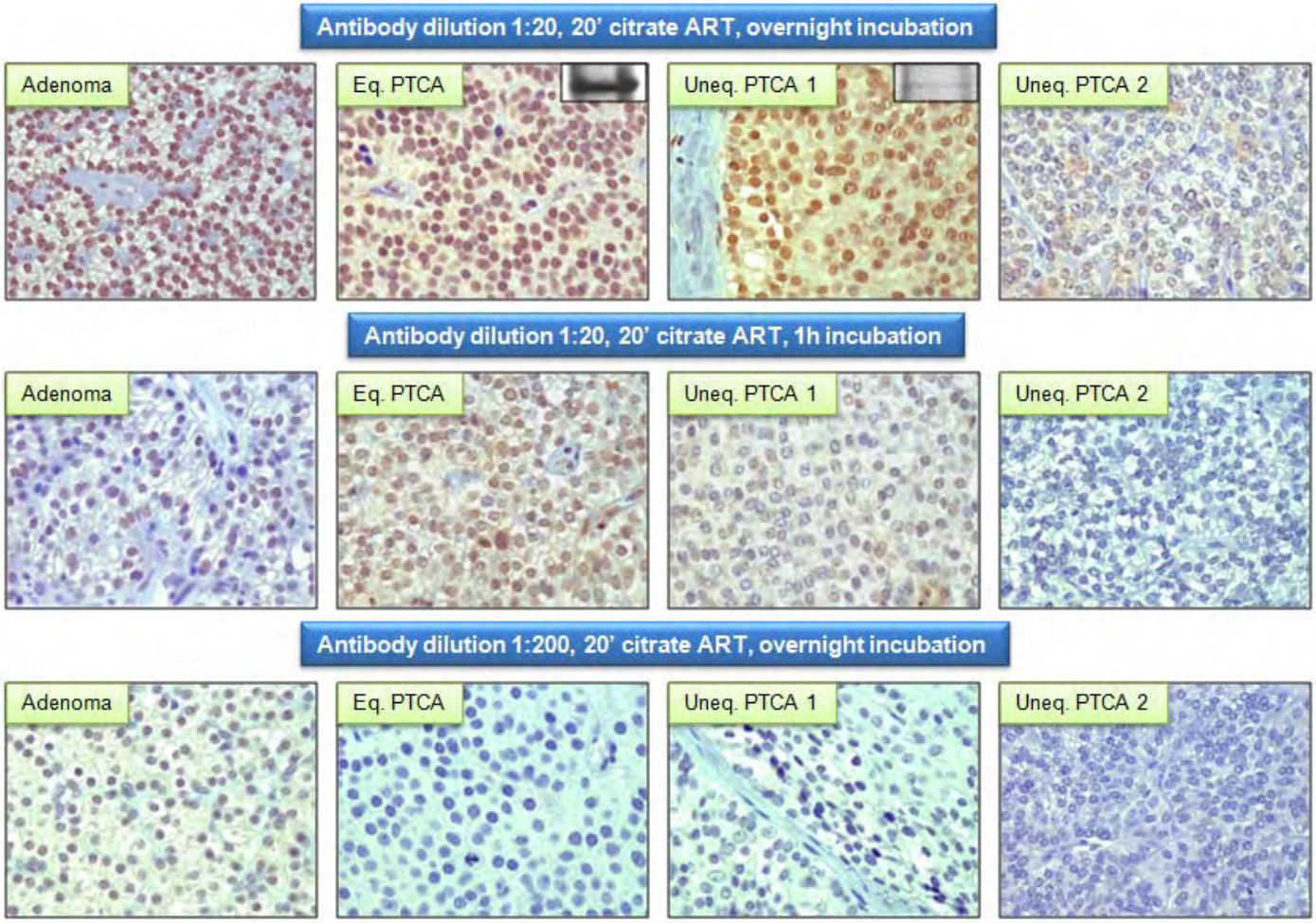

Figure 15 Different IHC methodologies give rise to different outcomes in staining interpretation. Top row denotes our standard settings when assessing the 2H1 parafibromin antibody, all samples are positive except for PTCA2 which demonstrate partial loss of parafibromin expression. Inserts are results from Western blot analyses. In the middle row, PTCA1 exhibits partial loss and PTCA2 is negative for parafibromin expression. In the third row the adenoma demonstrates partial loss, while the other three samples are negative for parafibromin immunoreactivity. 1:20 dilution denotes 10 μg/ml.

Our study indicates that a majority of parathyroid carcinomas have reduced parafibromin expression which is fully in line with previous reports. However, since the vast majority of cases with deranged parafibromin immunoreactivity in our study displayed reduced expression rather than a complete loss of protein as suggested from previous studies, methodological differences could possibly explain the divergent findings herein. In support of our results, all cases were completely negative when analyzed by peptide neutralization tests, thereby confirming the specificity of our positive findings in a subset of tumour nuclei. Moreover, we could observe that a more pronounced dilution (1:200) of the 2H1 parafibromin antibody gave rise to a partial loss phenomenon in the Western blot positive cancers T4 and T23, whereas the two other carcinomas changed from partial loss to complete absence of parafibromin immunoreactivity using the same dilution (Figure 15). However, the initial findings of positive immunoreactivity in cancers T4 and T23 were verified by peptide neutralization tests, suggesting that the positive stainings were specific but could be lost in a focal pattern when increasing the primary antibody dilution. Since the different parafibromin antibodies yielded similar results regarding parafibromin immunoreactivity, our data indicate that full-length parafibromin is physically present in the positive nuclei of partial loss cases and that the functionally important CID and PAF domains are physically present in these nuclei. Subsequent studies regarding parafibromin IHC demonstrate similar observations, in that the majority of parathyroid carcinomas examined display positive or reduced expression rather than a complete absence of parafibromin (Tominaga *et al.* 2008, Fernandez-Ranvier *et al.* 2009).

Although all adenomas in our study were positive for parafibromin expression, the specificity for reduced/absent parafibromin immunoreactivity in detecting malignant tumours is believed to be somewhat reduced since loss of parafibromin has been demonstrated for a small subset of parathyroid adenomas (paper II, Gill *et al*. 2006, Cetani *et al*. 2007). The sensitivity in our study was 68%, which is similar to previous studies with the important exception that cases with aberrancies in parafibromin almost exclusively displayed partial loss rather than complete absence of expression. Theoretical NPV and PPV values would be high and low respectively, indicating that positive parafibromin immunoreactivity strongly implies a benign parathyroid tumour, whereas a negative or partial loss finding could indicate carcinoma as well as an adenoma likely demonstrating an *HRPT2* gene mutation. As of this, parafibromin would be of value as a complementary marker in the clinical setting, but cannot be used as a single distinguishing marker for parathyroid malignancy. Cases with reduced or negative

parafibromin expression should probably undergo *HRPT2* mutational screening since reduced or absent parafibromin expression is connected to aberrations in the *HRPT2* gene. Hence an *HRPT2* screening in these instances might detect hereditary disease in cases with a negative family history.

To summarize, parafibromin displays value as an adjunct marker in parathyroid tumour classification, where positive expression clearly suggests benign disease. Nevertheless, negative or reduced expression of parafibromin has been found in both carcinomas and adenomas and should not be interpreted as a definite sign of malignancy. However, cases with aberrant parafibromin staining could possibly benefit from *HRPT2* gene analyses in constitutional tissues.

APC is a novel marker for the detection of parathyroid cancer (paper IV)

Parathyroid carcinomas exhibit frequent aberrations in the Wnt signaling pathway

If applied as a sole marker for malignancy, parafibromin would suffer from reduced specificity. To explore additional molecular events in parathyroid carcinomas that could be of diagnostic utility, we turned to the parafibromin-associated Wnt pathway. Of immediate interest, APC and GSK3-β are two regulatory proteins with key functions within the canonical branch of the Wnt pathway. Together with axin, APC and GSK3-β will keep the central oncoprotein β-catenin phosphorylated and subsequently degraded, and thus APC and GSK3-β exhibit negative growth regulating properties. The *APC* gene is mutated in the familial adenomatous polyposis (FAP) syndrome in which the afflicted members early on presents with multiple benign polyps of the colorectum, lesions which ultimately lead to the development of colorectal cancer. Interestingly, a few FAP families have also been associated with parathyroid tumours as well as radio-opaque jaw lesions, features also present in the HPT-JT syndrome (Sakai *et al*. 2002, Wijn *et al*. 2007).

Using monoclonal antibodies for APC and GSK3-β, we established the methodology by assessing a control panel of four parathyroid adenomas by Western blot and IHC. The control cases were all positive for the respective protein expression by both methods with comparable

intensity for each case. We then investigated a total of 30 parathyroid tumours, including 12 carcinomas and 18 adenomas, for APC and GSK3-β, immunoreactivity using IHC analysis. In addition, normal rims present on the slides for 12 of the adenoma cases were separately evaluated. Positive controls consisted of normal colon tissue, colon cancer and cases of parathyroid adenomas mounted on the same slide, and negative controls consisted of omission of the primary antibodies for each case. We demonstrate complete absence of APC immunoreactivity in 9 out of 12 parathyroid carcinomas (75%), whereas all 18 parathyroid adenomas and 12 normal rims stained positive for APC. For GSK3-β, 4 out of 12 carcinomas and 1 out of 18 adenomas stained negative (33% and 6% respectively).

These findings suggest that inactivation of APC is a common molecular event in parathyroid malignant tumours, while the expression is retained in parathyroid adenomas. The findings are in line with the recent results by Björklund *et al.* who detected APC expression in normal parathyroid, parathyroid adenomas as well as in cases of SHPT, thereby supporting the notion that loss of APC expression occurs exclusively in malignant parathyroid tumours (Björklund *et al*. 2007a). In our series, loss of APC immunoreactivity was significantly associated with parathyroid carcinomas ($p < 0.001$), and the resulting sensitivity (75%) and specificity (100%) yielded NPV and PPV values of 99.7% and 100% correspondingly. These values, although preliminary and in need of additional evaluation in an independent series of parathyroid tumours, indicate that APC immunoreactivity could be used as a discriminating marker for the detection of parathyroid carcinoma. To validate our findings, an additional monoclonal antibody targeting C-terminal APC was employed. Using the identical IHC protocol, 6 out of 12 parathyroid carcinomas were negative for APC expression (50%) whereas all adenomas stained positive (100%). The resulting NPV and PPV values were 99.5% and 100% respectively, supporting the notion that APC immunohistochemistry is of value for detection of parathyroid malignant tumours.

Loss of APC does not confer an increase in ser-37-unphosphorylated (active) β-catenin levels

We subsequently turned our attention to the Wnt target oncoproteins cyclin D1 and β-catenin, speculating that an eventual loss of APC and/or GSK3-β expression in parathyroid malignant tumours could lead to an increase in Wnt target protein expression. We employed three monoclonal antibodies, a cyclin D1 antibody previously used in paper II, an antibody directed

at C-terminal β-catenin (measuring total levels of β-catenin) and one β-catenin antibody targeting an epitope containing a serine residue at aa position 37. When β-catenin is dephosphorylated at residue 37 (ser-37) in exon 3, the protein is stabilized (activated) and translocated to the nucleus to initiate Wnt target gene transcription. The latter antibody thus detects unphosphorylated (active) β-catenin only.

