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Genetic and Epigenetic Mechanisms in Primary Hyperparathyroidism

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*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!

ABSTRACT

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

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* Both authors contributed equally

LIST OF ABBREVIATIONS

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

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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).

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].

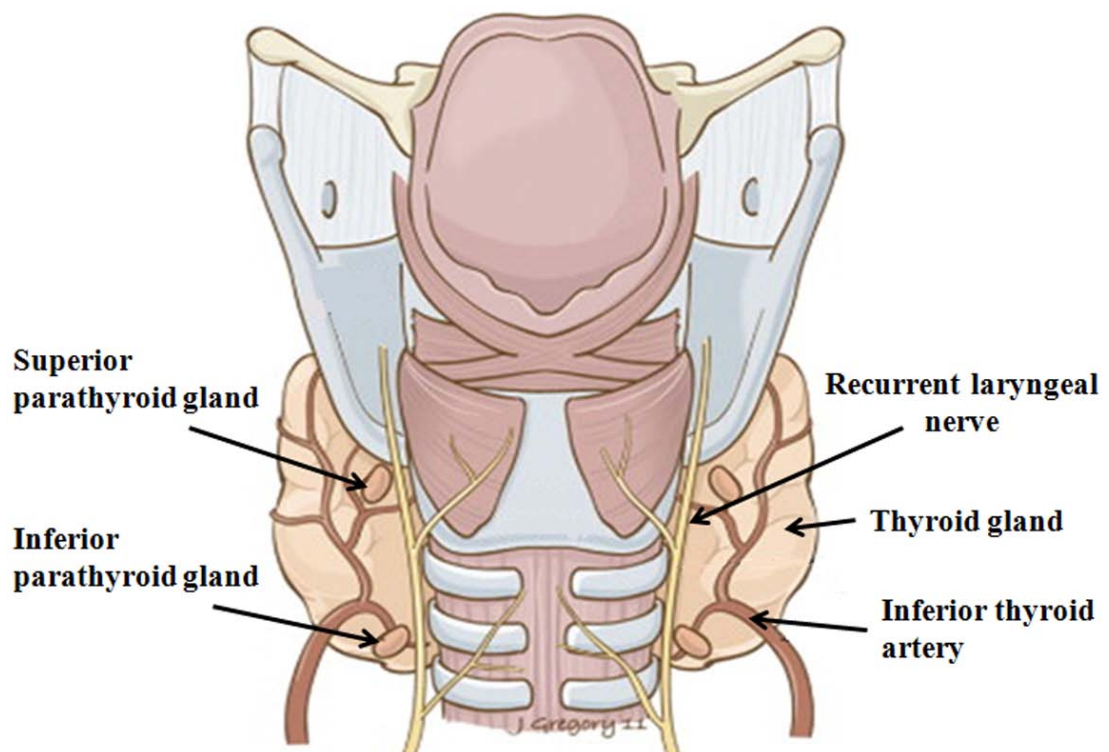


Figure 2. Locations of the parathyroid glands in the neck (posterior view). *Modified from reference [9].*

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.

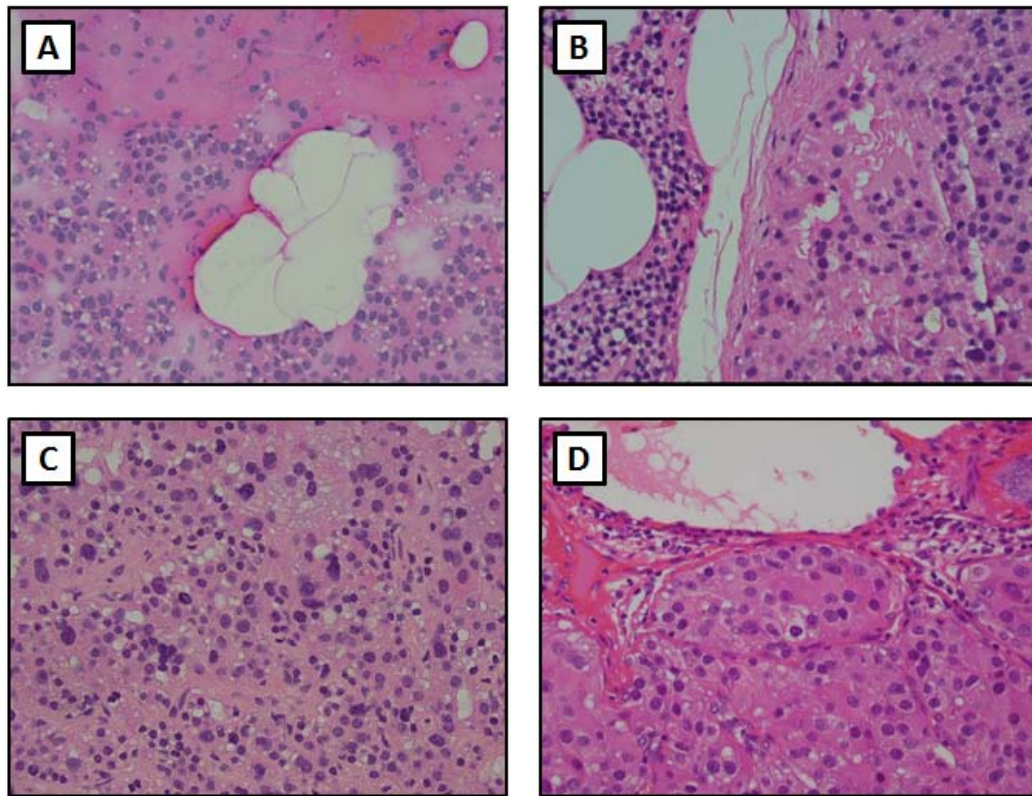


Figure 3. (A) Normal parathyroid gland. (B) Parathyroid adenoma with normal rim. (C) Parathyroid carcinoma. (D) Atypical parathyroid adenoma.

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 ($S\text{-Ca}^{2+}$) levels. PTH secretion has an inverse sigmoidal relationship with $S\text{-Ca}^{2+}$ 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 $S\text{-Ca}^{2+}$ 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 $S\text{-Ca}^{2+}$ 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 blood stream 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 $S\text{-Ca}^{2+}$ 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 $S\text{-Ca}^{2+}$ within the normal physiological level.

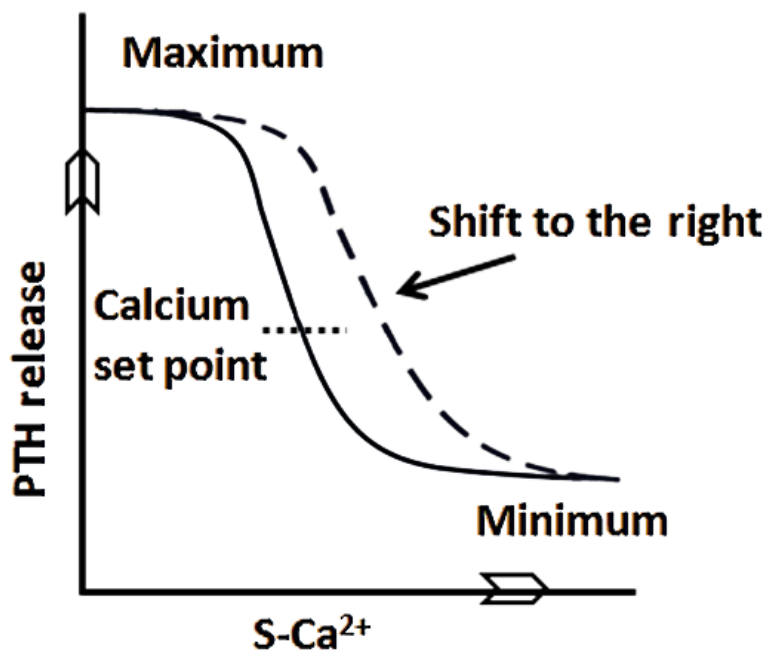


Figure 4. Illustration of the sigmoidal relationship between PTH release and level of $S\text{-Ca}^{2+}$.

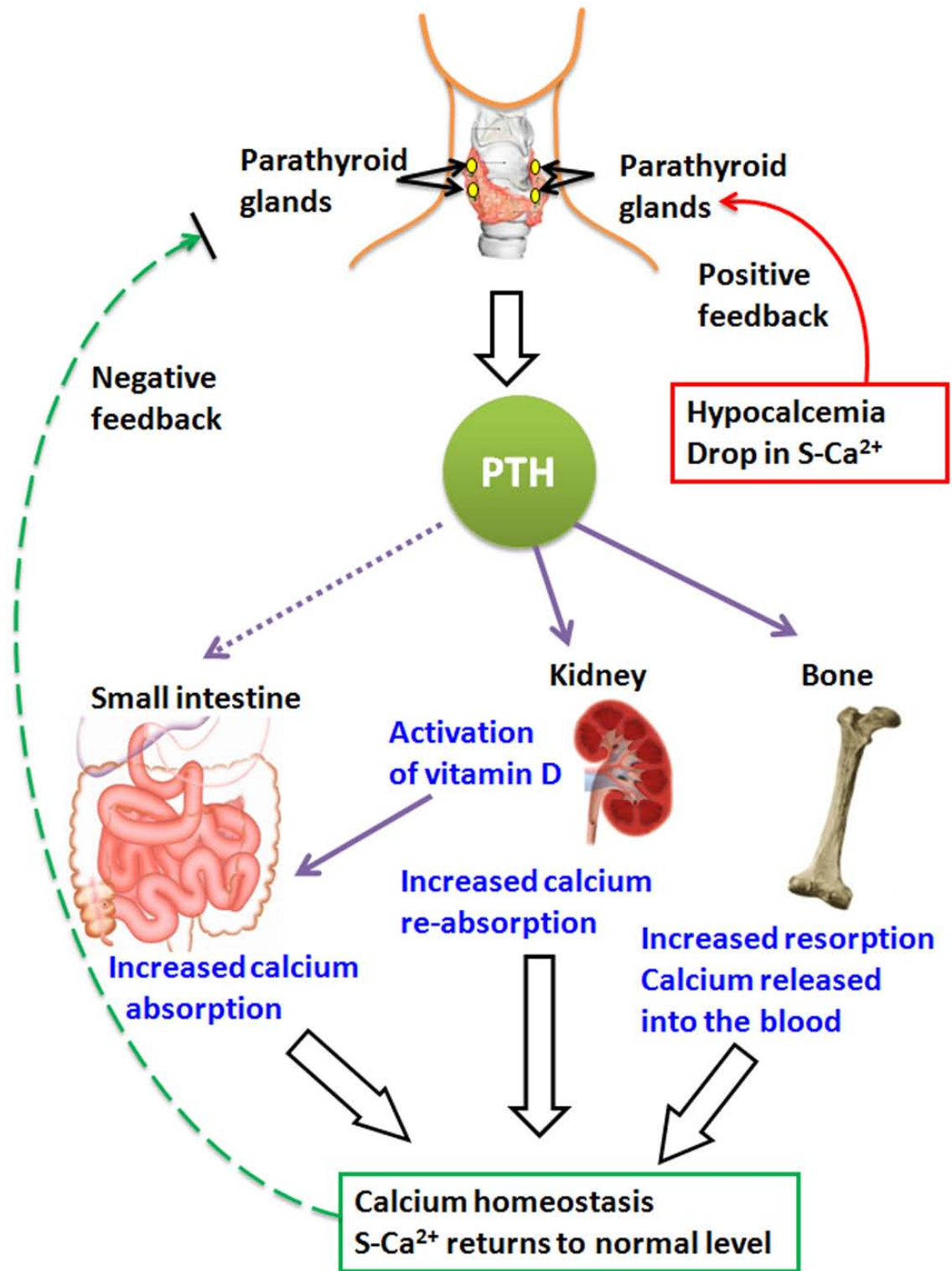


Figure 5. Calcium homeostasis. Regulation of extracellular calcium by PTH.

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 $S\text{-Ca}^{2+}$. 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 $S\text{-Ca}^{2+}$ [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 reflect the population studied as well as $S\text{-Ca}^{2+}$ 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 $S\text{-Ca}^{2+}$ 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 $S\text{-Ca}^{2+}$ [33, 34].

The diagnosis of PHPT starts with the clinical suspicion in any patients with elevated $S\text{-Ca}^{2+}$ (Normal ionized $S\text{-Ca}^{2+}$ range 1.15-1.33 mmol/L). The combination of high $S\text{-Ca}^{2+}$ 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 Technetium99 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 straight forward; 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-Ca²⁺ 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-Ca²⁺, 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-Ca²⁺ 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-Ca²⁺ 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-Ca²⁺ 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 persists 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 $S\text{-Ca}^{2+}$, 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 HPT-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-Ca²⁺ 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 $S\text{-Ca}^{2+}$. 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-Ca²⁺ (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 *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 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].

