New Insights in Genetic and Epigenetic Mechanisms Involved in Parathyroid Tumorigenesis

LEE STARKER
Primary hyperparathyroidism (pHPT) is a pathology associated with one or multiple hyperfunctioning parathyroid glands. The disease prevalence occurs in roughly 1-2% of the population primarily post-menopausal women. The molecular pathology of the disease is poorly understood. Elevated serum calcium levels in the setting of an inappropriately elevated parathyroid hormone level are indicative of the disease process. The ultimate treatment of the disease is to remove the hyperfunctioning gland.

The aim of this thesis was to examine potential genetic and epigenetic aberrations that are potentially disease causing.

The methylation signature of normal and pathological parathyroid tissue has yet to be investigated. DNA was bisulphite modified and analyzed using the Infinium HumanMethylation27 BeadChip. Distinct hierarchical clustering of genes with altered DNA methylation profiles in normal and pathologic parathyroid tissue was evident. DNA hypermethylation of CDKN2B, CDKN2A, WT1, SFRP1, SFRP2, and SFRP4 known to be important in the development of parathyroid tumors were associated with reduced gene expression in both benign and malignant parathyroid tumors.

Familial primary hyperparathyroidism (FPHPT) may occur due to an underlying germ-line mutation in the MEN1, CASR, or HRPT2/CDC73 genes. Eighty-six young (≤45 years of age) patients with clinically non-syndromic PHPT underwent genetic analysis. Eight of 86 (9.3%) young patients with clinically non-familial PHPT displayed deleterious germ-line mutations in the susceptibility genes (4 MEN1, 3 CASR, and 1 HRPT2/CDC73).

Accumulation of non-phosphorylated active β-catenin has been reported to commonly occur in parathyroid adenomas from patients with primary hyperparathyroidism (pHPT). We assessed possible β-catenin stabilizing mutations in a large series of parathyroid adenomas. A total of one hundred and eighty sporadic parathyroid adenomas were examined for mutations in exon 3 of the CTNNB1 gene. The mutation S33C (TCT>TGT) was detected by direct-DNA sequencing of PCR fragments in 1 out of 180 sporadic parathyroid adenomas (0.68%).

Eight matched tumor-constitutional DNA pairs from patients with sporadic parathyroid adenomas underwent whole-exome capture and high-throughput sequencing. Four of eight tumors displayed a frame shift deletion or nonsense mutations within the MEN1 gene, which was accompanied by loss of heterozygosity of the remaining wild-type allele. One tumor harbored a Y641N mutation of the histone methyltransferase EZH2 gene, previously linked to myeloid and lymphoid malignancy formation. Targeted sequencing in the additional 185 parathyroid adenomas revealed a high rate of MEN1 mutations (35%).

Keywords: Parathyroid, tumorigenesis, mutation, exome sequencing

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To my wife Christina, my parents and my siblings, without their love and support, none of this would have been possible.
List of Papers

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


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Related Manuscripts by the Author

1) **Starker, L.F.,** et al., *4D Parathyroid CT as the Initial Localization Study for Patients with De Novo Primary Hyperparathyroidism.* Ann Surg Oncol, 2010.


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### Abbreviations

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<thead>
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<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>ADMH</td>
<td>Autosomal dominant mild hyperparathyroidism</td>
</tr>
<tr>
<td>Aza</td>
<td>5-Aza-2’-deoxycytidine</td>
</tr>
<tr>
<td>c-Myc</td>
<td>v-myv avian myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>CASR</td>
<td>Calcium Sensing Receptor</td>
</tr>
<tr>
<td>CCND1</td>
<td>Cyclin D1</td>
</tr>
<tr>
<td>CDKN2A/B</td>
<td>Cyclin dependent kinase inhibitor 2A/B</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytocine-phosphate-guanine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA Methyltransferase</td>
</tr>
<tr>
<td>EZH2</td>
<td>Enhancer of Zeste Homolog 2</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FHH</td>
<td>Familial Hypocalciuric Hypercalcemia</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HPT</td>
<td>Hyperparathyroidism</td>
</tr>
<tr>
<td>HPT-JT</td>
<td>Hyperparathyroidism-jaw tumour syndrome</td>
</tr>
<tr>
<td>HRPT2/CDC73</td>
<td>cell division cycle 73/Parafibromin</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of Heterozygosity</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia type 1</td>
</tr>
<tr>
<td>MEN2A/B</td>
<td>Multiple endocrine neoplasia type 2A/B</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NSHPT</td>
<td>Neonatal severe primary hyperparathyroidism</td>
</tr>
<tr>
<td>pHPT</td>
<td>Primary hyperparathyroidism</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RET</td>
<td>Receptor tyrosine kinase Proto-oncogene</td>
</tr>
<tr>
<td>RIZ1</td>
<td>Retinoblastoma protein-interacting zinc-finger</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SFRP</td>
<td>Secreted Frizzled-related proteins</td>
</tr>
<tr>
<td>sHPT-1</td>
<td>Parathyroid tumor cell line</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D Receptor</td>
</tr>
<tr>
<td>WNT1, WNT3, WNT3A</td>
<td>wingless-type MMTV integration site family, member 1, 3 and 3A</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumor 1</td>
</tr>
</tbody>
</table>
Introduction

The Parathyroid Glands

The parathyroid glands were first discovered by a Swedish medical student named Ivar Sandström[1]. The parathyroid glands derive from the endoderm of the third and fourth pharyngeal pouches, starting in the fifth week of development. Typically there are a total of four parathyroid glands with two being located superiorly and two inferiorly, often in symmetric locations dorsal to the thyroid gland.