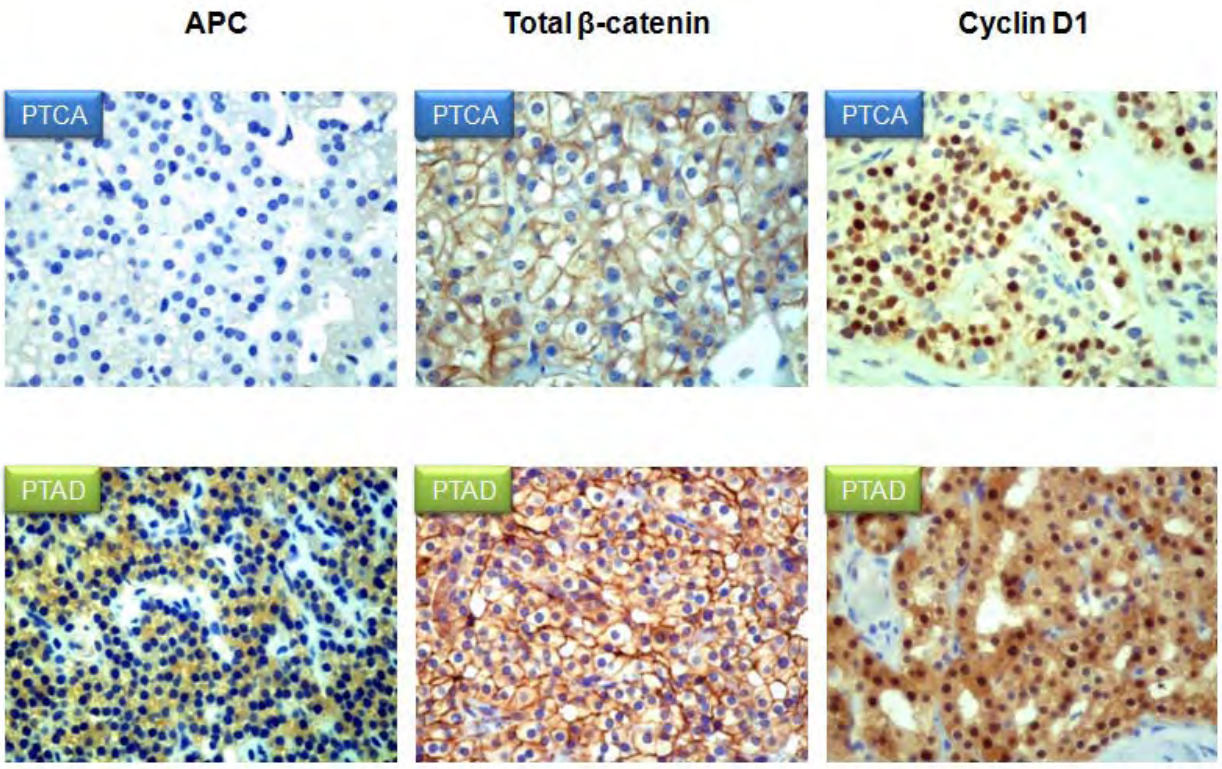

Figure 16 Immunohistochemical staining for APC, C-terminal β-catenin and cyclin D1 in a parathyroid carcinoma (PTCA) and adenoma (PTAD) respectively. APC is uniformly lost in the carcinoma but strongly expressed in the adenoma. Although endowed with membranous and cytoplasmic staining, no nuclear accumulation of β-catenin is evident in either the carcinoma or adenoma. The levels of cyclin D1 nuclear immunoreactivity are equally strong in both tumours, as was the proportion of positive nuclei.

By IHC analyses, we demonstrate clear cytoplasmic and membranous expression of C-terminal β-catenin in all parathyroid tumours. All normal rims similarly displayed clear membranous and cytoplasmic staining (with a single exception for a case that lacked the membranous signal). No differences in intensity between normal samples or parathyroid

tumours were noticed. Two parathyroid carcinomas displayed a mixed pattern of negatively and positively stained nuclei, but apart from these two tumours, all normal rims and tumour tissues were completely devoid of nuclear C-terminal β-catenin expression (Figure 16). The findings are fully in line with previous and succeeding publications (Ikeda *et al*. 2002, Cetani *et al*. 2009).

Regarding active β-catenin, all normal parathyroid rims exhibited positive cytoplasmic staining as well as nuclear expression in a mixed pattern. Nuclear and cytoplasmic expression of unphosphorylated β-catenin was similarly detected in all parathyroid benign and malignant tumours investigated. To confirm our findings, unrelated cases of parathyroid adenomas as well as pooled non-tumorous parathyroid tissues were assessed for β-catenin expression by Western blot analysis. Normal parathyroid tissue as well as 6 parathyroid adenomas displayed equally strong ~90 kDa bands corresponding to the size of β-catenin by both the C-terminal as well as the anti-active β-catenin antibodies. These data indicate that normal parathyroid and parathyroid tumours express similar amounts of ser-37-unphosphorylated (active) β-catenin as well as total levels of β-catenin, suggesting that β-catenin ser-37 related aberrations do not play a major role in parathyroid tumorigenesis. Indeed, the high levels of active β-catenin in nuclei of normal parathyroid cells are not believed to stimulate proliferation, and one explanation can be due to the "just-right" hypothesis of Wnt signaling, suggesting that high β-catenin expression might stimulate apoptosis in addition to proliferation (Bordnonaro *et al*. 2008). Interestingly, all parathyroid carcinomas with loss of APC expression displayed β-catenin levels comparable in intensity to that of adenomas and normal rims. In addition, cyclin D1 levels were equally high in both benign and malignant tumours (Figure 16). These findings suggest that loss of APC confer a proliferative advantage to parathyroid carcinomas by other means than through up-regulation of the canonical Wnt cascade. For example, APC has been shown to directly regulate DNA replication as well as apoptotic processes in a β-catenin independent manner, providing two examples of how APC could regulate proliferation autonomously of canonical Wnt signaling (Hanson and Miller 2005, Qian *et al*. 2008).

To summarize, our data suggest that APC and GSK3-β as central players of the canonical Wnt pathway are involved in the development of parathyroid malignant disease. APC could become a novel marker for confident detection of parathyroid carcinomas as it was found absent in the majority of carcinomas while retained in all benign tumours. In addition,

upregulation of the β-catenin or cyclin D1 oncoproteins are not expected to be essential components in the development of the parathyroid tumours investigated in our series.

APC and parafibromin – a joint venture in the hunt for potentially malignant PHPT (paper V)

Atypical parathyroid adenomas display molecular similarities to carcinomas

APC and parafibromin are both considered as promising distinguishing markers between malignant and benign parathyroid disease. In this study we evaluated 5 cases of atypical adenomas, a single carcinoma as well as 33 adenomas for APC and parafibromin expression using IHC and Western blot analysis. Since atypical adenomas harbour unknown malignant potential and sometimes recur as fully malignant in the same patient after surgery, we sought to determine whether APC or parafibromin or both markers combined could detect an eventual malignant potential in these tumours prior to required histopathological characteristics.

We employed the 2H1 monoclonal parafibromin antibody used in several preceding publications as well as an APC antibody targeting N-terminal APC previously assessed in paper IV. The results demonstrate that atypical parathyroid adenomas may display negative APC immunoreactivity together with reduced expression of nuclear parafibromin, as this IHC profile was evident in 2 out of 5 atypical adenomas. Furthermore, the single parathyroid carcinoma examined exhibited the same molecular phenotype (APC negative, partial loss of parafibromin), whereas all parathyroid reference adenomas (devoid of atypical findings) displayed positive APC expression, including two cases endowed with *HRPT2* gene mutations and loss of parafibromin expression.

Interestingly, the molecular similarity of atypical adenomas with carcinoma regarding parafibromin and APC expression in this study and in previous publications suggests that subsets of atypical adenomas share a similar molecular profile with fully malignant parathyroid tumours. Although in need of reproduction, our data implies that atypical adenomas ought to be subjects to vigilant follow-up since a substantial fraction of parathyroid tumours presenting with atypical histological findings probably possess malignant potential although not yet phenotypically manifestated. Furthermore, atypical cases could possibly

benefit from APC and parafibromin expression screening to detect tumours with a molecular risk profile which is so commonly encountered among parathyroid carcinomas (Figure 17).

Absence of recurrence in the study group

Since two of the five atypical adenomas investigated displayed complete loss of APC immunoreactivity in concert with reduced parafibromin expression, we wanted to establish whether or not these two patients had relapsed since initial surgery. The follow-up time for the patients presenting with atypical adenoma ranged from 23 to 84 months after the initial surgery. None of the five patients exhibited symptoms that pointed towards malignant disease and all cases have been normocalcemic since initial surgery. This could indicate that both cases with APC and parafibromin aberrancies were in fact benign at surgery, alternatively that the tumours had not yet spread prior to excision.

For the reference adenoma group, the time of follow-up ranged from one day post-surgically to 195 months. None of the patients displayed indications of malignant disease; nonetheless one case exhibited persistent hypercalcemia and one case developed recurrent hypercalcemia. Both cases exhibited very modest hypercalcemia, so no additional neck surgery was performed based on the benign clinical profile.

Combined APC and parafibromin screening – uniting the benefits

The main advantage of using APC as a marker for parathyroid carcinoma as opposed to parafibromin is attributable to its high specificity in determining parathyroid malignant tumours from benign. Including this study, more than 60 parathyroid adenomas have so far been examined for APC expression using immunohistochemistry or Western blot analysis, all positive (Björklund *et al*. 2007a, paper IV, paper V). Moreover, normal parathyroid controls as well as ten cases of SHPT have also demonstrated positive APC expression. These data together with our findings indicate that loss of APC occurs exclusively in parathyroid atypical adenomas and parathyroid carcinomas. An additional benefit of employing APC as a separating marker is that APC immunoreactivity appears to be homogeneously lost in the preponderance of parathyroid carcinomas in preference to parafibromin which frequently demonstrate reduced expression of the protein rather than a total deficiency.

The advantage of employing parafibromin as a marker stems from its ability to detect cases with *HRPT2* gene aberrations. For example, in paper II loss of parafibromin was found in three parathyroid adenomas, all carrying HRPT2 gene alterations.

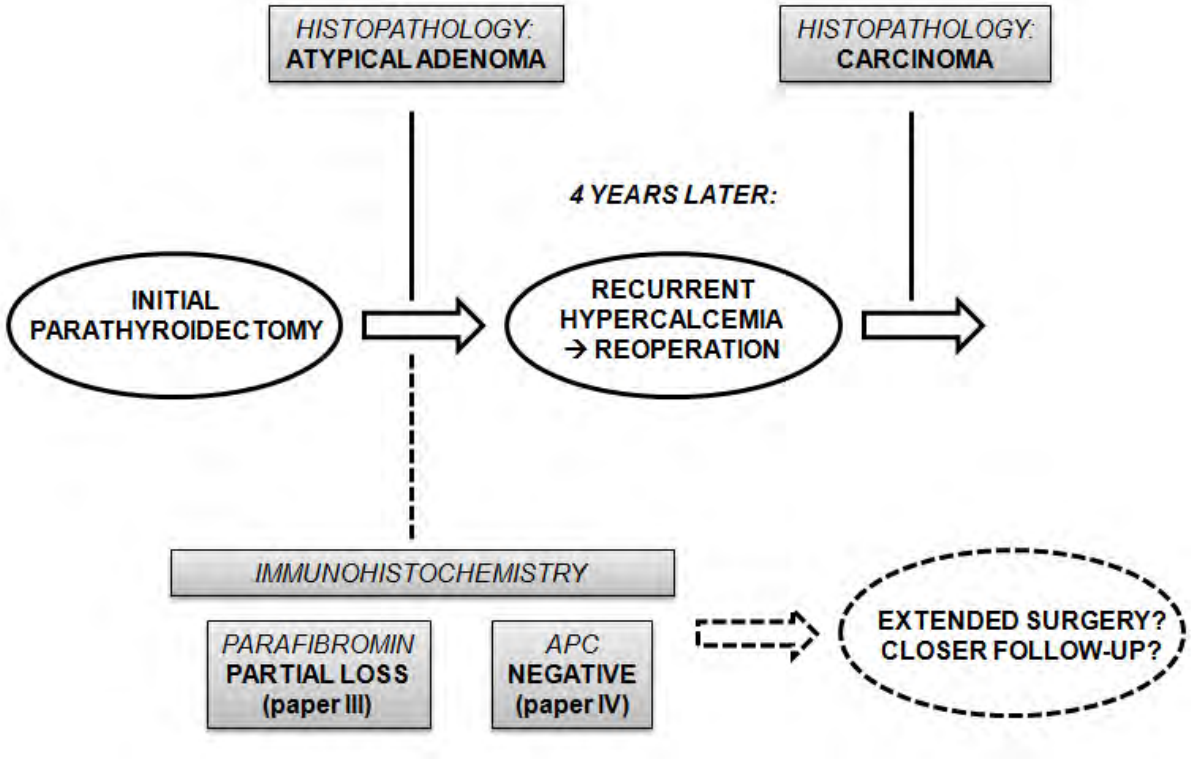

Figure 17 Highlighting the benefits of APC and parafibromin IHC. The following scheme represents the handling of a patient operated for PHPT at Karolinska University Hospital. The patient was originally diagnosed with an atypical adenoma at initial histopathological examination. Four years later the patient relapsed with recurrent hypercalcemia and was referred for additional surgery. The reoccurring lesion was excised, and the diagnosis was consistent with parathyroid carcinoma. Re-examination of the initial lesion found no standpoints for malignancy. The initial tumour has since been analyzed for parafibromin (paper III) and APC (paper IV), represented by the dashed line, and was found to exhibit reduced expression and total loss of the respective proteins. If applied after the initial surgery, parafibromin and APC could have altered the course for this patient possibly in terms of extended surgery or more vigilant follow-up, since the markers indicated malignant potential in this tumour prior to acquired histopathological requirements (i.e. invasion or metastasis).