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

Genes	protein encoded	Associated hyperparathyroid syndrome: main syndromic manifestations	Features of syndromic parathyroid tumors	Defect in sporadic parathyroid tumors
<i>MEN1</i>	Menin	Multiple endocrine neoplasia type 1: anterior pituitary, parathyroid, enteropancreatic, foregut carcinoid tumors	Multiple, assymetric tumors typical (>99% benign)	Inactivation in about 35% of benign tumors; mutations exceedingly rare in cancer
<i>HRPT2/CDC73</i>	Parafibromin	Hyperparathyroidism-jaw tumor syndrome: fibro-osseous jaw. Parathyroid, uterine tumors; renal cysts	single tumor common (about 15% malignant)	Inactivation in about 70% of cancers; mutations are rare in sporadic adenomas
<i>CASR</i>	Calcium sensing receptor	Familial hypocalcemic hypercalcemia (FHH) with heterozygous inactivation; neonatal severe Hyperparathyroidism (NSHPT) with homozygous inactivation	FHH: near-normal size and surgical pathology; altered serum calcium set point for PTH release NSHPT: Marked enlargement of multiple glands	Decreased expression common; mutation exceedingly rare
<i>RET</i>	c-Ret	Multiple endocrine neoplasia type 2A: medullary thyroid cancer, pheochromocytoma, parathyroid tumors	Single tumor common (>99% benign)	Mutation exceedingly rare
<i>CCND1/PRAD1</i>	Cyclin D1	NA	NA	Overexpression results from DNA rearrangement involving PTH gene

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].

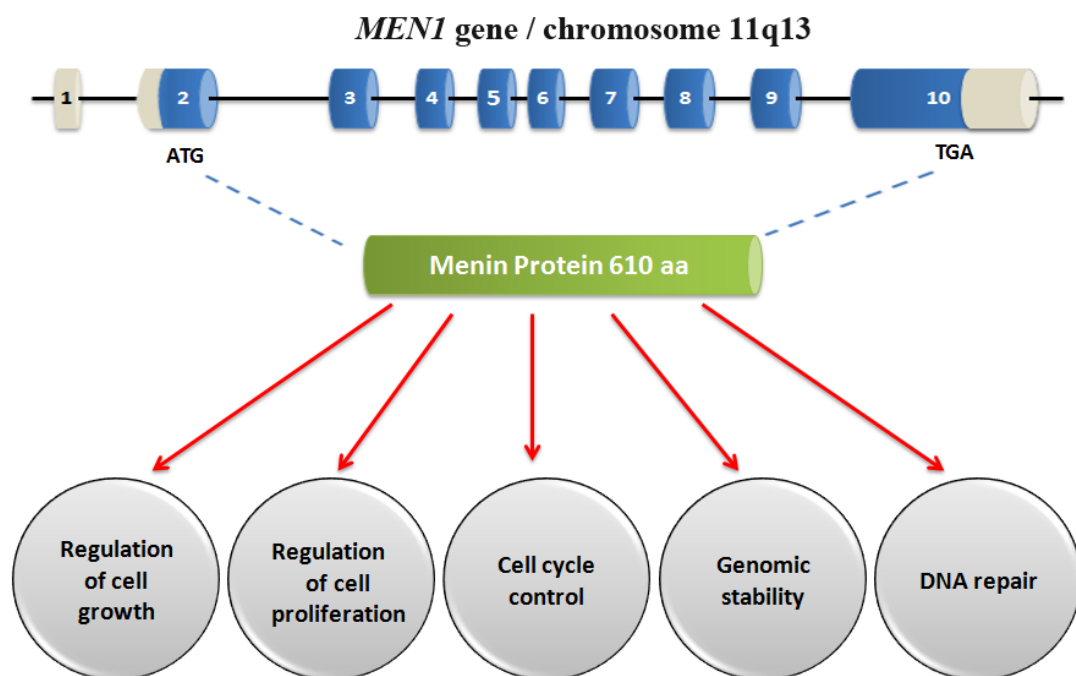


Figure 7. Diagrammatic representation of *MEN1* gene and its product, menin with proposed functions.

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].

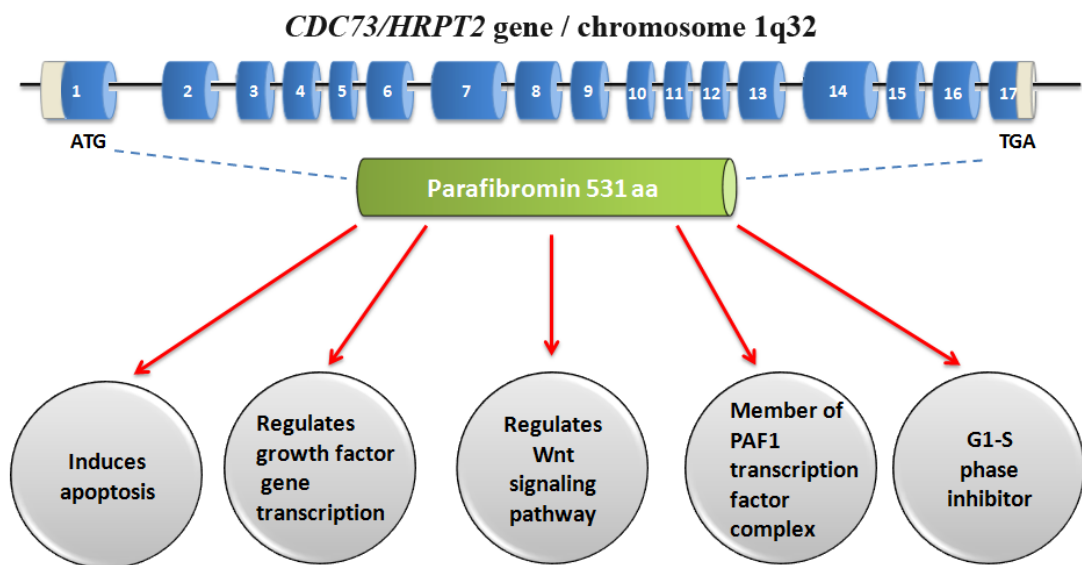


Figure 8. Diagrammatic representation of *HRPT2/CDC73* gene and its product, parafibromin with proposed functions. *Modified from reference* [91].

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.

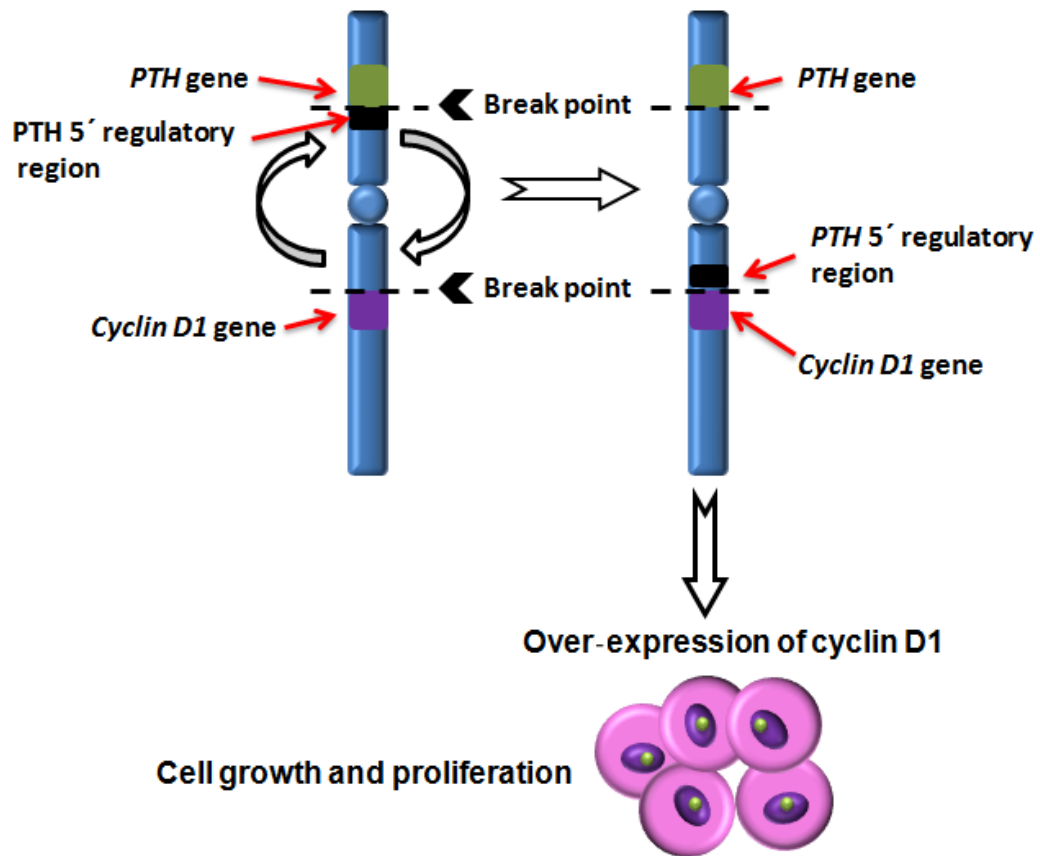


Figure 9. Illustration of *cyclin D1/PRAD1* chromosomal rearrangement. *Modified from reference [137].*

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].

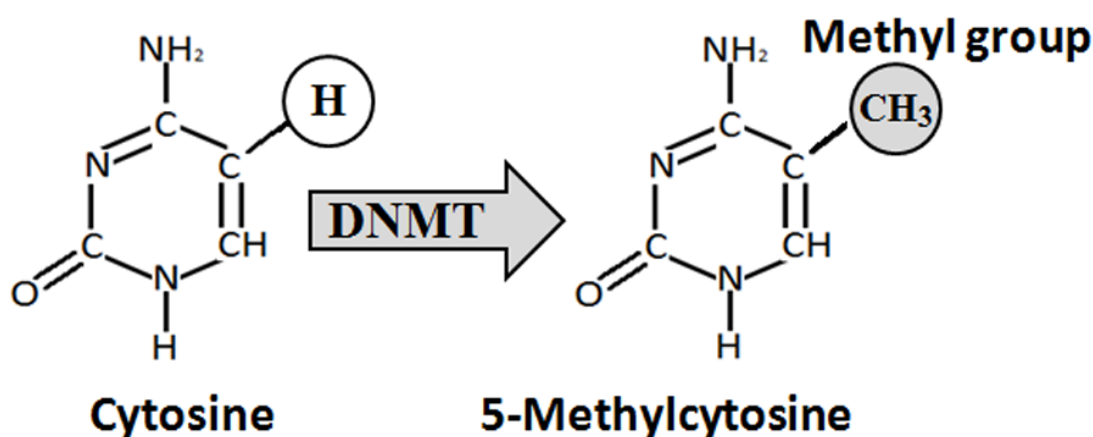


Figure 10. DNA 5-methylcytosine methylation. *Modified from reference [161].*

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 *p16^{INK4a}* [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].

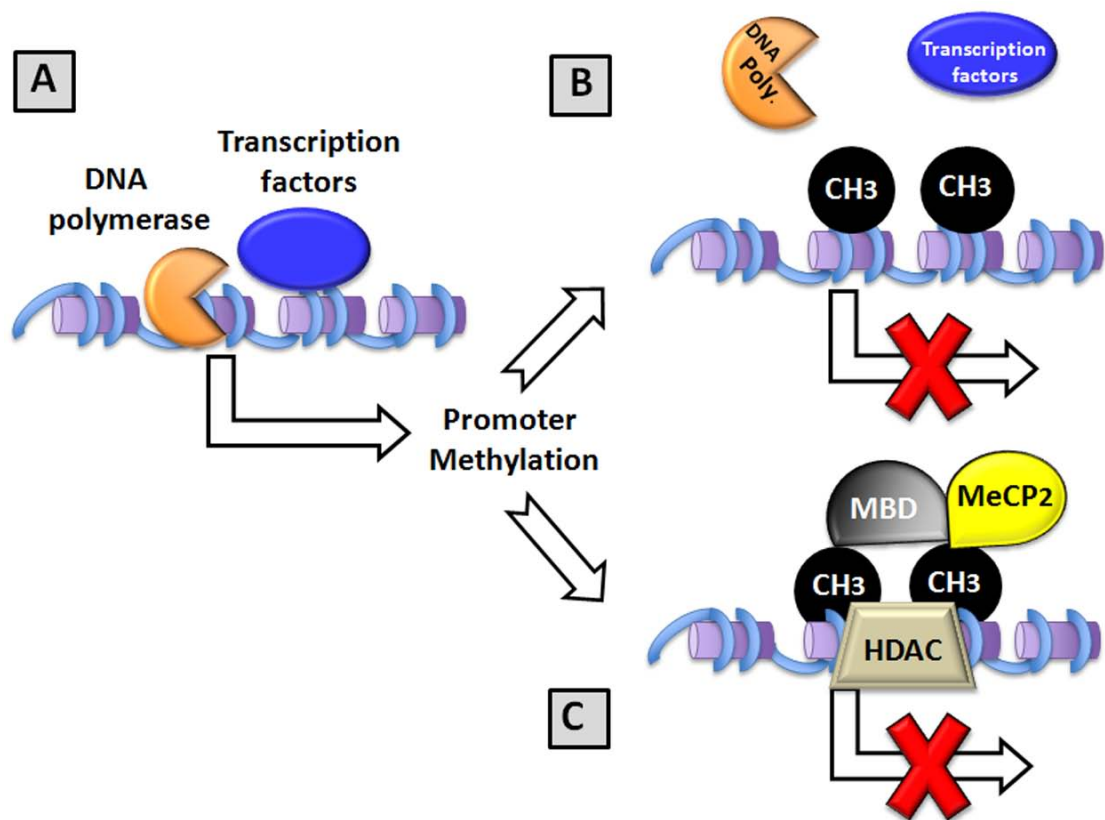


Figure 11. Mechanisms of gene silencing by DNA methylation. (A) Transcriptionally active, unmethylated gene promoter. (B) Silenced gene due to promoter hypermethylation. (C) Silenced gene due to methylation-recruited transcription repressors. *Modified from reference [179].*

Histone modifications and chromatin 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].