The inferior parathyroid glands arise from the third pharyngeal pouch endoderm and have a common origin and migration with the thymus. The fourth pharyngeal pouch gives rise to the superior parathyroid glands, which have a much shorter embryologic descent than their inferior counterparts. After losing contact with the pharynx in the sixth week of development it attaches to the caudally migrating thyroid, and remains in contact with the posterior midportion of the thyroid lobe. This limited course leads to a smaller variability in location compared with the inferior gland, and in 85% of cases the superior parathyroid can be found at the posterior aspect of the thyroid lobe in a 2-cm diameter area centered 1 cm above the crossing of the inferior thyroid artery and the recurrent nerve. Each gland typically weighs 35 to 40 mg, measures 3 to 8 mm in all 3 dimensions, and can vary in color from light yellow to reddish brown[2].

Their sole task is the production of parathyroid hormone (PTH), which regulates the distribution of calcium in the blood stream, bone and urine. They accomplish this goal by being the only gland in the entire body that can produce PTH. This peptide hormone consists of a total of 84 amino acids[3], which signals for bone destruction and increased reabsorption via the gastrointestinal and renal systems[4-6]. Also affected is the serum vitamin D metabolism, which also aids in the ultimate goal of a constant environment of eucalcemia[7, 8]. Vitamin D receptors on parathyroid cells and mediate the overall amount of PTH production through a negative feedback mechanism. Therefore, theoretically, as the serum calcium levels begin to climb, the amount of PTH secreted into the bloodstream declines, according to a sigmoidal relation curve[5, 6]. Another way of stating this is that the extracellular concentration required to decrease PTH secretion to 50% of maximum, is defined as the set-point, and normally corresponds to a physiological free calcium concentration of 1.10-1.25 mM[9]. Overall serum calcium
sensing is regulated within the parathyroid glands by the calcium sensing receptor (CasR), a cell surface receptor. Upon binding of extracellular calcium it affects intracellular inositol phosphate levels and regulates PTH secretion and mRNA levels[10-12]. The CasR was eventually mapped to a location on chromosome 3, after it was cloned from bovine parathyroid cells[13].

Parathyroid Tumors

Hyperparathyroidism (HPT)

Hyperparathyroidism occurs in three forms, primary, secondary, and tertiary. The most common manifestation of the disease is by far primary hyperparathyroidism (pHPT). pHPT can occur in two distinct fashions, either sporadic or familial, with the sporadic form being much more common than the inherited. HPT can be secondary in nature, in which there is an inherent defect in the renal system, usually end-stage renal disease. Regardless of the subdivision of the disease the treatment of choice is surgical resection of the hyperfunctioning gland or glands.

Primary Hyperparathyroidism (pHPT)

pHPT occurs sporadically in the vast majority of cases. However, it may also be part of a group of familial disorders with a high degree of disease penetrance, these diseases include multiple endocrine neoplasia type 1 (MEN1), MEN2A, the HPT-jaw tumor syndrome (HPT-JT), familial isolated hyperparathyroidism (FIHPT), autosomal dominant mild hyperparathyroidism (ADMH), and neonatal severe HPT (NSHPT)[14].

pHPT is a relatively common disease, occurring in 2.1% of the population[15, 16]. The peak age of diagnosis is during the fifth to sixth decade of life, with a gender predominance towards females [17]. The disease is characterized by an elevated serum calcium level in the face of an physiologically inappropriately elevated PTH level. Patients can present with the typical signs and symptoms of decreased bone density, nephrolithiasis, pancreatitis, gastro-esophageal reflux disease, and vague symptoms of fatigue and difficulty with concentration and even significant cardiac abnormalities [17, 18].

Sporadic pHPT occurs predominantly due to a single hyperfunctioning gland (85%), but can be due to multi-glandular disease in 10-15%[19] and is rarely from carcinoma (<1%)[20]. The sporadic nature of the disease has been linked to direct exposure of radiation to the head and neck area, especially if the exposure occurred during childhood[21, 22].

Parathyroid carcinoma is rare and despite some recent efforts,[23] is often exceedingly difficult to definitively distinguish from "atypical" adenoma
unless extensive loco-regional invasion or distant metastases are present. Investigation of the RB tumor suppressor gene at chromosome 13q identified LOH in all parathyroid carcinomas of 5 investigated patients, and was associated with an absence of immunostaining for the Rb protein.[24] Subsequent studies confirmed that allelic loss on 13q, appears to be frequent in clinically and histopathologically aggressive parathyroid tumors, including parathyroid carcinoma.[25, 26] Somatic mutations of the HRPT2 gene (encoding parafibromin) seem common in parathyroid carcinomas.[27] Most recently aberration with the WNT/B-Catenin pathway has been demonstrated.[28] Studies surveying the genomes of parathyroid cancers have identified a very different pattern of somatic DNA gains and losses than are characteristic of benign adenomas. These differences, including evidence that MEN1/11q13 defects are rare in carcinomas, suggest that carcinomas generally arise de novo rather than from preexisting adenomas, and that genetic analysis may assist in the often difficult distinction between malignant and benign parathyroid neoplasia.[29]