Likewise, in paper III we found reduced parafibromin expression in half the carcinoma with known *HRPT2* mutations. Given the fact that a subset of patients with seemingly sporadic PHPT displays germline *HRPT2* gene mutations, parafibromin screening might increase the detection rate of HPT-JT and FIHP kindreds without a perceptible family history.

To conclude, atypical parathyroid adenomas are tumours with uncertain malignant potential, and subsets recur as fully malignant. Since atypical adenomas might exhibit negative APC immunoreactivity in unison with reduced expression of parafibromin, we propose that both APC and parafibromin are included in the screening process of parathyroid tumours with unclear malignant potential. APC confers a higher specificity than parafibromin and therefore excels in terms of predicting parathyroid malignancy; however parafibromin immunohistochemistry might detect cases with germline *HRPT2* gene aberrations which could be of immense value to the treating physician. Even though our sample size is small and no atypical cases devoid of APC expression were shown to recur, our results suggest that parathyroid atypical adenomas absent of APC expression necessitate vigilant follow-up, since this molecular profile is frequently detected in parathyroid malignant tumours.

Regional and global hypermethylation in parathyroid tumours (paper VI)

The APC and RASSF1A promoters are hypermethylated in parathyroid tumours

Epigenetic mechanisms are increasingly identified as contributing factors underlying human tumorigenesis, still little is known regarding the role of epigenetic aberrancies regarding *bona fide* tumour suppressor genes in parathyroid tumours. In addition, previous methylation assays concerning parathyroid tumours have been based on the non-quantitative MS-PCR methodology (Carling *et al*. 2003, Hewitt *et al*. 2007, Takeuchi *et al*. 2007). As of this, we sought to determine the quantitative levels of promoter methylation in a group of parathyroid tumours by bisulphite Pyrosequencing. We collected a series of 55 parathyroid tumours with known *MEN1* and/or *HRPT2* status, including 34 cases of cystic parathyroid adenomas, 19 cases of regular parathyroid adenomas, 2 tumours with constitutional *HRPT2* gene mutations and a case of SHPT. As non-tumorous controls, two samples of normal parathyroid tissue,

human leukocyte DNA as well as thymic tissue were employed. We selected four different tumour suppressor genes widely hypermethylated in various human tumours and with theoretical connections to parathyroid tumorigenesis; namely *APC*, *ras association domain family protein 1* (*RASSF1A*), $p16^{INK4A}$ and retinoic acid receptor-beta (*RAR-β)*. In addition, *LINE-1* repeats were also assessed as a marker for global methylation.

We demonstrate hypermethylation of *APC* promoter 1A and the *RASSF1A* promoter in the majority of parathyroid tumours investigated (71% and 98% respectively) as compared to the controls (normal parathyroid samples, SHPT, leukocytes and thymic tissue) which all displayed very low levels of promoter methylation. The mean methylation density for each sample ranged from 1.5% to 77.5% for *APC* and from 8.0% to 80% for *RASSF1A.*

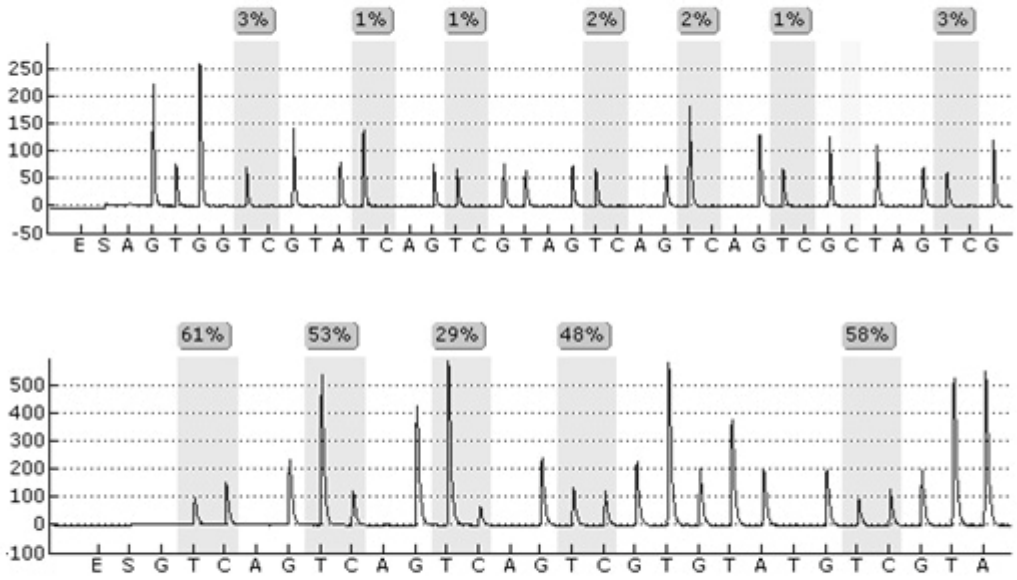

Figure 18 Pyrograms for the $p16^{INK4A}$ (above) and *RASSF1A* (below) promoters analyzed in a parathyroid adenoma, demonstrating average methylation densities of ~2% and ~50% respectively.

There was a significant correlation between *APC* and *RASSF1A* methylation levels, suggesting that these events are coupled proceedings in parathyroid tumours. Regarding $p16^{INK4A}$, a single adenoma was found to be hypermethylated as compared to non-tumorous controls while all other cases (98%) displayed mean levels of promoter methylation densities below 10%. *RAR-β* did not exhibit promoter hypermethylation in any case examined.

APC promoter 1A hypermethylation correlates to MEN1 mutational status

Since our tumour panel previously has been extensively investigated for *MEN1* and/or *HRPT2* mutations, we could correlate the levels of promoter methylation to the genotype for each of these two tumour suppressors. Interestingly, while there were no correlations between *HRPT2* mutational status and methylation levels of any of the four genes examined, a significant correlation between *MEN1* gene mutations and APC promoter 1A methylation levels was identified. The findings are particularly appealing since recent studies point towards an association between the menin protein and the parathyroid-associated canonical Wnt pathway, and theoretically suggest that menin constitute a putative epigenetic regulator of the *APC* gene (Chen *et al*. 2008).

Parathyroid tumours exhibit genome-wide hypermethylation of LINE-1 elements

Most tumours with few exceptions display relative hypomethylation as compared to their respective normal tissue counterpart. We therefore investigated all tumours for *LINE-1* methylation, which is a widely accepted and sensitive instrument for the assessment for global methylation. Normal parathyroid tissues demonstrated methylation densities of 60.7% and 59.7% respectively, whereas most investigated parathyroid tumours displayed relative hypermethylation, ranging from 60.7% to 78.3%. These findings were unanticipated, and suggest that parathyroid tumours display a more heavily methylated genome than normal parathyroid tissue. Interestingly, *LINE-1* methylation levels correlated strongly to the presence of an *HRPT2* gene mutation. For instance, the two tumours in our study with the highest levels of *LINE-1* methylation both carried germ-line *HRPT2* mutations. These data suggest that parafibromin, as a known member of a histone methyltransferase (HMTase)

complex, might directly or indirectly regulate the levels of DNA methylation in addition to its histone modification properties.

To conclude, we demonstrate *APC* and *RASSF1A* promoter hypermethylation in the preponderance of parathyroid tumours examined. APC promoter hypermethylation correlated to the presence of an *MEN1* mutation, suggesting that menin might regulate APC expression through epigenetic modifications. Moreover, the habitually observed *RASSF1A* promoter hypermethylation in parathyroid adenomas suggest the involvement of the Ras signaling pathway in parathyroid tumorigenesis. Lastly, *LINE-1* pyrosequencing demonstrate parathyroid global hypermethylation as compared to normal parathyroid tissue and imply that *HRPT2* and parafibromin play important direct or indirect roles in epigenetic regulation of parathyroid cells.

CONCLUDING REMARKS

Although not widely known to the general public, the parathyroid glands are frequently endowed with tumorous growth both in sporadic as well as in familial settings. As more sophisticated molecular methods are persistently being developed, the amount of research data regarding parathyroid aberrant growth is correspondingly augmented. As a consequence of increased understanding regarding parathyroid tumorigenesis, clinical adjuncts such as *MEN1* and *HRPT2* genetic screening as well as parafibromin immunostaining have been developed to facilitate both diagnostic procedures and treatment options for patients suffering from primary hyperparathyroidism. These studies were performed to further characterize the multifaceted molecular and genetic events underlying parathyroid tumorigenesis, as well as to extrapolate these findings to clinical use. The main findings comprise:

1. The recognition of 1p, 6q and 11q allelic losses in subsets of patients with primary hyperparathyroidism. Loss of 1p and 11q appear to be more prominent in asymptomatic hyperparathyroidism whereas loss of chromosomal material at 6q might confer a more symptomatically pronounced disease.

2. The identification of the *HRPT2* gene and its product parafibromin as ubiquitously expressed in human tissues. Parafibromin localizes mainly to the nuclear compartment, and total loss of its expression is strongly indicative of *HRPT2* gene mutations as evident in a small subset of sporadic parathyroid adenomas.

3. The recognition of parafibromin as an adjunct marker facilitating parathyroid tumour classification. Positive parafibromin expression point to benign disease, but cannot fully exclude the possibility of a parathyroid carcinoma. Reduced expression of parafibromin in a parathyroid tumour indicates *HRPT2* gene aberrations which require germline *HRPT2* gene screening.

4. The discovery of APC as a novel marker for the proper recognition of parathyroid carcinoma. Uniform loss of APC was exclusively found in parathyroid malignant tumours, and all adenomas investigated displayed retained APC expression.

5. The suggestion to include both parafibromin and APC screening for parathyroid tumours with unknown malignant potential, i.e. atypical adenomas. APC confer a superior specificity to parafibromin in detecting malignant cases, but parafibromin might detect cases with germline *HRPT2* mutations even if absent of family history.

6. The identification of regional *APC* and *RASSF1A* promoter hypermethylation as well as global hypermethylation in the majority of parathyroid benign tumours. *APC* promoter hypermethylation was associated to the presence of an *MEN1* mutation and genome-wide hypermethylation correlated strongly to *HRPT2* mutations respectively, suggesting the theoretical involvement of menin and parafibromin as epigenetic regulators of parathyroid cells.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bisköldkörtlarna (på latin *glandulae parathyroidea*) är normalt sett fyra risgrynsstora, gulbruna körtlar belägna i anslutning till sköldkörtelns baksida framtill på halsen strax under adamsäpplet. Bisköldkärtlarna har en huvudsaklig uppgift; att reglera halterna av kalcium i blodet, och de medierar denna funktion genom att insöndra bisköldkörtelhormon (PTH) till blodbanan. Bisköldkörtlarna besitter en förmåga att med mycket hög känslighet kunna läsa av kalciumnivåerna i blodet genom ett avancerat molekylärt system, och de korrigerar ett sjunkande kalciumvärde genom att insöndra PTH som i sin tur leder till att mer kalcium når blodet via ökad skelettnedbrytning, ökat tarm- samt njurupptag. Om kalciumvärdet blir för högt kommer insöndringen av PTH från bisköldkörtlarna att avta, och på så sätt uppnås balans i systemet.

Primär hyperparathyroidism (förkortas PHPT) är en sammanfattande term som omfattar sjukdomstillstånd som kännetecknas av överfunktion i en eller flera bisköldkörtlar. PHPT är ett vanligt förekommande tillstånd, exempelvis uppskattas ca. 2-3% av alla postmenopausala kvinnor i Sverige lida av denna åkomma men även yngre människor, kvinnor som män, kan drabbas. PHPT leder vanligen till förhöjda halter av kalcium i blodet, och symptomen är oftast relaterade till denna s.k. hypercalcemi. Symptom inkluderar trötthet, ökad törst och muskelsvaghet men kan även ta sig allvarligare uttryck som exempelvis benskörhet, allvarlig depression eller gall- och njurstenar. De allra flesta patienter med PHPT är dock helt utan symptom. Nyligen publicerad forskning visar även att PHPT-patienter har en ökad risk för hjärtkärlsjukdom såväl som cancer i olika organsystem. Den vanligaste orsaken till PHPT är ett adenom, dvs en godartad tumörtillväxt av en enskild bisköldkörtel, som utgör ca. 80-85% av fallen med PHPT. Den näst vanligast orsaken är så kallad flerkörtelsjukdom (eller hyperplasi) där flera körtlar är förstorade samtidigt. Denna form utgör ca. 15% av all PHPT och är ofta kopplad till en ärftlig sjukdom där bisköldkörtelsjukdom är det vanligaste inslaget, så kallad multipel endokrin neoplasi typ 1 (MEN 1). Slutligen har vi den potentiellt dödliga formen av PHPT, bisköldkörtelcancer. Till skillnad från adenomen så kan en cancertumör sprida sig bortom halsen och orsaka dottersvulster, metastaser. Bisköldkörtelcancer är en ovanlig form av PHPT (ca. 1%), och har visat sig vara kopplad till ett ärftligt tillstånd vid namn hyperparathyroidism-jaw tumour- syndromet (HPT-JT), där PHPT är ett vanligt inslag och där bisköldkörtelcancern är överrepresenterad (ca 15% av HPT-JT-fallen).