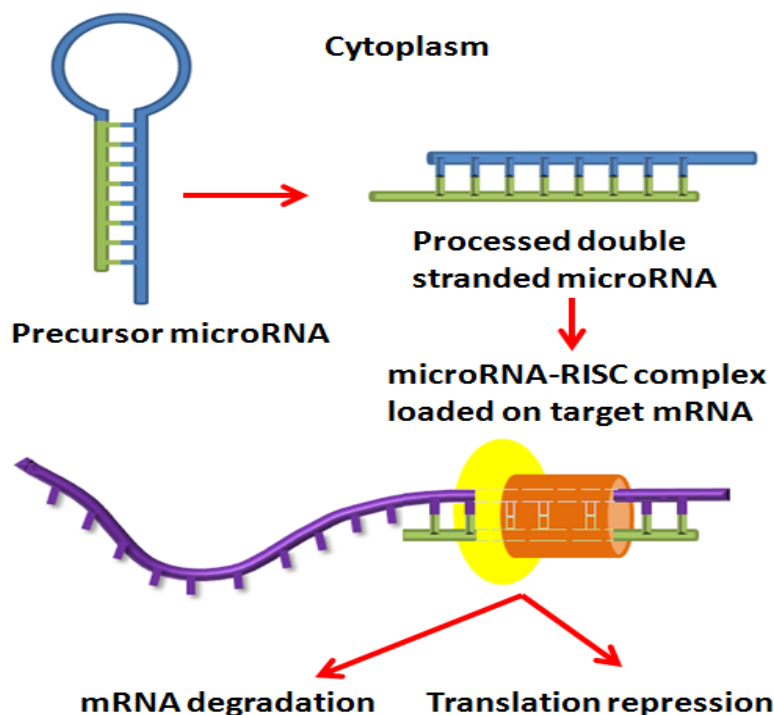


Figure 12. MicroRNA and posttranscriptional regulation of gene expression via RISC (RNA-induced silencing complex). *Modified from reference [189].*

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-Ca²⁺, PTH and tumor weight [198]. Furthermore, this study also found C19MC promoter hypomethylation in 50% of the tumors which significantly associated with S-Ca²⁺ 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.

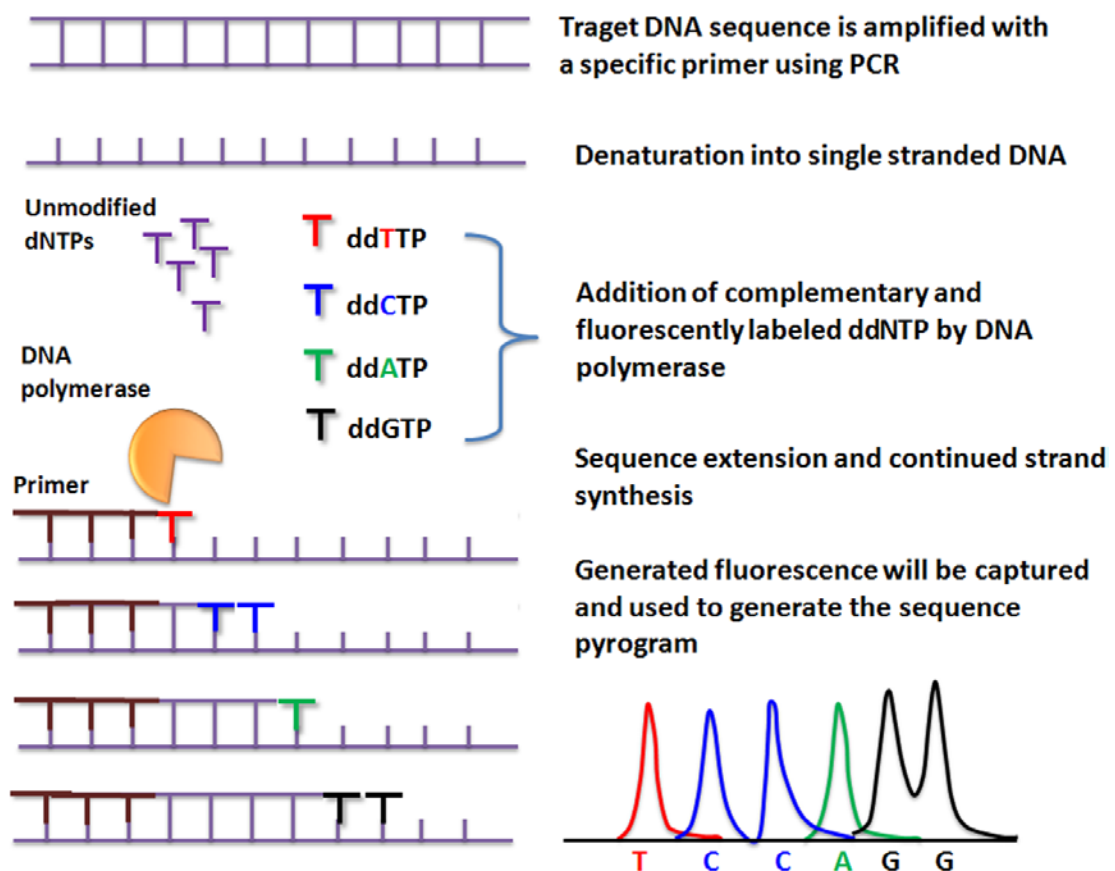


Figure 13. Principle of chain terminator DNA sequencing.

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].

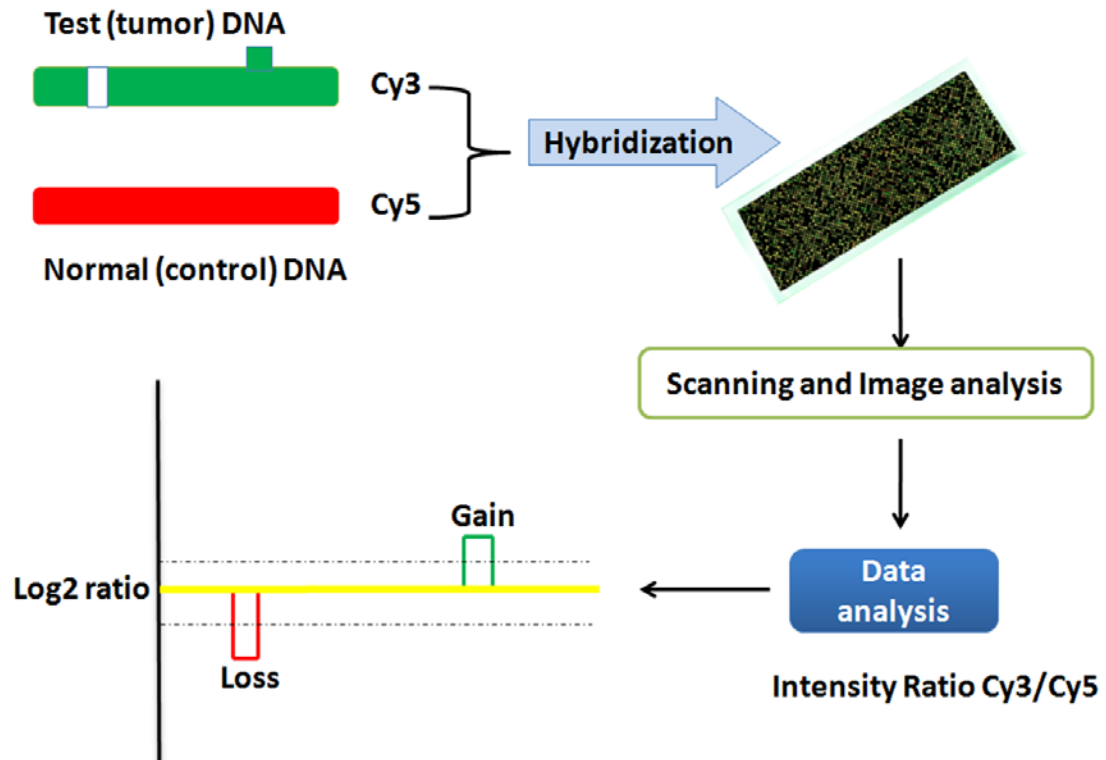


Figure 14. Principle of a-CGH

Single nucleotide polymorphism microarray (SNP array) for loss of heterozygosity (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.

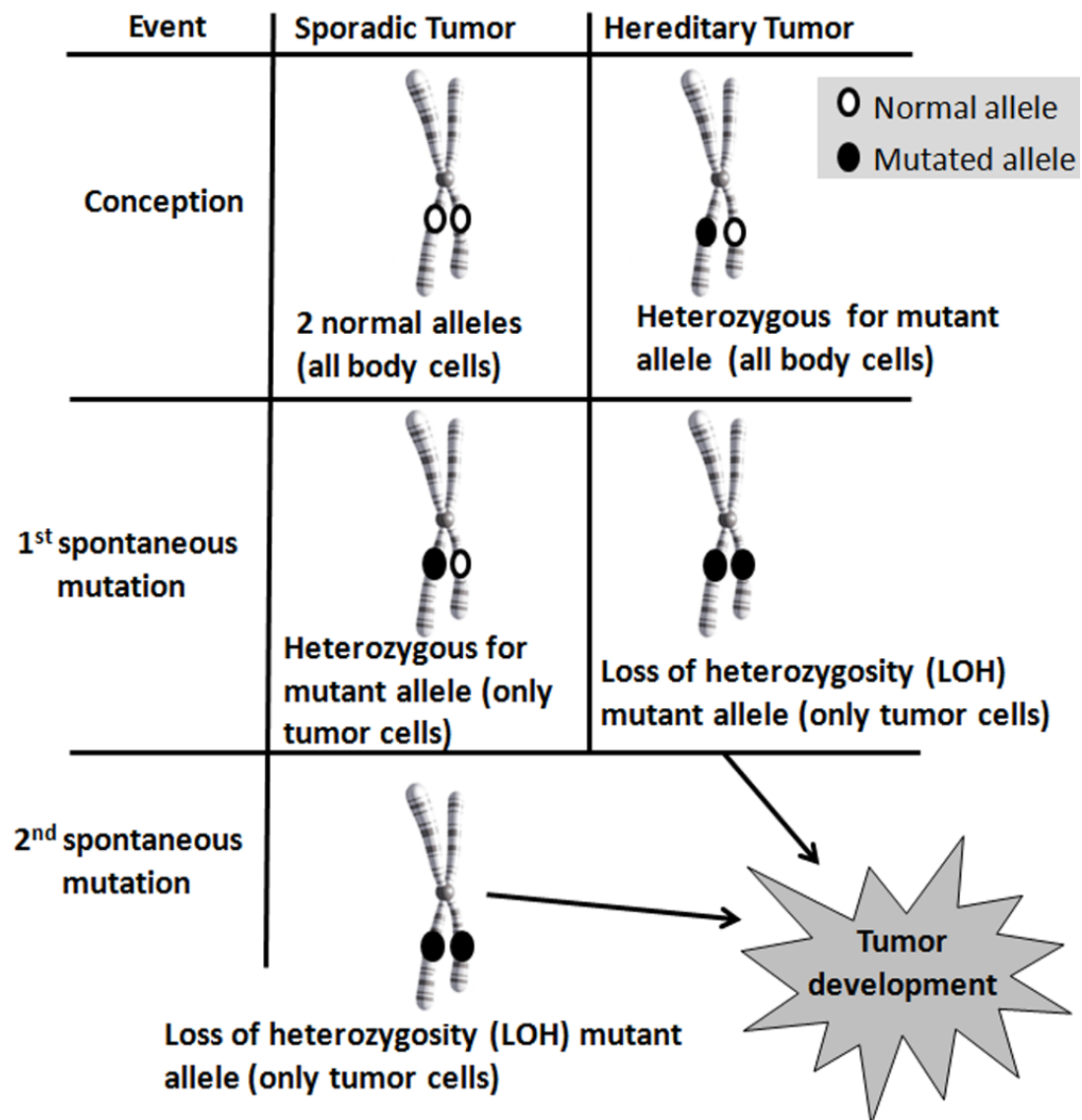


Figure 15. Principle of loss of heterozygosity (LOH). *Redrawn from reference [208].*

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).