During the past decade and a half, studies of genetic predisposition, parathyroid tumorigenesis, and molecular genetics of familial hyperparathyroid disorders have started to unveil the molecular basis of pHPT. There are reasons to believe that the etiology of clinically apparently non-familial cases of pHPT can be dependent upon a genetic predisposition. The first genetic association study in endocrine tumor disease was performed in patients with sporadic pHPT[30]. The study demonstrated that certain naturally occurring genotypes of the vitamin D receptor (VDR) were overrepresented in pHPT, especially in postmenopausal female patients. Furthermore, these genotypes were associated with enhanced dysregulation of the calcium-controlled PTH secretion and reduced expression of VDR mRNA in parathyroid adenomas.[31, 32] Thus, an individual's VDR genotype may induce reduced parathyroid VDR expression causing impaired inhibitory effects by 1,25(OH)2D3 on parathyroid cell proliferation and PTH secretion, and thereby contribute to a higher life-time risk of developing pHPT. The association between VDR polymorphisms and primary HPT was subsequently shown in other cohorts of patients from the USA and Germany.[33] The precise molecular mechanism of these associations are not known and requires further exploration. With the completion of the Human Genome Project in 2003 and the International HapMap Project in 2005, large-scale genome-wide association studies (GWAS) have proved to be a powerful tool in identifying genetic loci of importance to genetic susceptibility to a number of disease processes lacking classical Mandelian inheritance.[34] To date, no GWAS has been performed to identify susceptibility loci for pHPT, but such studies are likely to bring important insights into the genetic predisposition of the disease.
Parathyroid oncogene activation

The *cyclin D1* oncogene, now recognized to have a central role in many forms of human neoplasia, was initially identified at the breakpoint of a parathyroid adenoma DNA rearrangement.[35, 36] This rearrangement is a pericentromeric inversion of chromosome 11 which juxtaposes the strong tissue-specific regulatory region of the *PTH* gene with the *cyclin D1/PRAD1* gene’s intact coding region, resulting in overexpression of cyclin D1. The oncogenicity of cyclin D1 overexpression has been further established in transgenic mice models. Tissue-specific enhancement of cyclin D1 expression in parathyroid glands results in development of tumors and abnormal PTH response to serum calcium.[37, 38] To date *PTH-cyclin D1* rearrangements have been documented in only a small subset of parathyroid adenomas, while overexpression of cyclin D1 protein has been substantiated in 18-40% of typical sporadic parathyroid adenomas.[39, 40], thus *PTH-cyclin D1* gene rearrangement appear to be but one of several mechanisms causing cyclin D1 overexpression.

Cyclin D1 is a target of the wnt/β-catenin signaling pathway.[41] Aberrations in the wnt/β-catenin signaling pathway have been shown in parathyroid tumors,[42-44] and 7% percent of examined tumors from a Swedish cohort showed activating mutations in exon 3 of β-catenin. However, other studies have failed to show any such mutations.[45-47] An aberrantly spliced, internally truncated variant of LRP5, a co-receptor for wnt ligands, resulting in stabilization and accumulation of β-catenin seems to be present in a majority of parathyroid tumors of pHPT.[48]

Additional parathyroid oncogenes are likely to be identified. The whole genome of 53 parathyroid adenomas was searched for DNA amplifications by the comparative genomic hybridization technique. DNA amplifications were detected at chromosomes 16p and 19p in about 10% of the parathyroid adenomas, suggesting the presence of oncogenes at these loci.[49]

Parathyroid tumor suppressor gene inactivation

Allelic loss (loss of heterozygosity; LOH) of chromosomal loci may identify tumor suppressor genes in neoplasia. LOH at the MEN type 1 locus on chromosome band 11q13 has been demonstrated in approximately 25-40% of sporadic parathyroid adenomas, and somatic homozygous mutations of the *MEN1* gene are found in 12-17% of adenomas, or about 50% of those tumors with LOH at 11q13.[50, 51] These findings clearly indicate that mutational aberrations in the *MEN1* gene contribute to parathyroid tumorigenesis, but also raise the possibility that 11q13 may harbor an additional parathyroid tumor suppressor gene. Functional aspects of the MEN1 gene product are addressed in the discussion of familial hyperparathyroidism below. Comprehensive LOH and comparative genomic hybridization studies
of parathyroid adenomas have identified locations for several other candidate tumor suppressor gene loci such as 1p, 1q, 6q, 9p, 11p, 15q.[49] To date no gene other than MEN1 and HRPT2 has been proven by somatic mutation to be a tumor suppressor in parathyroid adenomas.[52]. Mutations in the well-characterized tumor suppressor genes p53 and RB do not appear to contribute to the development of parathyroid adenomas.[24, 53] However, hypermethylation of the retinoblastoma interacting zinc finger gene (RIZ1) is common in parathyroid tumors.[54] p15INK4d, p16INK4a, and p18INK4c, members of the INK-4 family of cyclin dependent kinase inhibitors which antagonize the actions of cyclin D1, have also been analyzed in parathyroid adenomas but no mutational aberrations were detected.[55, 56] Immunohistochemical studies of the p27, a member of CIP/KIP family of cyclin-dependent kinase inhibitors, showed a decreasing level of protein expression in normal, hyperplastic, adenomatous, and malignant parathyroid glands, respectively.[57, 58] While it has been speculated that p27 possesses tumor suppressor activity and diminished protein expression could be involved in the progression of parathyroid tumors, pathogenetic mutations in the p27KIP1 gene are rare in human tumors and have been only reported in very few human parathyroid tumors.[59, 60] The theoretically appealing possibility that somatic inactivating mutations in the CasR and VDR genes are involved in parathyroid tumor formation has been investigated, but no such mutations were detected.[61] Genes involved in basic cellular processes such as repair of damaged DNA and replication are of interest, given the association of parathyroid neoplasia with radiation exposure. Two such genes investigated to date, RAD51 and RAD54, are not somatically mutated in parathyroid adenomas and are unlikely to contribute to parathyroid tumorigenesis.[62, 63].

