Genetic and Epigenetic Mechanisms in Primary Hyperparathyroidism

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Stockholm 2013
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ISBN 978-91-7549-009-0
When you only live for yourself, you are dead for the others!

Luqman

To my family
Thesis defense

Radiumhemmet Lecture Hall, Floor 01

Karolinska University Hospital, Solna

Friday, 11th of January 2013 at 09:00

Scan me for the location!
Primary hyperparathyroidism (PHPT) is a common endocrine disorder characterized by abnormally excessive secretion of parathyroid hormone (PTH) and elevated serum calcium. PHPT patients can develop a wide range of complications affecting many body organs such as the skeleton, kidneys and heart. In the majority of patients, PHPT is due to a solitary adenoma and less frequently due to multiglandular disease. Very rarely PHPT is caused by a parathyroid carcinoma.

This thesis aimed at a better understanding of the genetic as well as epigenetic mechanisms involved in this disease in order to improve future patients management.

In **study I** we have investigated large parathyroid adenomas (≥ 4 grams) and detected frequent MEN1, but rare HRPT2/CDC73 mutations and low MIB1 proliferation index. The majority of the tumors had loss of parafibromin expression and positive APC expression. Furthermore, gain of chromosome 5 was the most unique and frequent copy number alteration detected in this group, while very rarely detected in unselected adenomas. We concluded that a subset of large parathyroid adenomas have distinct genetic profile and pronounced clinical features reflected by significantly higher serum calcium.

In **study II** we examined the role of constitutional APC mutations in parathyroid tumors from two patients with APC mutation-associated familial colorectal cancers. Pathological revision confirmed the benign nature of both tumors. None of them had somatic mutations or DNA copy number alterations of the APC gene and both tumors displayed strong APC and parafibromin expression with low MIB index. Although the APC 1A promoter was hypermethylated, promoter APC 1B was unmethylated and this was consistent with normal APC mRNA expression. Our results supported the benign nature of the parathyroid tumors and excluded a possible association between constitutional APC mutations and parathyroid tumorigenesis.

In **study III** we defined the molecular cytogenetic profile of CDC73/HRPT2-mutated parathyroid tumors. All the carcinomas displayed frequent DNA copy number losses on chromosome 1p and 13 while the adenomas did not display any significant alterations. All the carcinomas were diploid at the CDC73 gene locus, but three adenomas had loss at this locus. The CDC73 promoter was unmethylated in all the tumors. The carcinomas displayed more loss of heterozygosity (LOH) events than the adenomas, and two carcinomas had LOH at the CDC73 locus. These results suggest that CDC73-mutated parathyroid adenomas exhibit a partly unique cytogenetic profile in addition to that of carcinomas and unselected adenomas.

In **study IV** analyses of promoter methylation status in a panel of benign and malignant parathyroid tumors revealed frequent hypermethylation of APC 1A, β-catenin and RASSF1A promoters in the adenomas which correlated with reduced mRNA expression. Parathyroid carcinomas were hypermethylated for APC 1A and exclusively for SFRP1. No changes in global methylation could be detected and tumor groups with known MEN1 or CDC73 mutations did not display different methylation profile. We concluded that aberrant promoter methylation of APC 1A, β-catenin, RASSF1A and SFRP1 can play a role in parathyroid tumorigenesis and hypermethylation of SFRP1 can act as a potential epigenetic marker for parathyroid carcinomas.
LIST OF PUBLICATIONS


* Both authors contributed equally
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<tr>
<td>CASR</td>
<td>Calcium sensing receptor</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
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<tr>
<td>CDC73</td>
<td>Cell division cycle 73</td>
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<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
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<tr>
<td>CGIs</td>
<td>CpG islands</td>
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<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
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<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatosis polyposis</td>
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<tr>
<td>FHH</td>
<td>Familial hypocalciuric hypercalcemia</td>
</tr>
<tr>
<td>FIHP</td>
<td>Familial isolated hyperparathyroidism</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence <em>in-situ</em> hybridization</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HPT-JT</td>
<td>Hyperparathyroidism-jaw tumor syndrome</td>
</tr>
<tr>
<td>HRPT2</td>
<td>Hyperparathyroidism 2 gene</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LPTA</td>
<td>Large parathyroid adenoma</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia type 1</td>
</tr>
<tr>
<td>MEN2A</td>
<td>Multiple endocrine neoplasia type 2A</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>PHPT</td>
<td>Primary hyperparathyroidism</td>
</tr>
<tr>
<td>PRAD1</td>
<td>Parathyroid adenomatosis 1</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RASSF1A</td>
<td>Ras association domain family 1 isoform A gene</td>
</tr>
<tr>
<td>S-Ca$^{2+}$</td>
<td>Serum ionized calcium</td>
</tr>
<tr>
<td>SFRP1</td>
<td>Secreted frizzled related-protein 1 gene</td>
</tr>
<tr>
<td>SHPT</td>
<td>Secondary hyperparathyroidism</td>
</tr>
<tr>
<td>SNHPT</td>
<td>Severe neonatal hyperparathyroidism</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>THPT</td>
<td>Tertiary hyperparathyroidism</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumor suppressor gene</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
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INTRODUCTION

DISCOVERY OF PARATHYROID GLANDS

The parathyroid gland is the last major organ identified and described in the human body [1]. This could be attributed to the anatomical position as well as the small size of these glands. Parathyroid glands were first observed by Richard Owen in 1850, however, Owen did not make any description of the nature of these glands and did not even name them. In his published article in Transactions in 1852, Owen described the glands as “small compact yellow glandular body attached to the thyroid to the point where the vein merges” [2]. Following Owen, the surgeon Robert Remark and the pathologist Rudolf Virchow have observed the glands, but again they did not make any descriptions apart from naming the anatomical location. It was not until 1852 when the medical student Ivar Sandström (Figure 1) from Uppsala University in Sweden made the first detailed description of the parathyroid glands and named them as “Glandulae Parathyroideae” [3].

![Figure 1. Ivar Victor Sandström (1852-1889).](image)

Ivar described the origin of the parathyroid glands as undeveloped embryonic remnants from the thyroid glands. His discovery was later published in the local journal *Uppsala*
Läkareförenings Förhandlingar [4]. Being parathyroid glands the last organs discovered, the recognition of parathyroid disease came late in 1908 when WG MacCallum and Carl Voegtlin attributed symptoms previously linked to the thyroid gland to the pathology of parathyroid glands [5]. Thereafter, signs and symptoms of parathyroid diseases were gradually recognized.

EMBRYOLOGY AND ANATOMY OF THE PARATHYROID GLANDS

Parathyroid glands develop during the fourth week of gestation. They originate from the third and the fourth pharyngeal pouches of the endoderm. They are usually four in number, two superior and two inferior and are situated in the anterior aspect of the neck just behind the thyroid gland (Figure 2). Supernumerary glands are not uncommon and have been reported in about 13% of cases [6]. Parathyroid glands are oval shaped, light yellow to reddish brown in color and are small in size with an average weight of 40 to 60 mg [7, 8]. The inferior glands have a common origin with the thymus, as they are both derived from the third pharyngeal pouch. They start to migrate together with the thymus inferiorly and medially in the neck and get separated from the thymus just before the thymus localizes into the anterior mediastinum [9].

Figure 2. Locations of the parathyroid glands in the neck (posterior view). Modified from reference [9].
The superior glands have a shorter migration path in the neck. They arise from the fourth pharyngeal pouch and migrate with the thyroid. They localize to the posterior mid-portion of the thyroid lobe [10]. The blood supply for the superior and the inferior parathyroid glands primarily comes from the inferior thyroid artery [11].

Ectopic localization of the parathyroid glands and especially the inferior glands are not uncommon [12]. This variation in the anatomical location can be simply explained by their origin and migration path in the neck. They can be found anywhere between the angle of the mandible and the upper mediastinum. In about 5% of the ectopic cases, the inferior parathyroid glands are found in the anterior mediastinum [9]. As the superior glands have a much shorter migration path in the neck, they are very seldom found at ectopic sites in the neck.

**HISTOLOGY OF NORMAL PARATHYROID GLANDS**

Parathyroid gland tissue is surrounded by a thin connective tissue capsule and consists mainly of two cell types, chief and oxyphil cells [10]. Chief cells are small polygonal cells with a rounded centrally located nucleus and a weak acidophilic cytoplasm and they are the predominant cell type (Figure 3A). In contrast, oxyphil cells are much less abundant, rounded, and larger in size and have more intense acidophilic cytoplasm.

While the main function of chief cells is to secrete parathyroid hormone (PTH), the function of the oxyphil cells is still unknown. However, oxyphil cells are proposed to secrete PTH in chronic kidney diseases (CKDs) [13]. Chief cells stain positive for PTH, glycogen, cytokeratin and chromogranin A [14, 15]. In addition to chief and oxyphil cells, the parathyroid gland also contains adipocytes. While the number of chief cells decreases by age, the number of oxyphil cells and adipocytes increases in number during adulthood and adipocytes may constitute up to 50% of the cells in older people.
FUNCTIONS OF THE PARATHYROID GLANDS

The endocrine function of the parathyroid glands is primarily calcium homeostasis (regulation of extracellular calcium level) through the secretion of PTH. The extracellular calcium is regarded as the main determinant of PTH secretion and parathyroid glands are very sensitive to even slight variations in baseline ionized serum calcium (S-Ca\(^{2+}\)) levels. PTH secretion has an inverse sigmoidal relationship with S-Ca\(^{2+}\) level and the midpoint between minimal and maximal PTH secretion is the set point of calcium which changes in parathyroid diseases (Figure 4) [16]. Similar to other endocrine organs, parathyroid glands lack ducts and they release PTH directly into the blood stream. PTH regulates S-Ca\(^{2+}\) mainly through its action on three major organs, namely the kidneys, skeleton and the small intestine (Figure 5). Any drops in the normal level of S-Ca\(^{2+}\) will be sensed by the calcium sensing receptor (CASR) expressed on parathyroid cell surface [17]. This will lead to downstream signaling and activation of the parathyroid glands to secrete or increase PTH secretion into the blood stream. PTH acts via the kidneys by enhancing calcium resorption from the distal tubules and reducing phosphate absorption from the proximal tubules [18].
In addition, PTH enhances the conversion of the inactive form of vitamin D (25-hydroxyvitamin D) to the active metabolite, 1, 25-dihydroxyvitamin D-3 via the activation of 1α-hydroxylase enzyme in the proximal tubules of the kidney. The active form of vitamin D in turn acts on the small intestine to increase intestinal absorption of calcium. The effect of PTH on skeleton is mainly through the induction of bone resorption which leads to increased release of calcium into the bloodstream and causes immediate elevation of the extracellular calcium. The net effect of secreted PTH on kidneys, bones and the small intestine is increase in the S-Ca\(^{2+}\) level which will be perceived by the parathyroid glands through the CASR as a negative feedback signal to reduce PTH secretion to a level keeping S-Ca\(^{2+}\) within the normal physiological level.

**Figure 4.** Illustration of the sigmoidal relationship between PTH release and level of S-Ca\(^{2+}\).
Figure 5. Calcium homeostasis. Regulation of extracellular calcium by PTH.
DISEASES OF THE PARATHYROID GLANDS – HYPERPARATHYROIDISM

Hyperparathyroidism is a disorder of increased parathyroid gland activity characterized by abnormally increased secretion of PTH and elevation of S-Ca\(^{2+}\). The PTH-calcium sigmoidal curve is shifted to the right and the calcium set point is increased (Figure 4). Based on the underlying pathology of hyperparathyroidism the parathyroid hyperactive state is classified into three different types: primary, secondary and tertiary hyperparathyroidism [19].