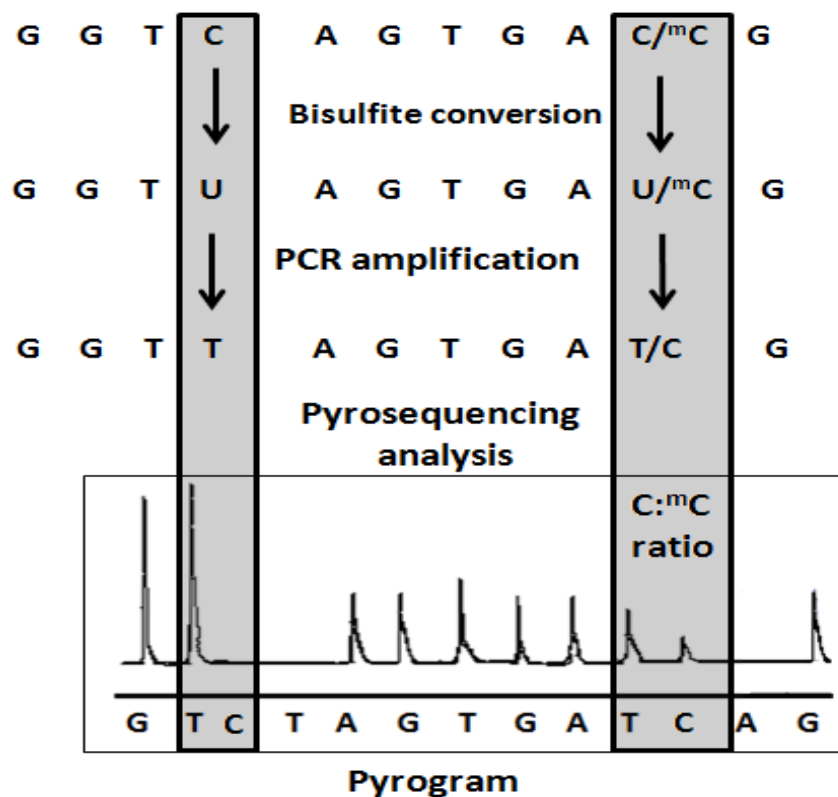


Figure 16. Bisulfite modification and subsequent processes in Pyrosequencing.
Redrawn from Pyrosequencing database, Qiagen.

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.

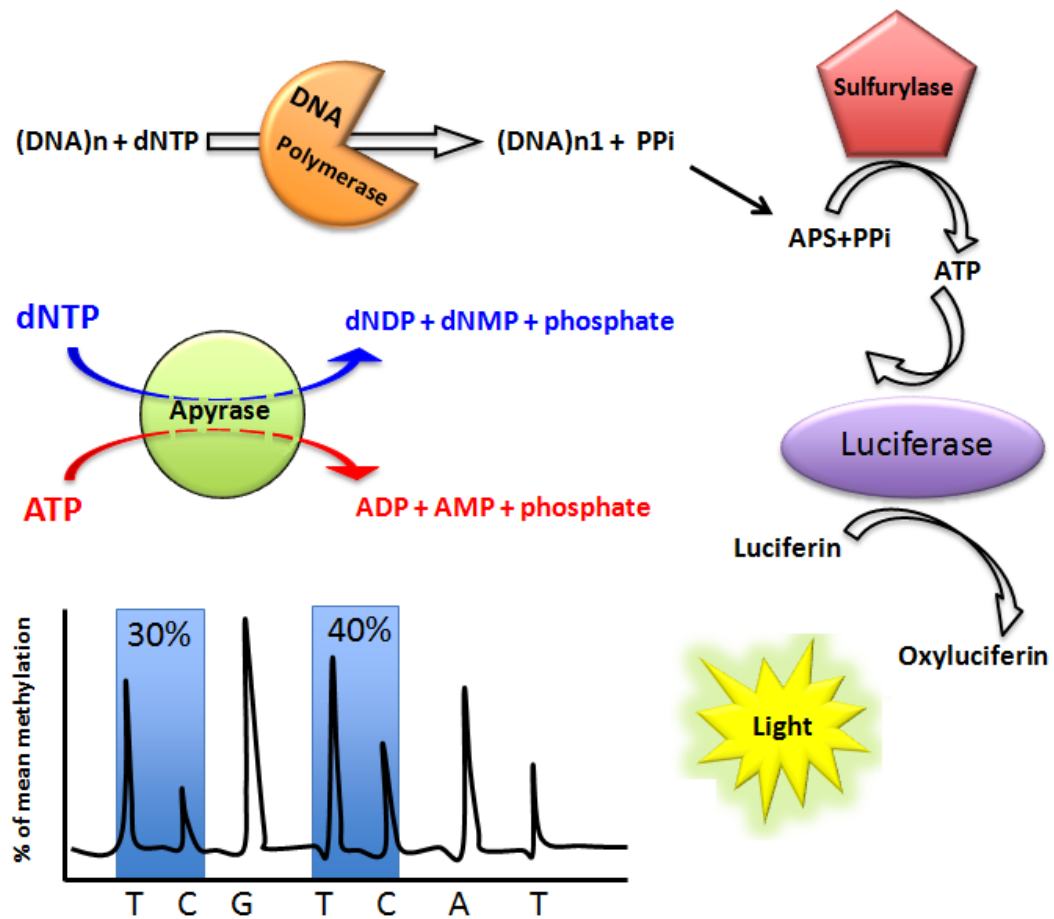


Figure 17. Principle of Pyrosequencing reaction.

Immunohistochemistry (IHC)

IHC is a well established and a widely used technique for the detection and sub-cellular 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 $5\mu\text{m}$ 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 where 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.

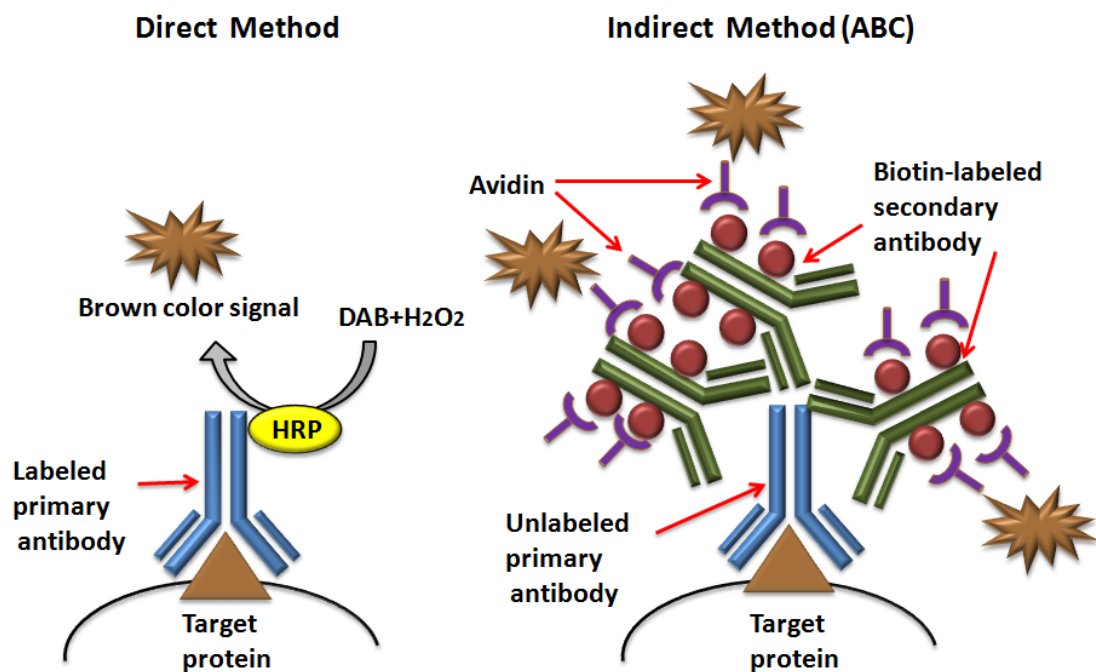


Figure 18. Immunohistochemistry (IHC). Direct and indirect methods of protein detection.

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 *CDC73* and *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 ($\Delta\Delta Ct$) [209]. In order to estimate $\Delta\Delta Ct$, 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 SYBR 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 $\Delta\Delta Ct = \Delta Ct (\text{sample}) - \Delta Ct (\text{calibrator})$, and ΔCt 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.

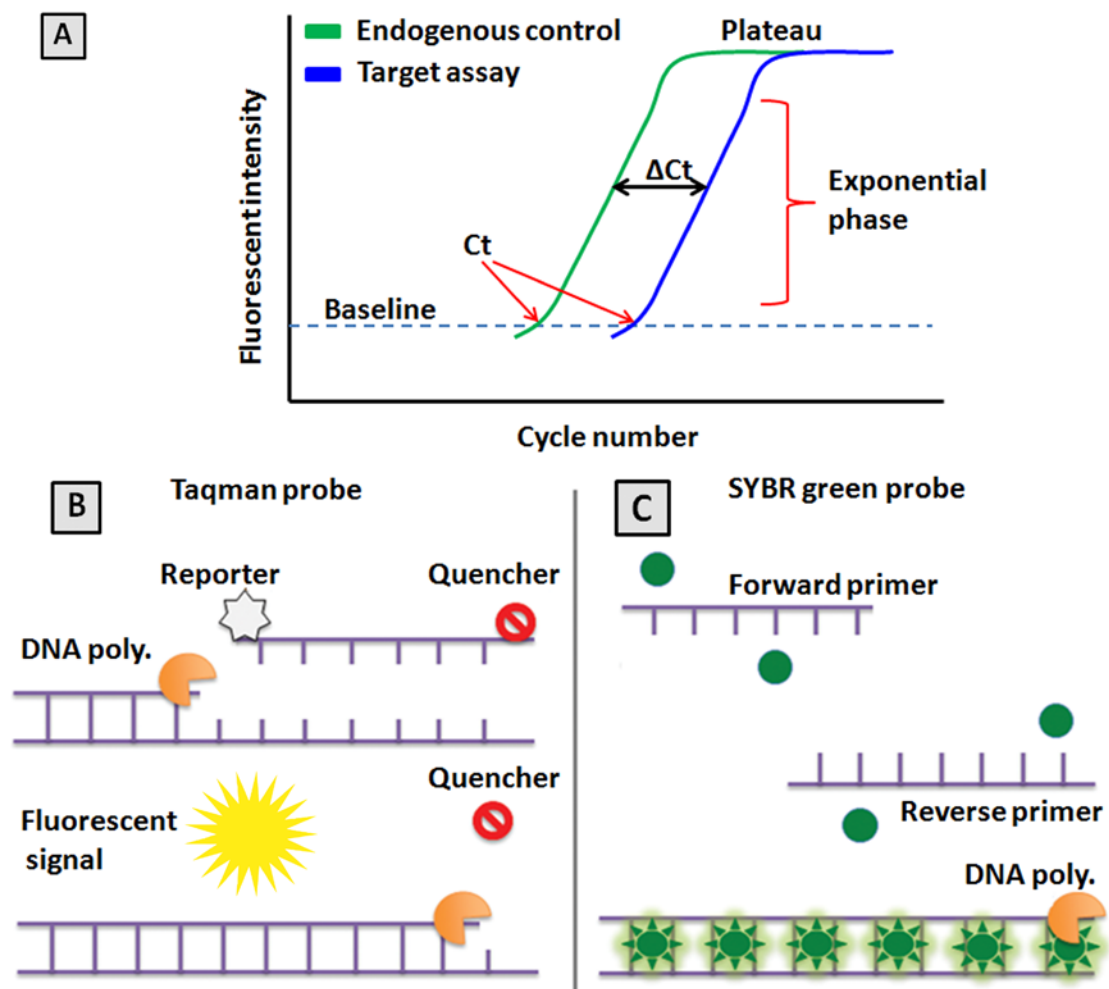


Figure 19. (A) Principle of QRT-PCR (B) Taqman probe (C) SYBR green probe.

MATERIALS

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 *APC*.

Study III

The tumor panel investigated in this study included 9 parathyroid tumors with established *CDC73/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.

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.