Recent studies showed involvement of Klotho in regulation of apoptosis,[64] and calcium homeostasis.[65] Klotho inhibits canonical Wnt signaling by binding to Wnt3.[66] Klotho functions as a co-receptor for FGF1 (IIIc) for FGF23, and modulates FGF23 signaling.[67] In parathyroid glands klotho seems to have a dual function. It stimulates secretion of PTH directly by recruitment of Na+/K+-ATPases,[65] and suppresses PTH secretion indirectly through FGF23.[68] Klotho expression is down regulated in parathyroid tumors of pHPT and its expression correlates with serum calcium and tumor size.[42] While klotho has been suggested to be a tumor suppressor gene involved in regulation of insulin-like growth factor (IGF) signaling in breast, ovarian and cervical cancer, its role in parathyroid tumorigenesis remains unclear.
Familial syndromes associated with Primary Hyperparathyroidism

Many insights into tumorigenesis have been gathered from studies on inherited tumor-susceptibility disorders, and parathyroid tumors are no exception in this respect. HPT is found in several distinct disorders with autosomal dominant inheritance such as in multiple endocrine neoplasia type 1 (MEN1), MEN2A, the HPT-jaw tumor syndrome (HPT-JT), familial isolated hyperparathyroidism (FIHPT), autosomal dominant mild hyperparathyroidism (ADMH), and neonatal severe HPT (NSHPT).[14, 69]

MEN1 is an autosomal dominant endocrine tumor susceptibility syndrome leading to the formation of tumors of the parathyroid, enteropancreatic endocrine tissue, and anterior pituitary[70]. MEN1 is relatively rare (approximately 1 in 30,000), and a consensus definition of MEN1 is used widely[71]. An MEN1 case has tumors in two of the three principal organs (parathyroid, enteropancreatic endocrine tissue, and anterior pituitary). Similarly, familial MEN1 is defined as one MEN1 case and one first-degree relative with one of the three principal tumors. A germline MEN1 mutation may be identified in 70 to 90% of typical MEN1 families, and some without an identified mutation may have large deletions not recognizable by polymerase chain reaction (PCR) or mutations the tested open reading frame and intron-exon junctions. Genetic testing with direct sequencing of the MEN1 gene is widely available and provides the best method of diagnosis.

pHPT is the most common and usually the initial endocrine manifestation in patients with MEN1, typically presenting in the third to fifth decades of life, although significant variation exist among affected kindreds[72]. The underlying germline mutation in the MEN1 gene is thought to render the parathyroid cells more susceptible to expressing a tumor after only one somatic mutation has silenced the second allele (the second hit)[33], but other secondary aberrations are probably common. Thus, it is not surprising that the abnormal parathyroid glands often occur metachronously, and usually asymmetrically involved. Diseased glands may occur with histologically normal ones and one or more parathyroid gland may appear grossly normal but still account for persistence of hypercalcemia in patients in whom inadequate resection was performed. A high incidence (up to 20%) of supernumerary glands have also been identified in these patients[73], and may be related to parathyroid glands that are chronically stimulated. It is possible that operative strategies are particularly meticulous in this patient population, thereby identifying supernumerary glands that would otherwise go unnoticed or that the genetic background makes the disease more aggressive and recurrence likely even with small amounts of remaining tissue provided follow-up is long enough.

The HPT-JT syndrome is due to inactivating mutations in the HRPT2 gene encoding for the protein parafibromin[74]. As in the case of HPT in
MEN1, HPT is the most common feature of the disease (as in MEN1) and occurs in approximately 80% of adults, generally with an early onset (mean age 32 years). The syndrome is associated with a high incidence of severe hypercalcemia, risk of parathyroid cancer (about 15%), and may be uniglandular[75-78]. In general, HPT stemming from HPT-JT may be treated with resection of grossly enlarged parathyroid glands unless parathyroid cancer is suspected. Glands are generally sequentially removed when found abnormal, though total parathyroidectomy may be preformed in the case of multiple tumors and a high risk of parathyroid carcinoma.

HPT in the setting of an underlying CASR mutation may either present as neonatal severe HPT (NSHPT), autosomal dominant mild hyperparathyroidism (ADMH) or familial hypocalciuric hypercalcemia (FHH). NSHPT presents in the neonate with severe hypercalcemia, very high PTH levels, and is typically lethal unless total parathyroidectomy is performed in the first months of life. The diagnosis of FHH as well as its distinction from HPT may be challenging. The combination of hypercalcemia, intact PTH levels in the mid-range, hypermagnesemia, relative hypocalciuria, and a family history of hypercalcemia is suggestive of the disease. Since the hypercalcemia for the most part is asymptomatic, a family screen may be necessary. A definite diagnosis can be made by the demonstration of germline heterozygous inactivating mutations in the CASR gene, but this is time- and labor consuming and not readily available[79]. The majority of patients with FHH are asymptomatic and do not benefit from surgical resection of their mildly enlarged parathyroid glands[80, 81]. However, some individuals with FHH may develop symptomatic HPT, probably due to a second hit of the underlying polyclonal parathyroid cell hyperproliferation caused by the heterozygous inactivation of CASR. Such patients are likely to be best treated with subtotal parathyroidectomy[82]. The identification of a large family (20 affected members) with familial hypercalcemia and hypercalciuria (or ADMH) provided new insights into the development and treatment of HPT with an underlying CASR mutation[83]. The disease is caused by a mutation in the cytoplasmic tail of the CASR and phenotypic features include hypercalcemia, inappropriately high serum PTH and magnesium levels and relative hypercalciuria, with nephrolithiasis in a subset a patients. Recent studies suggest that heterozygous inactivating mutations in the CASR gene is more common than previously anticipated (1 in 16,000) and that hypercalciuria, not hypocalciuria, is seen in a significant portion of these patients[79]. Additionally, almost a third of patients with mild HPT may indeed have relative hypocalciuria in the setting of hypercalcemia. Traditionally, a urinary calcium/creatinine clearance ratio was used to distinguish between HPT and FHH, with the former having a ratio above 0.01. However, these studies were performed more than 30 years ago, when patient with HPT usually presented with significant symptoms and
hypercalciuria. Relying solely on urinary calcium excretion is unlikely to
distinguish FHH from HPT.