**Primary hyperparathyroidism (PHPT)**

PHPT is defined as excessive production of PTH by the parathyroid glands due to disorders arising from within the glands themselves and causing abnormally high S-Ca\(^{2+}\) [19]. The most common cause of PHPT is a single parathyroid adenoma which is detected in about 85% of the cases. Less commonly (~10-15%), PHPT is due to multiglandular disease. Fortunately, PHPT is rarely (< 1%) due to an underlying parathyroid carcinoma [20]. PHPT is the third most common endocrine disorder following diabetes and thyroid diseases [21]. Based on published data, there is a wide variation in the prevalence of PHPT. This variation can reflects the population studied as well as S-Ca\(^{2+}\) cut offs used for disease recognition. While the most recent Swedish study estimated a prevalence of 3.4% in postmenopausal women [22], other studies estimated a prevalence of 0.3% in the general population [23, 24]. The exact etiology of PHPT is still unknown; however a number of risk factors are identified such as female gender, age, family history and history of radiation exposure. The prevalence increases with age and postmenopausal women are specifically more prone to develop PHPT [22, 25].

**Clinical features and diagnosis of PHPT**

The clinical presentation of PHPT has changed significantly over the last 20-25 years. This is attributed to the introduction of automated calcium measurement which has contributed dramatically to the easy and early detection of the disease. Symptomatic patients usually represent a minority (~ 20%) of PHPT patients. They usually present with clinical features secondary to abnormally high S-Ca\(^{2+}\) such as weakness, lethargy and signs and symptoms of renal diseases, most commonly renal stones [26, 27].
The skeleton is an important target for PTH and skeletal complications such as bone pain and pathological fractures due to loss of bone mass can also be present [28, 29]. Other skeletal complications such as osteitis fibrosa cystica are very rarely detected nowadays as most patients are diagnosed early in the course of the disease. Psychological manifestations such as depression and dementia are also common in PHPT patients [30]. Disturbances in the cardiovascular system such as hypertension and arrhythmias have been described more commonly in severe forms of PHPT [31, 32]. However, the majority of PHPT patients are asymptomatic and diagnosed incidentally based on high S-Ca\(^{2+}\) [33, 34].

The diagnosis of PHPT starts with the clinical suspicion in any patients with elevated S-Ca\(^{2+}\) (Normal ionized S-Ca\(^{2+}\) range 1.15-1.33 mmol/L). The combination of high S-Ca\(^{2+}\) and high or upper normal limit serum PTH (normal PTH range 10-65 ng/L) should always indicate hyperparathyroidism until proved otherwise [35]. Except for hyperparathyroidism caused by lithium and thiazide treatment, all other causes of hypercalcemia are associated with suppressed PTH. Other laboratory findings include reduced or lower limit serum phosphate and increased or upper limit 1,25 dihydroxy vitamin D. Concurrent vitamin D deficiency may also be detected and can exacerbate the hyperparathyroid status [36, 37]. Imaging studies such as ultrasound and Technitium99 sestamibi scanning are used to localize the enlarged gland prior to the operation [38]. Other imaging techniques such as CT-scan and MRI are also used, but less frequently.

Biochemical investigations such as measurement of serum vitamin D, phosphate and creatinine as well as 24-hour urinary calcium should be part of the initial evaluation of PHPT patients. In addition, as PTH has significant anabolic effect on the skeleton, measurement of bone mass (bone densitometry) using dual energy X-ray absorptiometry (DXA) has become an important and integral part in the management of hyperparathyroidism patients and is recommended for all PHPT patients.

**Surgical treatment**

Surgical removal of the pathologically enlarged parathyroid gland/glands (parathyroidectomy) is the only curative treatment available so far. The decision for parathyroidectomy in symptomatic PHPT patients with no concurrent contraindications
for anesthesia is usually straightforward; however, since the majority of PHPT patients are asymptomatic, controversy exist regarding parathyroidectomy. A practical clinical guideline was established to help in selecting patients for surgical treatment [39]. Based on this guideline, patients with the following criteria are recommended for parathyroidectomy: (1) elevated S-Ca\(^{2+}\) 1 mg above the upper normal limit; (2) markedly reduced cortical bone density (T score < -2.5); or (3) age < 50 years. Patients having any of these criteria should be entitled for surgery. The details of the surgical approach depend on the underlying pathology. The half life of PTH is short about 3-5 minutes and intraoperative PTH measurement within 5-10 minutes following gland removal has proven to be of clinical use [40, 41]. Several studies have suggested a reduction of 50% of intraoperative PTH from baseline measurements as an indication for a successful operation [42, 43].

About 30% of asymptomatic patients will have disease progression on follow-up [44]. Bone mass density is not stable over time and monitored patients may experience more bone mass loss over time. Parathyroidectomized patients have increased bone mass density due to reduced bone turnover [39]. Evidences from postoperative patients indicate increased bone mass and reduced fractures [45, 46], reduced incidence of renal stones in those with a history of nephrolithiasis and improved neuromuscular symptoms. Furthermore, advances in surgical approaches and techniques combined with reduced postoperative complications all support the clinical benefits of parathyroid surgery over medical and/or monitoring strategies in asymptomatic patients.

Non-surgical treatment

Parathyroidectomy is not always possible or indicated in patients with PHPT. When patients do not meet the criteria for surgical removal or have concurrent co-morbid conditions, or refuse surgery due to personal decisions, they are monitored regularly by annual measurements of S-Ca\(^{2+}\), serum creatinine and bone density and they may be offered some sort of medical treatment [39].

Vitamin D supplementation is required for all the patients with a goal of a serum level of 20 ng/dl of 25-hydroxyvitamine D and it has been shown to reduce PTH level without significant increase in S-Ca\(^{2+}\) level [47]. Dietary calcium restriction is not recommended as this may further elevate the serum PTH level [48]. However,
excessive intake of calcium should also be avoided. In general, it is advisable to keep the dietary calcium intake within the range of 1000-1500 mg/day. In addition to these measures, some patients may be offered medical treatment with one or more of the following agents: estrogen, selective estrogen receptor blockers, calcimimetic cinacalcet and bisphosphonates. However, none of the currently available medical treatments are regarded as an advisable alternative to surgery. Furthermore, all the available medical therapies need further extended studies to make a final recommendation of any of them.

Long term follow up of non-surgically treated patients have showed that about 20-30% of them will eventually have disease progressions either as elevated S-Ca\(^{2+}\) or urinary calcium and/or reduced bone mass density [46]. Therefore, these patients will have one or more of the criteria required for parathyroidectomy and may need to proceed to surgery. This finding re-enforces the need for regular long term follow-up of PHPT patients treated conservatively.

**Secondary hyperparathyroidism (SHPT)**

Chronic and persistent stimulation of the parathyroid glands by concurrent decreased level of the active form of vitamin D and of S-Ca\(^{2+}\) along with elevated serum phosphate leads to hyperplasia of the parathyroid glands and abnormally elevated PTH level, a condition called Secondary hyperparathyroidism (SHPT) [49]. The overactivation of the parathyroid glands in SHPT represents a compensatory mechanism toward any external factor causing deregulation of calcium homeostasis.

The most common cause of SHPT is CKDs where three well recognized biochemical changes drive the hyperactivation of the parathyroid glands in these patients and further exacerbated by reduced sensitivity of both CASR and vitamin D receptor (VDR) to PTH. These changes are represented by declining level of 1,24 dihydroxy vitamin D which acts as the earliest stimulus for PTH secretion, followed by hyperphosphatemia and hypocalcemia. The sequences of the main biochemical changes are believed to correspond to the drop in the glomerular filtration rate (GFR) and therefore decline in renal function [49-51].
Patients with SHPT usually suffer from higher morbidity and mortality as compared to PHPT patients and this is attributed to the underlying pathology which in most cases is a long standing CKD. Therefore signs and symptoms of SHPT patients are those of the CKD. These patients usually have higher levels of serum PTH, increased resistance to PTH and more severe biochemical disturbances accompanied by severe skeletal and sometimes cardiovascular complications. It is very frequent to find some degree of SHPT in patients undergoing renal replacement therapy with uncontrolled hyperphosphatemia and/or vitamin D replacement [52]. A high incidence of SHPT ranging from 30 to 57% among patients with low vitamin D has been reported [53, 54].

Treatment of SHPT is primarily aimed at preventing the deterioration of the condition by correcting the biochemical changes and treating the underlying pathology such as CKD. Replacement of vitamin D, correction of calcium and phosphate imbalances by controlling dietary intake and dialysis as well as treatment with calcimimetics are important cornerstones in the prevention of irreversible, tertiary hyperparathyroidism. Today, surgical removal of parathyroid glands is reserved for severe cases of SHPT patients in which medical interventions fail or when hyperparathyroidism persist after renal transplantation.

**Tertiary hyperparathyroidism (THPT)**

Patients with long standing SHPT can go through a state of progressive hyperactive parathyroid gland associated with elevated serum PTH and S-Ca\(^{2+}\), even when the underlying pathology was treated. This progressive state of hyperactivity is called tertiary hyperparathyroidism (THPT) [52]. This is a very infrequent condition and the frequency is even further reduced with improved therapeutic approaches of SHPT.

**Familial hyperparathyroidism**

Although in the majority of PHPT patients the disease is sporadic, in about 5%, PHPT is part of familial syndromes such as multiple endocrine neoplasia type 1 (MEN1) and type 2A (MEN2A), hyperparathyroidism-jaw tumor (HPT-JT) syndrome, familial isolated hyperparathyroidism (FIHP), familial hypocalciuric hypercalcemia (FHH) and severe neonatal hyperparathyroidism (SNHPT). In the following section, these familial conditions will be discussed individually.
Multiple endocrine neoplasia type 1 (MEN1)

MEN1 is a heritable autosomal dominant syndrome characterized by tumors involving the parathyroid glands, the pituitary and endocrine pancreas (MEN1; OMIM # 131100) [55]. It is a rare syndrome with a prevalence of 2-3/100,000. However, it is the most common cause of familial PHPT and constitutes about 2% of all the causes [56]. The penetrance for parathyroid adenoma is very high in this group of patients and may reach up to 90% by the age of 50 years. The syndrome has a similar sex distribution and usually occurs between the second and the fourth decades of life. In contrast to sporadic adenomas, parathyroid disease in patients with MEN1 syndrome is usually multiglandular (multiple adenomas or four-gland hyperplasia). These patients are also treated surgically; however tumors are more likely to recur following removal [57, 58].

The etiology behind this syndrome is a constitutional mutation of the tumor suppressor gene (TSG) MEN1 on chromosome 11q13 (discussed later) [59]. While germline mutations inactivate one allele of MEN1 gene, the other allele is usually inactivated by the second sporadic hit. Mutations in MEN1 gene can be identified in 70-95% of MEN1 patients [60]. In a small percentage of MEN1 patients no mutations can be detected in MEN1 gene and this could be due to mutations in the non-coding regions or mutations in other related genes which inactivate the menin protein (encoded by MEN1 gene) as a result.