No.	Age (years)	Sex	Diagnosis	<i>MEN1</i>	<i>CDC73</i>	Study
1	62	F	Adenoma	Wt	Wt	I and IV
2	58	F	Adenoma	Wt	Wt	I and IV
3	66	M	Adenoma	Wt	Wt	I and IV
4	74	F	Adenoma	Wt	Wt	I and IV
5	83	F	Adenoma	Wt	Wt	I and IV
6	65	F	Adenoma	Wt	Wt	I and IV
7	72	M	Adenoma	Wt	Wt	I and IV
8	54	F	Adenoma	Wt	Wt	I and IV
9	38	F	Adenoma	Wt	Wt	I and IV
10	78	F	Adenoma	Wt	Wt	I and IV
11	51	F	Adenoma	Wt	Wt	I and IV
12	66	M	Adenoma	Wt	Wt	I and IV
13	36	F	Adenoma	Mut	Wt	I and IV
14	57	F	Adenoma	Mut	Wt	I and IV
15	64	F	Adenoma	Mut	Wt	I and IV
16	37	M	Adenoma	Mut	Wt	I and IV
17	41	M	Adenoma	Wt	Mut	I and IV
18	75	F	Atypical Adenoma	Wt	Wt	I and IV
19	69	M	Atypical Adenoma	Wt	Wt	I and IV
20	59	M	Atypical Adenoma	Wt	Wt	I, II and IV
21	83	M	Adenoma	Mut	Wt	I, II and IV
22	78	F	Adenoma	Wt	Wt	II
23	59	F	Atypical Adenoma	-	-	II
24	64	M	Atypical Adenoma	-	Mut	III
25	81	F	Adenoma	-	-	IV
26	42	F	Adenoma	-	-	IV
27	70	F	Adenoma	-	-	IV
28	60	M	Adenoma	-	-	IV
29	65	F	Adenoma	-	-	IV
30	88	M	Adenoma	-	-	IV
31	60	F	Adenoma	-	-	IV
32	63	F	Adenoma	-	-	IV
33	36	M	Adenoma	-	-	IV
34	37	F	Adenoma	-	-	IV
35	52	F	Adenoma	-	-	IV
36	55	F	Adenoma	-	-	IV
37	52	F	Adenoma	-	-	IV
38	84	F	Adenoma	-	-	IV

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

Continued on next page...

No.	Age (years)	Sex	Diagnosis	<i>MEN1</i>	<i>CDC73</i>	Study
39	49	M	Adenoma	-	-	IV
40	64	F	Adenoma	-	-	IV
41	45	M	Adenoma	-	-	IV
42	60	M	Adenoma	-	-	IV
43	54	F	Adenoma	-	-	IV
44	52	F	Adenoma	-	-	IV
45	67	F	Adenoma	-	-	IV
46	63	F	Adenoma	-	-	IV
47	60	F	Adenoma	-	-	IV
48	63	M	Adenoma	-	-	IV
49	43	M	Adenoma	-	-	IV
50	56	F	Adenoma	-	-	IV
51	54	F	Adenoma	-	-	IV
52	54	F	Adenoma	-	-	IV
53	54	F	Adenoma	-	-	IV
54	57	F	Adenoma	-	-	IV
55	55	F	Adenoma	-	-	IV
56	70	F	Adenoma	-	-	IV
57	58	M	Adenoma	-	-	IV
58	75	M	Adenoma	-	-	IV
59	52	F	Adenoma	-	-	IV
60	61	F	Adenoma	-	-	IV
61	38	F	Adenoma	-	-	IV
62	43	M	Adenoma	-	-	IV
63	71	F	Adenoma	-	-	IV
64	59	F	Adenoma	-	-	IV
65	75	F	Adenoma	Wt	Wt	IV
66	60	M	Adenoma	Wt	Wt	IV
67	51	M	Adenoma	Wt	Wt	IV
68	63	M	Adenoma	Wt	Wt	IV
69	82	M	Adenoma	-	Mut	III and IV
70	46	F	Adenoma	-	Mut	III and IV
71	47	M	Adenoma	-	Mut	III and IV
72	53	F	Adenoma	-	Mut	III and IV
73	61	F	carcinoma	-	Mut	III and IV
74	54	M	carcinoma	-	Mut	III and IV
75	62	M	carcinoma	-	Mut	III and IV

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 ≥ 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-Ca²⁺, 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- κ B 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 *CDC73* gene. These results might suggest that parathyroid tumors can develop malignant features even with one remaining functional copy of *CDC73*.

Global and CDC73 promoter methylation analyses

All the tumors were unmethylated at the *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 *CDC73* gene inactivation and *CDC73* mutation itself does not induce alterations in global methylation assessed by *LINE-1* analysis.

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 *CDC73* gene mutations possibly direct the parathyroid adenomas towards a different genetic pathway.

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 *CDC73* mutational status and for which no candidate genes have been found [224, 225]. Furthermore, LOH at the *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 *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 suggest 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 توێژینه‌وه به زمانی کوردی

شیرپه نجه , یه‌کیکه له‌و کیشه هه‌ره مه‌زن و ترسناکانه ته‌ندروستیانه وه یه‌کیکه له‌ هویه سه‌ره‌کیه‌کانی مردن له‌ جیهاندا . له‌ گه‌ل له‌و هه‌موو پیشکه‌وتنه زانستیانه له‌ بواری پزیشکی و له‌و به‌ره‌وپیشچوانه‌ی له‌ چاکردنی چاره‌سه‌رکردنی هه‌ندیک جو‌ر له‌ نه‌خۆشیه‌کانی شیرپه نجه له‌و ۳۰ تا ۵۰ سائهی له‌م دوایه‌دا , تا ئیستاش پیوستیه‌کی زۆر هه‌یه به‌ باشت‌کردنی که‌رسته‌ی ده‌ستنی‌شان‌کردنی نه‌خۆشیه‌که وه باشت‌کردنی ریگای چاره‌سه‌رکردنی . تا به‌شداریی بکه‌ین له‌م بواره , ئیمه هه‌ستاین به‌ پشکنینی ژماره‌کی زۆری له‌و شیرپه‌نجانیه‌ی که‌وا تووشی گلاندی پارا‌تایروید ده‌بن وه‌ک نمونه‌ک له‌ شیرپه نجه به‌ ئامانجی باشت‌تی‌گه‌یشتن له‌و میکانیزمانه‌ی که‌وا شیرپه نجه‌ی پی‌دروست ده‌بی‌ت و به‌م شیوه‌یه‌ش بو‌گه‌یشتن به‌ چاره‌سه‌ری باشت‌ری له‌و نه‌خۆشیه .

وه تا بتوانین له‌و نه‌رکه‌ی که‌وا له‌سه‌ره‌وه ئاماژه‌مان پیکرد نه‌نجام ده‌ین , هه‌ستاین به‌ پشکنینی چری ژماره‌کی یه‌کجار زۆر له‌ شیرپه نجه بی‌ زیان و به‌زیانه‌کان ی گلاندی

پارائايرۆيد له بنكهى تويژينهوهى شيرپه نجه له نه خوشخانهى زانكوى كارولينسكا له

ستوكهولم , سويد.

دوو جور له ميكانيزمى پيدا بوون و تهشه نه كردنى شيرپه نجه جيگه بايه خدان و مهيلي

ئيمه بوو . درسه تگردنى ميكانيزمى بوماوهيى يارمه تيمان ديدات له دوزينه وهى ئهو

پازدانانهى (گورانكاريانهى له ريزبه ندى دى ان اى) كهوا بويان ههيه هوى پيدا بوونى

شيرپه نجه بن . له لايهكى ترهوه , درسه تگردنى ايبيجينييتك , كه تيبايدا ئهو

ميكانيزمانه دهگريتهوه كه گورانكارى ئه نجامدهدن له جينه كان بى هيچ گورانكاريبهك

له ريزبه ندى دى ان اى.

ئهو دوو ميكانيزمه تهواو كهرى يهكترن . لهو تويژينه وانهى ئيمه ئه نجامان دان توانيمان

زانيارى زور گرنگ و نوو به دهست بهينين كهوا يارمه تى دهرن له جيا كردنه وهى شيرپه نجهى

به زيان و بى زيان وه ههروهها هه لبراردنى باشترين شيوازى چاره سه ركردن به گويرهى

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REFERENCES

1. Eknoyan, G., *A history of the parathyroid glands*. American journal of kidney diseases : the official journal of the National Kidney Foundation, 1995. **26**(5): p. 801-7.
2. Taylor, S., *Hyperparathyroidism: retrospect and prospect*. Ann R Coll Surg Engl, 1976. **58**(4): p. 255-65.
3. DeLellis, R.A., *Tumors of the parathyroid gland*. 1993: American Registry of Pathology.
4. Seipel, C.M., *An English translation of Sandström's glandulae parathyroideae*. Bull Inst Hist Med, 1938. **6**: p. 192-222.
5. Thompson, R.L. and D.L. Harris, *A Consideration of the Pathological Histology of the Parathyroid glandules, and a Report of a Parathyroid-Like tumor*. J Med Res, 1908. **19**(1): p. 135-152 1.
6. Akerström, G., J. Malmaeus, and R. Bergstrom, *Surgical anatomy of human parathyroid glands*. Surgery, 1984. **95**(1): p. 14-21.
7. Fancy, T., D. Gallagher, 3rd, and J.D. Hornig, *Surgical anatomy of the thyroid and parathyroid glands*. Otolaryngologic clinics of North America, 2010. **43**(2): p. 221-7, vii.
8. Yao, K., et al., *Weight of normal parathyroid glands in patients with parathyroid adenomas*. Journal of Clinical Endocrinology & Metabolism, 2004. **89**(7): p. 3208-3213.
9. Policeni, B.A., W.R. Smoker, and D.L. Reede, *Anatomy and embryology of the thyroid and parathyroid glands*. Semin Ultrasound CT MR, 2012. **33**(2): p. 104-14.
10. Wenig, B.M., *Embryology, anatomy, and histology*, in *Atlas Of Head And Neck Pathology*. 2008, Elsevier Health Sciences. p. 1012-1028.
11. Mohebati, A. and A.R. Shaha, *Anatomy of thyroid and parathyroid glands and neurovascular relations*. Clin Anat, 2012. **25**(1): p. 19-31.
12. Gomes, E.M., et al., *Ectopic and extranumerary parathyroid glands location in patients with hyperparathyroidism secondary to end stage renal disease*. Acta Cir Bras, 2007. **22**(2): p. 105-9.
13. Tanaka, Y., et al., *Oxyphil cell function in secondary parathyroid hyperplasia*. Nephron, 1996. **73**(4): p. 580-6.

14. Schmid, K.W., et al., *An immunological study on chromogranin A and B in human endocrine and nervous tissues*. Histochem J, 1989. **21**(6): p. 365-73.
15. DeLellis, R.A., *Parathyroid tumors and related disorders*. Mod Pathol, 2011. **24 Suppl 2**: p. S78-93.
16. Malberti, F., M. Farina, and E. Imbasciati, *The PTH-calcium curve and the set point of calcium in primary and secondary hyperparathyroidism*. Nephrol Dial Transplant, 1999. **14**(10): p. 2398-406.
17. Brown, E.M., et al., *Calcium-ion-sensing cell-surface receptors*. N Engl J Med, 1995. **333**(4): p. 234-40.
18. Tfelt-Hansen, J. and E.M. Brown, *The calcium-sensing receptor in normal physiology and pathophysiology: a review*. Crit Rev Clin Lab Sci, 2005. **42**(1): p. 35-70.
19. Fraser, W.D., *Hyperparathyroidism*. Lancet, 2009. **374**(9684): p. 145-58.
20. DeLellis RA, et al., *Pathology and Genetics of the Tumours of Endocrine Organs, WHO Classification of Tumours*. 2004: Lyon: IARC Press.
21. Melton, L.J., 3rd, *The epidemiology of primary hyperparathyroidism in North America*. J Bone Miner Res, 2002. **17 Suppl 2**: p. N12-7.
22. Lundgren, E., et al., *Primary hyperparathyroidism revisited in menopausal women with serum calcium in the upper normal range at population-based screening 8 years ago*. World J Surg, 2002. **26**(8): p. 931-6.
23. Wermers, R.A., et al., *Incidence of Primary Hyperparathyroidism in Rochester, Minnesota, 1993–2001: An Update on the Changing Epidemiology of the Disease*. Journal of Bone and Mineral Research, 2006. **21**(1): p. 171-177.
24. Mackenzie-Feder, J., et al., *Primary hyperparathyroidism: an overview*. Int J Endocrinol, 2011. **2011**: p. 251410.
25. Ljunghall, S., et al., *Primary hyperparathyroidism: epidemiology, diagnosis and clinical picture*. World J Surg, 1991. **15**(6): p. 681-7.
26. Silverberg, S.J., *Non-classical target organs in primary hyperparathyroidism*. J Bone Miner Res, 2002. **17 Suppl 2**: p. N117-25.
27. Rejnmark, L., P. Vestergaard, and L. Mosekilde, *Nephrolithiasis and renal calcifications in primary hyperparathyroidism*. J Clin Endocrinol Metab, 2011. **96**(8): p. 2377-85.
28. Mazzaglia, P.J., et al., *The changing presentation of hyperparathyroidism over 3 decades*. Arch Surg, 2008. **143**(3): p. 260-6.

29. Khosla, S., et al., *Primary hyperparathyroidism and the risk of fracture: a population-based study*. J Bone Miner Res, 1999. **14**(10): p. 1700-7.
30. Solomon, B.L., M. Schaaf, and R.C. Smallridge, *Psychologic symptoms before and after parathyroid surgery*. Am J Med, 1994. **96**(2): p. 101-6.
31. Stefanelli, T., et al., *Cardiac abnormalities in patients with primary hyperparathyroidism: implications for follow-up*. J Clin Endocrinol Metab, 1997. **82**(1): p. 106-12.
32. Stefanelli, T., et al., *Primary hyperparathyroidism: incidence of cardiac abnormalities and partial reversibility after successful parathyroidectomy*. Am J Med, 1993. **95**(2): p. 197-202.
33. Parfitt, A.M., D.S. Rao, and M. Kleerekoper, *Asymptomatic primary hyperparathyroidism discovered by multichannel biochemical screening: clinical course and considerations bearing on the need for surgical intervention*. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research, 1991. **6 Suppl 2**: p. S97-101; discssion S121-4.
34. Bilezikian, J.P., M. Rubin, and S.J. Silverberg, *Asymptomatic primary hyperparathyroidism*. Arq Bras Endocrinol Metabol, 2006. **50**(4): p. 647-56.
35. Juppner, H. and J.T. Potts, Jr., *Immunoassays for the detection of parathyroid hormone*. J Bone Miner Res, 2002. **17 Suppl 2**: p. N81-6.
36. Harinarayan, C.V., N. Gupta, and N. Kochupillai, *Vitamin D status in primary hyperparathyroidism in India*. Clin Endocrinol (Oxf), 1995. **43**(3): p. 351-8.
37. Grey, A., et al., *Vitamin D repletion in patients with primary hyperparathyroidism and coexistent vitamin D insufficiency*. J Clin Endocrinol Metab, 2005. **90**(4): p. 2122-6.
38. Allendorf, J., et al., *The impact of sestamibi scanning on the outcome of parathyroid surgery*. J Clin Endocrinol Metab, 2003. **88**(7): p. 3015-8.
39. Bilezikian, J.P., A.A. Khan, and J.T. Potts, Jr., *Guidelines for the management of asymptomatic primary hyperparathyroidism: summary statement from the third international workshop*. The Journal of clinical endocrinology and metabolism, 2009. **94**(2): p. 335-9.
40. Irvin, G.L., 3rd, C.C. Solorzano, and D.M. Carneiro, *Quick intraoperative parathyroid hormone assay: surgical adjunct to allow limited parathyroidectomy, improve success rate, and predict outcome*. World J Surg, 2004. **28**(12): p. 1287-92.

41. Inabnet, W.B., *Intraoperative parathyroid hormone monitoring*. World J Surg, 2004. **28**(12): p. 1212-5.
42. Carneiro, D.M., et al., *Comparison of intraoperative iPTH assay (QPTH) criteria in guiding parathyroidectomy: which criterion is the most accurate?* Surgery, 2003. **134**(6): p. 973-9; discussion 979-81.
43. Inabnet, W.B., 3rd, et al., *Targeted parathyroidectomy in the era of intraoperative parathormone monitoring*. World J Surg, 2002. **26**(8): p. 921-5.
44. Silverberg, S.J. and J.P. Bilezikian, *The diagnosis and management of asymptomatic primary hyperparathyroidism*. Nat Clin Pract Endocrinol Metab, 2006. **2**(9): p. 494-503.
45. Nomura, R., et al., *Marked and sustained increase in bone mineral density after parathyroidectomy in patients with primary hyperparathyroidism; a six-year longitudinal study with or without parathyroidectomy in a Japanese population*. Clin Endocrinol (Oxf), 2004. **60**(3): p. 335-42.
46. Vestergaard, P. and L. Mosekilde, *Parathyroid surgery is associated with a decreased risk of hip and upper arm fractures in primary hyperparathyroidism: a controlled cohort study*. J Intern Med, 2004. **255**(1): p. 108-14.
47. Silverberg, S.J., *Vitamin D deficiency and primary hyperparathyroidism*. J Bone Miner Res, 2007. **22 Suppl 2**: p. V100-4.
48. Silverberg, S.J., et al., *A 10-year prospective study of primary hyperparathyroidism with or without parathyroid surgery*. N Engl J Med, 1999. **341**(17): p. 1249-55.
49. Cunningham, J., F. Locatelli, and M. Rodriguez, *Secondary hyperparathyroidism: pathogenesis, disease progression, and therapeutic options*. Clinical journal of the American Society of Nephrology : CJASN, 2011. **6**(4): p. 913-21.
50. Rodriguez, M., E. Nemeth, and D. Martin, *The calcium-sensing receptor: a key factor in the pathogenesis of secondary hyperparathyroidism*. Am J Physiol Renal Physiol, 2005. **288**(2): p. F253-64.
51. Rodriguez, M., et al., *Regulation of parathyroid function in chronic renal failure*. J Bone Miner Metab, 2006. **24**(2): p. 164-8.
52. Ahmad, R. and J.M. Hammond, *Primary, secondary, and tertiary hyperparathyroidism*. Otolaryngol Clin North Am, 2004. **37**(4): p. 701-13, vii-viii.

53. Harris, S.S., E. Soteriades, and B. Dawson-Hughes, *Secondary hyperparathyroidism and bone turnover in elderly blacks and whites*. J Clin Endocrinol Metab, 2001. **86**(8): p. 3801-4.
54. Thomas, M.K., et al., *Hypovitaminosis D in medical inpatients*. N Engl J Med, 1998. **338**(12): p. 777-83.
55. Schussheim, D.H., et al., *Multiple endocrine neoplasia type 1: new clinical and basic findings*. Trends Endocrinol Metab, 2001. **12**(4): p. 173-8.
56. Arnold, A., S.J. Marx, and Asbmr, *Chapter 76. Familial Hyperparathyroidism (Including MEN, FHH, and HPT-JT)*, in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. 2009, John Wiley & Sons, Inc. p. 361-366.
57. Hellman, P., et al., *Primary and reoperative parathyroid operations in hyperparathyroidism of multiple endocrine neoplasia type 1*. Surgery, 1998. **124**(6): p. 993-9.
58. Rizzoli, R., J. Green, 3rd, and S.J. Marx, *Primary hyperparathyroidism in familial multiple endocrine neoplasia type 1. Long-term follow-up of serum calcium levels after parathyroidectomy*. Am J Med, 1985. **78**(3): p. 467-74.
59. Larsson, C., et al., *Multiple endocrine neoplasia type 1 gene maps to chromosome 11 and is lost in insulinoma*. Nature, 1988. **332**(6159): p. 85-7.
60. Marini, F., et al., *Multiple Endocrine Neoplasia Type 1 (MEN1) Syndrome*. 2009.
61. Delellis, R.A., *Challenging lesions in the differential diagnosis of endocrine tumors: parathyroid carcinoma*. Endocr Pathol, 2008. **19**(4): p. 221-5.
62. Mulligan, L.M., et al., *Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A*. Nature, 1993. **363**(6428): p. 458-60.
63. DeLellis, R.A., *Multiple endocrine neoplasia syndromes revisited. Clinical, morphologic, and molecular features*. Lab Invest, 1995. **72**(5): p. 494-505.
64. Chen, J.D., et al., *Hyperparathyroidism-jaw tumour syndrome*. J Intern Med, 2003. **253**(6): p. 634-42.
65. Bradley, K.J., et al., *Uterine tumours are a phenotypic manifestation of the hyperparathyroidism-jaw tumour syndrome*. J Intern Med, 2005. **257**(1): p. 18-26.
66. Carpten, J.D., et al., *HRPT2, encoding parafibromin, is mutated in hyperparathyroidism-jaw tumor syndrome*. Nat Genet, 2002. **32**(4): p. 676-80.

67. Krebs, L.J., T.M. Shattuck, and A. Arnold, *HRPT2 mutational analysis of typical sporadic parathyroid adenomas*. J Clin Endocrinol Metab, 2005. **90**(9): p. 5015-7.
68. Shattuck, T.M., et al., *Somatic and germ-line mutations of the HRPT2 gene in sporadic parathyroid carcinoma*. N Engl J Med, 2003. **349**(18): p. 1722-9.
69. Cetani, F., et al., *Genetic analyses of the HRPT2 gene in primary hyperparathyroidism: germline and somatic mutations in familial and sporadic parathyroid tumors*. The Journal of clinical endocrinology and metabolism, 2004. **89**(11): p. 5583-91.
70. Marx, S.J., et al., *The hypocalciuric or benign variant of familial hypercalcemia: clinical and biochemical features in fifteen kindreds*. Medicine (Baltimore), 1981. **60**(6): p. 397-412.
71. Law, W.M., Jr. and H. Heath, 3rd, *Familial benign hypercalcemia (hypocalciuric hypercalcemia). Clinical and pathogenetic studies in 21 families*. Ann Intern Med, 1985. **102**(4): p. 511-9.
72. Heath, H., 3rd, et al., *Genetic linkage analysis in familial benign (hypocalciuric) hypercalcemia: evidence for locus heterogeneity*. Am J Hum Genet, 1993. **53**(1): p. 193-200.
73. Lloyd, S.E., et al., *Localization of familial benign hypercalcemia, Oklahoma variant (FBHOk), to chromosome 19q13*. Am J Hum Genet, 1999. **64**(1): p. 189-95.
74. Pollak, M.R., et al., *Mutations in the human Ca(2+)-sensing receptor gene cause familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism*. Cell, 1993. **75**(7): p. 1297-303.
75. Simonds, W.F., et al., *Familial isolated hyperparathyroidism: clinical and genetic characteristics of 36 kindreds*. Medicine (Baltimore), 2002. **81**(1): p. 1-26.
76. Simonds, W.F., et al., *Familial isolated hyperparathyroidism is rarely caused by germline mutation in HRPT2, the gene for the hyperparathyroidism-jaw tumor syndrome*. The Journal of clinical endocrinology and metabolism, 2004. **89**(1): p. 96-102.
77. Warner, J.V., et al., *Familial isolated hyperparathyroidism is linked to a 1.7 Mb region on chromosome 2p13.3-14*. J Med Genet, 2006. **43**(3): p. e12.

78. Pearce, S.H., et al., *A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor*. N Engl J Med, 1996. **335**(15): p. 1115-22.
79. Sunthornthepvarakul, T., S. Churesigaew, and S. Ngowngarmratana, *A novel mutation of the signal peptide of the preproparathyroid hormone gene associated with autosomal recessive familial isolated hypoparathyroidism*. J Clin Endocrinol Metab, 1999. **84**(10): p. 3792-6.
80. Rubin, M.R. and J.P. Bilezikian, *Hypoparathyroidism: clinical features, skeletal microstructure and parathyroid hormone replacement*. Arq Bras Endocrinol Metabol, 2010. **54**(2): p. 220-6.
81. Shoback, D., *Clinical practice. Hypoparathyroidism*. N Engl J Med, 2008. **359**(4): p. 391-403.
82. Verdonk, C.A. and A.J. Edis, *Parathyroid "double adenomas": fact of fiction?* Surgery, 1981. **90**(3): p. 523-6.
83. Almquist, M., et al., *Changing biochemical presentation of primary hyperparathyroidism*. Langenbecks Arch Surg, 2010. **395**(7): p. 925-8.
84. Power, C., et al., *Unusual presentation of a giant parathyroid adenoma: report of a case*. Surgery today, 2005. **35**(3): p. 235-7.
85. Carlson, D., *Parathyroid pathology: hyperparathyroidism and parathyroid tumors*. Arch Pathol Lab Med, 2010. **134**(11): p. 1639-44.
86. DeLellis, R.A., *Parathyroid carcinoma: an overview*. Adv Anat Pathol, 2005. **12**(2): p. 53-61.
87. Brown, S., et al., *Parathyroid carcinoma: increasing incidence and changing presentation*. ANZ J Surg, 2011. **81**(7-8): p. 528-32.
88. Rodriguez, C., et al., *Parathyroid carcinoma: a difficult histological diagnosis*. Eur Ann Otorhinolaryngol Head Neck Dis, 2012. **129**(3): p. 157-9.
89. Juhlin, C.C., et al., *Parafibromin immunoreactivity: its use as an additional diagnostic marker for parathyroid tumor classification*. Endocr Relat Cancer, 2007. **14**(2): p. 501-12.
90. Tan, M.H., et al., *Loss of parafibromin immunoreactivity is a distinguishing feature of parathyroid carcinoma*. Clin Cancer Res, 2004. **10**(19): p. 6629-37.
91. Juhlin, C.C., et al., *Parafibromin and APC as screening markers for malignant potential in atypical parathyroid adenomas*. Endocr Pathol, 2010. **21**(3): p. 166-77.

92. Abbona, G.C., et al., *Proliferative activity in parathyroid tumors as detected by Ki-67 immunostaining*. Hum Pathol, 1995. **26**(2): p. 135-8.
93. Johnson, S.J., *Changing clinicopathological practice in parathyroid disease*. Histopathology, 2010. **56**(7): p. 835-51.
94. Seethala, R.R., M.A. Virji, and J. Ogilvie, *Pathology of the Parathyroid Glands*, in *Surgical Pathology of the Head and Neck*. 2008. p. 1429-1474.
95. Parfitt, A.M., et al., *Cell kinetics in parathyroid adenomas: evidence for decline in rates of cell birth and tumour growth, assuming clonal origin*. Clinical endocrinology, 1991. **35**(2): p. 151-7.
96. Arnold, A., et al., *Monoclonality and abnormal parathyroid hormone genes in parathyroid adenomas*. The New England journal of medicine, 1988. **318**(11): p. 658-62.
97. Chandrasekharappa, S.C., et al., *Positional cloning of the gene for multiple endocrine neoplasia-type 1*. Science, 1997. **276**(5311): p. 404-7.
98. Lemmens, I., et al., *Identification of the multiple endocrine neoplasia type 1 (MEN1) gene*. The European Consortium on MEN1. Hum Mol Genet, 1997. **6**(7): p. 1177-83.
99. Miedlich, S., et al., *Frequency of somatic MEN1 gene mutations in monoclonal parathyroid tumours of patients with primary hyperparathyroidism*. Eur J Endocrinol, 2000. **143**(1): p. 47-54.
100. Farnebo, F., et al., *Alterations of the MEN1 gene in sporadic parathyroid tumors*. J Clin Endocrinol Metab, 1998. **83**(8): p. 2627-30.
101. Newey, P.J., et al., *Whole-exome sequencing studies of nonhereditary (sporadic) parathyroid adenomas*. J Clin Endocrinol Metab, 2012. **97**(10): p. E1995-2005.
102. Guo, S.S. and M.P. Sawicki, *Molecular and genetic mechanisms of tumorigenesis in multiple endocrine neoplasia type-1*. Mol Endocrinol, 2001. **15**(10): p. 1653-64.
103. Starker, L.F., et al., *Frequent germ-line mutations of the MEN1, CASR, and HRPT2/CDC73 genes in young patients with clinically non-familial primary hyperparathyroidism*. Horm Cancer, 2012. **3**(1-2): p. 44-51.
104. Carling, T., et al., *Parathyroid MEN1 gene mutations in relation to clinical characteristics of nonfamilial primary hyperparathyroidism*. J Clin Endocrinol Metab, 1998. **83**(8): p. 2960-3.

105. Dwight, T., et al., *Loss of heterozygosity in sporadic parathyroid tumours: involvement of chromosome 1 and the MEN1 gene locus in 11q13*. Clin Endocrinol (Oxf), 2000. **53**(1): p. 85-92.
106. Dionisi, S., et al., *Concurrent parathyroid adenomas and carcinoma in the setting of multiple endocrine neoplasia type 1: presentation as hypercalcemic crisis*. Mayo Clin Proc, 2002. **77**(8): p. 866-9.
107. Shih, R.Y., et al., *Parathyroid carcinoma in multiple endocrine neoplasia type 1 with a classic germline mutation*. Endocr Pract, 2009. **15**(6): p. 567-72.
108. Haven, C.J., et al., *Identification of MEN1 and HRPT2 somatic mutations in paraffin-embedded (sporadic) parathyroid carcinomas*. Clinical Endocrinology, 2007. **67**(3): p. 370-376.
109. Agha, A., et al., *Parathyroid carcinoma in multiple endocrine neoplasia type 1 (MEN1) syndrome: two case reports of an unrecognised entity*. J Endocrinol Invest, 2007. **30**(2): p. 145-9.
110. Sharretts, J.M. and W.F. Simonds, *Clinical and molecular genetics of parathyroid neoplasms*. Best Pract Res Clin Endocrinol Metab, 2010. **24**(3): p. 491-502.
111. Agarwal, S.K., et al., *Menin molecular interactions: insights into normal functions and tumorigenesis*. Horm Metab Res, 2005. **37**(6): p. 369-74.
112. Agarwal, S.K., et al., *Menin interacts with the AP1 transcription factor JunD and represses JunD-activated transcription*. Cell, 1999. **96**(1): p. 143-52.
113. Agarwal, S.K., et al., *Transcription factor JunD, deprived of menin, switches from growth suppressor to growth promoter*. Proc Natl Acad Sci U S A, 2003. **100**(19): p. 10770-5.
114. Kim, H., et al., *Menin, a tumor suppressor, represses JunD-mediated transcriptional activity by association with an mSin3A-histone deacetylase complex*. Cancer Res, 2003. **63**(19): p. 6135-9.
115. Huang, J., et al., *The same pocket in menin binds both MLL and JUND but has opposite effects on transcription*. Nature, 2012. **482**(7386): p. 542-6.
116. Hughes, C.M., et al., *Menin associates with a trithorax family histone methyltransferase complex and with the hoxc8 locus*. Mol Cell, 2004. **13**(4): p. 587-97.
117. Kaji, H., et al., *Inactivation of menin, a Smad3-interacting protein, blocks transforming growth factor type beta signaling*. Proc Natl Acad Sci U S A, 2001. **98**(7): p. 3837-42.

118. Sowa, H., et al., *Menin inactivation leads to loss of transforming growth factor beta inhibition of parathyroid cell proliferation and parathyroid hormone secretion*. Cancer Res, 2004. **64**(6): p. 2222-8.
119. Howell, V.M., et al., *HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours*. J Med Genet, 2003. **40**(9): p. 657-63.
120. Bradley, K.J., et al., *Parafibromin mutations in hereditary hyperparathyroidism syndromes and parathyroid tumours*. Clin Endocrinol (Oxf), 2006. **64**(3): p. 299-306.
121. Guarnieri, V., et al., *CDC73 mutations and parafibromin immunohistochemistry in parathyroid tumors: clinical correlations in a single-centre patient cohort*. Cell Oncol (Dordr), 2012.
122. Masi, G., et al., *Clinical, genetic, and histopathologic investigation of CDC73-related familial hyperparathyroidism*. Endocr Relat Cancer, 2008. **15**(4): p. 1115-26.
123. Newey, P.J., et al., *Cell division cycle protein 73 homolog (CDC73) mutations in the hyperparathyroidism-jaw tumor syndrome (HPT-JT) and parathyroid tumors*. Hum Mutat, 2010. **31**(3): p. 295-307.
124. Rozenblatt-Rosen, O., et al., *The parafibromin tumor suppressor protein is part of a human Paf1 complex*. Molecular and cellular biology, 2005. **25**(2): p. 612-20.
125. Woodard, G.E., et al., *Parafibromin, product of the hyperparathyroidism-jaw tumor syndrome gene HRPT2, regulates cyclin D1/PRAD1 expression*. Oncogene, 2005. **24**(7): p. 1272-6.
126. Lin, L., et al., *The parafibromin tumor suppressor protein inhibits cell proliferation by repression of the c-myc proto-oncogene*. Proc Natl Acad Sci U S A, 2008. **105**(45): p. 17420-5.
127. Agarwal, S.K., W.F. Simonds, and S.J. Marx, *The parafibromin tumor suppressor protein interacts with actin-binding proteins actinin-2 and actinin-3*. Mol Cancer, 2008. **7**: p. 65.
128. Lin, L., et al., *Nuclear localization of the parafibromin tumor suppressor protein implicated in the hyperparathyroidism-jaw tumor syndrome enhances its proapoptotic function*. Molecular cancer research : MCR, 2007. **5**(2): p. 183-93.

129. Wang, P., et al., *Parafibromin, a component of the human PAF complex, regulates growth factors and is required for embryonic development and survival in adult mice*. Mol Cell Biol, 2008. **28**(9): p. 2930-40.
130. Arnold, A., et al., *Molecular cloning and chromosomal mapping of DNA rearranged with the parathyroid hormone gene in a parathyroid adenoma*. J Clin Invest, 1989. **83**(6): p. 2034-40.
131. Friedman, E., et al., *Genetic abnormalities in sporadic parathyroid adenomas*. The Journal of clinical endocrinology and metabolism, 1990. **71**(2): p. 293-7.
132. Mallya, S.M. and A. Arnold, *Cyclin D1 in parathyroid disease*. Front Biosci, 2000. **5**: p. D367-71.
133. Hsi, E.D., et al., *Cyclin D1/PRAD1 expression in parathyroid adenomas: an immunohistochemical study*. J Clin Endocrinol Metab, 1996. **81**(5): p. 1736-9.
134. Tominaga, Y., et al., *Expression of PRAD1/cyclin D1, retinoblastoma gene products, and Ki67 in parathyroid hyperplasia caused by chronic renal failure versus primary adenoma*. Kidney Int, 1999. **55**(4): p. 1375-83.
135. Vasef, M.A., et al., *Expression of cyclin D1 in parathyroid carcinomas, adenomas, and hyperplasias: a paraffin immunohistochemical study*. Mod Pathol, 1999. **12**(4): p. 412-6.
136. Pestell, R.G., et al., *The cyclins and cyclin-dependent kinase inhibitors in hormonal regulation of proliferation and differentiation*. Endocr Rev, 1999. **20**(4): p. 501-34.
137. Hosokawa, Y., et al., *Absence of cyclin D1/PRAD1 point mutations in human breast cancers and parathyroid adenomas and identification of a new cyclin D1 gene polymorphism*. Cancer letters, 1995. **93**(2): p. 165-70.
138. Pausova, Z., et al., *Role of the RET proto-oncogene in sporadic hyperparathyroidism and in hyperparathyroidism of multiple endocrine neoplasia type 2*. J Clin Endocrinol Metab, 1996. **81**(7): p. 2711-8.
139. Carling, T., et al., *Familial hypercalcemia and hypercalciuria caused by a novel mutation in the cytoplasmic tail of the calcium receptor*. J Clin Endocrinol Metab, 2000. **85**(5): p. 2042-7.
140. Egan, A.M., et al., *Primary hyperparathyroidism in a patient with familial hypocalciuric hypercalcaemia due to a novel mutation in the calcium-sensing receptor gene*. J Bone Miner Metab, 2012.
141. Frank-Raue, K., et al., *Inactivating calcium-sensing receptor mutations in patients with primary hyperparathyroidism*. Clin Endocrinol (Oxf), 2011.

142. Witteveen, J.E., et al., *Downregulation of CASR expression and global loss of parafibromin staining are strong negative determinants of prognosis in parathyroid carcinoma*. *Mod Pathol*, 2011. **24**(5): p. 688-97.
143. Cryns, V.L., et al., *Loss of the retinoblastoma tumor-suppressor gene in parathyroid carcinoma*. *The New England journal of medicine*, 1994. **330**(11): p. 757-61.
144. Yoshimoto, K., et al., *Role of p53 mutations in endocrine tumorigenesis: mutation detection by polymerase chain reaction-single strand conformation polymorphism*. *Cancer Res*, 1992. **52**(18): p. 5061-4.
145. Cryns, V.L., et al., *p53 abnormalities in human parathyroid carcinoma*. *The Journal of clinical endocrinology and metabolism*, 1994. **78**(6): p. 1320-4.
146. Hakim, J.P. and M.A. Levine, *Absence of p53 point mutations in parathyroid adenoma and carcinoma*. *The Journal of clinical endocrinology and metabolism*, 1994. **78**(1): p. 103-6.
147. Tamura, G., et al., *Parathyroid carcinoma with anaplastic feature: association of a p53 gene mutation with anaplastic transformation*. *Pathol Int*, 2009. **59**(2): p. 107-10.
148. Agarwal, S.K., et al., *Comparative genomic hybridization analysis of human parathyroid tumors*. *Cancer Genet Cytogenet*, 1998. **106**(1): p. 30-6.
149. Farnebo, F., et al., *Alternative genetic pathways in parathyroid tumorigenesis*. *J Clin Endocrinol Metab*, 1999. **84**(10): p. 3775-80.
150. Garcia, J.L., et al., *Chromosomal imbalances identified by comparative genomic hybridization in sporadic parathyroid adenomas*. *Eur J Endocrinol*, 2002. **146**(2): p. 209-13.
151. Hemmer, S., et al., *Deletion of 11q23 and cyclin D1 overexpression are frequent aberrations in parathyroid adenomas*. *Am J Pathol*, 2001. **158**(4): p. 1355-62.
152. Palanisamy, N., et al., *Novel chromosomal abnormalities identified by comparative genomic hybridization in parathyroid adenomas*. *J Clin Endocrinol Metab*, 1998. **83**(5): p. 1766-70.
153. Correa, P., et al., *Allelic loss in clinically and screening-detected primary hyperparathyroidism*. *Clin Endocrinol (Oxf)*, 2002. **56**(1): p. 113-7.
154. Y. Yi, et al., *Chromosome 11 genomic changes in parathyroid adenoma and hyperplasia: Array CGH, FISH, and tissue microarrays*. *Genes, Chromosomes and Cancer*, 2008. **47**(8): p. 639-648.