With the technological explosion in molecular genetics and cancer genet-
ics, it is likely that the next decade will continue to bring important insights
in to the molecular pathology of primary hyperparathyroidism. The ability to
perform whole genome genotyping, copy number variation analysis, whole
exome capture and massively parallel DNA sequencing of matched parathy-
roid tumor and germline DNA as well as quantitative whole genome DNA
methylation analysis represents just a few recent genomic techniques to fur-
ther characterize the molecular basis of pHPT.

Epigenetics

Although there are a significant number of cell- surface and intracellular
mechanisms that maintain tight control on the physiologic levels of serum
calcium, there also seems to be important epigenetic regulation of these re-
ceptors and proteins. Epigenetics has been described as “any heritable in-
formation that is carried by the genome that is not coded by DNA” which
encompasses changes in gene expression that occurs independent of changes
in the primary DNA sequence, including DNA methylation, histone modifi-
cations, nucleosome positioning, and non-coding RNA's, specifically mi-
croRNA expression. Most commonly epigenetics refers to stable, reversible
alterations to the genome that affect gene expression and genome function,
the most studied mechanisms are DNA methylation and histone modifica-
tions. DNA methylation is a covalent modification of the cytosine ring at the
5' position of a CpG dinucleotide, whereby a methyl group is deposited on
the carbon 5 of that ring using S-adenosyl methionine as a methyl donor.
These modifications occur when DNA methyltransferases (DNMTs) add
methyl-groups to cytosines, while histone methyltransferases (HMTs), his-
tone demethylases (HDMTs), histone acetyltransferases (HATs) and histone
decacytelylases (HDACs) all modify the core histone tail, which affects the
chromatin structure. CpG dinucleotides, the usual targets of DNA methyla-
tion in mammals, are scattered throughout the genome and present at a lower-
than-expected abundance. This has been explained over evolution by the
spontaneous deamination of the cytosine in the CpG site into a thymine[84].
In certain areas of the genome, a high concentration of these CpG
dinucleotides is found, and these are referred to as "CpG islands" (CGIs).
These CGIs average 1000 base pairs and can be found at the 5' promoter
region of approximately 50% of genes. In a normal differentiated cell, CpG
loci disseminated across the genome are highly methylated, whereas most
promoter CGIs are protected from the spreading of methylation inside their
boundaries. DNA methylation is traditionally recognized as a repressor of
gene expression, and can function in this regard through two main mecha-
nisms, firstly, by interfering with the assembly of the transcription machinery and/or by causing a change in chromatin structure via various methyl-CpG binding proteins. Silencing by direct inhibition of transcription factor binding to their relative sites and by recruitment of methyl-binding domain proteins (MBDs) has also been hypothesized[85]. These MBDs are present in transcription corepressor complexes involving several other members of the epigenetic machinery such as histone deacetylases (HDAC) and histone methyltransferases, resulting in chromatin reconfiguration and gene silencing. Throughout evolution, DNA methylation has been used to silence the expression of endogenous repeats and infecting retrotransposons, keeping them from disrupting normal gene expression. Over recent years there has been rapid progress to elucidate the nature and role of the mechanisms involved in promoter hypermethylation during carcinogenesis. Current evidence is divided between the hypotheses that hypomethylation is either an important early cancer-causing aberration or that it is a passive inconsequential side effect of carcinogenesis[86].

Tumorigenesis is a result of the activation of oncogenic and/or inactivation of proapoptotic or tumor suppressor pathways. Initially, these were believed to result exclusively from genetic events such as mutations, amplifications, gene rearrangements, or deletions. We now understand that DNA methylation is an alternate way of silencing tumor suppressor genes, in a manner equivalent to genetic mutations[87]. DNA methylation effects on pathway alterations can be either direct, by affecting promoters of tumor suppressor genes, or indirect, by silencing known inhibitors of oncogenes, such as the silencing of the secreted frizzled-related protein (SFRP) family of genes, leading to the activation of the Wnt pathway in colorectal carcinogenesis. Similar to mutations, silencing of tumor suppressor genes confers a selective proliferative advantage to corresponding cells, mediates invasiveness, and facilitates metastasis[88].

DNA hypermethylation is an early event in tumorigenesis, most likely playing a major role in tumor initiation and progression, and creating a fertile ground for the accumulation of a multitude of simultaneous genetic and epigenetic aberrations. This is supported by the finding of a "field defect," in which normal tissue adjacent to a tumor is found to harbor several "epi-mutations" as well, most notably in colorectal cancers, but also in gastric cancer and liver cancer. Another example is MGMT hypermethylation, which plays a direct role in the accumulation of G-to-A mutations in the KRAS gene in colorectal tumors. These data led to a new thinking regarding the mechanisms behind tumor initiation and progression, even at the earliest stages of carcinogenesis.

On the other end of the spectrum, we find global DNA hypomethylation, the first epigenetic alteration noted in cancer cells. In various cancers, 5 methyl-cytosine content was found to decrease by an average of 10%. One potential consequence of profound hypomethylation is genomic instability,
predisposing patients to mutations, deletions, amplifications, inversions, and translocations[89]. Indeed, hypomethylation correlates with a higher rate of chromosomal changes in patients with colon cancer and is associated with a poor prognosis. Another potential consequence of DNA hypomethylation is the reactivation of normally silenced genes. This could lead to the disruption of normal gene expression and potential activation of growth-promoting and antiapoptotic pathways. Furthermore, promoter hypomethylation can lead to reactivation of miRNAs embedded in the coding regions of certain genes, resulting in silencing or aberrant expression of the corresponding protein.