Forskare har på senare år lyckats identifiera flertalet gener som uppvisar mutationer i bisköldkörteltumörer. En mutation kännetecknas av att genens DNA-sekvens (och därmed också koden för hur det respektive proteinet skall byggas) förändras. Dessa gener kallas tumörsuppressor-gener eftersom de kodar för proteiner som när de uttrycks i sin oskadade form på olka sätt bromsar bisköldkörtelcellens tillväxt. När genen skadas, exempelvis genom mutation, så uttrycks proteinet felaktigt eller inte alls, och cellerna ökar således sin delningshastighet. Dessa mekanismer utgör några av de fundamentala grunderna för tumörtillväxt. Mycket av det vi idag vet gällandes den genetiska bakgrunden till PHPT baseras på fynd från familjer med ärftlig sjukdom där PHPT är en huvud- eller sidomanifestation. Exempelvis visade man på början av nittiotalet att mutationer i en gen på

kromosom 11 orsakade MEN 1-syndromet, och genen fick således namnet *MEN1*. Sedermera har man visat att inaktiverande mutationer i *MEN1* även orsakar icke-familjär (så kallad sporadisk) PHPT, och *MEN1*-mutationer har visat sig finnas i ca 10-20% av alla bisköldkörteladenom och i ett fåtal hyperplasier. *MEN1* kodar för menin, ett protein med flera kända tillväxtbromsande funktioner i cellen. Vidare har man identifierat *HRPT2* som genen bakomliggande det ärftliga HPT-JT-syndromet, och *HRPT2*-mutationer återfinns i majoriteten av alla sporadiska fall av bisköldkörtelcancer så väl som i ett fåtal sporadiska adenom. *HRPT2* kodar för parafibromin, ett relativt okänt protein med tumörsuppressor-egenskaper. Utöver *MEN1* och *HRPT2* känner man till ett flertal andra genetiska defekter som förmodligen bidrar till uppkomsten av PHPT, exempelvis så uppvisar en stor andel av bisköldkörteltumörer överuttryck av ett protein vid namn cyklin D1 som till skillnad från menin och parafibromin stimulerar celltillväxt, dvs ett så kallat onkoprotein. I ett fåtal fall har detta överuttryck visat sig bero på en kromosomal omförflyttning av genen som kodar för bisköldkörtelhormonet PTH, så att dess reglerande sekvens sätts framför cyklin D1-genen. Med andra ord, varje gång bisköldkörteln ska producera mer PTH så produceras istället cyklin D1-proteinet, som stimulerar celltillväxt. Dock är detta förmodligen bara toppen av ett genetiskt isberg, och mycket återstår att utreda avseende de molekylära mekanismer som ligger till grunden för utvecklandet av tumörer i bisköldkörtlarna. Denna avhandling syftar till att kasta nytt ljus på detta intrikata fält, främst inom områden där de moleyklärbiologiska forskningsresultaten direkt kan överföras till klinisk applicering.

I **arbete I** har vi identifierat förlust av genetiskt material vid korta änden av kromosom 1 (1p) och långa ändarna av kromosom 6 (6q) och 11 (11q) i 56 stycken bisköldkörteltumörer. Tumörerna hämtades från två olika grupper av patienter, dels från asymptomatiska patienter som hälsoscreenats för PHPT och dels från sedvanlig klinsik verksamhet där många uppvisar symptom från sin PHPT. Denna studie bygger på en så kallad loss of heterozygozity-teknik, där man med hjälp av radioaktiva eller fluorescerande markörer som binder DNA kan upptäcka om en tumör har förlorat en av två DNA-kopior i ett specifikt område. Förlust av genkopior är nämligen utöver genmutationer en vanlig bidragande orsak till tumöruppkomst. Förlusten av genetiskt material vid respektive kromosom 1p, 6q och 11q återfanns i drygt en fjärdedel av tumörerna och indikerar att en eller flera tumörsuppressorgener är lokaliserade i dessa områden. Gällandes 11q13 så vet vi redan att detta område ofta uppvisar förlust av genetiskt material, eftersom *MEN1*-genen är lokaliserad här. Intressant nog så såg vi ett samband mellan förlust vid 1p och 11q och utvecklandet av asymptomatisk PHPT, eftersom betydligt fler patienter i hälsoscreeningsgruppen uppvisade denna genetiska profil än i gruppen hämtad från kliniken. På samma sätt sågs ett samband mellan förlust vid 6q och utvecklandet av mer symptomgivande PHPT, eftersom signifikant fler patienter ifrån klinikgruppen uppvisade 6q-förlust än från den asymptomatiska screeningsgruppen. Detta tyder på att förlust av olika kromosomregioner i bisköldkörteltumörer kan resultera i olika kliniska yttringar.

I **arbete II** har vi i detalj studerat *HRPT2*-genen och dess proteinprodukt parafibromin, som ju nyligen visade sig vara inblandade i uppkomsten av bisköldkörteltumörer. Vi visar här att *HRPT2*-genen uttrycks (dvs är aktiv) i en lång rad vävnadstyper i människokroppen och

således troligen har en eller flera funktioner för olika celler runt om i kroppen, ex. hjärta, hjärna, lever och lunga för att nämna några exempel. Vidare visar vi genom att fästa en fluorescerande signal till parafibromin och sedan med hjälp av mikroskop studera vart parafibromin tar vägen i en levande cell att parafibromin framförallt återfinns i cellkärnan. Slutligen undersökte vi 46 bisköldkörteladenom med avseende på parafibromin-uttryck, och fann att parafibromin-uttrycket var är utplånat i tre adenom som dessutom uppvisar *HRPT2*-mutationer. Detta stödjer den härskande terorin att *HRPT2* är en tumörsuppressorgen, och att förlusten av parafibromin har haft en inverkan på tumörtillväxten i dessa tre tumörer. Intressant är även att resterande 43 adenom med normal *HRPT2*-sekvens uppvisade parafibromin, vilket förmodligen innebär att uttrycksförlust av parafibromin är relativt ovanligt som fenomen bland bisköldkörteladenom.

Vi fortsatte därefter våra studier kring parafibromin i **arbete III**. Som tidigare nämnts så är *HRPT2*-genen inaktiverad i majoriteten av sporadiska bisköldkörtelcarcinom, och initiala studier kring parafibromin visade att dess uttryck var förlorat eller reducerat i de allra flesta cancerfallen. Detta rönte speciellt stor uppmärksamhet, då bisköldkörtelcarcinom är notoriskt svårdiagnosticerade. Cancer i bisköldkörtlarna kräver nämligen mikroskopiskt bevis på dirext inväxt i kärl eller nerver, alternativt inväxt på andra intilliggande organ eller fjärrmetastaser. Om någon av dessa parametrar ej föreligger så kan man således ej säga att det rör sig om en elakartad bisköldkörteltumör. När något av kriterierna väl är uppfyllt är det i regel för sent då tumören redan spridit sig, och man har därför länge letat efter en molekylär markör som identifierar elakartade tumörer innan de hinner sprida sig. Således ingav fynden kring parafibromin-förlust i bisköldkörtelcancer hopp om att här finna en särskiljande markör mellan elakartad och benign sjukdom, och vi undersökte således en stor samling bisköldkörtelcancerfall samt adenom för parafibrominuttryck med en metod vid namn immunohistokemi. I denna metod låter man en antikropp (som fungerar som en slags målsökande missil) binda till ett ultratunt snitt av tumörvävnaden som fästs på ett objektglas. Därefter låter man antikroppen reagera i en kemisk reaktion, och färg utvecklas där antikroppen bundit in. På så sätt skvallrar färgen om att vårt protein av intresse återfinns just här. Man undersöker glaset i ett mikroskop, och kan således visualisera färgen. I detta arbete upptäckte vi att majoriteten av alla studerade parathyroideacarcinom uppvisade reducerat parafibrominuttryck jämfört med de undersökta adenomen som samtliga uppvisade positivt parafibrominuttryck. Resultaten indikerar att immunhistokemisk färgning med parafibromin kan vara av nytta som en diagnostisk markör, då positiv färgning tyder på benign sjukdom. Dock är sensitiviteten och specificiteten inte är såpass höga att parafibromin skulle kunna användas som en enskild markör för särskiljande av tumörer i bisköldkörteln, eftersom förlust av parafibromin även återses i ett fåtal adenom och en negativ färgning kan betyda både så väl benign som malign sjukdom.

Parafibromin har visat sig interagera med protein inom en specifik signalkaskad i cellen, den så kallade Wnt-kaskaden. I **arbete IV** undersöktes därför ett antal bisköldkörtelcarcinom samt adenom m.a.p. immunohistokemi för ett antal protein inom Wnt-signalkaskaden. En minoritet av cancerfallen uppvisade förlust av GSK3-β, medan en majoritet var negativa för APC – det sistnämda proteinet en känd tumörsuppressor inom Wnt-kaskaden och ofta nedreglerat i

ändtarms- samt tjocktarmscancer. Resultaten indikerar att förlust av APC är unikt för bisköldkörtelcancer, och uppvisar högre sensitivitet och specificitet än parafibromin visade i delarbete III. Således har APC potential att utnyttjas som särskiljande markör mellan benign och malign sjukdom i bisköldkörtlarna.

I **arbete V** undersöktes fem fall av så kallade atypiska adenom med avseende på immunohistokemisk färgning av APC och parafibromin, de två kandidatmarkörer vi utrett i delarbeten III och IV. Atypiska adenom är tumörer med oklar elakartad potential, då de i mikroskopet uppvisar en eller flera egenskaper som många bisköldkörtelcarcinom också innehar, exempelvis olikstora cellkärnor, många celldelningar (mitoser) et.c. Utfallet korrelerades till kliniska uppföljningsdata. Som kontroll undersöktes 33 adenom för APC-uttryck. Resultaten visar att förlust av APC inskränktes till de atypiska tumörerna, medan samtliga adenom i denna studie var positiva för APC, inklusive två fall med *HRPT2*-mutation och parafibrominbortfall. Slutsatsen är att negativa APC-färgningar enligt denna studie och gängse litteratur ebart återfinns inom malign och atypisk bisköldkörtelsjukdom, medan parafibromin kan ses i reducerat/negativt uttryck även i fåtalet benigna fall. Således finns anledning att implementera både APC samt parafibromin i screeningprocessen av bisköldkörteltumörer med atypiskt växtsätt för att tidigt upptäcka malign potential och ev. familjär sjukdom, då förlust av APC är så tätt kopplat till malign sjukdom och då reduktion av parafibromin-uttryck kan ses hos patienter med ärftliga *HRPT2*-mutationer. Vidare kräver APC-negativa atypiska fall förmodligen en noggrannare uppföljning än vad som sker idag, eftersom de molekylärt liknar carcinomen.

Slutligen, i **arbete VI** analyserades 55 bisköldkörteltumörer m.h.a. pyrosekvensering. Metoden bygger på sekvensanalys där man kan avläsa om en gens reglerande DNA-sekvens (så kallad promotor) är metylerad, där metylerade promotorregioner oftast leder till nedsatt uttryck av den respektive genen. Således är metylering ett sätt att effektivt tysta en gen som cellen ej vill uttrycka just då. Resultaten visar att majoriteten av tumörerna uppvisar hypermetylering av *APC*- samt *RASSF1A*-promotorregionerna, två tumörsuppressor-gener som således teoretiskt nedregleras. Intressant nog så fanns ett signifikant samband mellan graden av APC-metylering och förekomsten av *MEN1*-mutation, något som indikerar att menin styr graden av APC-metylering direkt eller indirekt. Vidare visade vi att bisköldkörteltumörer i helhet har hypermetylerat genom jämfört med normal bisköldkörtelvävnad, och denna hypermethylering korrelerade starkt till förekomsten av en *HRPT2*-mutation. Således finns även fog att tro att parafibromin på ett större plan styr graden av metylering i bisköldkörtelceller. Fortsatta studier avseende dessa hypotetiska mekanismer kan hjälpa oss förstå hur parafibromin och menin i detalj styr tillväxthastigheten i bisköldkörteln så väl som i andra vävnader.

ACKNOWLEDGEMENTS

This thesis is dedicated to all patients suffering from cancer.

I would like to express my sincere and outermost gratitude and appreciation to all who so enthusiastically and magnanimously conferred visions and implemented ideas to my thesis; and for all of your guidance, advice and support during the compilation of this work. Without you, this booklet would not exist.

The studies were financially supported by the Swedish Cancer Foundation, the Cancer Society in Stockholm, the Gustav V Jubilee Foundation, the Swedish Society of Medical Research, the Swedish Medical Association, the Göran Gustavsson Foundation for Research in Natural Sciences and Medicine, the Knut and Alice Wallenberg Foundation, the Vera and Emil Cornell Foundation, the Foundation of the National Board of Health and Welfare, Karolinska Institutet and the Stockholm City Council.

The author is obliged to the support conferred by the MD/PhD program of Karolinska Institutet and the MD Research Intern program at Karolinska University Hospital Solna, Stockholm, Sweden.

My supervisors

Catharina Larsson, my always smiling professor and main supervisor; pure scientific brilliance and outstanding social skills merged into one mind. I am truly blessed and honored to have been given the opportunity to explore parathyroid genetics under your masterful and prudent guiding. Most of all, thank you for combining your distinguished scientific expertise with a particularly bighearted and humorous attitude which spread throughout the group and creates a great surrounding atmosphere. Working in your group might be demanding at times, but never ever dull.

Anders Höög, my first co-supervisor. Most people meet their first pathologist when it's already too late. I met you just in time. You are a true role-model for PhD students, and I can surely say that without your everlasting patience and vast knowledge in tumour pathology the papers wouldn't have been as meticulous as they turned out. You let me conduct the research reasonably autonomously, which sometimes was a rather challenging and frustrating experience - but also very rewarding in the end. Thank you for scrutinizing my immunostaining analyses with your "histology hawk-eye" and demanding countless control runs and carefully planned blocking experiments, something that made the papers a good deal stronger in the final run (although Felix nicknamed our research lab "the control group" as a consequence). I am also exceptionally grateful for all those extra hours by the microscope, studying the fundamentals of endocrine pathology (as well as dissecting the meaning of life) with such a skilled and sociable physician as you.

Andrea Villablanca, my second co-supervisor, for welcoming me to the lab as a young molecular biologist/M.D. student and for introducing me to the current projects. Thank you for teaching me the basics of parathyroid genetics as well as the required methodology in a superb manner, and for your top-notch ability to combine your pedagogic skills with a friendly and humorous touch. Your warm personality (and hot temper) helped instigate a very special aura to our lab which I believe is still present, later nick-named "the Larsson factor". Sin usted, este lugar perdió algo de su encanto!

Scientific collaborators

Robert Bränström, for your abilities to combine scientific thinking with a first rate sense of humor, for all those esteemed propositions that improved our overall work, and for being genuinely interested in my dawning clinical career in addition to my scientific one.

Lars Forsberg, for your sparkling skills in the field of molecular biology that spiced up our manuscripts, for introducing a solution to virtually every technical setback that arose (”Har du provat med sladden i?”), for being a truly hilarious chap and for your undying passion for the Hammarby football team. And did I mention Bajen?

Ming Lu, the always smiling endocrinologist – for excellent research collaborations and interesting discussions during the parathyroid group meetings.

Lars-Ove Farnebo, for your ever-lasting and compassionate interest in parathyroid tumorigenesis, for contributing prudent and insightful ideas during the parathyroid group meetings, for kindly teaching me the basics of parathyroid surgery and finally for your embodiment of the proverb "the friendly endocrine surgeons".

Kerstin Sandelin, at all times kind and with a knife-sharp intellect, for all the hours you put into our projects although literary drowning with work.

Jörgen Nordenström, for your vast knowledge regarding the parathyroid glands, for our particularly motivating discussions during the parathyroid group meetings and for your commitment and encouraging attitude.

Andrew Arnold, my transatlantic unofficial mentor, for all your positive energy and confidence that you brought into our work, for all your astute and sensible comments and for the rewarding collaborations. It has been an absolute honor to work with you.

Göran Åkerström, **Gunnar Westin**, **Jonas Rastad**, **Tobias Carling** and **Pamela Correa** as well as all other members at the Dept. of Surgical Sciences, Uppsala University Hospital anno 1998-1999: **Anders Knutson**, **Steven Lucas**, **Ola Hessman**, **Per Hellman**, **Eva Szabo**, **Birgitta Bondesson** and **Peter Lillhage**, for welcoming me as a "practical working experience student" (PRAO) during lower 2nd school, as well as guiding me through my high school exam project and finally for letting me work as a junior scientist at your lab for a full year. I could not have dreamt of a better way to start my scientific profession.

Tatyana Yakoleva, **Günther Weber, Ingo** and **Barbara Leibiger**, for excellent collaborations, especially Günther for teaching me qRT-PCR and for pleasant company in Maryland during the MEN 1 meeting in 2004.

Takao Obara and **Kenth Johansson**, for providing us with much needed parathyroid samples and for rapid correspondence.

Former and present members of the Medical Genetics Unit at the Centre for Molecular Medicine (CMM)

Adam Andreasson, genuinely gifted medical student for brilliant support working as a summer student. **Andrei Alimov**, for your vast knowledge in chemistry, sub-cloning, lab foods and freezer doors. And of course, the art of growing a proper mustache. **Mattias Berglund**, for Uppsala reminiscence and friendly chats. **Andrii "Mr. 5 min thyroidectomy" Dinets**, at all times friendly, cheerful and ready to lend a hand. **Pernilla "Pärlan" Garberg**, for first-rate support operating as a summer student and for being truly sociable and humorous. **Janos Geli**, the CMM rooster, for all entertaining conversations and medical in-house tales that just never goes out of style. **Emma Flordal-Thelander**, for your radiant sense of humor and hilarious talks. **Theodoros "Theo" Foukakis**, the "comet kid" of oncology, an outermost affable chap who can talk for hours and hours about peripheral stem cells – thereby proving the fact that the Greeks really invented the Marathon. **Luqman Sulaiman**, brilliant parathyroid researcher, for interesting genetic discussions as well as hilarious beer nights in Denver. "God bless America!" **Svetlana Lagercrantz**, **Jamileh Hashemi** and **Jia-Jing Lee**, for collaborations and for smiling and laughing all the way through my doctoral degree. **Shasha Lu**, for keeping the lab phone warm and functional. **Weng-Onn Lui**, for expert advice on DNA techniques and for spreading the Swedish art of *fika* around the globe. **Anastasios "Anestis" Sofiadis**, for hilarity discussing Hellenic and Swedish cultures, for S100A6 collaborations and for careful "Prussian style" supervision of lab supplies. **Srinivasan Thoppe**, for your ability to drink boiling water straight from the cup ("good for stomach") during the scorching summer months when me and Felix were hibernating in the cold room. **Wendy Weng**, **Stiina Välimäki, Chen Zhao, Hong Xie, Deniz Özata, Pinar Akçakaya, Iryna Kolosenko** and **Jan Åhlén**, for your outgoing and friendly attitude; particularity Stiina for your hard work collecting parathyroid carcinoma sample specimen.

My office mates

Stefano Caramuta, the first ever observed case of intestinal sub-blockage due to excessive intake of Ahlgren's Bilar. The indisputable king of RNA techniques and an exceptionally outgoing and entertaining italiano. Thank you for all the laughs my friend, e non dica prego chiunque che sia pazzesco. Grazie!

Nimrod "Nimmie" Kiss, the man that proves the myth – pipetting gives you strong arms. Thank you for your professional help in the lab covering technical issues, especially pyrosequencing, and for carrying on the office tradition of practical jokes (I especially

appreciated the package from Cheektown). An exceptionally wide-ranging gentleman, broader than his own sideburns; musically, literary, in wit and in mind. Todaloo IT!

Felix Haglund, my dear friend and ever-so-humoristic co-worker. Thank you for providing our parathyroid research group with steady supplies of great ideas since day one. In my eyes you are an astounding research talent, and I am truly delighted to have you on the team – especially since I know that our journey together does not end with this thesis. I believed it just took off.

Other CMM related members: **Susanna Brauner**, for your encouraging and sociable attitude, the doctors and patients within the specialty you'll choose should really congratulate themselves, **Anna-Maria Marino**, for your ever-present humor and warm personality, **Yin-Choy "Kato" Chuan**, for your lovely morning greetings in the corridor – stop pretending, you're not fooling anyone with that lab coat. **Sven Degerman**, for proficient assistance in installing a much needed IHC scorer in our lab. **David Velasquez-Fernandez**, for your exceedingly sociable and witty attitude, **Mohsen Karimi**, "what's so funny, guys?", **Zahidul Khan**, **Monira Akhtar**, **Tomas Ekström**, **Eva Rudd**, **Delphi Post**, **Louisa Cheung**, **Sofia Johansson**, **Daniel Johansson**, **Agneta Gunnar** for being genuinely friendly people. **Erik Björck** for interesting chats revolving hereditary parathyroid syndromes and for letting me in on a genetic consultant meeting with a Swedish HPT-JT family. **Caterina Zannin**, my only co-worker at 07.00 a.m in the morning, for humorous discussions regarding "zum-zum", "pistolas", Berlusconi and the pope, for blessing my experiments with holy water (double distilled) and for being the most bighearted human being within the whole of CMM.

Other staff from the Karolinska University Hospital in Solna

Martin Bäckdahl, for the adrenocortical tumour collaborations and superb supervision during my weeks in the endocrine surgery department. **Lars Grimelius**, for all your help with the parathyroid tumour classification, it has been a true honor to work in conjunction with a living legend. **Magnus Kjellman**, for enjoyable E.R/O.R collaborations. **Erik Linnander**, for brilliant supervision during my month at your emergency ward unit, for all your fantastic African stories and for guiding me throughout my first appendectomy. **Per "Pelle" Mattsson**, for your wonderful sense of humor and for arranging additional endocrine surgery during my research months. **Inga-Lena Nilsson**, always friendly - for the rewarding group effort regarding atypical tumours, for parathyroid epidemiologic discussions and for teaching me the crude basics of neck surgery (haken… släpp aldrig haken). **Per J Nilsson**, colorectal surgeon, for brilliant support during my stay at your unit, you are an astounding role-model for juvenile physicians. **Christian Danielsson**, **Peter Johansson**, **Emma Larsson**, **Krister Sjödahl** and **Olof Wallin** – intern colleagues and friends, for enduring the pain together. **Jan Zedenius**, the Godfather of Surgery, for hilarious morning meetings at the surgical department (mjältmousse), for arranging extracurricular endocrine surgery during my in-turn months, for organizing the much appreciated molecular wine seminars and for "making me an offer I could not refuse".

To the **administrators** at the Dept. of Molecular Medicine and Surgery, especially **Ann-Britt Wikström** and **Britt-Marie Witasp** for being tremendously helpful and proficient with

administrative and organizational dealings, and by God - there are many papers to fill out at this department… M-A-N-Y. Finally, thanks to the personnel at **Glada Restaurangen**, especially **Tomas**, **Lotta** and **Dennis**, for preparing the best "Dagens" there is.

My friends

Peter "Petta" Johansson and **Sven "Benny" Karlström,** my oldest friends from junior high school, for all years of intense football playing and gangster pranks around the Sunnersta neighborhoods. **Niklas "Poijan" Poijes**, "Tjuren från Zinken", walking legend - for all your hilarious stories that cracks me up just about every time and for being a genuinely great friend. "Utan dig hade det aldrig gått – high five!" **Olle Procopé**, for all guitar sessions and for getting used to the phrase "Soon done, promise". Well, guess what; now I'm done! **Niklas Schüllerqvist** and **Lina Tormod**, for all the laughs and uproarious evenings. All my friends from the Molecular Biology Program at Stockholm University: **Marcus Boman, Mats Ensterö, Måns Grundsten, Per Nyberg, Klas Strååt**, **Richard Ullstrand**, you made all those hours at KÖL/BÖL so worth it. Jag kunde inte drömt om mer stabila snubbar bredvid mig när vi räknade klyvöppningar på klibbal (*Alnus glutinosa*) eller körde molekylärbiologisk roulette (rullindunstning av bromsyra utan skyddsglasögon för den oinsatte).

All friends –and by now practicing physicians- from the med school years at Karolinska institutet, especially **Andreas Bartley**, **Lars Bergqvist**, **Johan Fridegren**, **Mikael** "Sluta förnedra dig, Dr. Juhlin" **Grumstedt**, **Bartek Jankowski**, **Mats Navrén**, **Erik Nyquist**, **Emil Sundler**, **Fredrik Thamsen, Hans Wirlind** and **Lars Öhrmalm**.

Linda Söderman&**Björn Sandell**, **Åsa Wennersten**, **Therese Gustafsson**, **Jannika Hernelius**, **Charlotte Roswall**&**Anders Molander**, **Emmy Dufvenmark**&**Simon Pontén**, **Heléne Riihonen**&**Daniel Lundqvist**, **Anna**&**Tom Abrahamsson**, **Emilia Hallin**, **Elisabeth Källén** and **Sandra Ohlsson** – for all the laughs and for constantly reminding me there are other things in life besides work.

Henrik "Hanky" Ljunggren, for your vast gastronomical interest, fabulous dinners and wonderfully dry sense of humor. **Ardalan "Ardy" Rahimi**, for all those hilarious years in med school, for your bizarre and outlandish sense of humor and for being my comical soul mate. **Måns "Måppe" Grundsten**, for everything and again; everything. Remember the late nights at the Chemical Practice Laboratory at Stockholm University in 1999. Thank you for being an out of this world companion throughout all these years. **Mikael "Koma" Mattsson**, the "Cosmo Kramer" character I always wished for in my life. For all those uproarious emergency fikas and late night strolls through Stockholm with me and Måns where the true meaning of life was dissected. Thank you for being a wonderful friend. **Nils "Ivica" Hedberg**, some people think you're crazy, I know you are. Thank you for being a splendid ally at all times, for arranging so many fanatical theme parties (but what happened to the despot theme?) and for sharing the same passion in TPM ("Tunnel På Mittplan") football ever since those early years in Sunnersta AIF. **Olof "Frukt" Hedberg**, ruling Wall Street since 2005 through 3.14159265… (Please feel free to continue yourself). Thank you for all the laughs, my friend. To **Anneli Flygare**, **Marie Peterson**, **Agneta Håkansson** and **Whitney J Davies**, for putting out.

Arbi Khachikian, “brorsan” – some things in life change, some things never will.

The **Rastad** family, **Jonas, Jane, Jens, Johanna** and **Jon**. My second family. Thank you for all those years at Rälsvägen, I could never have dreamt of a better childhood. Growing up with you as next-door neighbors was the ultimate “snorunge” experience.

My family

The Norwegian side**:**

All my cousins; **Emma, Ida-Marie, Mathilde, Marthe, Haakon, Robin** and **Kenneth.** My aunts with husbands: **Hege** and **Tron**, **Line** and **Roger**, **Monica** and **Jan-Åge**. For all your warm humor, kindness and for all those wonderful summers spent in beautiful Sandefjord during my childhood. My uncle **Kjell-Vidar**, my great grandmother **Marie** and my grandmother **Berit**, for all the love and laughter you passed our way. I miss you. My grandfather **Kjell**, for the early jogs at Ula beach, for your wonderful sense of humor and your warm heart. I will never forget our very last conversation on the porch at Hystadveien, and I will never break the promise I made that day.

The Swedish side:

“Moster” Marianne Schück, for being a great inspiration to all of us in that you are always so exceptionally optimistic in addition to being a walking encyclopedia. **Ann** and **Göran Insulander**, for all superb dinners at Grottestigen and wonderful crayfish fishing in Boda. My cousins **Carl** and **Henric Insulander**, I have followed your journeys from tiny, bald babies in Mickey Mouse kick suites to 2 meter back-slicked grown-ups in Eton shirts. Thank you for spreading laughter and warmth with your exceptional humorous manners, you will do great in life no matter what quest you’ll embark on.

My family in law; **Lars** and **Ann-Mari**, **Cecilia**, **Ulf** and **Agnes**, **Ulla** and **Göte**, **Signe**, for your tremendously gracious and considerate attitude, at all times genuinely relaxed and reliable people displaying so much warmth and heart. Thank you all for welcoming me so nicely into the Helmersson family, I feel just like home with you guys (except for the dialect perhaps).

Ingrid and **Lennart**, for all your consideration and love that you send our way, everyday. Thank you farfar for learning me so much about science, biology, nature and history as a young boy. If I’d become half the doctor and a third of the gentleman you were, I’d be happy. Thank you farmor for your everlasting generosity, for your witty attitude and for learning me and my sister how to behave properly (God knows what would have happened if you had left the noble art of infant upbringing to my irrational parents). You have the biggest heart.

Min fenomenale far **Claes**, för att du är den bästa av pappor och medicinska förebilder i en och samma person. Ditt stöd under den här tiden har betytt så otroligt mycket för mig. Tack för allt du lärt mig genom åren och fortfarande lär mig, såväl om medicin som om livet. Min underbara mor **Marianne**, för din oändliga omtänksamhet, all kärlek, värme och fantastisk humor du skänker mig och vår familj varje dag. Du får mig alltid att glömma det som man

tror betyder något och istället anamma det som faktiskt spelar roll här i livet. Min fantastiska lillasyster **Camilla**, som med sitt gudomligt sydländska temperament och sylvassa humor alltid lyckas få mig att skratta högre än någonsin annars. Tack för att du alltid står på min sida och är precis den du är; min älskade syster.

Sofia, du är det bästa som någonsin hänt mig! Det finns helt enkelt inte ord för att beskriva hur lycklig jag är som har dig i mitt liv. Tack för alla dagar du räddat och för att du fått mig att inse vad livet egentligen handlar om. Jag älskar dig så oändligt mycket.

REFERENCES

Agarwal SK, Schröck E, Kester MB, *et al*. Comparative genomic hybridization analysis of human parathyroid tumors. *Cancer Genet Cytogenet* **106**: 30-36, 1998.

Agarwal SK, Guru SC, Heppner C, *et al*. Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription. *Cell* **96**: 143-152, 1999.

Agarwal SK, Simonds WF and Marx SJ. The parafibromin tumor suppressor protein interacts with actin-binding proteins actinin-2 and actinin-3. *Mol Cancer* **7**: 65, 2008.

Agarwal SK, Mateo CM and Marx SJ. Rare germline mutations in cyclin-dependent kinase inhibitor genes in multiple endocrine neoplasia type 1 and related states. *J Clin Endocrinol Metab* **94**: 1826-1834, 2009.

Arnold A. The cyclin D1/PRAD1 oncogene in human neoplasia. *J Investig Med* **43**: 543-549, 1995.

Arnold A, Brown MF, Ureña P, *et al*. Monoclonality of parathyroid tumors in chronic renal failure and in primary parathyroid hyperplasia. *J Clin Invest* **95**: 2047-2053, 1995.

Bassett JH, Forbes SA, Pannett AA, *et al*. Characterization of mutations in patients with multiple endocrine neoplasia type 1. *Am J Hum Genet* **62**: 232-244, 1998.

Bell A and Rickinson AB. Epstein-Barr virus, the TCL-1 oncogene and Burkitt's lymphoma. *Trends Microbiol* **11**: 495-497, 2003.

Bjerneroth G, Juhlin C, Gudmundsson S, *et al*. Major histocompatibility complex class II expression and parathyroid autoantibodies in primary hyperparathyroidism. *Surgery* **124**: 503-509, 1998.

Björklund P, Åkerström G and Westin G. Accumulation of nonphosphorylated β-catenin and c-myc in primary and uremic secondary hyperparathyroid tumors. *J Clin Endocrinol Metab* **92**: 338-344, 2007a.

Björklund P, Åkerström G and Westin G. An LRP5 receptor with internal deletion in hyperparathyroid tumors with implications for deregulated WNT/beta-catenin signaling. *PLoS Med* **4**: e328, 2007b.

Bond GL, Hu W, Bond EE, *et al*. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* **119**: 591-602, 2004.

Bondeson L, Sandelin K and Grimelius L. Histopathological variables and DNA cytometry in parathyroid carcinoma. *Am J Surg Path* **17**: 820-829, 1993.

Bordonaro M, Lazarova DL and Sartorelli AC. Hyperinduction of Wnt activity: a new paradigm for the treatment of colorectal cancer? *Oncol Res* **17**: 1-9, 2008.

Brown EM, Gamba G, Riccardi D, *et al*. Cloning and characterization of an extracellular Ca(2+)-sensing receptor from bovine parathyroid. *Nature* **366**: 575-580, 1993.

Bhuiyan MM, Sato M, Murao K, *et al*. Expression of menin in parathyroid tumors. *J Clin Endocrinol* Metab **85**: 2615-2619, 2000.

Carling T, Kindmark A, Hellman P, *et al*. Vitamin D receptor genotypes in primary hyperparathyroidism. *Nat Med* **1**: 1309-1311, 1995.

Carling T, Correa P, Hessman O, *et al*. Parathyroid MEN1 gene mutations in relation to clinical characteristics of nonfamilial primary hyperparathyroidism. *J Clin Endocrinol Metab* **83**: 2960–2963, 1998.

Carling T, Szabo E, Bai M, *et al*. Familial hypercalcemia and hypercalciuria caused by a novel mutation in the cytoplasmic tail of the calcium receptor. *J Clin Endocrinol Metab* **85**: 2042-2047, 2000.

Carling T, Du Y, Fang W, *et al*. Intragenic allelic loss and promoter hypermethylation of the RIZ1 tumor suppressor gene in parathyroid tumors and pheochromocytomas. *Surgery* **134**: 932-939, 2003.

Carpten JD, Robbins CM, Villablanca A, *et al*. HRPT2, encoding parafibromin, is mutated in hyperparathyroidism-jaw tumor syndrome. *Nat Genet* **32**: 676-680, 2002.

Cerni C. Telomeres, telomerase, and myc. An update. *Mutat Res* **462**: 31-47, 2000.

Cetani F, Pardi E, Viacava P, *et al*. A reappraisal of the Rb1 gene abnormalities in the diagnosis of parathyroid cancer. *Clin Endocrinol (Oxf)* **60**: 99-106, 2004a.

Cetani F, Pardi E, Borsari S, *et al*. Genetic analyses of the HRPT2 gene in primary hyperparathyroidism: germline and somatic mutations in familial and sporadic parathyroid tumors. *J Clin Endocrinol Metab* **89**: 5583-5591, 2004b.

Cetani F, Ambrogini E, Viacava P, *et al*. Should parafibromin staining replace HRTP2 gene analysis as an additional tool for histologic diagnosis of parathyroid carcinoma? *Eur J Endocrinol* **156**: 547-554, 2007.

Cetani F, Pardi E, Banti C, *et al*. β-catenin activation is not involved in sporadic parathyroid carcinomas and adenomas. *Endocr Relat Cancer* Sep 15 [Epub ahead of print], 2009.

Chadwick RB, Jiang GL, Bennington GA, *et al*. Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. *Proc Natl Acad Sci USA* **97**: 2662-2667, 2000.

Chandrasekharappa SC, Guru SC, Manickam P, *et al*. Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* **276**: 404-407, 1997.

Chaudhary K, Deb S, Moniaux N, *et al*. Human RNA polymerase II-associated factor complex: dysregulation in cancer. *Oncogene* **26**: 7499-7507, 2007.

Chen G, A J, Wang M, *et al*. Menin promotes the Wnt signaling pathway in pancreatic endocrine cells. *Mol Cancer Res* **6**: 1894-1907, 2008.

Corbetta S, Vicentini L, Ferrero S, *et al*. Activity and function of the nuclear factor kappaB pathway in human parathyroid tumors. *Endocr Relat Cancer* **12**: 929-937, 2005.

Costa-Guda J and Arnold A. Absence of stabilizing mutations of beta-catenin encoded by CTNNB1 exon 3 in a large series of sporadic parathyroid adenomas. *J Clin Endocrinol Metab* **92**: 1564-1566, 2007.

Costa-Guda J, Tokura T, Roth SI, *et al*. Mitochondrial DNA mutations in oxyphilic and chief cell parathyroid adenomas. *BMC Endocr Disord* **7**: 8, 2007.

Cryns VL, Rubio MP, Thor AD, *et al*. p53 abnormalities in human parathyroid carcinoma. *J Clin Endocrinol Metab* **78**:1320-1324, 1994a.

Cryns VL, Thor A, Xu HJ, *et al*. Loss of the retinoblastoma tumor-suppressor gene in parathyroid carcinoma. *N Engl J Med* **330**: 757-761, 1994b.

DeLellis RA, Lloyd RV, Heitz PU and Eng C. Pathology and genetics of the tumours of endocrine organs. In: *WHO classification of tumours*. DeLellis RA, Lloyd RV, Heitz PU and Eng C (ed.) Lyon: IARC Press, pp. 124-127, 2006.

Drummond IA, Madden SL, Rohwer-Nutter P, *et al*. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. *Science* **257**: 674-678, 1992.

Egbuna OI and Brown EM. Hypercalcaemic and hypocalcaemic conditions due to calcium-sensing receptor mutations. *Best Pract Res Clin Rheumatol* **22**: 129-48, 2008.

Eng C, Clayton D, Schuffenecker I, *et al*. The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. *JAMA* **276**: 1575-1579, 1996.

Farnebo F, Enberg U, Grimelius L, *et al*. Tumor-specific decreased expression of calcium sensing receptor messenger ribonucleic acid in sporadic primary hyperparathyroidism. *J Clin Endocrinol Metab* **82**: 3481-3486, 1997a.

Farnebo F, Teh BT, Dotzenrath C, *et al*. Differential loss of heterozygosity in familial, sporadic, and uremic hyperparathyroidism. *Hum Genet* **99**: 342-349, 1997b.

Farnebo F, Teh BT, Kytölä S, *et al*. Alterations of the MEN1 gene in sporadic parathyroid tumours. *J Clin Endocrinol Metab* **83**: 2627–2630, 1998a.

Farnebo F, Höög A, Sandelin K, *et al*. Decreased expression of calcium-sensing receptor messenger ribonucleic acids in parathyroid adenomas. *Surgery* **124**: 1094-1098, 1998b.

Farnebo F, Auer G, Farnebo L-O, *et al*. Evaluation of retinoblastoma and Ki-67 as diagnostic markers in benign and malignant parathyroid disease. *World J Surg* **23**: 68-74, 1999.

Fernandez-Ranvier GG, Khanafshar E, Tacha D, *et al*. Defining a molecular phenotype for benign and malignant parathyroid tumors. *Cancer* **115**: 334-344, 2009.

Forsberg L, Björck E, Hashemi J, *et al*. Distinction in gene expression profiles demonstrated in parathyroid adenomas by high-density oligoarray technology. *Eur J Endocrinol* **152**: 459-470, 2005.

Fraser WD. Hyperparathyroidism. *Lancet* **374**:145-158, 2009.

Gasparre G, Porcelli AM, Bonora E, *et al*. Disruptive mitochondrial DNA mutations in complex I subunits are markers of oncocytic phenotype in thyroid tumors. *Proc Natl Acad Sci USA* **104**: 9001-9006, 2007.

Gialmanidis IP, Bravou V, Amanetopoulou SG, *et al*. Overexpression of hedgehog pathway molecules and FOXM1 in non-small cell lung carcinomas. *Lung Cancer* **66**: 64-74, 2009.

Gill AJ, Clarkson A, Gimm O, *et al*. Loss of nuclear expression of parafibromin distinguishes parathyroid carcinomas and hyperparathyroidism-jaw tumor (HPT-JT) syndrome-related adenomas from sporadic parathyroid adenomas and hyperplasias. *Am J Surg Pathol* **30**: 1140-1149, 2006.

Gujral TS, van Veelen W, Richardson DS, *et al*. A novel RET kinase-beta-catenin signaling pathway contributes to tumorigenesis in thyroid carcinoma. *Cancer Res* **68**: 1338-1346, 2008.

Guru SC, Goldsmith PK, Burns AL, et al. Menin, the product of the MEN1 gene, is a nuclear protein. *Proc Natl Acad Sci USA* **95**:1630-1634, 1998.

Hahn MA and Marsh DJ. Identification of a functional bipartite nuclear localization signal in the tumor suppressor parafibromin. *Oncogene* **24**: 6241-6248, 2005.

Hanahan D and Weinberg RA. The hallmarks of cancer. *Cell.* **100**: 57-70, 2000.

Hashimoto M, Kyo S, Hua X, et al. Role of menin in the regulation of telomerase activity in normal and cancer cells. Int J Oncol **33**: 333-340, 2008.

Hanson CA and Miller JR: Non-traditional roles for the adenomatous polyposis coli (APC) tumor suppressor protein. *Gene* **361**: 1-12, 2005.

Haven CJ, Howell VM, Eilers PH, *et al*. Gene expression of parathyroid tumors: molecular subclassification and identification of the potential malignant phenotype. *Cancer Res* **64**:7405-7411, 2004.

Haven CJ, van Puijenbroek M, Tan MH, et al. Identification of MEN1 and HRPT2 somatic mutations in paraffin-embedded (sporadic) parathyroid carcinomas. *Clin Endocrinol* (Oxf) **67**: 370-376, 2007.

Hayward NK. Genetics of melanoma predisposition. *Oncogene* **22**: 3053-3062, 2003.

Heppner C, Kester MB, Agarwal SK, *et al*. Somatic mutation of the MEN1 gene in parathyroid tumours. *Nat Genet* **16**: 375–378, 1997.

Hewitt KM, Sharma PK, Samowitz W, *et al*. Aberrant methylation of the HRPT2 gene in parathyroid carcinoma. *Ann Otol Rhinol Laryngol* **116**: 928-933, 2007.

Hollstein M, Sidransky D, Vogelstein B, et al. p53 mutations in human cancers. *Science* **253**: 49-53, 1991.

Howell VM, Haven CJ, Kahnoski K, *et al*. HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours. *J Med Genet* **40**: 657–663, 2003.

Hsi ED, Zukerberg LR, Yang WI, *et al*. Cyclin D1/PRAD1 expression in parathyroid adenomas: an immunohistochemical study. *J Clin Endocrinol Metab* **81**: 1736-1739, 1996.

Huang SM, Duh QY, Shaver J, *et al*. Familial hyperparathyroidism without multiple endocrine neoplasia. *World J Surg* **21**: 22-28, 1997.

Igreja S, Chahal HS, Akker SA, et al. Assessment of p27 (cyclin-dependent kinase inhibitor 1B) and aryl hydrocarbon receptor-interacting protein (AIP) genes in multiple endocrine neoplasia (MEN1) syndrome patients without any detectable MEN1 gene mutations. *Clin Endocrinol (Oxf)* **70**: 259-264, 2009.

Ikeda S, Ishizaki Y, Shimizu Y, *et al*. Immunohistochemistry of cyclin D1 and beta-catenin, and mutational analysis of exon 3 of beta-catenin gene in parathyroid adenomas. *Int J Oncol* **20**: 463-466, 2002.

Jagadeesh S and Banerjee PP. Telomerase reverse transcriptase regulates the expression of a key cell cycle regulator, cyclin D1. *Biochem Biophys Res Commun* **347**:774-780, 2006.

Johnson RL, Rothman AL, Xie J, *et al*. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272**: 1668-1671, 1996.

Juhlin C, Johansson H, Holmdahl R, *et al*. Monoclonal anti-parathyroid antibodies interfering with a Ca2+-sensor of human parathyroid cells. *Biochem Biophys Res Commun* **143**: 570-574, 1987.

Juhlin C, Rastad J, Klareskog L, *et al*. Parathyroid histology and cytology with monoclonal antibodies recognizing a calcium sensor of parathyroid cells. *Am J Pathol* **135**: 321-328, 1989.

Kaji H, Canaff L, Goltzman D, *et al*. Cell cycle regulation of menin expression. *Cancer Res* **59**: 5097-5101, 1999.

Kamb A. Cell-cycle regulators and cancer. *Trends Genet.* **11**: 136-140, 1995.

Kassem M, Kruse TA, Wong FK, *et al*. Familial isolated hyperparathyroidism as a variant of multiple endocrine neoplasia type 1 in a large Danish pedigree. *J Clin Endocrinol Metab* **85**: 165-167, 2000.

Kayath MJ, Martin LC, Vieira JG, *et al*. A comparative study of p53 immunoexpression in parathyroid hyperplasias secondary to uremia, primary hyperplasias, adenomas and carcinomas. *Eur J Endocrinol* **139**: 78-83, 1998.

Kim JK and Diehl JA. Nuclear cyclin D1: an oncogenic driver in human cancer. *J Cell Physiol* **220**:292-296, 2009.

Knudson A. Retinoblastoma: teacher of cancer biology and medicine. *PLoS Med* **2:**e349, 2005.

Knutson A, Hellman P, Akerström G, *et al*. Characterization of the human Megalin/LRP-2 promoter in vitro and in primary parathyroid cells. *DNA Cell Biol* **17**: 551-560, 1998.

Kytölä S, Farnebo F, Obara T, *et al*. Patterns of chromosomal imbalances in parathyroid carcinomas. *Am J Pathol* **157**: 579-586, 2000.

La Perle KM, Jhiang SM and Capen CC. Loss of p53 promotes anaplasia and local invasion in ret/PTC1-induced thyroid carcinomas. *Am J Pathol* **157**: 671-677, 2000.

Larsson C, Skogseid B, Oberg K, *et al*. Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma. *Nature* **332**: 85-87, 1988.

Lemmens I, Van de Ven WJ, Kas K, *et al*. Identification of the multiple endocrine neoplasia type 1 (MEN1) gene. The European Consortium on MEN1. *Hum Mol Genet* **6**:1177-1183, 1997.

Lin L, Czapiga M, Nini L, *et al*. Nuclear localization of the parafibromin tumor suppressor protein implicated in the hyperparathyroidism-jaw tumor syndrome enhances its proapoptotic function. *Mol Cancer Res* **5**: 183-193, 2007.

Lin L, Zhang JH, Panicker LM, *et al*. The parafibromin tumor suppressor protein inhibits cell proliferation by repression of the c-myc proto-oncogene. *Proc Natl Acad Sci USA* **105**: 17420-17425, 2008.

Lloyd RV, Carney JA, Ferreiro JA, *et al*. Immunohistochemical analysis of the cell cycle-associated antigens Ki-67 and retinoblastoma protein in parathyroid carcinomas and adenomas. *Endocr Pathol* **6**: 279-287, 1995.

Lodish MB and Stratakis CA. RET oncogene in MEN2, MEN2B, MTC and other forms of thyroid cancer. *Expert Rev Anticancer Ther* **8**: 625-632, 2008.

Lum L and Beachy PA. The Hedgehog response network: sensors, switches, and routers. *Science* **304**:1755-1759, 2004.

McCarthy RA, Barth JL, Chintalapudi MR, *et al*. Megalin functions as an endocytic sonic hedgehog receptor. *J Biol Chem* **277**: 25660-25667, 2002.

Malkin D, Li FP, Strong LC, *et al*. Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* **250**:1233-1238, 1990.

Marx SJ, Stock JL, Attie MF, *et al*. Familial hypocalciuric hypercalcemia: recognition among patients referred after unsuccessful parathyroid exploration. *Ann Intern Med* **92**: 351-6, 1980.

Marx SJ, Simonds WF, Agarwal SK, *et al*. Hyperparathyroidism in hereditary syndromes: special expressions and special managements. *J Bone Miner Res* **17**: 37-43, 2002.

MacDonald BT, Tamai K and He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* **17**: 9-26, 2009.

Miedlich S, Krohn K, Lamesch P, *et al*. Frequency of somatic MEN1 gene mutations in monoclonal parathyroid tumours of patients with primary hyperparathyroidism. *Eur J Endocrinol* **143**:47-54, 2000.

Miyoshi Y, Iwao K, Nagasawa Y, *et al.* Activation of the beta-catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res* **58**: 2524-7, 1998.

Mosimann C, Hausmann G and Basler K. Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. *Cell* **125**: 327-341, 2006.

Mosimann C, Hausmann G and Basler K. The role of Parafibromin/Hyrax as a nuclear Gli/Ci-interacting protein in Hedgehog target gene control. *Mech Dev* **126**: 394-405, 2009.

Müller-Tidow C, Metzelder SK, Buerger H, *et al*. Expression of the $p14^{ARF}$ tumor suppressor predicts survival in acute myeloid leukemia. *Leukemia* **18**:720-726, 2004.

Murakami H, Furihata M, Ohtsuki Y, *et al.* Determination of the prognostic significance of cyclin B1 overexpression in patients with esophageal squamous cell carcinoma. *Virchows Arch* **434**: 153-158, 1999.

Naccarato AG, Marcocci C, Miccoli P, *et al*. Bcl-2, p53 and MIB-1 expression in normal and neoplastic parathyroid tissues. *J Endocrinol Invest* **21**: 136-141, 1998.

Nemeth EF and Scarpa A. Cytosolic Ca2+ and the regulation of secretion in parathyroid cells. *FEBS Lett* **203**: 15-19, 1986.

Nesbit CE, Tersak JM and Prochownik EV. MYC oncogenes and human neoplastic disease. *Oncogene* **18**: 3004-16, 1999.