Multiple endocrine neoplasia type 2A (MEN2A)

PHPT develops in about 20-30% of patient with type 2A of the autosomal dominant multiple endocrine syndrome (MEN2A; OMIM #171400). Patients with this syndrome carry germline mutations of the RET proto-oncogene [61] and develop tumors in multiple endocrine glands such as medullary thyroid carcinoma (MTC), pheochromocytoma, and parathyroid adenomas [62]. Hyperparathyroidism in MEN2A is usually mild, clinically resembles sporadic cases of PHPT and is almost always caused by underlying benign parathyroid tumors [63].
**Hyperparathyroidism-jaw tumor (HPT-JT) syndrome**

HPT-JT is an autosomal dominant syndrome characterized by tumors of parathyroid glands, jaw bones, uterus as well as various kidney lesions (HPT-JT; OMIM 607393). Most of PHT-JT patients are presenting with features of PHPT and in about 10-15% of the cases, it is due to a parathyroid carcinoma [64-66]. This syndrome is caused by constitutional mutation of *CDC73/HRPT2* gene (discussed later) [66] which is identified in about 50-75% of the patients. Numerous mutations, most of which are expected to cause inactivation of the gene, have been identified scattered through the *CDC73* gene, but no hotspot has been reported thus far [67]. In contrast to MEN1, somatic mutations in *CDC73* are uncommon in parathyroid adenomas while they are frequently detected in parathyroid carcinomas [66-69].

**Familial hypocalciuric hypercalcemia (FHH)**

FHH is an autosomal dominant condition (FHH; OMIM #145980) characterized by slightly elevated or upper normal serum PTH, elevated S-Ca\(^{2+}\) and reduced excretion of urinary calcium. Patients with FHH carry a heterozygous germline mutation in the *CASR* gene rendering the receptor much less sensitive to changes in the extracellular calcium [70, 71]. Two other loci on chromosome 19p and 19q have been reported to be linked to other families with FHH [72, 73].

**Severe neonatal hyperparathyroidism (SNHPT)**

This is a very rare variant of FHH due to homozygous mutations in *CASR* and results in a fatal condition in the newborn [74].

**Familial isolated hyperparathyroidism (FIHP)**

FIHP is a clinical entity identified when there is hyperparathyroidism without any specific features of MEN1, HPT-JT or FHH syndromes (OMIM #146200) [75]. In a subset of families the patients exhibit mutations of *MEN1, CDC73* or *CASR* [76]. However, in most families no causative mutations have been identified. One study has suggested an additional locus on chromosome 2 as potentially involved in the pathology of FIHP [77].
DISEASES OF THE PARATHYROID GLANDS – HYPOPARATHYROIDISM

In contrast to hyperparathyroidism, the condition hypoparathyroidism refers to reduced glandular activity with abnormally reduced PTH secretion leading to hypocalcemia. Hypoparathyroidism is most commonly encountered after neck surgery due to inadvertent damage to the parathyroid glands or removal of all parathyroid glands with failure of re-implantation. In the majority of the cases, hypoparathyroidism is sporadic, however, it also occurs as a part of complex autoimmune diseases or as an isolated entity such as the autosomal dominant familial isolated hypoparathyroidism [78, 79]. The clinical signs and symptoms of hypoparathyroidism are predominantly related to hypocalcemia such as muscle spasms, numbness and paresthesias. In severe cases, the patient may experience life threatening complications such as seizures, tetany and laryngeal spasm [80]. The diagnosis is mainly based on the detection of concurrently low PTH and low S-Ca$^{2+}$. The standard treatment of hypoparathyroidism is correction of hypocalcemia and the accompanying reduced vitamin D by the supplementation of oral calcium and vitamin D [81].

TUMORS OF THE PARATHYROID GLANDS

Tumors of the parathyroid glands can be benign or malignant and are broadly classified as adenomas, carcinomas and atypical adenomas.

Parathyroid adenoma

Parathyroid adenoma is the most common type of parathyroid tumors and responsible for about 85% of PHPT cases [20]. In sporadic cases, one single parathyroid gland is usually enlarged and has hyperactive state causing excessive secretion of PTH. Double adenomas are also reported, but they are rare [82]. Parathyroid adenomas are more frequently detected in postmenopausal women [20]. The median glandular weight reported is 650 mg [83]; however, parathyroid adenomas as large as 110 grams have been reported [84]. Typical parathyroid adenomas appear as reddish brown in color with soft consistency encapsulated in a thin fibrous layer (Figure 6). In about 50-60% of the cases there is also a yellowish brown rim of glandular tissue most often found close to the vascular hilus of the gland and it represents the remnant of the normal parathyroid gland commonly referred to as “normal rim” [20].
The majority of parathyroid adenomas are composed of chief cells, with a smaller proportion consisting mainly of oxyphil cells [85]. Microscopically, the cells usually have mild degree of nuclear polymorphism with reduced intracytoplasmic fat contents as well as an overall reduction in the number of adipocytes. Identification of an intact thin fibrous capsule surrounding the parenchyma cells and the presence of a “normal rim” can be of great diagnostic value in differentiating adenomas from malignant parathyroid tumors and hyperplasia, respectively (Figure 3B) [20].

Figure 6. Macroscopic appearance of a parathyroid adenoma.

Parathyroid carcinoma

Carcinoma of the parathyroid gland is a rare malignancy and it accounts for less than 1% of PHPT causes [86, 87]. Parathyroid carcinoma has no gender preferences and it is usually diagnosed at younger ages as compared to adenomas. However, it can occur at any age. The clinical presentation of patients with parathyroid carcinoma is usually very aggressive and characterized by a palpable neck mass, hoarseness of voice, very high S-Ca^{2+} (3.5 mmol/L) and serum PTH (usually 4 fold normal). Patients may also have skeletal, kidney and other body organs complications. However, none of the clinical features are exclusive and parathyroid adenomas could have any of these features. Certain pathological features such as large tumor size, marked nuclear atypia, trabecular growth pattern and thick fibrous bands are more frequently seen in
parathyroid carcinomas (Figure 3C), but again these features can be also seen in atypical adenomas (discussed below) which further complicate the diagnosis. However, only capsular penetration and invasion of the surrounding tissue, local recurrence and distant metastasis can set the final diagnosis. Unfortunately, these diagnostic criteria can only be detected in advanced stages of the disease [88]. The difficulty in correct diagnosis and therefore difficulty in selecting best treatment modality and follow-up protocol urged the need for molecular markers which can identify carcinomas in pre or intraoperative time. Studies have identified a number of potential markers each with different sensitivity and specificity for the detection of carcinoma cases. Loss of parafibromin expression, the protein product of \textit{HRPT2} gene, is a well studied marker which is integrated into the diagnostic work up whenever parathyroid carcinoma is suspected [89-91]. Along with parafibromin, the proliferation marker Ki67 is also used as additional diagnostic aid as parathyroid carcinomas usually have a higher proliferation index than adenomas [92].

Atypical parathyroid adenoma

Atypical adenomas refer to a group of parathyroid tumors with histopathological features that overlap with parathyroid carcinomas [93, 94]. The differentiation between parathyroid carcinoma and atypical parathyroid adenoma is a diagnostic challenge and yet very important since parathyroid carcinoma requires a more extensive surgery and a closer follow-up [93]. The diagnosis of atypical adenoma is based on the identification of any two of the following histological features: incomplete capsular invasion, fibrous bands, pronounced trabecular growth pattern, mitotic activity more than one mitosis per 10 high-power fields and tumor necrosis (Figure 3D) [94].

Atypical parathyroid adenomas were previously referred to as equivocal carcinomas and several studies raised the question whether these atypical cases carry any malignant potential [20]. These uncertainties necessitate close follow-up of patients diagnosed with atypical adenomas in order to avoid any possible misdiagnosed parathyroid carcinoma [61].
MOLECULAR GENETIC BACKGROUND OF PARATHYROID TUMORS

Clonal development is a prominent feature of cancer as it provides selective growth advantage to the tumor cell. The debate on whether parathyroid tumors are monoclonal or polyclonal in growth lasted for a long time until a number of studies showed experimentally the monoclonal nature of parathyroid tumors using chromosome X inactivation [95, 96].

Many studies investigated the molecular genetic mechanisms involved in parathyroid tumors development and they had considerable contribution to the current insight into this disease (Table 1). Studies of familial cases of PHPT provided valuable information on the genetic of sporadic cases of parathyroid tumors and have led to the identification of two important TSGs which are:

Multiple endocrine neoplasia type 1 (MEN1) gene

The tumor suppressor MEN1 is a located in chromosomal region 11q13. MEN1 has 10 exons and it is about 9.8 kb in size and encodes for a 610 amino-acid nuclear protein called menin (Figure 7). The MEN1 gene was first mapped to 11q13 in family studies [59]. This gene was subsequently identified as the underlying etiology in MEN1 patients and MEN1 mutations were detected in the majority of the cases [97, 98]. This has motivated the search for MEN1 mutations in sporadic cases of PHPT which identified mutations in about 25% of the cases [99, 100]. A more recent study using whole exome sequencing has identified MEN1 mutation in 6/16 (35%) of sporadic parathyroid adenomas [101]. Mutational analyses have revealed more than 400 different MEN1 mutations in parathyroid tumors, most commonly of the type that inactivates the protein such as truncating deletions or insertions and/or nonsense type which inactivate the gene [102]. Constitutional MEN1 mutations have been also reported in clinically apparent sporadic cases of parathyroid adenomas [103, 104]. Furthermore, loss of heterozygosity (LOH) at the MEN1 gene locus is frequently detected in parathyroid adenomas in about 30% of cases, half of which have MEN1 mutations [100, 105]. In contrast to adenomas, MEN1 mutations are rare in parathyroid carcinomas and it has only been reported in four cases so far [106-109].

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Table 1. Summary of known genetic background of parathyroid tumors. *Adopted from reference [110].*

<table>
<thead>
<tr>
<th>Genes</th>
<th>protein encoded</th>
<th>Associated hyperparathyroid syndrome: main syndromic manifestations</th>
<th>Features of syndromic parathyroid tumors</th>
<th>Defect in sporadic parathyroid tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>MEN1</em></td>
<td>Menin</td>
<td>Multiple endocrine neoplasia type 1: anterior pituitary, parathyroid, enteropancreatic, foregut carcinoid tumors</td>
<td>Multiple, assymetric tumors typical (&gt;99% benign)</td>
<td>Inactivation in about 35% of benign tumors; mutations exceedingly rare in cancer</td>
</tr>
<tr>
<td><em>HRPT2/CDC73</em></td>
<td>Parafibromin</td>
<td>Hyperparathyroidism-jaw tumor syndrome: fibro-osseous jaw. Parathyroid, uterine tumors; renal cysts</td>
<td>Single tumor common (about 15% malignant)</td>
<td>Inactivation in about 70% of cancers; mutations are rare in sporadic adenomas</td>
</tr>
<tr>
<td><em>CASR</em></td>
<td>Calcium sensing receptor</td>
<td>Familial hypocalcic hypercalcemia (FHH) with heterozygous inactivation; neonatal severe Hyperparathyroidism (NSHPT) with homozygous inactivation</td>
<td>FHH: near-normal size and surgical pathology; altered serum calcium set point for PTH release NSHPT: Marked enlargement of multiple glands</td>
<td>Decreased expression common; mutation exceedingly rare</td>
</tr>
<tr>
<td><em>RET</em></td>
<td>c-Ret</td>
<td>Multiple endocrine neoplasia type 2A: medullary thyroid cancer, pheochromocytoma, parathyroid tumors</td>
<td>Single tumor common (&gt;99% benign)</td>
<td>Mutation exceedingly rare</td>
</tr>
<tr>
<td><em>CCND1/PRAD1</em></td>
<td>Cyclin D1</td>
<td>NA</td>
<td>NA</td>
<td>Overexpression results from DNA rearrangement involving PTH gene</td>
</tr>
</tbody>
</table>
The nuclear protein menin is involved in key cellular functions such as growth, proliferation and cell cycle control as well as DNA repair [111]. Menin acts as a transcription repressor through the interaction with Jun D, a member of the transcription factor family AP-1/fos Jun and also through recruitment of histone deacetylase complex [112-115]. Menin has histone methyltransferase activity which is involved in controlling cell growth through upregulation of the cyclic dependant kinase inhibitors p16 and p18 [116]. In addition, menin promotes gene transcription via the interaction with an important member of the TGF-beta family, Smad3. While in parathyroid glands, TGF-b inhibits cellular proliferation and PTH production, inactivation of menin will remove this inhibition and will therefore enhances cellular proliferation and increases PTH secretion [117, 118].