Understanding the complexity of the epigenome and of all the actors involved in modulating its interactions with genomic sequences is of fundamental importance in health and disease. This understanding will allow us to reach newer horizons in our search for the mechanisms governing cellular fate. On the tumorigenic spectrum, the time when we switch from untargeted cytotoxicity to reversion of the malignant phenotype is drawing near.
Aims of Current Investigation

The overall aim of this thesis study was to ascertain the potential mechanistic processes within Parathyroid tumorigenesis. Our goal was to decipher which genes or epigenetic factors could be associated with the alteration of normal parathyroid glands into hyperfunctioning glands. In consideration of this goal, various mechanistic processes were incorporated in our overall hypothesis.

Specific Aims

- To perform an unbiased, systemic investigation of the role of DNA methylation of CpG islands, an epigenetic mechanism, which has not been investigated in parathyroid tumorigenesis. (*Paper I*)

- Analysis of the most common genes associated the Familial primary hyperparathyroidism (FPHPT), by investigating the presence of underlying germ-line mutations in the *MEN1*, *CASR*, or *HRPT2/CDC73* genes. (*Paper II*)

- To investigate the implication of aberrant accumulation of β-catenin in parathyroid neoplasms. This can be caused by stabilizing mutation of β-catenin (*CTNNB1*, exon3) or by mutation or deregulated expression of other components of the WNT/β-catenin signaling pathway. (*Paper III*)

- To investigate the occurrence of somatic mutations driving tumor formation and progression in sporadic parathyroid adenoma using whole-exome sequencing. (*Paper IV*)
Materials and Methods

One hundred and eighty patients with surgically verified, non-familial pHPT due to a single adenoma, with variable levels of hypercalcemia were included in the studies. Tumors were meticulously evaluated by an experienced endocrine pathologist prior to use in any of the studies. Tumors were dissected prior to processing to minimize contamination by normal cells. The tissue was snap frozen in liquid nitrogen and stored at -80 C°. None of the patients demonstrated evidence of familial disease or had a history of previous neck irradiation. Blood was collected after an overnight fast and serum (s-) calcium (reference range 2.20-2.60 mmol/L) and intact s-PTH (reference range 10-65 ng/l) were determined preoperatively. All patients were normocalcemic during routine follow-up, which lasted for at least 6 months (Table 1). Informed consent was obtained and all studies were approved by the institutional review board.

Amplification of all coding regions of MEN1, CASR and HRPT2 (Paper II)

Whole blood was obtained through phlebotomy and genomic DNA from leukocytes isolated by standard methods. 200 ng of genomic DNA was PCR-amplified using specific primers for exons 1-10 of the MEN1 gene, exons 1-10 of the CASR gene and exons 1-17 of the HRPT2/CDC73 gene, as described previously. Primer sequences are provided in supplementary Table 1.

Genomic DNA Extraction (Papers I-IV)

Genomic DNA was isolated from surgically resected and then snap-frozen fresh tumor samples and matched blood using standard protocols included in the commercially available Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). All specimens were quality control checked for purity utilizing the nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).
DNA sequencing and analysis (Paper II, III)

PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA) according to the instructions from the manufacturer. Each sample (40 ng of DNA) underwent both forward and reverse Sanger sequencing at the Keck DNA Sequencing Facility at Yale University. Data were analyzed with the use of Sequencing Analysis and AutoAssembler software (Applied Biosystems), and publically available web based resources (NCBI-Blast, GenBank). All novel DNA variants were verified by re-sequencing using genomic DNA from a separate extraction. All variants were analyzed for predicting damaging effects of missense mutations using the PolyPhen 2 software (http://genetics.bwh.harvard.edu/pph2), as described. The frequency of single nucleotide polymorphisms (SNP) in the examined cases was compared to the HapMap CEU population (http://www.ncbi.nlm.nih.gov/projects/SNP).

Bisulfite Modification (Paper I)

High molecular weight genomic DNA was isolated from normal and pathological parathyroid as previously described. Genomic DNA (500 ng) from normal parathyroid tissue (n=3), parathyroid adenoma (n=14), and cancer (n=7) were simultaneously bisulphite modified using the EZ DNA Methylation kit (Zymo Research, Orange, CA).

Methylation-Specific PCR Analysis Using SYBR Green (Paper I)

To verify the findings from the methylation arrays, 6 highly hypermethylated genes were analyzed using methylation-specific PCR (MSP). Methylated and unmethylated specific primers were designed using the Methyl Primer Express software (Applied Biosystems, Foster City, CA, USA) and the primer sequences are presented in supplementary Table 2. Both unmethylated and methylated specific primers displayed an identical target amplicon. Semi-quantitative PCR was performed using SYBR-Green PCR Master Mix (#4309155) and results were analyzed using StepOne Software v2.1 (Applied Biosystems). Human methylated DNA (Epitect Control DNA; Qiagen, Valencia, CA, USA) was utilized as the reference DNA to quantitatively assess the methylation status of the target CpG island, when using methylated specific primers. Similarly, human demethylated DNA (Epitect Control DNA; Qiagen) was used as reference for the unmethylated specific primers. The relative percentage of the values from the methylated and unmethylated measurements was calculated.
Quantitative RT-PCR Analysis (paper I)

cDNA was synthesized using 1 μg of total RNA and iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc. Hercules, CA). Quantitative real-time PCR was performed on StepOnePlus™ Real-Time PCR systems (Applied Biosystems) using assays for CDKN2B (Hs 00793225_m1), CDKN2A (Hs 00233365_m1), WT1 (Hs 01103754_m1), SFRP4 (Hs 00180066_m1), SFRP1 (Hs 00610060_m1), SFRP2 (Hs 293258_m1) and GAPDH (Hs99999905_m1; all from Applied Biosystems). Each cDNA sample was analyzed in triplicate. Standard curves for each experiment were established by amplifying a purified PCR fragment covering the sites for probes and primers.