Nilsson IL, Zedenius J, Yin L, *et al*. The association between primary hyperparathyroidism and malignancy: nationwide cohort analysis on cancer incidence after parathyroidectomy. *Endocr Relat Cancer* **14**:135-140, 2007.

Okawa ER, Gotoh T, Manne J, *et al*. Expression and sequence analysis of candidates for the 1p36.31 tumor suppressor gene deleted in neuroblastomas. *Oncogene* **27**: 803-810, 2008.

Owens M, Stals K, Ellard S, *et al*. Germline mutations in the CDKN1B gene encoding p27 Kip1 are a rare cause of multiple endocrine neoplasia type 1. *Clin Endocrinol (Oxf)* **70**: 499-500, 2009.

Palanisamy N, Imanishi Y, Rao PH, *et al*. Novel chromosomal abnormalities identified by comparative genomic hybridization in parathyroid adenomas. *J Clin Endocrinol Metab* **83**: 1766-1770, 1998.

Palmer M, Adami HO, Krusemo UB *et al*. Increased risk of malignant diseases after surgery for primary hyperparathyroidism. A nationwide cohort study. Am J Epidemiol **127**: 1031–1040, 1988.

Pausova Z, Soliman E, Amizuka N, *et al*. Role of the RET proto-oncogene in sporadic hyperparathyroidism and in hyperparathyroidism of multiple endocrine neoplasia type 2. *J Clin Endocrinol Metab* **81**: 2711-2718, 1996.

Pearce SH, Trump D, Wooding C, *et al*. Calcium-sensing receptor mutations in familial benign hypercalcemia and neonatal hyperparathyroidism. *J Clin Invest* **96**: 2683-2692, 1995.

Pellegata NS, Quintanilla-Martinez L, Siggelkow H, *et al*. Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia syndrome in rats and humans. *Proc Natl Acad Sci USA* **103**: 15558-15563, 2006.

Poetsch M, Dittberner T and Woenckhaus C. Frameshift mutations of RIZ, but no point mutations in RIZ1 exons in malignant melanomas with deletions in 1p36. *Oncogene* **21**: 3038-3042, 2002.

Polakis P. The many ways of Wnt in cancer. *Curr Opin Genet Dev* **17**: 45-51, 2007.

Potocnik U, Glavac MR, Golouh R, *et al*. The role of P-glycoprotein (MDR1) polymorphisms and mutations in colorectal cancer. *Pflugers Arch* **442**: R182-183, 2001.

Qian J, Sarnaik AA, Bonney TM, *et al*. The APC tumor suppressor inhibits DNA replication by directly binding to DNA via its carboxyl terminus. *Gastroenterology* **135**: 152-162, 2008.

Rodgers SE and Perrier ND: Parathyroid carcinoma. *Curr Opin Oncol* **18**: 16-22, 2006.

Rozenblatt-Rosen O, Hughes CM, Nannepaga SJ, *et al*. The parafibromin tumor suppressor protein is part of a human Paf1 complex. *Mol Cell Biol* **25**: 612-620, 2005.

Sakai Y, Koizumi K, Sugitani I, *et al*. Familial adenomatous polyposis associated with multiple endocrine neoplasia type 1-related tumors and thyroid carcinoma: a case report with clinicopathologic and molecular analyses. *Am J Surg Pathol* **26**: 103-110, 2002.

Sandelin K, Auer G, Bondeson L, *et al*. Prognostic factors in parathyroid cancer: a review of 95 cases. *World J Surg* **16**: 724-731, 1992.

Satyanarayana A and Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. *Oncogene* **28**: 2925-2939, 2009.

Segouffin-Cariou C and Billaud M. Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. *J Biol Chem* **275**: 3568-3576, 2000.

Shane E: Clinical review 122: parathyroid carcinoma. *J Clin Endocrinol Metab* **86**: 485-493, 2001.

Shen M, Feng Y, Gao C, *et al*. Detection of cyclin b1 expression in g(1)-phase cancer cell lines and cancer tissues by postsorting Western blot analysis. *Cancer Res* **64**:1607-1610, 2004.

Sherr CJ and Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* **13**: 1501-1512, 1999.

Shattuck TM, Välimäki S, Obara T, *et al*. Somatic and germ-line mutations of the HRPT2 gene in sporadic parathyroid carcinoma. *N Engl J Med* **349**: 1722-1729, 2003a.

Shattuck TM, Kim TS, Costa J, *et al*. Mutational analyses of RB and BRCA2 as candidate tumour suppressor genes in parathyroid carcinoma. *Clin Endocrinol (Oxf)* **59**: 180-189, 2003b.

Shi SR, Cote RJ and Taylor CR. Antigen retrieval techniques: current perspectives. *J Histochem Cytochem* **49**: 931-937, 2001.

Shi G, Perle MA, Mittal K, *et al*. Let-7 repression leads to HMGA2 overexpression in uterine leiomyosarcoma. *J Cell Mol Med* Oct 13 [Epub ahead of print], 2008.

Simonds WF, Robbins CM, Agarwal SK, *et al*. Familial isolated hyperparathyroidism is rarely caused by germline mutation in HRPT2, the gene for the hyperparathyroidism-jaw tumor syndrome. *J Clin Endocrinol Metab* **89**: 96-102, 2004.

Slamon DJ, Clark GM, Wong SG, *et al*. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science **235**: 177-182, 1987.

Soria JC, Jang SJ, Khuri FR, *et al*. Overexpression of cyclin B1 in early-stage non-small cell lung cancer and its clinical implication. *Cancer Res* **60**: 4000-4004, 2000.
Stojadinovic A, Hoos A, Nissan A, *et al*. Parathyroid neoplasms: clinical, histopathological, and tissue microarray-based molecular analysis. *Hum Pathol* **34**: 54-64, 2003.

Subramaniam P, Wilkinson S and Shepherd JJ. Inactivation of retinoblastoma gene in malignant parathyroid growths: a candidate genetic trigger? *Aust N Z J Surg* **65**: 714-716, 1995.

Tahara H, Smith AP, Gas RD, *et al*. Genomic localization of novel candidate tumor suppressor gene loci in human parathyroid adenomas. *Cancer Res* **56:** 599-605, 1996a.

Tahara H, Smith AP, Gaz RD, *et al*. Loss of chromosome arm 9p DNA and analysis of the p16 and p15 cyclin-dependent kinase inhibitor genes in human parathyroid adenomas. *J Clin Endocrinol Metab* **81**: 3663-3667, 1996b.

Tahara H, Smith AP, Gaz RD, *et al*. Parathyroid tumor suppressor on 1p: analysis of the p18 cyclin-dependent kinase inhibitor gene as a candidate. *J Bone Miner Res* **12**: 1330-1334, 1997.

Takeuchi H, Greep NC, Hoon DS, *et al*. Hypermethylation of adenosine triphosphate-binding cassette transporter genes in primary hyperparathyroidism and its effect on sestamibi imaging. *J Clin Endocrinol Metab* **92**: 1785-90, 2007.

Tan MH, Morrison C, Wang P, *et al*. Loss of parafibromin immunoreactivity is a distinguishing feature of parathyroid carcinoma. *Clin Cancer Res* **10**: 6629-6637, 2004.

Teh BT, Farnebo F, Twigg S, *et al*. Familial isolated hyperparathyroidism maps to the hyperparathyroidism-jaw tumor locus in 1q21-q32 in a subset of families. *J Clin Endocrinol Metab* **83**: 2114-2120, 1998.

Thomas WD, Chen J, Gao YR, *et al*. Patched1 deletion increases N-Myc protein stability as a mechanism of medulloblastoma initiation and progression. *Oncogene* **28**: 1605-1615, 2009.

Thompson DB, Samowitz WS, Odelberg S, *et al*. Genetic abnormalities in sporadic parathyroid adenomas: loss of heterozygosity for chromosome 3q markers flanking the calcium receptor locus. *J Clin Endocrinol Metab* **80**: 3377-3380, 1995.

Tominaga Y, Tsuzuki T, Matsuoka S, *et al*. Expression of parafibromin in distant metastatic parathyroid tumors in patients with advanced secondary hyperparathyroidism due to chronic kidney disease. *World J Surg* **32**: 815-821, 2008.

Trump D, Farren B, Wooding C, *et al*. Clinical studies of multiple endocrine neoplasia type 1 (MEN1). *QJM* **89**: 653-669, 1996.

Uchino S, Noguchi S, Sato M, *et al*. Screening of the Men1 gene and discovery of germ-line and somatic mutations in apparently sporadic parathyroid tumors. Cancer Res 60:5553-5557, 2000.

Vasef MA, Brynes RK, Sturm M, *et al*. Expression of cyclin D1 in parathyroid carcinomas, adenomas, and hyperplasias: a paraffin immunohistochemical study. *Mod Pathol* **12**:412-416, 1999.

Villablanca A, Farnebo F, Teh BT, *et al*. Genetic and clinical characterization of sporadic cystic parathyroid tumours. *Clin Endocrinol (Oxf)* **56**: 261-269, 2002.

Villablanca A, Calender A, Forsberg L, *et al*. Germline and de novo mutations in the HRPT2 tumour suppressor gene in familial isolated hyperparathyroidism (FIHP). *J Med Genet* **41**: e32, 2004.

Volpato CB, Martínez-Alfaro M, Corvi R, *et al*. Enhanced sensitivity of the RET proto-oncogene to ionizing radiation in vitro. *Cancer Res* **68**: 8986-8992, 2008.

Walker MD and Silverberg SJ. Cardiovascular aspects of primary hyperparathyroidism. *J Endocrinol Invest* **31**: 925-931, 2008.

Wang P, Bowl MR, Bender S, *et al*. Parafibromin, a component of the human PAF complex, regulates growth factors and is required for embryonic development and survival in adult mice. *Mol Cell Biol* **28**: 2930-2940, 2008.

Wang Q, Williamson M, Bott S, *et al*. Hypomethylation of WNT5A, CRIP1 and S100P in prostate cancer. *Oncogene* **26**:6560-6565, 2007.

Wang W, Johansson H, Kvasnicka T, *et al*. Detection of apoptotic cells and expression of Ki-67 antigen, Bcl-2, p53 oncoproteins in human parathyroid adenoma. *APMIS* **104**: 789-796, 1996.

Warner JV, Nyholt DR, Busfield F, *et al*. Familial isolated hyperparathyroidism is linked to a 1.7 Mb region on chromosome 2p13.3-14. *J Med Genet* **43**: e12, 2006.

Watanabe S, Fukumoto S, Chang H, *et al*. Association between activating mutations of calcium-sensing receptor and Bartter's syndrome. *Lancet* **360**: 692-694, 2002.

Weitzman JB, Fiette L, Matsuo K, *et al*. JunD protects cells from p53-dependent senescence and apoptosis. *Mol Cell* **6**: 1109-1119, 2000.

Wijn MA, Keller JJ, Giardiello FM, *et al*. Oral and maxillofacial manifestations of familial adenomatous polyposis. *Oral Dis* **13**: 360-365, 2007.

Willeke F, Hauer MP, Buchcik R, *et al*. Multiple endocrine neoplasia type 2-associated RET proto-oncogene mutations do not contribute to the pathogenesis of sporadic parathyroid tumors. *Surgery* **124**: 484-490, 1998.

Woodard GE, Lin L, Zhang JH, *et al*. Parafibromin, product of the hyperparathyroidism-jaw tumor syndrome gene HRPT2, regulates cyclin D1/PRAD1 expression. *Oncogene* **24**: 1272-1276, 2005.

Xie J, Murone M, Luoh SM, *et al*. Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391**: 90-92, 1998.

Yang AS, Estécio MR, Doshi K, *et al*. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* **32**: e38, 2004.

Yart A, Gstaiger M, Wirbelauer C, *et al*. The HRPT2 tumor suppressor gene product parafibromin associates with human PAF1 and RNA polymerase II. *Mol Cell Biol* **25**: 5052-5060, 2005.

Zardawi SJ, O'Toole SA, Sutherland RL, *et al*. Dysregulation of Hedgehog, Wnt and Notch signalling pathways in breast cancer. *Histol Histopathol* **24**: 385-398, 2009.

Zeller C, Hinzmann B, Seitz S, *et al*. SASH1: a candidate tumor suppressor gene on chromosome 6q24.3 is downregulated in breast cancer. *Oncogene* **22**: 2972-2983, 2003.

Zhang C, Kong D, Tan MH, *et al*. Parafibromin inhibits cancer cell growth and causes G1 phase arrest. *Biochem Biophys Res Commun* **350**: 17-24, 2006.

Åkerström G, Malmaeus J and Bergström R. Surgical anatomy of human parathyroid glands. *Surgery* **95**: 14-21, 1984.