CHAPTER 10

Molecular Mechanisms in Parathyroid Tumorigenesis

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Primary hyperparathyroidism (1 HPT) is a relatively common disorder, with an estimated prevalence of 1:500-1:1000.[1] In about 80%-85% of 1 HPT cases, a single enlarged parathyroid gland with a distinct capsule that separates the tumorous from the nontumorous tissue (=adenoma or uniglandular disease) is detected.[1] In the majority of remaining individuals with 1 HPT, more than one parathyroid gland is enlarged, and at times, no distinct capsule can be detected (hyperplasia or multiglandular disease).[2] Both single adenomas and multiple hyperplastic parathyroid tumors are benign lesions, capable of local growth with no metastasizing potential. There are rare cases of parathyroid carcinoma, which account for less than 1% of all 1 HPT cases, a tumor with both local invasion and distant metastasis capabilities.[3] Uniglandular 1 HPT is usually noted in a single individual within a family, where there are no additional family members with either 1 HPT or other endocrinopathies (=sporadic 1 HPT). In about 10% of 1 HPT cases there are other family members affected with either 1 HPT (familial HPT) or other endocrinopathies, and these cases are at times associated with multiglandular disease. Several distinct inherited neoplastic disorders have 1 HPT as part of the spectrum of tumors that occur in affected families: multiple endocrine neoplasia type I (MEN1) (MIM # 131100), multiple endocrine neoplasia type 2A (MEN2A) (MIM # 171400), and familial 1 HPT with (MIM #145001) or without (MIM # 145000) jaw tumors.

The majority of human neoplasms, malignant as well as benign, are considered monoclonal tumors: they originate from a single cell, the clone precursor cell that acquires a genetic mutation which leads to an increased proliferation rate of the resulting clone, via a variety of molecular mechanisms. In order to continue and accelerate the uncontrolled proliferative activity, the clone precursor cell acquires subsequent mutations in different genes from different gene families. These subsequent genetic alterations lead to subclonal expansion of the original clone, and result in a clinically detectable tumor mass. Several gene families are intimately involved in promoting and facilitating uncontrolled cellular proliferation: oncogenes, tumor suppressor genes, telomerase genes, and DNA mismatch repair genes (reviewed in refs. 4,5).

The clonal origin of parathyroid tumors was evaluated by several investigators over the years, using a variety of technologies. Initial studies from the late 1970's and early 1980's were based on a limited number of tumors and utilized patterns of X chromosome inactivation by measurements of protein isoforms of glucose 6-phosphate dehydrogenase (G-6-PD), an X-linked gene product.[6,7] These studies surprisingly reported that parathyroid adenomas are polyclonal tumors, rather than the expected monoclonal neoplasms. In 1988, Arnold and coworkers[8] have readdressed the issue of monoclonal origin of parathyroid tumors, and applied both a DNA-based technique that measures the X-inactivation pattern in heterozygous women, and

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genetic analysis (Southern blotting) to look for major gene rearrangements involving the parathyroid hormone (PTH) gene, as surrogate markers heralding the monoclonal origin of parathyroid adenomas. This breakthrough study clearly demonstrated that the majority, if not all, parathyroid tumors are in fact monoclonal. This changed concept about the clonal origin of sporadic, uniglandular parathyroid tumors, paved the way for other studies that directly or indirectly addressed the issue of clonality of parathyroid tumors encountered in different clinical settings. Indeed, MEN1-associated parathyroid tumors,[9] nonfamilial, primary parathyroid hyperplasia,[10] parathyroid hyperplasia seen in uremic patients,[10,11] and parathyroid carcinoma,[12,13] were all shown to contain a significant monoclonal component.

The well-established monoclonal origin of parathyroid tumors, prompted a search for tumor-specific genetic alterations and the precise genes that are involved in parathyroid tumorigenesis. As the tumorigenic paradigm of monoclonal tumors stipulates somatic mutations in specific genes initiate uncontrolled parathyroid cellular proliferation and somatic mutations in other genes, sustains and promotes this unregulated proliferation. Finding a coding region mutation that clearly alters the gene function (activating mutations within oncogenes or inactivating mutations in tumor suppressor genes) is the "gold standard" of defining the involvement of a gene in tumorigenesis. Yet, altered expression of the gene's product (at the mRNA or protein levels) by epigeneic mechanisms (e.g., methylation alterations, loss of imprinting, chromatin modification)[14] may also contribute to tumorigenesis.

In addition to the genes that play pivotal role in parathyroid tumorigenesis by virtue of somatic mutations or "expression altering" mechanisms, there are genes that predispose to parathyroid tumorigenesis, in the context of familial or inherited neoplastic syndromes. Individuals who harbor germline mutations within these genes have a significantly increased risk for developing parathyroid tumors during their lifetime, a risk that at times approaches 100%. This subset of genes includes the genes for MEN1,[15] MEN2A,[16] and for familial hyperparathyroidism with or without jaw tumors.[17] In addition to their role in conferring an increased lifetime risk for parathyroid tumor development, these genes are also somatically involved in parathyroid tumorigenic process.[18,19] The definition of the precise genes involved in initiation and promotion of parathyroid tumorigenesis, as well as the genes that predispose to parathyroid tumor development has gained momentum over the past few years, and is the topic of this book chapter. Noteworthy, several recent reviews of the molecular mechanism involved in parathyroid tumorigenesis are recommended for reading.[20-22]

Oncogenes Involved in Parathyroid Tumor Development

Protoncogenes are normal cellular proteins involved primarily in the signal transduction cascade initiated by binding of a ligand to a cell membrane receptor which via a series of phosphorylations of cytoplasmic proteins results in the activation of transcription factors and cellular proliferation (reviewed in refs. 23-24). When either an activating point mutation or overexpression of a nonmutated allele of a protooncogene occurs (by either DNA amplification or chromosomal translocation), the protooncogene is converted to an oncogene that promotes ligand-independent cellular proliferation. One of the oncogenes notably involved in parathyroid tumorigenesis is PRAD1 (parathyroid adenomatosis gene 1)/ cyclin D1/CCND1 (MIM# 168461). The PRAD1 gene normally localizes to the long arm of chromosome 11 (at 11q13). In a small subset of parathyroid tumors (about 5%), a major gene rearrangement occurs, involving pericentromeric inversion of one of the chromosome 11 alleles, leading to a juxtaposition of the PRAD1 gene and the 5' region of the PTH gene (localized to 11p15). This juxtaposition leads to unregulated overexpression of the PRAD1 protein, specifically in parathyroid tissue, by having the nonmutated PRAD1 gene under the control of tissue specific enhancers of the PTH gene.[25] Overexpression of PRAD1 with no demonstrable gene rearrangement has been shown by immunohistochemistry in 20-40% of parathyroid adenomas, as

well as parathyroid hyperplasia and carcinoma.[26-28] The underlying mechanism of overexpression of the nonmutated, nonrearranged PRAD1 in parathyroid tumors is probably trans-acting regulatory derangement. To date, no somatic PRAD1 activating point mutations have been reported in parathyroid tumors,[29] but in a transgenic mouse model where the transgene construct mimicked the human PTH-cyclin D1 rearrangement, the resulting phenotype was that of hyperproliferative parathyroids, biochemical evidence of hypersecretion of PTH and hypercalcemia, with bone morphological changes that are similar to those of human 1 HPT.[30] The PRAD1 protein contains 295 amino acids, and belongs to a family of cyclins (hence the alternative name cyclin D1), proteins that play a pivotal role in regulation of cell cycle progression, specifically, progression through the G1 phase and G1-S transition, thus regulating whether a new cell cycle occurs (reviewed in ref. 31). The involvement of cyclins in cell cycle control is mediated by binding to and activation of cyclin dependent kinases (CDK's). In Contrast, beta catenin, a known regulator of cyclin D1 transcription, was not shown to harbor somatic mutations in exon 3 or to have an altered expression pattern in parathyroid tumors.[32]

Several oncogenes have been shown to be overexpressed in benign parathyroid tumors. The involvement of these oncogenes in parathyroid tumorigenesis was evaluated because it was either biologically plausible, or they have been isolated from parathyroid tumors and displayed transforming capability in vitro. An oncogene that was shown to be overexpressed at the protein level in sporadic uni- and multiglandular parathyroid tumors, is the Int-2 gene product, fibroblast growth factor 3 (FGF-3), a known growth promoter of parathyroid cells in culture.[33] Similarly, Epidermal growth factor receptor (EGFR) protein was overexpressed by immunostaining in 5/12 (41.6%) sporadic parathyroid tumors.[34] A novel, alternatively spliced form of the keratinocyte growth factor receptor (KGFR) isolated from parathyroid adenoma has been shown to possess oncogenic activity and transform NIH 3T3 cells.[35] In contrast, activating point mutations in Ha-Ki- and N- RAS genes, oncogenes involved in the pathogenesis of a large variety of human cancers, have not been detected in benign parathyroid tumors.[36,37] Similarly, *gsp* and *gip2* mutations, activating oncogenic mutations that have been described in other endocrine tumors, most notably in pituitary and thyroid tumors (*gsp*), adrenal and ovarian tumors (*gip2*),[38] have not been detected in benign parathyroid tumors.[39] Missense activating germline mutations of the RET proto-oncogene have been identified in the hereditary cancer syndrome MEN2A (MIM# 171400), characterized by the cooccurrence and familial clustering of medullary thyroid carcinoma, pheochromocytoma and parathyroid tumors. In addition, somatic RET proto-oncogene mutations have been identified in a subset of sporadic medullary carcinomas and pheochromocytomas (reviewed in ref. 40). Yet, no activating somatic point mutations have been detected in the RET protooncogene in sporadic parathyroid tumors.[41,42]

Indications as to the putative chromosomal regions that may contain oncogenes involved in parathyroid tumorigenesis have emerged from cytogeneic analyses. In a single parathyroid adenoma, a cytogenetically visible translocation between chromosome 1 and 5 has been reported.[43] Applying comparative genomic hybridization (CGH) to benign parathyroid tumors and targeting chromosomal regions that display an increase in DNA copy number (=amplification) may reveal novel oncogenes involved in parathyroid tumorigeneis. In the few CGH studies published,[44-46] regions on chromosomes 7, 16, and 19 have been consistently amplified. Yet, the specific genes that localize to these regions have not yet been defined.

Tumor Suppressor Genes Involved in Parathyroid Tumorigenesis

Inactivation of both alleles of a tumor suppressor gene (=bi-allelic inactivation) is a prerequisite for tumor development. Such inactivation serves to deplete the cell of the antiproliferative control that the gene product normally exerts, and uncontrolled cellular proliferation ensues. In most cases, a point mutation inactivates one allele (usually by causing a

frameshift, nonsense mutation, or creating a premature stop codon), whereas the other allele is inactivated by a gross somatic deletion of the wild type bearing allele. This somatic deletion is heralded by allelic loss (Loss Of Heterozygosity—LOH), seen when the genotype of the tumor DNA is compared with the genotype of the nontumorous DNA from the same individual, using DNA markers linked to the chromosomal region that bears the tumor suppressor gene (reviewed in ref. 47). Allelotyping of parathyroid tumors revealed that there are nonrandom, chromosomal regions that display LOH, thus putatively harbor tumor suppressor genes that are relevant to parathyroid tumorigenesis.

One of the regions that most commonly display LOH in parathyroid tumors is 11q13, at the MEN1 locus.[48-50] Multiple Endocrine neoplasia type 1 (MEN1) is a rare syndrome characterized by the clustering in families (in an autosomal dominant inheritance pattern) or in individuals (sporadic cases) of benign parathyroid tumors, pancreaticoduodenal endocrine tumors (mostly benign) and pituitary adenomas. Other tumors, such as carcinoids, lipomas, nonfunctioning adrenal tumors, and angiofibromas also occur at a high rate in MEN1 patients (reviewed in refs. 51-52). Using a combined approach of allelotyping of malignant insunlinomas from MEN1 patients and subsequent family linkage analyses, Larsson and coworkers,[53] localized the MEN1 gene to the long arm of chromosome 11 (11q13). Subsequent linkage studies verified this location,[54,55] and allelotyping of MEN1-associated parathyroid tumors revealed that the majority of tumors displayed LOH using MEN1-linked markers.[9] Additionally, using the same set of genetic markers, LOH was also present in about one third of sporadic parathyroid tumors.[9] With the cloning of the MEN1 gene, somatic inactivating mutations have been detected in both MEN1-associated (in most tumors) and sporadic parathyroid tumors (in about to 20% of sporadic tumors).[8,19,56,57] The rate of LOH at 11q13 in sporadic parathyroid tumors is almost double that of the rate of MEN1 gene somatic mutations,[8,56,57] raising the possibility that there is yet an unidentified parathyroid related tumor suppressor gene on 11q13 that is targeted for inactivation by LOH.[58]

The MEN1 gene product, MENIN, is a 610 amino acid nuclear protein with no strong homologies to any known proteins, which has been shown to play a role in regulating Jun D transcription factor.[59] Protein-protein interactions with other proteins have been shown: Smad3 (a protein that acts in the TGF-β pathway) and NF-κB transcription factors.[60-62] These latter proteins have been shown to bind to the Cyclin D1 promotor and increase its transcriptional activity, whereas binding to MENIN inhibits their activity. Thus, parenthetically and speculatively, loss of MENIN in parathyroid tissue (by biallelic inactivation) may result in more binding of NF-kβ to Cyclin D1 promotor, which leads to an increase in its parathyroid pro-proliferative activity.

In support of the tumor suppressor activity of the *MEN1* gene, a mouse model was generated, where the mouse homologue of the *Men1* gene was heterozygously knocked out. The resulting phenotype included parathyroid hyperplasia (without hypercalcemia), as well as pancreatic endocrine, pituitary and adrenal tumor formation,[63] all part of the tumor spectrum of human MEN1 phenotype.

Allelotyping studies revealed other regions in the genome that are nonrandomly, clonally deleted in parathyroid tumors: 1p,[64,65] 1q,[66] 6q, 9p, and 15q.[67,68] These regions putatively contain tumor suppressor genes involved in parathyroid tumorigenic process. Applying CGH to parathyroid tumors, 20q12-13 displayed a nonrandom decrease in DNA copy numbers, indicative of the existence of tumor suppressor gene(s) relevant to parathyroid tumor development in that chromosomal region, in addition to 11q, 1p and the other regions displaying LOH detected by allelotyping.[69]

Of the chromosomal loci showing LOH, the most common region is at 1p, a region displaying LOH in about 40% of sporadic parathyroid tumors.[12,64] The region that defined the minimally deleted area localizes to 1p36, a region that is also deleted in a subset of

medullary thyroid tumors, and pheochromocytomas,[70,71] tumors that form the clinical spectrum of MEN2A. Thus, it would appear, that tumor suppressor gene(s) that locate to 1p36 are important in parathyroid tumorigenesis as well as the pathogenesis of other endocrine tumors. There are several candidate genes that localize to that region, two of whom, the p18, cyclin dependent kinase inhibitor,[66] and the p73 tumor suppressor gene[72] were excluded as involved in parathyroid tumorigenesis, as no somatic inactivating mutations were detected in either gene in the analyzed parathyroid tumors. Intriguingly, the region on 1p shown to be deleted in benign parathyroid adenomas is distinct from a more proximal region on 1p that displays allelic loss in parathyroid carcinomas.[12] This latter finding may indicate that different tumor suppressor genes that localize to 1p are involved in benign and malignant parathyroid tumor development. Two genes that localize to 9p, p15 p16, both cyclin kinase inhibitors, and the PPP2R1B gene that localizes to 11q23, were also excluded as contributors to parathyroid tumorigenesis.[66,73] Another candidate tumor suppressor gene whose involvement was invoked in parathyroid tumorigenesis is the *Smad3* gene: it localizes to 15q, a region displaying nonrandom LOH, and the encoded protein is a TGF-β signaling molecule, a known binding partner of MENIN.[60] Yet, no somatic inactivating mutations could be shown in this gene as well.[74]

Another class of tumor suppressor genes that were evaluated as potential contributors to parathyroid tumorigenesis, are "classical tumors genes": tumor suppressor genes that have been shown to be involved in a variety of tumors, including some of endocrine origin: p53 (MIM# 191170), Rb (MIM# 180200). While no somatic p53 inactivating mutations or positive immunostaining of mutant p53 protein could be shown in most parathyroid tumors analyzed,[75-78] inactivation of the Rb gene seems to significantly contribute to parathyroid carcinoma, but not to benign parathyroid tumors.[79,80] Additionally, loss of Rb expression seems to be limited to aggressive parathyroid tumors.[81] Given this seemingly intimate involvement of the Rb gene in malignant parathyroid tumorigenesis, it was surprising that no somatic inactivating mutations could be shown in the coding region of the Rb gene in parathyroid carcinomas.[13]

There is a well-established association between external ionizing irradiation exposure and the subsequent development of head and neck tumors, including parathyroid neoplasms (reviewed in ref. 82). Thus, genes that are involved in mediating radiation damage repair and meiotic recombination have become candidate genes to be involved in parathyroid tumorigenesis. Three genes, *RAD51* (MIM# 179617), *RAD54* (MIM# 603615) and *BRCA2* (MIM# 600185) are involved in DNA damage repair and localize to 15q, 1p, and 13q, respectively, regions that display nonrandom allelic loss in parathyroid adenomas. Thus, these genes were considered candidate parathyroid tumor suppressor genes. However, there is currently no evidence that any of these genes is somatically inactivated in benign or malignant parathyroid tumors.[13,83,84]

Other Molecular Pathways Involved in Parathyroid Tumorigenesis

A number of genes have been suggested as contributors to parathyroid tumorigenesis by virtue of their known biological function, disease-associated and normal tissue expression patterns, chromosomal locations, and/or involvement in familial syndrome relevant to parathyroid pathological states. Some of these genes were evaluated for harboring somatic mutations in parathyroid tumors, but none proved to harbor such pathogenic mutations. Thus, while these genes do not have an established role in parathyroid tumorigenesis, they may still be important indirect contributors to hyperparathyroidism.

Microsatellite Instability in Parathyroid Tumors

The hallmark of involvement of DNA mismatch repair (DNA-MMR) genes in the tumorigenic pathway, is the demonstration of somatic genomic instability, heralded by microsatellite instability (MSI) (reviewed in ref. 85): comparison of tumor and nontumorous DNA from the same individual shows the existence of novel, different size alleles in the tumor tissue. When the DNA mismatch repair system is nonfunctional by mutational inactivation of one or more of the participating genes or by epigenetic mechanisms, the rate of random accumulation of DNA replication errors increases by two to three orders of magnitude (reviewed in ref. 85). This increased rate of base mismatching usually does not result in a tumorous phenotype, unless an activation of an oncogene or inactivation of a tumor suppressor gene, occurs. The involvement of an abnormal DNA-MMR as determined by the demonstration of MSI is most notable in colon cancer, especially in the context of hereditary nonpolyposis colon cancer (HNPCC) (MIM# 114500),[86] but a variety of other tumors have also shown to display MSI.[87] Only a handful of reports that document MSI in parathyroid tumors exist. In a single large parathyroid adenoma from a young Brazilian girl, MSI was shown in 9/23 markers from chromosomes 1, 10 and 11.[88] MSI was also documented in 6/14 single parathyroid adenomas, especially with chromosome 11 markers, and in 5/6 multiglandular hyperplastic lesions, especially with 17p markers.[89] No mutation detection was attempted in either study, so the precise gene(s) underlying this apparent MSI in parathyroid tumorigenesis are presently unknown. Furthermore, the rarity of the reports on MSI in parathyroid tumors, combined with the extensive allelotyping studies reported in the same tumor types,[67,68] probably indicate that DNA-MMR gene involvement in parathyroid tumorigenesis is a rare event.

Telomerase Activity in Parathyroid Tumors

A teleomere is composed of a large tract of a short (6 bases) repeat—TTAGGG—located at the ends of chromosomes.[90] The size of the telemores in human chromosomes ranges from 5,000 to 15,000,[91] and their putative function is to protect against degeneration by exonucleases and ligases, protect against chromosomal end fusion, protect from activation of DNA damage check points, and also to play a role in homologous pairing.[92,93] Normal dividing somatic cells lose telomeric sequences progressively with each cell division. When the telomeres have been shortened to a critical length, the cell recognizes the DNA damage and enters a senescence phase.[94] Thus, normal cells have a finite number of cell replications in vitro (about 40-70) before senescence occurs, a feature that is related to the decrease in telomere length.[95] The length of the telomere is in part controlled by the enzyme telomerase, a ribonucleoprotein capable of maintaining telomere length. In normal adult tissues, its levels are negligible, but it is overexpressed in about 90% of human cancers (reviewed in ref. 96). The activity of telomerase can be measured in vivo by the Telomeric Repeat Amplification Protocol (TRAP),[97] whereas the consequences of its activity can be quantified by measuring and comparing telomere lengths in tumorous and nontumorous tissue. Few studies have looked at telomeres as contributors to parathyroid tumor development. Falchetti and coworkers[98] reported that a single metastasis from a parathyroid carcinoma and primary cell culture derived from the primary tumor exhibit a high telomerase activity. Subsequently, Kammori et al[99] demonstrated that telomere length was significantly shorter in benign (uni and polyglandular disease) and malignant parathyroid tumors compared with normal parathyroid tissue, but that telomerase activity was only observed in the malignant parathyroid tumors and in none of the benign parathyroid adenomas. These results may indicate that telomerase activity may distinguish benign from malignant parathyroid tumors, but that telomere shortening is observed in all parathyroid neoplasms, regardless of biological behavior and specific histological features.

Calcium Set Point and the Regulatory Genes Involved

Familial hypocalciuric hypercalcemia (FHH) (MIM# 145980) is an autosomal dominant disorder characterized by parathyroid hyperfunction resulting from reduced sensitivity to extracellular calcium.[100,101] In most cases, a heterozygous inactivating mutation in the calcium sensing receptor (CaSR) gene (localized to 3q13.3-21) (MIM# 601199) can be found.[102-104] Although this disorder is usually not accompanied by parathyroid cellular proliferation, a specific heterozygous mutation was associated with an FHH variant characterized by parathyroid adenomas and hypercalciuria,[105] and a homozygous mutation in CaSR causes severe neonatal hyperparathyroidism (MIM# 239200), a disorder associated with parathyroid hypercellularity.[106] These observations pertaining to inherited disorders associated with parathyroid proliferation and perturbations of calcium metabolism, made it plausible that acquired, biallelic, somatic inactivation of the CaSR gene may contribute to parathyroid tumorigenesis. However, several investigators analyzed sporadic parathyroid tumors for such mutations, with negative results.[107-109] These findings are indicative that inactivating mutations in the CaSR gene do not contribute to parathyroid tumorigenesis in a classical tumor suppressor gene manner, and probably do not offer growth advantage to parathyroid cells. However, decreased expression in CaSR protein assessed by immunohistochemistry was shown in about half of parathyroid adenomas tested.[110-112] Combined with the lack of demonstrable somatic mutations, one plausible interpretation of these results is that mutations in genes involved in the regulation of the calcium sensing pathway (or even epigenetic mechanisms affecting CaSR expression levels) may contribute to parathyroid tumorigenesis, and perhaps play a pivotal role in the well established insensitivity of parathyroid tumors to extracellular calcium.

Vitamin D Receptor

1, 25 dihydroxyvitamin D3, the ligand of the vitamin D receptor (VDR) is capable of inhibiting parathyroid proliferation in vitro.[113,114] This observation, prompted a search for inactivating mutations in the VDR gene in sporadic parathyroid tumors, with negative results in both sporadic primary hyperparathyroidism and in secondary hyperparathyroidism of uremia.[115] Thus, akin to the CaSR gene, the VDR gene does not function as a classical tumor suppressor gene in parathyroid tissue, and its inactivation is noncontributory to parathyroid proliferation. Yet, its involvement in parathyroid tumorigenesis is inferred from the altered expression pattern at the mRNA and protein levels,[116] and the known effects of the ligand, 1, 25 OH VitD3, on PTH secretion, as well as the known effects of vitamin D deficiency on parathyroid cellular proliferation.[117]

Summary

Over the past decade a more comprehensive understanding of the molecular mechanisms involved in initiation and progression of benign and malignant parathyroid tumors has been achieved. These new insights provide better fundamental understanding of the biology and interaction of endocrine tumor related genes and have the potential of providing more accurate, biologically rational diagnostic and therapeutic tools. The application of novel technologies such as microarrays for concomitant analyses of thousands of genes at the RNA and protein levels as well as proteomic technology over the next few years will hopefully lead to new and exciting revelations in parathyroid tumorigenesis.

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