Primary Cell Cultures, and Treatment With 5-Aza-2'-Deoxycytidine (Paper I)

Parathyroid carcinoma (n=1) and adenoma cells (n=18) were prepared fresh, directly after operation according to published procedures with minor modifications. Briefly collagenase digestion was performed for 1 h. Cells were then cultured in 35-mm dishes in DMEM, containing 10% fetal bovine serum and penicillin/fungizone/L-glutamine (Sigma-Aldrich St.Louis, MO) and were treated in triplicates with 5 μM 5-aza-2'-deoxycytidine (Sigma-Aldrich). Cell viability was measured on PC1 cells distributed in triplicates (2×10^4) onto 96 well plates using the cell proliferation reagent WST-1 (Roche Diagnostics GmbH, Mannheim, Germany). Fresh 5-aza-2'-deoxycytidine was added every 24 h. Cells were harvested after 24 h and 48 h for RNA extraction based upon previously published literature. Primary cell cultures were utilized due to the lack of either parathyroid adenoma or carcinoma cell lines.

Immunohistochemistry (Paper III)

Immunohistochemical staining was preformed on the lone pathologic specimen with a β-catenin mutation and compared to normal parathyroid tissue which had been incidentally removed during thyroid surgery. Paraffin-embedded specimens were cut into fixed cyrosections and fixed utilizing acetone. Sections were then blocked with an avidin-biotin blocking kit (Vector Laboratories, INC., Burlingame, CA) using normal horse serum. The immunostaining was then carried out using an anti-β-catenin goat polyclonal antibody with an epitope mapping at the C-terminus (Santa Cruz Biotechnology, INC., Santa Cruz, CA; catalog no. sc-1496).
Whole exome capture (Paper IV)

Constitutional and tumor DNA was captured on a NimbleGen Sequence Capture 2.1M Human Exome Array following the manufacturer's protocols with modifications at the W. M. Keck Facility at Yale University. DNA was sheared and adaptors were ligated onto the resulting fragments. Fragments were amplified by ligation-mediated PCR, purified, and hybridized to the array. After washing, fragments were eluted and purified. Resulting fragments were then subjected to high-throughput sequencing.

Quantification of contamination using quantitative-PCR (Paper IV)

Surgically resected tumor tissue often contains constitutional cells surrounding the tumor in vivo. Though unavoidable, it is possible to estimate the level of contamination, which can facilitate correction for such during data analysis. Commercially available probes for the TaqMan Custom SNP Genotyping Assay were designed using the Applied Biosystems website. Quantitative-PCR using probes overlapping a homozygous nonsense MEN1 mutation identified in one of the tumors differentiated between mutant and wild type alleles. Fluorophores FAM and VIC corresponded to amplification of the somatically-mutated tumor allele and the wild type allele, respectively. This estimated the presence of tumor DNA in the constitutional sample to be negligible, but constitutional DNA in the tumor sample to be 41.3±1.0%.

Methylation-specific-PCR (Paper I & IV)

Genomic DNA isolated from constitutional and tumor samples from nine patients was bisulfite-converted using the Qiagen EpiTect Bisulfite Kit. Methyl Primer Express software (Applied Biosystems, Foster City, CA, USA) predicted gene of interest promoter CpG islands and designed primers allowing PCR amplification specific to the presence or absence of methyl protection. Amplification of a product of appropriate size was identified via agarose gel electrophoresis.

High-throughput sequencing (Paper IV)

Captured libraries were sequenced on the Illumina Genome Analyzer as 74bp single-end reads and 74bp or 99bp paired-end reads following the manufacturer's protocols. Image analysis and base-calling was performed by Illumina pipeline versions 1.3 and 1.4 with default parameters. Analysis of
applicable raw data including sequence reads were mapped to the reference genome (hg18) using the Maq program. Reads outside the targeted sequences were discarded. Statistics on coverage were collected from the remaining reads using perl scripts. For indel detection, BWA was used to allow gapped alignment to the reference genome. SAMtools was used to call targeted bases with any base-call deviating from the reference sequence regarded as a potential variation. Identified variants were annotated based on novelty, impact on the encoded protein, and conservation using an automated pipeline.
Results and Discussion