155. Örndal, C., et al., *Parathyroid adenoma with t(1;5)(p22;q32) as the sole clonal chromosome abnormality*. Cancer Genet Cytogenet, 1990. **48**(2): p. 225-8.
156. Sammarelli, G., et al., *A translocation t(4; 13)(q21;q14) as single clonal chromosomal abnormality in a parathyroid adenoma*. Tumori, 2007. **93**(1): p. 97-9.
157. Bird, A., *Perceptions of epigenetics*. Nature, 2007. **447**(7143): p. 396-8.
158. Esteller, M., *Epigenetics in evolution and disease*. The Lancet, 2008. **372**: p. S90-S96.
159. Esteller, M., *Cancer epigenomics: DNA methylomes and histone-modification maps*. Nat Rev Genet, 2007. **8**(4): p. 286-98.
160. Crews, D. and J.A. McLachlan, *Epigenetics, evolution, endocrine disruption, health, and disease*. Endocrinology, 2006. **147**(6 Suppl): p. S4-10.
161. Richardson, B., *Primer: epigenetics of autoimmunity*. Nat Clin Pract Rheumatol, 2007. **3**(9): p. 521-7.
162. Doi, A., et al., *Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts*. Nat Genet, 2009. **41**(12): p. 1350-3.
163. Saxonov, S., P. Berg, and D.L. Brutlag, *A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters*. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(5): p. 1412-7.
164. Goelz, S.E., et al., *Hypomethylation of DNA from benign and malignant human colon neoplasms*. Science, 1985. **228**(4696): p. 187-90.
165. Wilson, A.S., B.E. Power, and P.L. Molloy, *DNA hypomethylation and human diseases*. Biochim Biophys Acta, 2007. **1775**(1): p. 138-62.
166. Bird, A., *DNA methylation patterns and epigenetic memory*. Genes Dev, 2002. **16**(1): p. 6-21.
167. Baylin, S.B., et al., *Alterations in DNA methylation: A fundamental aspect of neoplasia*, in *Advances in Cancer Research*, Vol 72. 1998. p. 141-196.
168. Ehrlich, M., *DNA methylation in cancer: too much, but also too little*. Oncogene, 2002. **21**(35): p. 5400-13.
169. Sandelin, K., et al., *Prognostic factors in parathyroid cancer: a review of 95 cases*. World J Surg, 1992. **16**(4): p. 724-31.
170. Flye, M.W. and M.F. Brennan, *Surgical resection of metastatic parathyroid carcinoma*. Ann Surg, 1981. **193**(4): p. 425-35.

171. Herman, J.G., et al., *Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma*. Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9700-4.
172. Yu, J., et al., *Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis*. BMC Cancer, 2002. **2**: p. 29.
173. Irizarry, R.A., et al., *The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores*. Nat Genet, 2009. **41**(2): p. 178-86.
174. Kuroda, A., et al., *Insulin gene expression is regulated by DNA methylation*. PLoS One, 2009. **4**(9): p. e6953.
175. Esteller, M., *Epigenetic gene silencing in cancer: the DNA hypermethylome*. Human Molecular Genetics, 2007. **16**: p. R50-R59.
176. Lopez-Serra, L. and M. Esteller, *Proteins that bind methylated DNA and human cancer: reading the wrong words*. Br J Cancer, 2008. **98**(12): p. 1881-5.
177. Goll, M.G. and T.H. Bestor, *Eukaryotic cytosine methyltransferases*. Annu Rev Biochem, 2005. **74**: p. 481-514.
178. Bostick, M., et al., *UHRF1 plays a role in maintaining DNA methylation in mammalian cells*. Science, 2007. **317**(5845): p. 1760-4.
179. Szyf, M., *Targeting DNA methylation in cancer*. Bull Cancer, 2006. **93**(9): p. 961-72.
180. Strahl, B.D. and C.D. Allis, *The language of covalent histone modifications*. Nature, 2000. **403**(6765): p. 41-5.
181. Jenuwein, T. and C.D. Allis, *Translating the histone code*. Science, 2001. **293**(5532): p. 1074-80.
182. Hake, S.B., A. Xiao, and C.D. Allis, *Linking the epigenetic 'language' of covalent histone modifications to cancer*. Br J Cancer, 2007. **96 Suppl**: p. R31-9.
183. Seligson, D.B., et al., *Global levels of histone modifications predict prognosis in different cancers*. Am J Pathol, 2009. **174**(5): p. 1619-28.
184. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function*. Cell, 2004. **116**(2): p. 281-97.
185. Gu, S. and M.A. Kay, *How do miRNAs mediate translational repression?* Silence, 2010. **1**(1): p. 11.

186. Bushati, N. and S.M. Cohen, *microRNA functions*. Annu Rev Cell Dev Biol, 2007. **23**: p. 175-205.
187. Iorio, M.V. and C.M. Croce, *microRNA involvement in human cancer*. Carcinogenesis, 2012. **33**(6): p. 1126-33.
188. Ng, E.K., et al., *MicroRNAs as New Players for Diagnosis, Prognosis, and Therapeutic Targets in Breast Cancer*. J Oncol, 2009. **2009**: p. 305420.
189. Chen, C.Z., *MicroRNAs as oncogenes and tumor suppressors*. N Engl J Med, 2005. **353**(17): p. 1768-71.
190. Levine, M.A., et al., *Tissue and gene specific hypomethylation of the human parathyroid hormone gene: association with parathyroid hormone gene expression in parathyroid glands*. Endocrinology, 1986. **119**(4): p. 1618-24.
191. Hahn, M.A., et al., *CDC73/HRPT2 CpG island hypermethylation and mutation of 5'-untranslated sequence are uncommon mechanisms of silencing parafibromin in parathyroid tumors*. Endocrine-Related Cancer, 2010. **17**(1): p. 273-282.
192. Mizusawa, N., et al., *Genetic analyses in patients with familial isolated hyperparathyroidism and hyperparathyroidism-jaw tumour syndrome*. Clin Endocrinol (Oxf), 2006. **65**(1): p. 9-16.
193. Hewitt, K.M., et al., *Aberrant methylation of the HRPT2 gene in parathyroid carcinoma*. Annals of Otology Rhinology and Laryngology, 2007. **116**(12): p. 928-933.
194. Juhlin, C.C., et al., *Frequent Promoter Hypermethylation of the APC and RASSF1A Tumour Suppressors in Parathyroid Tumours*. Plos One, 2010. **5**(3): p. 10.
195. Starker, L.F., et al., *The DNA methylome of benign and malignant parathyroid tumors*. Genes Chromosomes Cancer, 2011. **50**(9): p. 735-45.
196. Rahbari, R., et al., *Identification of differentially expressed microRNA in parathyroid tumors*. Ann Surg Oncol, 2011. **18**(4): p. 1158-65.
197. Corbetta, S., et al., *Differential expression of microRNAs in human parathyroid carcinomas compared with normal parathyroid tissue*. Endocrine-Related Cancer, 2010. **17**(1): p. 135-146.
198. Vaira, V., et al., *The microRNA cluster C19MC is deregulated in parathyroid tumours*. J Mol Endocrinol, 2012. **49**(2): p. 115-24.
199. Smith, L.M., et al., *Fluorescence detection in automated DNA sequence analysis*. Nature, 1986. **321**(6071): p. 674-9.

200. Albertson, D.G., et al., *Chromosome aberrations in solid tumors*. Nat Genet, 2003. **34**(4): p. 369-76.
201. Callagy, G., et al., *Identification and validation of prognostic markers in breast cancer with the complementary use of array-CGH and tissue microarrays*. J Pathol, 2005. **205**(3): p. 388-96.
202. Paris, P.L., et al., *Whole genome scanning identifies genotypes associated with recurrence and metastasis in prostate tumors*. Hum Mol Genet, 2004. **13**(13): p. 1303-13.
203. Schwaenen, C., et al., *Automated array-based genomic profiling in chronic lymphocytic leukemia: development of a clinical tool and discovery of recurrent genomic alterations*. Proc Natl Acad Sci U S A, 2004. **101**(4): p. 1039-44.
204. Kallioniemi, A., et al., *Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors*. Science, 1992. **258**(5083): p. 818-21.
205. Solinas-Toldo, S., et al., *Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances*. Genes Chromosomes Cancer, 1997. **20**(4): p. 399-407.
206. Snijders, A.M., et al., *Assembly of microarrays for genome-wide measurement of DNA copy number*. Nature genetics, 2001. **29**(3): p. 263-4.
207. Greenwald, B.D., et al., *Loss of heterozygosity affecting the p53, Rb, and mcc/apc tumor suppressor gene loci in dysplastic and cancerous ulcerative colitis*. Cancer Res, 1992. **52**(3): p. 741-5.
208. Ponder, B.A., *Molecular genetics of cancer*. BMJ, 1992. **304**(6836): p. 1234-6.
209. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method*. Methods, 2001. **25**(4): p. 402-8.
210. Nolan, T., R.E. Hands, and S.A. Bustin, *Quantification of mRNA using real-time RT-PCR*. Nat Protoc, 2006. **1**(3): p. 1559-82.
211. Chiofalo, M.G., et al., *Huge parathyroid carcinoma: clinical considerations and literature review*. World J Surg Oncol, 2005. **3**: p. 39.
212. Lallanne-Mistrih, M.L., et al., *Giant parathyroid tumors: characterization of 26 glands weighing more than 3.5 grams*. Ann Chir, 2002. **127**(3): p. 198-202.
213. Juhlin, C., et al., *Loss of parafibromin expression in a subset of parathyroid adenomas*. Endocr Relat Cancer, 2006. **13**(2): p. 509-23.

214. Juhlin, C.C., et al., *Loss of expression for the Wnt pathway components adenomatous polyposis coli and glycogen synthase kinase 3-beta in parathyroid carcinomas*. Int J Oncol, 2009. **34**(2): p. 481-92.
215. Kytölä, S., et al., *Patterns of chromosomal imbalances in parathyroid carcinomas*. Am J Pathol, 2000. **157**(2): p. 579-86.
216. Reutzel, D., et al., *Genomic imbalances in 61 renal cancers from the proximal tubulus detected by comparative genomic hybridization*. Cytogenet Cell Genet, 2001. **93**(3-4): p. 221-7.
217. Kjellman, M., et al., *Genetic aberrations in adrenocortical tumors detected using comparative genomic hybridization correlate with tumor size and malignancy*. Cancer Res, 1996. **56**(18): p. 4219-23.
218. Voortman, J., et al., *Array comparative genomic hybridization-based characterization of genetic alterations in pulmonary neuroendocrine tumors*. Proc Natl Acad Sci U S A, 2010. **107**(29): p. 13040-5.
219. Aggarwal, B.B., *Nuclear factor-kappaB: the enemy within*. Cancer Cell, 2004. **6**(3): p. 203-8.
220. Corbetta, S., et al., *Activity and function of the nuclear factor kappaB pathway in human parathyroid tumors*. Endocr Relat Cancer, 2005. **12**(4): p. 929-37.
221. Lloyd, R.V., et al., *Immunohistochemical Analysis of the Cell Cycle-Associated Antigens Ki-67 and Retinoblastoma Protein in Parathyroid Carcinomas and Adenomas*. Endocr Pathol, 1995. **6**(4): p. 279-287.
222. Svedlund, J., et al., *Aberrant WNT/beta-catenin signaling in parathyroid carcinoma*. Mol Cancer, 2010. **9**: p. 294.
223. Segditsas, S., et al., *Promoter hypermethylation leads to decreased APC mRNA expression in familial polyposis and sporadic colorectal tumours, but does not substitute for truncating mutations*. Exp Mol Pathol, 2008. **85**(3): p. 201-6.
224. Välimäki, S., et al., *Distinct target regions for chromosome 1p deletions in parathyroid adenomas and carcinomas*. Int J Oncol, 2002. **21**(4): p. 727-35.
225. Cryns, V.L., et al., *Frequent loss of chromosome arm 1p DNA in parathyroid adenomas*. Genes Chromosomes Cancer, 1995. **13**(1): p. 9-17.
226. Esteller, M., et al., *Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer*. Cancer Res, 2000. **60**(16): p. 4366-71.
227. Dong, S.M., et al., *Epigenetic inactivation of RASSF1A in head and neck cancer*. Clinical cancer research : an official journal of the American Association for Cancer Research, 2003. **9**(10 Pt 1): p. 3635-40.

228. Suzuki, H., et al., *Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer*. Nat Genet, 2004. **36**(4): p. 417-22.
229. Marsit, C.J., et al., *Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma*. Int J Cancer, 2006. **119**(8): p. 1761-6.
230. Veeck, J., et al., *Aberrant methylation of the Wnt antagonist SFRP1 in breast cancer is associated with unfavourable prognosis*. Oncogene, 2006. **25**(24): p. 3479-88.
231. Sulaiman, L., et al., *Genome-Wide and Locus Specific Alterations in CDC73/HRPT2-Mutated Parathyroid Tumors*. PLoS One, 2012. **7**(9): p. e46325.
232. Rosen, J.E., et al., *Gland size is associated with changes in gene expression profiles in sporadic parathyroid adenomas*. Ann Surg Oncol, 2005. **12**(5): p. 412-6.