![Diagram of MEN1 gene and its product, menin with proposed functions.](image)

**Figure 7.** Diagrammatic representation of *MEN1* gene and its product, menin with proposed functions.

**Hyperparathyroidism 2 (HRPT2)/CDC73 gene**

The *CDC73* gene is a known TSG located on chromosome 1q32 and has 17 coding exons. Similar to *MEN1*, mutations of the *CDC73* gene were first detected in familial forms of PHPT, namely the HPT-JT syndrome where the majority of patients were found to harbor an inactivating *CDC73* mutation [66]. As parathyroid tumors within
HPT-JT syndrome carry a high risk of malignancy, *CDC73* mutations were also expected to have a role in parathyroid carcinomas. This speculation was then supported by studies showing *CDC73* mutations in the majority of sporadic parathyroid carcinomas [119]. The reported frequencies of somatic *CDC73* mutations detected in sporadic parathyroid carcinomas vary between 67% up to 100% [68, 69, 119]. In contrast to carcinomas, *CDC73* mutation is rare (~4%) in sporadic parathyroid adenoma [68, 120, 121]. Constitutional mutations of *CDC73* have been also detected in clinically apparent sporadic cases of parathyroid carcinomas [122, 123].

![Diagrammatic representation of HRPT2/CDC73 gene and its product, parafibromin with proposed functions. Modified from reference [91].](image)

The *CDC73* gene encodes a 531 amino-acid tumor suppressor protein called parafibromin. Parafibromin has multiple important cellular functions (Figure 8). It is part of the of RNA polymerase II-regulatory Paf1 complex which is involved in gene transcription mediated by histone methylation in the promoter and coding regions of specific genes [124]. It also regulates the cell cycle via the regulation of cyclin D1 expression [125]. Parafibromin is a ubiquitously expressed protein and it is mainly located in the nucleus where it acts as a transcription factor and regulates transcription of certain genes such as *cMYC* [126]. It can be also found in the cytoplasm where it is involved in the organization of the cytoskeleton through binding to actin binding proteins actinin-2 and actinin-3 [127]. Studies have shown that over-expression of parafibromin by transfected cells can induce apoptosis [128] while parafibromin null mice do not survive and die *in utero* [129].
Other genetic alterations reported in parathyroid tumors

One of the earliest genetic abnormalities identified in parathyroid adenomas is the chromosomal rearrangement of *cyclin D1/PRAD1* (parathyroid adenomatosis 1) proto-oncogene [130-132]. This rearrangement is a pericentromeric inversion on chromosome 11 that positions the 5' PTH regulatory region, originally located on 11p, to the upstream of the *cyclin D1* gene located on 11q, leading to over-expression of *cyclin D1* (Figure 9). Although this genetic alteration has been reported infrequently, overexpression of *cyclin D1* is more commonly observed in about 20-40% of sporadic parathyroid adenomas and even more frequently in parathyroid carcinomas [133-135]. *Cyclin D1* is a proto-oncogene that plays a major role in regulation of the cell cycle and over-expression of this gene leads to proliferation and cell growth [136]. However, no *cyclin D1* mutations have been reported in parathyroid tumors [137].

Mutations of *RET* (REarranged during Transfection) oncogene is detected in patients with MEN2A. Although no *RET* mutations have been reported in sporadic parathyroid tumors, over-expression of this gene has been demonstrated in sporadic cases [138].

Reduced expression of *CASR* has been repeatedly reported in parathyroid tumors. However, only very few studies have reported mutations in this gene [75, 139-141]. In a recent retrospective study, reduced *CASR* expression was associated with significantly poorer prognosis in parathyroid carcinoma patients [142].

A number of potential candidate genes known to be commonly mutated in other tumor types such as *RAS, RB, TP53* have also been investigated in parathyroid tumors and apart from only one study which reported *TP53* mutation in a single anaplastic parathyroid carcinoma, no other mutations have been reported [131, 143-147].

In addition to mutations, other genetic aberrations such as recurrent DNA copy number alterations (CNAs) and LOH have been also reported in parathyroid tumors. Examples of the most commonly detected recurrent CNAs and LOH are losses in 1p, 3q, 6q, 9p, 11p, 11q, 13 and 15q and gains of 16p and 19p [148-154]. Furthermore, two different chromosomal translocations have been reported in parathyroid adenomas including a t(1;5)(p22;q32) [155] and a t(4;13)(q21;q14) [156]. However, the functional consequences of these translocations have not been identified yet.
Figure 9. Illustration of cyclin D1/PRAD1 chromosomal rearrangement. Modified from reference [137].

EPIGENETICS

Epigenetics is the study of heritable and potentially reversible changes in cellular information which are not due to alterations in DNA sequence [157]. The field of epigenetics has undergone a rapid and considerable development over the last 20 years and its role is clearly demonstrated in both normal and pathological conditions including human cancers [158]. Epigenetic mechanisms regulate gene function independent of the DNA sequence and can be regarded as the interface between genotype and phenotype. Consequently, epigenetics can explain the functional and morphologic differences between different cell types in any organism, although these cells all have identical genetic material.

Epigenetic mechanisms are involved in many important cellular processes such as embryonic development, cellular growth and differentiation, X chromosome
inactivation and genomic imprinting as well as protection against viral genome integration [159, 160]. Different epigenetic mechanisms exist, all of which are essential for regulation of gene function. These mechanisms include: DNA methylation, histone modifications and chromatid remodeling, and RNA-mediated gene silencing.

**DNA methylation**

DNA methylation is the most studied and the best characterized epigenetic mechanism. It involves covalent binding of a methyl group to the C-5 position of cytosine base of a cytosine-guanine (CpG) dinucleotide (Figure 10) [158].

![DNA Methylation Diagram](image)

**Figure 10.** DNA 5-methylcytosine methylation. *Modified from reference* [161].

DNA methylation usually occurs at clusters of CpG dinucleotides known as CpG islands (CGIs) [158]. They are defined as clusters of CpG dinucleotides stretching for about 200 to 500 bp with GC contents of over 50% and observed/expected GC contents of 60%. CGIs are localized at the promoters of more than half of the human genes and they are usually unmethylated under normal conditions [162, 163]. The co-localization of the CGIs with gene promoters provide a more chromatin permissive state [164] and can act as a distinguishing mark for transcriptional start sites [165]. While CGI promoter hypermethylation leads to transcriptional repression and gene silencing [166], hypomethylation of CGIs is associated with reactivation of repressed genes [167]. However, the later mechanism is less well characterized. Many TSGs involved in different cellular processes such as DNA repair, cell cycle, apoptosis and angiogenesis are frequently silenced by aberrant DNA promoter methylation in various human
cancers and at different stages of tumor development [168]. There are many examples of TSGs that are inactivated by promoter methylation, for e.g. RB, VHL, hMHL1, BRCA1 and p16INK4a [169-172]. Aberrant CGI methylation is recognized as a hallmark of cancer [159]. In addition to gene-specific DNA methylation changes, alterations in DNA methylation can also occur at genomic regions other than gene promoters and cause alterations in global methylation status [164]. Global hypomethylation occurs mainly at repetitive sequences such as long interspersed nuclear elements-1 family member L1 (LINE-1), inducing chromosomal instability, translocations, gene disruption and reactivation of endoparasitic sequences [164]. Hypomethylation of LINE-1 has been reported in many human cancers such as cancers of breast, lung, bladder and liver [165]. More recent genome-wide methylation analyses in colon cancer have revealed frequent hypermethylation of CpGs at genomic regions of less dense GC contents located at about 0.5-2 kb upstream to the promoter and have been termed “CpG shores” [173]. This study found that most tissue-specific DNA methylation occurs at CGI shores, rather than on the islands themselves.

DNA methylation induced gene transcriptional repression occurs through a number of proposed mechanisms. Two different, but biologically relevant DNA methylation mechanisms are described (Figure 11). First methylation of CGIs at gene promoters can act as a physical hindrance and block the access of transcription factors to the transcription starting site and prevent the initiation of transcription [174]. A second proposed mechanism suggests that DNA methylation will recruit histone modifying and chromatin-remodeling complexes to methylated sites which act as repressor proteins and therefore trigger gene silencing [175, 176]. Among these repressor proteins are four members of the methyl binding proteins (MBD1, MBD2, MBD3 and MeCP2), which act as methylation-dependant transcription repressors [161].

Methylation of CpGs is mediated by members of the DNA methyltransferase (DNMTs) family of enzymes which catalyze the transfer of a methyl group from S-adenosyl methionine to DNA. DNMTs are mainly classified into de novo and maintenance DNMTs [177]. De novo DNMTs include DNMT3A and DNMT3B which are highly expressed in embryonic stem cells and downregulated in differentiated cells and are responsible for establishing the pattern of methylation during embryonic development [175]. DNMT1 acts as a maintenance DNMT and is responsible for maintaining the methylated state during cell division [178].
**Figure 11.** Mechanisms of gene silencing by DNA methylation. (A) Transcriptionally active, unmethylated gene promoter. (B) Silenced gene due to promoter hypermethylation. (C) Silenced gene due to methylation-recruited transcription repressors. *Modified from reference* [179].

**Histone modifications and chromatid remodeling**

This describes posttranscriptional modifications of N-terminal of histone tail protruding from the nucleosomes. Various histone modifications are identified and include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation and ADP ribosylation which will modify the chromatin state to be active or repressive (according to the type of the modification) [180]. Studies have shown that histone modifications are correlated and occur in connection to each other, a process that has later been termed as the “histone code” [181]. Although histone modifications are not as well studied as DNA methylation, the interest to investigate this modification is growing. Different patterns of histone modifications have been identified in a variety of human tumors and are found to be correlated with tumor stage and prognosis [182, 183].
RNA-mediated gene silencing

Posttranscriptional silencing by small non-coding RNAs has become increasingly recognized as an epigenetic mechanism of gene function control. Example of non-coding small RNAs are microRNA (miRNA) (Figure 12) which are about 20-22 nucleotides long usually located in the introns or exons of the protein coding genes in 70% of the cases [184]. They can also be found in the intergenic regions in about 30% of the cases [158, 160]. miRNA can directly interact with mRNA causing either its degradation or repression [185] and they have important functions such as regulation of cellular proliferation, differentiation, apoptosis, and development [186]. The number of known miRNAs has dramatically increased in the last 5-10 years. Interestingly, miRNAs can function as tumor suppressors or as oncogenes and many studies have shown that deregulation of miRNAs are involved in different pathological conditions including cancer [187]. This class of small non-coding RNAs is now being explored to have great cancer diagnostic and prognostic potentials as emerging evidence revealed that the pattern of miRNAs expression correlate well with clinicopathological characteristics and outcome of different cancer types [188].

![Figure 12](image.png)

**Figure 12.** MicroRNA and posttranscriptional regulation of gene expression via RISC (RNA-induced silencing complex). *Modified from reference* [189].
Epigenetic background of parathyroid tumors

Studies of epigenetic mechanisms have also been conducted in parathyroid tumors, however with a much lesser frequency than genetic studies. Among the earliest published report is a study which investigated tissue and gene-specific expression of PTH by parathyroid glands using a restriction enzyme-based method [190]. They reported hypomethylation of DNA sequences in proximity to the \( PTH \) gene while the same sequences were hypermethylated in control non-PTH expressing tissues. Although this study analyzed only normal parathyroid tissues and did not find any correlation with PTH secretion in the parathyroid glands themselves, it suggested a role of DNA methylation in association with \( PTH \) gene and tissue-specific expression. Few studies have specifically analyzed methylation of \( CDC73/HRPT2 \) and all [191, 192], but one [193], excluded hypermethylation as a silencing mechanism. Taken together, the overall data do not support regulation of \( HRPT2 \) by methylation. This will be further discussed in this thesis (study III and IV). We have analyzed parathyroid tumors for promoter methylation of several genes and we found frequent hypermethylation of \( APC \) and \( RASSF1A \) [194]. On a genome-wide scale, a recently published study identified a number of hypermethylated genes with possible involvement in parathyroid tumorigenesis [195]. Among the significantly hypermethylated genes were \( CDKN2B, CDKN2A, WT1, SFRP1, SFRP2, \) and \( SFRP4 \).

In addition to DNA methylation, genome-wide miRNA profiling of parathyroid tumors has revealed interesting patterns with potential capability of distinguishing carcinomas from benign parathyroid tumors [196, 197]. For example, one study found the expression of miR-296 and miR-222 was significantly different between carcinomas and adenomas. More interestingly, a recent study has identified a miRNA cluster called C19MC located on 19q13.4 which was significantly associated with parathyroid carcinoma and positively correlated with S-Ca\(^{2+}\), PTH and tumor weight [198]. Furthermore, this study also found C19MC promoter hypomethylation in 50% of the tumors which significantly associated with S-Ca\(^{2+}\) and metastatic disease, further supporting the role of epigenetic mechanisms in parathyroid tumor development. More studies are required to validate and identify potential genes that are regulated by methylation as well as to define the methylation profile of different parathyroid tumor types and subtypes.
AIMS OF THE STUDIES

The general aim of this thesis was to contribute for better understanding of the genetic as well as epigenetic alterations involved in parathyroid tumors development. More specifically, the studies have aimed at the following:

- Defining the genetic profile of large parathyroid adenomas (LPTAs) and determining whether LPTAs display malignant potential (study I).

- Identifying recurrent minimal regions of DNA copy number alterations which may harbor potential tumor suppressor genes and oncogenes (study I).

- Investigating a possible association between constitutional $APC$ mutations and parathyroid tumors (study II).

- Characterizing the genetic alterations in parathyroid tumors with established $CDC73$ mutations (study III).

- Understanding mechanisms of $CDC73$ gene inactivation in parathyroid tumors (study III).

- Studying the role of DNA promoter methylation in the regulation of genes potentially involved in parathyroid tumorigenesis and determining whether the mutational status of $MEN1$ or $CDC73$ gene can alter gene-specific as well as global methylation profile in parathyroid tumors (study IV).
METHODS

Mutational analysis by Sanger sequencing

DNA sequencing is the golden standard approach for mutation screening. In our studies (study I, II and III) we have used the dye-terminator Sanger sequencing method for mutational screening of *APC*, *MEN1* and *CDC73*. The principle of this method relies on the use of fluorescently labeled chain terminator dideoxynucleotides (ddNTP’s) (Figure 13) [199]. This ddNTP differs from normal deoxynucleotides as it has a hydrogen group at the 3’ carbon instead of a hydroxyl group. The advantage of this modification is that when ddNTP is incorporated into the synthesized DNA sequence it immediately terminates the reaction and blocks further addition of any ddNTPs. The protocol starts with amplification of the target sequence with specific primers using PCR. The PCR product is then purified to remove any unincorporated dNTPs using either chemical or enzymatic cleanup processing.

Figure 13. Principle of chain terminator DNA sequencing.
The sequencing reaction mixture contains: DNA polymerase, the four ddNTPs (G, A, T and C) labeled in one fluorescent dye color each, the single stranded template of the specifically amplified PCR product, a specific sequencing primers, unmodified dNTPs, and other buffers required for the sequencing reaction. The sequencing reaction is run in the sequencing machine and it is a semi-automated process. When incorporated by DNA polymerase each labeled ddNTP will emit light at different wavelengths that will be captured and reported as a colored peak in the chromatogram. The reaction continues until the specified region has been fully sequenced. The resulted chromatogram will be cross-referenced with a reference genome and any mismatch in the target sequence will be further analyzed for possible mutations.

**Array comparative genomic hybridization (a-CGH)**

Tumors are characterized by DNA copy number alterations (CNAs) that are the consequences of structural chromosomal alterations of various types and complexity such as amplifications, deletions, translocations or gain and loss of a chromosome [200]. Knowledge of CNAs can have immediate clinical use in diagnosis and in some cases provide useful prognostic information such as in assessing the prognosis in breast [201] and prostate cancers [202]. Furthermore, it can aid in therapeutic judgments such as in chronic lymphocytic leukemia treatment [203].

Different techniques exist for the study of CNAs, each with its own advantages and disadvantages. Examples of such techniques are fluorescence *in-situ* hybridization (FISH), karyotyping and CGH. In the recent years tremendous improvement in the resolution and throughput has occurred. While next generation sequencing currently provide the most comprehensive information of the whole genome, some of these techniques are still successfully used in both research and clinical practice such as FISH and microarray.

Array CGH (a-CGH) is a powerful and a precise tool developed to detect and quantify genomic aberrations and map them directly onto the sequences of the human genome. It is a tool for cancer gene discovery and understanding disease pathogenesis. The main advantage of a-CGH is that the entire genome can be scanned for CNAs in a single experiment [204, 205]. The primary goal is to identify regions of recurrent CNAs of losses and gains where TSGs and oncogenes can
reside, respectively. Different a-CGH platforms exist. The main differences lie in the type, size and spacing of the genomic sequence printed on the microarray slides. These features will determine the final resolution of the applied microarray platform. An early platform which we also used in our studies is BAC arrays. This type of microarray platform implement large inserts of DNA produced from bacterial artificial chromosome (BAC) inserts. The resolution varies based on the type of the BAC arrays and it has developed from 24K to 38K which is the latest for BAC tiling arrays. We have used human BAC 38K arrays (study I & III) generated at the SCIBLU Genomics Centre at Lund University, Sweden (www.lu.se/sciblu). These arrays contain about 38,000 BAC clones (CHORI BACPAC resources) (http://bacpac.chori.org/genomicRearray.php) arranged in a tiling fashion resulting in a final resolution of about 100-150 kb. This platform can provide sufficient signal intensity to quantitatively detect single copy number changes as well as homozygous deletions and high-level amplifications. However, one main disadvantage of a-CGH in general, is the inability to detect balanced translocations and other copy number neutral alterations.

Array CGH method basically relies on hybridization where DNA will only bind to complementary genomic sequences spotted on the array slides (Figure 14). Tumor and normal DNA are fluorescently labeled in different colors. Both tumor and normal DNA will compete for binding to the genomic sequences on the slide. When there are no CNAs in the tumor, i.e. diploid, theoretically the combined color will give a yellow fluorescent signal. When the tumor has a deletion or a gain, the fluorescent color will be either red or green based on the initial labeling. In a-CGH, genomic DNA is labeled in vitro by random priming to incorporate fluorescently labeled nucleotides, usually Cy3 (green) or Cy5 (red). The hybridized microarray slide will be scanned and the features will be extracted using special feature extractions software. The extracted raw data will be further analyzed after several steps of normalization and smoothing to remove unwanted background and avoiding false positive effects. The signal intensity log2 ratio will be calculated for each single feature on the array slide and the expected log2 ratio of a single copy gain in a diploid genome, when hybridized versus normal genomic DNA, would be + 0.58, and -1 for a single copy loss. In practice, log2 ratios can range from −4 for homozygous deletions to log2 ratio > 6 for high-level amplifications [206].
Single nucleotide polymorphism microarray (SNP array) for loss of heterozygocity (LOH) analysis

LOH is a common type of alteration and refers to a change from heterozygous to homozygous state in a paired tumor genome as compared to constitutional genome. LOH is a common mechanism of TSG inactivation and are frequently identified in human tumors (Figure 15) [207]. Several methods are available for identifying LOH, for example SNP-based analysis, microsatellite DNA analysis and SNP-based Pyrosequencing. SNPs are very common human genetic variations and are highly conserved during evolution making these polymorphic loci excellent markers for studying LOH. SNP-based microarrays provide a high throughput genome-wide technique for screening LOH events. This technique can also simultaneously provide genome-wide DNA copy number information in a single experiment. This method involves hybridization-based microarray analysis, where the chip contains highly condensed SNPs and is hybridized to fluorescently labeled samples. The resolution of SNP microarrays rely on the density of the SNPs printed on the microarray chip.
We have used the Affymetrix 250K genotyping chip (Affymetrix Inc., Santa Clara, CA, USA) in our study (study III). This platform has about 25,000 SNPs distributed randomly throughout the genome. Four rows of 25-mer oligonucleotides are used as a detector for each SNP loci in the chip. Two of them perfectly match SNP allele A or SNP allele B, while the other two contain single-base mismatch at various positions. SNP-based LOH analysis requires that tumor and normal samples being allelotyped on separate chips. However, with current SNP databases, comparison to non-paired online SNP databases generated from normal individuals is feasible. Following hybridization, the chips are scanned and the genotyping calls (LOH, retention of heterozygosity, uninformative or no call) are made using a special software which will calculate the ratio of allelic imbalance at each individual loci investigated.

![Figure 15. Principle of loss of heterozygosity (LOH). Redrawn from reference [208].](image)
Bisulfite Pyrosequencing

Different techniques are available for DNA methylation analysis. However, the majority of these methods lack enough sensitivity and have time consuming labor-intensive protocols. In addition, some of these methods such as restriction enzyme-based techniques require large amount of DNA for analysis and the sensitivity is limited to those sites recognized by the enzyme. Bisulfite (BS) Pyrosequencing was developed to circumvent those issues and has become the standard method for quantitative DNA methylation analysis. Pyrosequencing is principally a sequencing by synthesis method which provides a powerful analytic tool for accurate quantification of multiple successive CpG dinucleotides with high resolution and reproducibility. This method uses a simple and easy to follow protocol which requires a very small amount of DNA for analysis. It relies on BS modification which will convert unmethylated cytosine bases in CpG dinucleotides to uracil (U) and then to thymine (T) in the subsequent PCR amplification step, while methylated cytosine will remain methylated (Figure 16).

**Figure 16.** Bisulfite modification and subsequence processes in Pyrosequencing.  
*Redrawn from Pyrosequencing database, Qiagen.*
BS converted DNA is amplified with specific methylation primers designed to specifically amplify the target CpG sites. For Pyrosequencing reaction, a single stranded BS modified DNA will be hybridized to specific sequencing primer and run in the Pyrosequencing machine. The Pyrosequencing reaction itself consists of a cascade of very well synchronized enzymatic reactions including four enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase (Figure 17).

Upon the addition of the first complementary nucleotide by DNA polymerase, there will be release of a pyrophosphate (ppi) in a quantity equimolar to the amount of the incorporated nucleotide. The released ppi will be converted to ATP by the action of ATP sulfurylase. The enzyme luciferase catalyzes the conversion of luciferin to oxyluciferin which will generate light in an amount proportional to the amount of ATP generated. The light is captured by a charge coupled device (CCD) camera and presented as a peak in the resulting Pyrogram. The height of the peak is proportional to the number of nucleotides incorporated and will be recorded graphically as a Pyrogram. The role of the apyrase enzyme is to remove any unincorporated nucleotides from the reaction mixture. This process will be continued until the target genomic region is sequenced. The resulting raw data generated from the Pyrosequencing reaction will be analyzed using pyromark software to quantify the methylation status. The ratio of the methylated to the unmethylated cytosine at each CpG dinucleotide will represent the percentage of methylation for that particular CpG site.

For our studies, we have compared the average methylation density of tumor samples for each gene to the methylation density of the normal parathyroid samples. A difference of more than 10% in the mean methylation density was regarded as significant.
Immunohistochemistry (IHC)

IHC is a well established and a widely used technique for the detection and subcellular localization of proteins using labeled antibodies specifically directed against the proteins of interest. The methodology of IHC can vary widely depending on the tissue type studied, the targeted protein as well as the required degree of detection sensitivity and specificity. However the basic principle of IHC remains the same involving a specific antigen-antibody reaction. In general, the protocol starts with fixation of the tissue most commonly using formalin fixation to produce formalin-fixed paraffin embedded tissue block. The paraffin block will be further processed and cut into sections of about 5μm thickness and fixed onto the surface of a glass slide. In order to uncover the antigenic sites, the slides are treated for what is called antigen retrieval were the slides are preheated allowing breakage of the protein cross-links created by the fixation step.
Two protein detection methods are used in IHC (Figure 18). The direct method uses only one labeled primary antibody to detect the target protein. This method has the advantages of being quick and short, but has the drawback of reduced sensitivity. In contrast, the indirect method uses two sets of antibodies. The first primary antibody is not labeled and used to detect the target antigen while the secondary antibody are labeled and directed to detect the first primary antibody. The indirect method has amplified signal intensity therefore has increased sensitivity; however it is more time consuming and has longer protocols. Stained IHC slides are examined under light microscope or scanned with special scanner for visualization, detection and sub-cellular localization. In study I and II, we used the indirect avidin-biotin complex (ABC) method.

![Diagram of Direct and Indirect Methods](image)

**Figure 18.** Immunohistochemistry (IHC). Direct and indirect methods of protein detection.

**TaqMan DNA copy number analysis**

This method is used for DNA copy number prediction at a specific genomic locus. We have used this method in our studies (study I, II and III) for validation of selected loci with recurrent CNAs as well as for \textit{CDC73} and \textit{APC} copy number estimations. This is a quantitative PCR-based method implementing Taqman
chemistry with a similar approach to standard real time based qPCR. The target and reference primers are labeled with FAM and VIC dyes, respectively and both are run simultaneously in a duplex real-time PCR. The reference primer is designed to detect genomic sequence with known diploid copy number and used as an internal control. The reaction is run in triplicates in a qRT-PCR machine using standard amplification method and target DNA copy number is predicted using relative quantification method (ΔΔ Ct) [209]. In order to estimate ΔΔ Ct, the difference between target Ct and reference Ct (ΔCt) is calculated and compared to a calibrator known to have two copies of the target loci. The copy number of the target is calculated to be two times the relative quantity. Raw data is extracted from the RT-PCR machine after the run using Sequence Detection Software SDS (Applied Biosystems) and automatically analyzed for DNA copy number prediction using a special software called CopyCaller software (Applied Biosystems).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Reverse transcriptase real-time PCR (qRT-PCR) is a very sensitive, specific and reproducible method for the detection and the quantification of mRNA [210]. This method allows real-time measurement of the PCR product while they accumulate during the exponential phase which is directly proportional to the amount of template prior to the start of the PCR process (Figure 19A).

There are two main chemistries used for probe labeling and mRNA detection in qRT-PCR, Taqman probes and SYBR green. Taqman probes only bind single stranded DNA molecules (Figure 19B) and is more specific, while SBYR green can bind any double stranded DNA molecule (Figure 19C) and hence is less specific. In qRT-PCR, mRNA is quantified using either a standard curve or relative quantification. The standard curve is usually used for absolute quantification such as for the quantification of viral loads. This method uses a series of dilutions of a known standard such as a plasmid for the target gene to generate a standard curve and the final mRNA quantity is estimated from the linear regression of the standard curve. In relative quantification, the gene expression is quantified relative to the reference samples such as normal tissue or untreated samples using $2^{\Delta\Delta Ct}$ method where $\Delta\Delta Ct = \Delta Ct$ (sample) – $\Delta Ct$ (calibrator), and $\Delta Ct$ is the Ct of the target gene subtracted from the Ct of the housekeeping gene [209].
The standard protocol for qRT-PCR starts with the cDNA library synthesis from total RNA using commercially available reverse transcriptase cDNA synthesis kits. The samples are run in triplicates with the target assay in a qRT-PCR machine. In order to quantify the target mRNA, the Ct value is measured using either a standard curve or relative quantification with an endogenous control. For our studies (study I, II and IV), we have used Taqman probes and relative quantification ($2^{\Delta\Delta Ct}$) method.

**Figure 19.** (A) Principle of QRT-PCR (B) Taqman probe (C) SYBR green probe.
This thesis was based on the study of parathyroid tumors. Parathyroid tumor tissues were obtained from patients operated for PHPT and immediately snap frozen. The final histopathological diagnoses were established according to World Health Organization (WHO) [20] criteria and aided by clinical, surgical and pathological findings. Unless mentioned otherwise, all parathyroid tumor samples were sporadic as none of the patients’ clinical features or family history were suggestive of familial parathyroid diseases. Three non-tumorous parathyroid tissues were used as reference samples (N1, N2 and N3) in all the four studies. None of the patients from whom normal parathyroid tissues obtained had hypercalcemia and/or elevated PTH level. All samples were collected with informed consent and approved by the local Research Ethics Committee. Table 2 summarizes all the tumor samples used for the studies included in this thesis.

Study I
This study included 21 parathyroid adenoma samples obtained from patients operated for primary hyperparathyroidism at Karolinska university hospital during 2005-2009. All the 21 adenomas weighed ≥ 4 grams and ranged between 4.07-12.30 grams. They were referred to as large parathyroid adenomas (LPTAs) in this study. Eight of the patients were males and 13 were females and their ages at diagnosis ranged from 37 to 84 years. The histopathological classification was based on the WHO criteria [20] which identified three cases of atypical adenomas among the 21 LPTAs.

Study II
In this study, we have investigated two parathyroid tumor samples obtained from two patients. The first patient was a 78 year old female with APC mutation-associated familial adenomatosis polyposis (FAP) diagnosed and operated on for an ectopic parathyroid tumor in Hospital de Santa Maria, Lisbon, Portugal. The excised parathyroid tumor measured 21 X 11 X 9 mm and histopathological examination established the diagnosis of a single parathyroid adenoma based on WHO criteria. The second patient was an 83 year old male with a history of colon polyps, colon cancer and Lynch syndrome and was operated for a parathyroid tumor in Karolinska
university hospital, Stockholm, Sweden. Similarly, this patient had histopathological diagnosis of parathyroid adenoma. The adenoma weighed 7.01 grams. Apart from the reference parathyroid samples (N1-3), this study also included two atypical parathyroid adenomas used as a negative control for APC immunostaining. The control parathyroid samples were also obtained from Karolinska university hospital, Stockholm, Sweden. Corresponding blood leukocytes were obtained from both patients for mutational analysis of \textit{APC}.

\textbf{Study III}

The tumor panel investigated in this study included 9 parathyroid tumors with established \textit{CDC73}/\textit{HRPT2} mutations. Three tumors had confirmed diagnosis of parathyroid carcinoma (one primary lesion, one from a local recurrence and one from a lung metastasis). The remaining tumors included 5 adenomas and one atypical adenoma. The atypical adenoma along with one adenoma were obtained from the same patient during different time intervals with the atypical tumor being excised last. Only one of the patients with parathyroid adenoma was regarded as familial and had FIH. The 3 carcinomas and the remaining tumors were obtained from Tokyo women’s medical hospital in Japan and Karolinska university hospital in Stockholm, Sweden, respectively, during 1994-2006.

\textbf{Study IV}

A panel of 72 parathyroid tumors including 66 adenomas, 3 atypical adenomas and 3 carcinomas was investigated in this study. Twenty-one tumors including the 3 atypical ones were from the previous study (study I). Six of the adenomas had cystic features. Apart from the three carcinomas which were obtained from Tokyo women’s medical hospital in Japan and used in study III too, all the other samples were from Karolinska university hospital, Stockholm, Sweden.
Table 2. All tumor samples included in the thesis.

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Age refers to age at diagnosis; M = Male; F = Female; Wt = Wild-type; Mut = Mutated.

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Age refers to age at diagnosis; M = Male; F = Female; Wt = Wild-type; Mut = Mutated.
RESULTS AND DISCUSSION

Study I: Genetic analysis of large parathyroid adenomas (LPTAs)

LPTAs were defined as the 5% largest sporadic parathyroid adenomas identified among the 590 cases operated in our institution during 2005–2009. In this study we asked two questions: What is the genetic profile of parathyroid adenomas with large glandular weight? And do large parathyroid adenomas carry malignant potential? To answer these questions, we investigated 21 LPTAs weighing \( \geq 4 \) grams.

**Correlation of adenoma weight with clinical and biochemical parameters**

LPTAs had a higher relative number of male cases and a more pronounced clinical picture reflected by significantly higher S-Ca\(^{2+}\), an observation supporting previous reports [211, 212].

**Screening for MEN1 and CDC73 mutations**

Mutational analyses revealed MEN1 mutation in 5 cases and CDC73 mutation in one case only, a finding in agreement with previous reports of frequent MEN1, but rare CDC73 mutations in parathyroid adenomas [68, 119, 120, 213].

**Staining for MIB-1, APC and Parafibromin**

IHC analyses revealed low MIB1 proliferation index of 1.5% in all the LPTAs favoring a benign nature of the tumors. On the other hand, loss of parafibromin staining was observed in 10 cases; two of them were also negative for APC. Since loss of APC and parafibromin expression are associated with parathyroid carcinoma [214], our observations suggest that a subset of LPTAs share molecular characteristics with this entity.

**CNAs in LPTAs**

Recurrent gross copy number losses of chromosome 1 and 11 and gains of chromosome 5 were the most frequently detected CNAs in the LPTAs screened using array CGH. Overall, gain of chromosome 5 was the most interesting and unique finding as frequent losses on chromosome 1 and 11, but not gain of chromosome 5, are known in parathyroid adenomas [150, 152]. Gain of chromosome 5 was reported in parathyroid carcinomas [148, 215] and in several other tumors such as low grade renal
cell carcinoma, adrenocortical tumors as well as bronchial and gastrointestinal carcinoids [216-218]. Unsupervised clustering of the array CGH data clearly identified those with gain of chromosome 5 as one group and those with loss of chromosome 11 as another group, further supporting the that gain of chromosome 5 reflect a distinct genetic pathway for a subset of LPTAs that is independent of chromosome 11 loss.

**CARD6 as a candidate oncogene**

Taqman DNA copy number analysis validated gain of several candidates genes located within minimal overlapping regions on chromosome 5, among them gain of the CARD6 gene. Over-expression of this gene detected by qRT-PCR further confirmed this finding. CARD6 plays an important role in activation of NF-kB signaling, involved in proliferation, differentiation and apoptosis [219] and is deregulated in many tumor types including parathyroid tumors [220]. The present observations of CARD6 over-expression as well as copy number gains suggest that evaluation of the protein expression could be valuable to further study the possible involvement of this gene in parathyroid tumors.

Finally, we concluded that LPTAs represent a group of patients with pronounced parathyroid hyperfunction and associated with specific genomic features.

### Study II: Molecular characterization of parathyroid tumors from two patients with hereditary colorectal cancer syndromes

The primary aim of this study was to investigate possible association between APC mutations in hereditary colorectal cancer syndromes and parathyroid tumor development. For this purpose, we investigated two parathyroid tumors obtained from two patients with familial colon cancers; one with familial adenomatous polyposis (FAP) and an established constitutional APC mutation, and second one with Lynch syndrome with an underlying germline MLH1 mutation and a non-classified missense alteration of the APC gene.

Histopathological re-examination confirmed the benign nature of both parathyroid tumors initially diagnosed as adenomas. To further assess any malignant potential, we studied the expression of Ki67, parafibromin and APC as potential molecular markers of parathyroid cancer [214, 221] using IHC. Both tumors had low MIB-1 proliferation index, negative parafibromin and strongly positive APC staining. Furthermore, while both patients carried constitutional APC mutations, no somatic APC mutation could be
detected. To further investigate other possible alterations of the APC gene, we analyzed APC gene copy number and promoter methylation using qPCR and bisulfite Pyrosequencing, respectively. Both tumors were diploid for APC. While promoter APC 1B was unmethylated in both tumors, promoter APC 1A was hypermethylated. Hypermethylation of the APC 1A promoter has been previously reported in parathyroid adenomas and carcinomas [194, 222]. However, this did not seem to affect the expression of APC as it was not altered when determined by qRT-PCR. This is possibly due to unmethylated transcriptionally active APC 1B promoter, a finding in agreement with previous studies [194, 223]. In addition, global methylation level represented by LINE-1 analysis did not show any alterations compared to reference parathyroid samples.

The findings in this study supported the benign nature of the parathyroid tumors and did not suggest a role for deregulated APC in parathyroid tumors from these two patients.

**Study III: Characterization of CDC73/HRPT2-mutated parathyroid tumors**

In this study we aimed to characterize the genetic profile of CDC73/HRPT2 mutated parathyroid tumors as well as to investigate possible mechanisms of the CDC73 gene inactivation. To accomplish that, we investigated 9 parathyroid tumors (3 carcinomas, 5 adenomas and one atypical adenoma) with established CDC73 gene mutations for CDC73-specific as well as genome-wide alterations.

**Screening for CDC73 mutations**

Bi-allelic mutations were detected in 3 tumors and the remaining tumors had monoallelic mutations. In 7 tumors the CDC73 mutations were predicted to prematurely truncate parafibromin. For the remaining two tumors the mutations were predicted to involve the first intron sequence and the first nucleotide of the consensus donor splice site of intron 1.

**DNA copy number analysis of CDC73 gene using qPCR**

DNA copy number analysis displayed only one copy of the CDC73 gene in 3 of the adenomas, which, in previous studies, also exhibited loss of parafibromin expression using Western blot and IHC [213] and hence representing the second hit. On the other
hand, all the carcinomas were diploid or presented with copy number gain of \textit{CDC73} gene. These results might suggest that parathyroid tumors can develop malignant features even with one remaining functional copy of \textit{CDC73}.

\textit{Global and CDC73 promoter methylation analyses}
All the tumors were unmethylated at the \textit{CDC73} promoter, a finding in line with a previous report [191]. On the global level, all the tumors were methylated and no global hypomethylation could be detected. These findings suggest that promoter methylation is an unlikely mechanism of \textit{CDC73} gene inactivation and \textit{CDC73} mutation itself does not induce alterations in global methylation assessed by \textit{LINE-1} analysis.

\textit{Array-CGH profile in the CDC73-mutated parathyroid tumors}
The a-CGH profiles of the carcinomas and the adenomas were significantly different and further confirmed using unsupervised clustering analysis where the carcinomas and the adenomas fell into two different clusters. The carcinomas displayed gross deletions of 1p, entire chromosome 13 deletions and gain of chromosome 20, with absence of any significant CNAs on chromosome 11. These findings are generally similar to previously published studies using conventional CGH [148, 215] and possibly reflect the aggressive clinical behavior and could correlate with the malignant nature of these tumors. On the other hand, and in contrast to previous CGH studies [148, 149, 152, 154], the adenomas displayed small extent of CNAs with the absence of any significant CNAs on chromosome 1 and 11. These results are interesting and support the hypothesis that \textit{CDC73} gene mutations possibly direct the parathyroid adenomas towards a different genetic pathway.

\textit{Genome-wide and CDC73 locus LOH analysis using SNP array}
The carcinomas displayed more frequent LOH events than the adenomas with chromosome 1 being the most commonly affected in both tumor types. Previous studies reported frequent losses on chromosome 1p in parathyroid tumors regardless of the \textit{CDC73} mutational status and for which no candidate genes have been found [224, 225]. Furthermore, LOH at the \textit{CDC73} locus was only detected in two tumors, one of which also displayed copy number loss by TaqMan copy number analysis. Our results suggest that \textit{CDC73} mutations drive parathyroid tumors into a partly distinct cytogenetic pathway different from unselected parathyroid tumors.
Study IV: DNA methylation profiling in parathyroid tumors

Here, we wanted to study the promoter methylation status of several candidate genes and see whether aberrant promoter methylation of these genes are involved in parathyroid tumorigenesis. For this purpose, we have determined gene-specific promoter methylation status of 10 candidate genes including APC (promoter 1A, and 1B), β-catenin (CTNNB1), CASR, CDC73/HRPT2, MEN1, P16 (CDKN2A), PAX1, RASSF1A, SFRP1, and VDR; as well as global methylation level (LINE-1) in 66 parathyroid adenomas, 3 atypical adenomas and 3 parathyroid carcinomas using bisulfite Pyrosequencing.

Gene-specific methylation analysis in parathyroid adenomas and atypical adenomas
We detected frequent hypermethylation of APC 1A, RASSF1A and β-catenin promoters in the adenomas. Frequent hypermethylation of APC 1A and RASSF1A are in line with previous studies [194, 195]. The majority of hypermethylated adenomas had reduced mRNA expression of the same gene. This association suggests a silencing effect by methylation on these genes. None of the other genes had altered methylation status. Promoter hypermethylation was detected in only one atypical adenoma for APC 1A.

Gene-specific methylation analysis in parathyroid carcinomas
Interestingly, all the 3 carcinomas were hypermethylated for RASSF1A and SFRP1, but only one was hypermethylated at the APC 1A promoter and none for β-catenin. Hypermethylation of APC 1A and RASSF1A have been reported in many other tumors such as colorectal [226] and head and neck cancers [227]. Hypermethylation of SFRP1 has been reported in parathyroid tumors [195] as well as in other tumors such as colorectal [228], head and neck [229] and as an independent poor prognostic feature in breast cancers [230]. SFRP1 is a potent antagonist of the Wnt signaling pathway and hypermethylation of this gene without hypermethylation of β-catenin, might suggests a constitutive activation of the canonical Wnt/β-catenin signaling in this subgroup.

Global methylation analysis using LINE-1
Regardless of tumor type, estimation of LINE-1 methylation density did not reveal any change in the global methylation status, a finding in line with our previous studies [194, 231].
Correlation of the methylation profile to the MEN1 and CDC73/HRPT2 mutational status

Tumors with known MEN1 (5 tumors) or CDC73 mutations (8 tumors) had similar methylation profiles suggesting that the mutational status of these two genes is unlikely to direct the tumors toward a different methylation profile.

Correlation between clinical/biochemical parameters and gene-specific methylation

The methylation status of APC 1A was significantly correlated with adenoma weight ($r = 0.306$, $P = 0.019$). This finding suggests that large parathyroid adenomas could have a different DNA methylation pattern in addition to previously reported specific genetic profile linked to adenoma size [232]. Furthermore, a statistically significant correlation was observed between the methylation status of RASSF1A and with both of APC 1A and β-catenin.

These findings support our previous study [194] and reinforce the role of aberrant hypermethylation of APC, RASSF1A and β-catenin in the tumorigenesis of a subgroup of parathyroid adenomas. It also motivates further studies into the role of aberrant methylation of SFRP1 in parathyroid carcinoma development and its role as a potential epigenetic marker.
CONCLUSIONS

- Large parathyroid adenomas may constitute a subset of parathyroid lesions associated with pronounced PHPT features and specific genomic aberrations.

- Large parathyroid adenomas are associated with significantly higher serum calcium and therefore more pronounced clinical features.

- The frequency of MEN1 and CDC73 mutations in large parathyroid adenomas is similar to those reported for parathyroid adenomas of smaller glandular weights.

- CARD6 gene could have oncogenic potential and may play a role on the development of a subset of large parathyroid tumors.

- APC gene is unlikely to be involved in parathyroid tumors development in patients with APC-mutated associated familial colorectal cancers.

- CDC73 mutations direct parathyroid adenomas in a different genetic pathway from those adenomas without established CDC73 mutations.

- Aberrant promoter methylation is an operational mechanism in parathyroid tumorigenesis.

- Methylation of APC A1, β-catenin and RASSF1A promoters are deregulated in parathyroid tumors and could trigger potential therapeutic targets in the future.

- SFRP1 is a potential epigenetic marker for parathyroid carcinomas and can be of important diagnostic aid along with already established molecular markers.
POPULAR SCIENTIFIC SUMMARY IN KURDISH

پوخته‌ی زانستی تویزیئن‌وی به زمانی نووی

شیرله‌که نجع، یه‌کیکه لهو گەچیشەه وەهری ەویز و ترسکانەکانی تەندروستیانە ەو یەکیکە لە هەویە

سەردەکیکەوەیە مەردن لە چیهاندا. ەو گەڵ نەوە هەموو پیشکوو یەمەنیانە لە چوارنیە

پژیشکی و نەوە بەرەوەچۆنەوەیە ڵە چاکردنی چارسەرکردنی ەژەکیدەکە چۆر لە

نەخۆشیەکانی شێڕلە پێ نجع لەوەیە 20 تا 30 سالانی نەم دوایەدا، تا نیستەش پێچەویستیەکە زۆر

ەویە لە باشتر کردەی کەرستیە دەستنیشانەکردنی نەخۆشیەکە وە باشترکردنی ڕێگای

چارسەرکردنی. تا بەشدرایی بەکەین لەم بوارە، نێەیە ەستایە یە پیشکێنی زەمارەکە

زۆری نەوە شێڕلە پێ نجعەیە کەوە تێوشی گەلەنەی پاراگریۆید دەبێت وەک نەمێنیکە لە

شێڕلە پێ نجع به یەمەنی یاکەوە باشترە تیگه‌یەکە لەوە میکانیزمانەی کەوە شێڕلە پێ نجع پێ دەوەست

ەکەیت و بەم شێوەمیش بۆ گەیشتیی بە چارسەرەی باشتری نەوە نەخۆشیە.

وە لە بتوانیت نەوە نەدەرکەی کەوە تەسەرەوە نەمادەمێن پێکرد نە نەوایەنەیی. ەستایە یە

پیشکێنی چەری زەمارەکی یەکەرەر پەڕە شێڕلە پێ نجع پێ نزیان و بەدزیانەکان ی گەلەندی
پارادایمی‌ی به بنکه‌ی تویزینه‌ودی شیر‌په نجمه له نه خوشگیانه‌ی زانگوئی کاردینیسکا له
ستوکهولم، سوید.

دورو جویر له میکانیزمی‌په یدابون و تشهنه کردنی شیر‌په نجمه چیگه بایه خدان و مهیلی
نیمه بیو. دراسه تکردنی میکانیزمی‌په‌موادی‌پیرام و تیمان ددات له دؤرینه‌ودی نهو
پازداننه‌ی (گُورانکاری‌انه) له ریزبه‌ندی دی ای ای (که‌وا بؤیان هدلی هدیه په‌یدابونی
شیر‌په نجمه بی. له لاپیک تروقی، دراسه تکردنی آپیسیتیک، که تیپ‌یی دا نهو
میکانیزمانه ددردیتیوه که گورانکاری‌نه نهامنه‌نه له جن‌نه کان پب‌هیج گورانکاری‌ودی
له ریز به‌ندی دی ای ای.

نهو دورو میکانیزمه ته‌واو کره‌ی پکرتی. له تویزینه‌ونه‌نه نیمه نه نجامان دان توانیمان
زایناری‌ی زور گرگ و نهو بدهست په‌ینین که‌وا پیرامته‌دی درن له جیاکردنو ودی شیر‌په نجمه
به زیان و پب‌زیان و هه‌یه‌مه‌ها له‌پژارتنی باشترین شیاوه‌ی چارس‌رکردن به گوپردی
جؤری شیر‌په نجمه.
ACKNOWLEDGEMENTS

I would like to take this opportunity to express my gratitude to the people who have been instrumental in the successful completion of this work as without their support this would not have been possible.

Dear Mr. Ahmad Ismail Nanakaly, the noble man from Erbil city of Kurdistan region of Iraq, I have never met you, but I have known you for your generosity, loyalty and love to your nation and humanity. Neither you nor any one from your family are known for big palaces or luxurious cars, but you are very well known for endless donations and support to many charity organizations in the region. Your 4 million US $ donation for sending Kurdish students abroad (including myself) is one among many of your outstanding works. I know for sure that you are not expecting anything from us but to transfer the knowledge and serve the nation in the best way, and this is what I promise.

My supervisors

Jamileh Hashemi, thanks very much for your continuous support, valuable guidance and advices. You have always been patient and encouraging. You have believed in me since the start and this has helped me very much during my PhD study. Your supervision may end now, but the friendship will last. Thanks very much for introducing me to such a wonderful and competent research group. I should also thank your wonderful family and especially your husband Ismail for being so kind with me and very helpful and friendly.

Catharina Larsson, you are the true definition of a super successful and a brilliant supervisor and a group leader. You have never ever stressed me, but instead you always relieved my anxiety and sorted out my problems in a very smart way. I felt always confident with your presence around. I would have had a much harder and probably a longer journey in my PhD without your support and encouragement. Your smile made me motivated. Your guidance, knowledge and supervision have broaden my professional experiences and prepared me for future challenges. Thank you very much
for having me in your group. I have learned so much from you. If there is any award in
this world that should be given to the best supervisor then should be awarded to you.

**Inga-Lena Nilsson**, I should say that I was extremely lucky to have you as a
supervisor. You broke the record of how fast you reply to me and always ready to help.
Your contribution to my work was outstanding. You have oriented and supported me
with promptness and care. Still I remember the day your ran to CMM from the
operation theater to bring me the “Normal” parathyroid sample! (I felt so embarrassed;
sorry, it should have been the other way around).

**Christofer Juhlin**, my very young talented supervisor and friend. To work with you
has been a real pleasure to me, with heaps of fun and excitement. Actually the research
work goes fast and very well with your supervision. I wish you a very successful carrier
in your life. Bring your beautiful daughter **Elmina** with you to the defense; she brings
me a good luck!

**Weng-Onn Lui**, yes, you were not officially my supervisor, but I have always thought
of you as one of my supervisors. You never let me down when I came to you asking for
help or advices. Even if you could not help me directly, you found me an alternative
solution. Thanks for arranging all the scientific and social activities. I’m deeply grateful
to you. You are a very dedicated capable young researcher and I wish one day you get
the Nobel Prize!

I am very grateful to my mentor, **Ingemar Ernberg**. Thanks for being my mentor and
accepting me as the PhD student representative in the Tumor Biology-Oncology board
at Karolinska Institute. This was a golden opportunity for me to learn from such a
wonderful and an experienced person as you.

Special thanks to all other current and previous members of Catharina Larsson group:
**Janos Geli** (smart and very social), **Anastasios Sofiadis** (very friendly), **Stefano
Caramuta** (very friendly, good luck with your post doc carrier), **Omid Fotouhi** (an
ambitious man) and his wife **Shler, Hong Xie** (a hard worker and a very well organized
person), **Na Wang** (very nice and social), **Pinar Akckakaya** (a smart young researcher),
**Ming Lu** (a very good parathyroid researcher), **Denis Özata** (a very friendly and a
social person), **Linkiat Lee** (very helpful and friendly, but too tidy!),

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**Patric Scicluna** (a very good guy, but sensitive to UV!), **David Fernandez** (Fantastic), **Roger** (very quiet), **Manal** (hababba kulish), **Jameela and Maral Adel**. Thank you all for your support, feedback, and friendship.

**Svetlana Lagercrantz**, you are a wonderful and a very kind person. I’m admired of your personality and knowledge in life. Thanks for your insightful comments and many motivating discussions and for letting me attend clinical sessions with you. Looking forward for future collaboration.

**Andrii Dinets**, the very young thyroid researcher and future endocrine surgeon. So glad I have shared the office room with you. When I was anxious and feeling my work goes slowly or my experiments did not work, your relaxing personality always made me feel better and relaxed too. We had many things in common and similar cultures. Thanks for all those lovely lunches and fikas. I’m looking forward to celebrating your PhD defense soon. Thanks for your lovely wife **Mariana** too and I wish you both the best life together.

**Nimrod Kiss**, I’m very happy that you couldn’t find a job in New Zealand (for time being only!) and you have to stay in the group (sorry for being selfish here!!). This was a good opportunity to know you more. I have learned and enjoyed from our frequent lunch discussions about life and exchanging the experiences. By the way, you look very serious!!!!!

**Felix Haglund**, the very smart, young and the very ambitious parathyroid researcher. It was a lot of fun to work together and thanks for the successful past and ongoing collaborations. Thanks for arranging lunches and times to watch movies together.

**Adam Andreasson**, the champion! a multitalented very friendly person. I’m so happy you have joined the group and had the chance to work together. Thanks for the arranging to watch movies and have dinner outside the lab! I feel bad that you won’t be here during my dissertation! By the way, you have missed the Top Ten most awkward situations happened in our group!! (Sorry, I can not discuss the list here). This awesome list is created by **Christofer, Nimrod and Felix**. I think the list should be handed to you so that you can keep updating it in the future!
I am also indebted to Anders Höög, one of the best endocrine pathologists who was always ready to answer my questions and offering me help when I needed it. Thanks for your collaborations, comments, fruitful discussions and support.

I would also like to thank Lisa Anfält, from the department of pathology, for helping me with samples collection and handling.

This PhD was also the reason behind knowing a group of wonderful Kurdish students whom otherwise I have not probably known, although many came from the same university that I have graduated from. Your friendship is very important for me. Thank you all for your support and all the fun we had together in the last few years and I wish you the very best of luck!

I would also like to express my gratitude to the Kurdish Organization for MedicAl Research (KOMAR) which has helped in recruiting me to the research program in Catharina’s group. This organization was founded by several Kurdish researchers (Jamileh Hashemi, Saleem Saeed, Adiba Isa, Kareem Arif and Alan Fotouhi) and had a fundamental role in establishing a scientific bridge between the universities in the Kurdistan region of Iraq and universities in the Scandinavian countries and continues to play an essential role in promoting and improving medical research in the Kurdistan region.

Thanks to the administration staffs of the molecular medicine and surgery department especially Ann-Britt Wikström, Kerstin Florell, Helena, Britt-Marie Witasp and Lennart Helleday for all the help.

Other previous co workers in CMM: Tomas Ekström (missing our frequent discussions about new technologies), Mohsen Karimi (you were very supportive from the early days), Atosa Estekizadeh, Mattias Berglund, Fahad Al-zadjali, Agneta, Vladana Vukojevic, Louise Sjöholm, Monira Akhtar and her husband Zahid, and Selim Sengul (you are an amazing person).

Special thanks to Jan Zedenius for arranging molecular genetic wine seminar (MGWS) and all the knowledge and fun we have.
My close friend Chaniya Leepiyasakulchai from Thailand thanks for sharing, encouragement and help. I loved the exotic fruit you brought me from your country. Wish you a very good luck with your PhD and life.

Bertha Brodin, moving to CCK gave me more chance to meet you and talk to you. Thanks for being on my halftime committee and for all the encouragements and discussions.

My friend, Anna Marino, you’re a very lovely and a strong person. Thanks for all the chats and jokes and I wish you a very good luck with your carrier.

I would also like to thank the coauthors: Jörgen Nordenström, Takao Obara, Sónia do Vale, João Martin Martins, Florbela Ferreira, Gabriel Miltenberger-Miltenyi, Lucas Batista and Erik Björck.

New colleagues and administrations staffs from CCK, thanks for welcoming us.

My deepest gratitude is also due to the patients and their families who have accepted to participate and donate tissue samples for research purposes. Wish them the best of health.

I would also like to convey thanks to the funding agencies Swedish Cancer Society, Swedish Research Council, Gustav V Jubilee Foundation, Karolinska Institutet and Stockholm County Council for providing the financial means.

Most importantly, I would like to thank my family for their endless support and encouragement. I was very lucky to be the youngest child (however, the tallest one!!) to have all the care and love from all of you while having the least amount of responsibility. Mom, your praying and blessing made my life much easier and more brightful. Without you mom I’m no body!

Finally, I would like to thank my wife Shaween. Your support, encouragement, quiet patience and unwavering love were undeniably the bedrock upon which the past few years of my life have been built. Your delicious food made me go to the gym more frequently!! You tolerance of my occasional vulgar moods is a testament in itself of
your unyielding devotion and love. I would not hesitate a single moment to give up my soul for you!

The list of names needs to be acknowledged is much longer than those mentioned here, sorry if I could not mention you, but be sure I have not forgotten you!
REFERENCES