Paper I.
The role of DNA methylation of CpG islands in parathyroid tumorigenesis has not been analyzed in an unbiased, systematic fashion. DNA was isolated from normal and pathologic parathyroid tissues, bisulphite modified and analyzed using the Infinium HumanMethylation27 BeadChip. Using the Illumina HumanMethylation27 platform, we characterized the DNA methylome of benign and malignant parathyroid tumors by interrogating 27,578 highly informative CpG sites from more than 14,000 genes throughout the genome. We found it to be a sensitive, robust and reproducible technique for mapping DNA methylation patterns in parathyroid tumor genomes. Normal parathyroid tissue, adenomas and carcinomas displayed a distinct DNA methylation profile. Genes involved in regulation of apoptosis and cell cycle control as well as ion channels were frequently altered in benign and malignant parathyroid tumors. Distinct hierarchical clustering of genes with altered DNA methylation profiles in normal and pathologic parathyroid tissue was evident. Comparing normal parathyroid tissue with parathyroid adenomas, 367 genes were significantly altered, while 175 genes significantly differed when comparing parathyroid carcinomas and normal parathyroid tissues. A comparison between parathyroid adenomas and parathyroid carcinomas identified 263 genes with significantly distinct methylation levels. Results were confirmed for certain genes in a validation cohort of 40 parathyroid adenomas by methylation-specific PCR. Genes of known or putative importance in the development of parathyroid tumors showed significant and frequent hypermethylation. DNA hypermethylation of CDKN2B, CDKN2A, WT1, SFRP1, SFRP2 and SFRP4 was associated with reduced gene expression in both benign and malignant parathyroid tumors. Treatment with 5-aza-2'-deoxycytidine of primary cell cultures restored expression of hypermethylated genes in benign and malignant parathyroid tumors. In conclusion, the unbiased, genome-wide study of the parathyroid tumor DNA methylome identified a number of genes with altered DNA methylation patterns of putative importance to benign and malignant parathyroid tumorigenesis.
Paper II.
Familial primary hyperparathyroidism (FPHPT) may occur due to an underlying germ-line mutation in the MEN1, CASR, or HRPT2/CDC73 genes. The disease may be undiagnosed in the absence of a history suggestive of FHPT. Young pHPT patients (≤ 45 years of age) are more likely to harbor occult FPHPT. A total of 1,161 (136 were ≤ 45 years of age) PHPT patients underwent parathyroidectomy from 2001 to 2009. Thirty-four patients declined participation. Sixteen patients were diagnosed in the clinical routine with FPHPT (11 MEN1, four MEN2A, and one HPT-JT) and were not included in the genetic analysis. Eighty-six young (≤ 45 years of age) patients with clinically non-syndromic PHPT underwent genetic analysis. Sanger sequencing of all coding regions of the MEN1, CASR, and the HRPT2/CDC73 genes was performed. Eight of 86 (9.3%) young patients with clinically non-familial PHPT displayed deleterious germ-line mutations in the susceptibility genes (4 MEN1, 3 CASR, and 1 HRPT2/ CDC73). There was one insertion, one deletion, two nonsense, and four missense mutations, all predicted to be highly damaging to protein function and absent in 3,244 control chromosomes. Germ-line mutations in known susceptibility genes within young patients with PHPT, including those diagnosed in the clinical routine, was 24/102 (23.5%; 15 MEN1, four RET, three CASR, and two HRPT2/CDC73).

Paper III.
The aim of this study was to determine the mutational frequency of the CTNNB1 gene, specifically exon 3 in a large series of parathyroid adenomas. One hundred and eighty sporadic parathyroid adenomas were examined for mutations in exon 3 of CTNNB1 by direct DNA sequencing, utilizing previously published primer sequences. The mutation S33C(TCT[T GT] was detected by direct-DNA sequencing of PCR fragments in 1 out of 180 sporadic parathyroid adenomas (0.68 %). Like serine 37, mutations of serine 33 have been reported in many neoplasms with resulting β-catnen stabilization, enhanced transcription, and oncogenic activities. Immunohistochemical analysis revealed an overexpression of the β-catenin protein in the lone mutant tumor. Similar to the four studies previously mentioned we were unable to identify any S37A mutation within exon 3 of the CTNNB1 gene. We were, however, able to identify a single somatic mutation within one parathyroid adenoma at codon 33, where a serine was substituted for cysteine. Serine 33 is one of the GSK-3b phosphorylation sites regulating β-catenin stability. A mutation at this site leads to protein accumulation, enhanced transcriptional and oncogenic activities. This novel parathyroid mutation has been implicated in a multitude of other neoplastic processes with
variable mutational frequency. Recently, this mutation was concomitantly identified in an Italian cohort, with a frequency of 1.8%. To determine the \( \beta \)-catenin protein expression level in normal parathyroid tissue and the pathological parathyroid gland, we performed immunohistochemical analysis on paraffin fixed tissue sections with a specific goat polyclonal peptide antiserum. Accumulation of \( \beta \)-catenin through stabilizing mutations or other mechanisms may result in deregulated transcription of Wnt signaling target genes and promotion of oncogenic signals that lead to tumor formation.

Paper IV.

The aim of the study was to investigate the occurrence of somatic mutations driving tumor formation and progression in sporadic parathyroid adenoma using whole-exome sequencing. Eight matched tumor-constitutional DNA pairs from patients with sporadic parathyroid adenomas underwent whole-exome capture and high-throughput sequencing. Selected genes were analyzed for mutations in an additional 185 parathyroid adenomas. Four of eight tumors displayed a frame shift deletion or nonsense mutation in MEN1, which was accompanied by loss of heterozygosity of the remaining wild-type allele. No other mutated genes were shared among the eight tumors. One tumor harbored a Y641N mutation of the histone methyltransferase EZH2 gene, previously linked to myeloid and lymphoid malignancy formation. Targeted sequencing in the additional 185 parathyroid adenomas revealed a high rate of MEN1 mutations (35%). Furthermore, this targeted sequencing identified an additional parathyroid adenoma that contained the identical, somatic EZH2 mutation that was found by exome sequencing. This study confirms the frequent role of the loss of heterozygosity of chromosome 11 and MEN1 gene alterations in sporadic parathyroid adenomas and implicates a previously unassociated methyltransferase gene, EZH2, in endocrine tumorigenesis.
Concluding remarks

Parathyroid tumorigenesis leading to the sequelae of clinical parathyroid disease continues to remain an issue for patients and clinicians alike. Although the above studies do not depict a flawless mechanism leading to the creation of parathyroid tumorigenesis we have made strides. We now know the distinct difference in the methylation signature of the different parathyroid pathologies. Multiple genes remain implicated in parathyroid tumorigenesis, yet the list of genes is unique and distinct for each of the three states researched.

Germline mutations have been implicated and associated with parathyroid tumorigenesis for multiple decades, especially in patients with known familial disease. Yet, these genes when investigated in a younger population without familial linkage demonstrate both previously described and novel aberrations. Given the high prevalence of germ-line mutations in clinically non-familial young pHPT patients, a more liberal use of DNA testing may be advocated for these patients even in the absence of a suspicious family history. This may aid the operating physician in their planning of the overall operative procedure, potentially preventing the need for recurrent disease and improved evaluation of the kindred.

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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine.