Independent Genetic Events Associated with the Development of Multiple Parathyroid Tumors in Patients with Primary Hyperparathyroidism

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Multiple parathyroid tumors, as opposed to hyperplasia, have been reported in a subset of patients with sporadic primary hyperparathyroidism (PHPT). It is not clear whether these multiple tumors are representative of a neoplastic process or whether they merely represent hyperplasia that has affected the parathyroid glands differentially and resulted in asynchronous growth. The molecular genetic techniques of comparative genomic hybridization (CGH), loss of heterozygosity (LOH), and *MEN1* mutation analysis were performed on a series of five patients with multiglandular PHPT, each of which had two parathyroid tumors removed. Analysis of these multiple parathyroid tumors from patients with PHPT revealed that independent genetic events were associated with the development of a subset of these tumors. The DNA sequence copy number changes, identified by CGH analyses, either involved different chromosomal regions in the paired glands of a patient (two patients), or those regions implicated in one gland were not changed in a second gland from the same patient (two patients). Each of the three patients exhibiting LOH demonstrated different changes between the paired glands. Where LOH was detected in one gland from a patient, the other gland from the same patient either exhibited no allelic loss or the loss detected was in another region. Each of the three tumors exhibiting LOH at 11q13 was found to

contain a somatic *MEN1* mutation in the remaining allele, however these mutations were not present in the germline or in the paired gland from the same patient. Although it is possible that a separate series of genetic changes has arisen randomly in two separate glands within the same individual, it seems more likely that the development of these multiple tumors has arisen because of the involvement of other unknown factors. These factors may be genetic [such as the involvement of one or more germline mutations in an unknown low-penetrance gene(s), germline mosaicism or alterations in calcium-sensing receptor gene(s)], epigenetic, physiological, or environmental. (*Am J Pathol* 2002, 161:1299–1306)

Hyperfunction of multiple parathyroid glands (encompassing hyperplasia and multiple tumors) is responsible for the development of sporadic primary hyperparathyroidism (PHPT) in ~15% of cases.^{1,2} Of these, multiple tumors, as opposed to hyperplasia, have been reported in 1.7 to 11.8% of cases.^{3–6} The histopathological classification of parathyroid hyperplasia or neoplasia cannot easily be differentiated¹ and it is not clear whether multiple parathyroid tumors are representative of a neoplastic process or whether they merely represent hyperplasia that has affected the glands differentially and resulted in asynchronous growth.⁶ Several studies have tried to determine whether these lesions represent true neoplasms or are hyperplastic through the use of clonality analyses.^{7,8} These studies however, have produced conflicting results and have not been able to clarify whether these lesions are monoclonal or polyclonal.

Loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) studies have implicated and/or confirmed chromosomal regions frequently altered in parathyroid tumorigenesis at 1p, 1q21-q32, 9p,

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Table 1. Summary of Clinical and Genetic Data for the 10 Parathyroid Tumors from 5 Patients with Multiglandular PHPT

Case number	Age at operation (years)	Sex (M/F)	s-Ca*		Pre-op PTH†	Follow-up (months)	Pathological glands	
			Pre-op (mmol/L)	Post-op (mmol/L)			Number	Location‡
M1	64	M	2.72	2.15	×3.17	16	2	LS RI
M2	51	F	2.90	2.38	×2.50	8	2	RS LS
M3	54	M	2.77	2.73	×1.24	11	2	RI RS
M4	46	M	2.73 2.75	2.30 2.47	— ×1.59	9	2	LS RS
M5	53	F	2.77	2.24	×3.23	15	2	LS RS

11q13, 13q, 15q, 17, 19, 22q, and the X chromosome.^{9–14} In addition, after the cloning of the *MEN1* gene,^{15,16} somatic mutations within *MEN1* have been shown in 7 to 27% of sporadic parathyroid tumors,^{14,17–22} and germline *MEN1* mutations have been identified in 49 to 100% of MEN 1 families.^{12,15,16,23–25}

In the present study, genetic changes were investigated in multiple tumors occurring in the same patients with a sporadic form of the disease. A series of five patients with multiglandular PHPT were studied by analyzing two parathyroid tumors removed from each patient. To investigate the genetic events that may contribute to the development of multiple tumors in patients with PHPT, CGH, LOH, and *MEN1* mutation analysis were used. CGH was used as a genome-wide screen to identify chromosomal regions likely to contain oncogenes or tumor suppressor genes that may be involved in the development of multiple parathyroid tumors. In addition, LOH studies of the tumors from patients with multiglandular PHPT were performed at regions previously associated with parathyroid tumorigenesis, to identify more discrete regions of loss that may have been beyond the resolution capabilities of CGH. The tumors were examined for LOH at distal 1p, the HPT-JT locus at 1q21-q32 and flanking regions, and the *MEN1* locus at 11q13. Further, in the tumors from patients with multiglandular PHPT the *MEN1* gene was screened for the presence of mutations.

Materials and Methods

Patients and Tumor Samples

Ten parathyroid tumor samples were obtained from five patients (two females and three males) who underwent parathyroidectomy at the Royal North Shore Hospital, Sydney, Australia, for sporadic PHPT and who were found to have multiglandular disease. The diagnosis of multiglandular PHPT was established based on criteria similar to those of Attie and colleagues,⁵ and were as follows: 1) identification of two or three enlarged parathyroid glands at surgery; 2) operative finding of at least one normal appearing parathyroid gland; 3) no indication of familial forms of PHPT, that is, MEN 1, HPT-JT, or FIHP; and 4) postoperative normalization of serum calcium levels, for at least 6 months, as suggested by Clark and colleagues²⁶ and Wadström and colleagues.²⁷

Four patients (M1, M2, M4, M5) had two parathyroid tumors removed at the time of surgery, with serum calcium levels returning to normal postoperatively in each of these cases, suggesting that all affected glands were removed. One patient, M3, remained hypercalcemic after the removal of a single parathyroid tumor and subsequently underwent further exploration, as the result of which a second parathyroid tumor was removed. The serum calcium levels in this patient (M3) returned to normal after the second operation, suggesting that all pathological glands had now been removed. Each of these patients remained normocalcemic, with follow-up periods ranging from 8 to 16 months. The relevant clinical information for each of the five patients with multiglandular sporadic PHPT is detailed in Table 1. None of the patients exhibited any other phenotypic features or had a family history suggestive of MEN 1, HPT-JT, or FIHP. All patients gave informed consent according to a protocol approved by the Royal North Shore Hospital Human Research Ethics Committee.

The parathyroid tumor tissue samples were frozen in liquid nitrogen immediately after surgical removal and stored at –70°C until use. Peripheral blood samples were also obtained from all patients for extraction of constitutional DNA. High molecular weight DNA was extracted from fresh-frozen tumor samples and peripheral blood leukocytes according to standard procedures.²⁸ At the time of DNA extraction, a representative section was cut from each tissue sample. After formalin-fixation, paraffin-embedding, and hematoxylin and eosin staining, the tumor status was confirmed by histopathological examination. Eight of the samples were thus shown to be composed of at least 80% tumor cells, and samples M2-LS and M5-LS were found to contain 50% and 60% tumor cells, respectively.

Table 1. Continued

Pathological glands		CGH imbalances		LOH results			MEN1 sequence [§]
Weight (mg)	Size (mm)	Losses	Gains	1p	1q	11q13	
2,500	20 (diameter)	nd**	19p	++	+	+	—
—	10 (diameter)	nd	nd	+	+	+	—
1,520	32 × 12 × 10	13q, 15q, 18, 21q, 22q	5, 6, 7, 16	+	+	+	wt [¶]
140	6 × 5 × 4	11	nd	+	+	LOH	c.1539delG
180	10 × 6 × 5	—	—	+	+	+	—
430	15 × 13 × 8	nd	nd	+	+	+	—
80	9 × 7 × 3	nd	nd	+	+	+	wt
350	20 × 8 × 8	12p, 13q	nd	+	+	LOH	c.911-912insT
<1,000	3 × 2 × 2	11	nd	+	+	LOH	c.318delG, c.311C>A
310	12 × 7 × 10	1p, 12q23-q24.3	12pter-q21	LOH ^{††}	+	+	wt

*Pre- and postoperative serum calcium (reference range 2.10 to 2.60 mmol/L).

†Pre-operative intact PTH-x times the upper normal limit.

‡LS, left superior; RS, right superior; LI, left inferior; RI, right inferior.

§Confirmed somatic mutations; nucleotide numbering based on sequence obtained from EMBL accession no. U93236.

¶wt, wildtype.

||—, not available/not analyzed.

**nd, none detected.

††LOH, loss of heterozygosity.

+++, Retention of heterozygosity.

CGH

Nine parathyroid tumors from five patients with multiglandular sporadic PHPT were analyzed by CGH, as previously described.¹³ Briefly, tumor DNA samples were labeled with fluorescein-12-dUTP (NEN, Life Science Products, Boston, MA) by nick translation, and normal reference DNA was labeled with SpectrumRed (Vysis, Inc., Downers Grove, IL). Tumor and reference DNA were mixed together with unlabeled Cot-1 DNA (Life Technologies, Inc., Gaithersburg, MD), denatured, and hybridized to normal male human metaphase chromosomes (Vysis, Inc.). After hybridization at 37°C for 72 hours, the slides were washed in 0.4× standard saline citrate/0.3% Nonidet P-40 (Boehringer Mannheim GmbH, Mannheim, Germany) at 74°C for 2 minutes, in 2× standard saline citrate/0.1% Nonidet P-40 at room temperature for 2 minutes, and finally in water at room temperature for 2 minutes before being air-dried. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole (Vysis, Inc.) to enable identification. For each hybridization, a control experiment was performed to ensure adequate hybridization conditions, in which normal male genomic DNA (Promega, Madison, WI) and normal female genomic DNA (Vysis, Inc.) were hybridized against normal metaphases. A minimum of 10 metaphases were captured for each analysis. Green to red ratios <0.80 were considered as losses, >1.20 as gains, and ratios >1.50 as amplifications of genetic material. Heterochromatic regions in the centromeric, paracentromeric, and telomeric parts of some chromosomes, the short arm of the acrocentric chromosomes, and the Y chromosome were not included in the analysis. Insufficient amount of DNA prevented CGH analysis of one of the tumors (M3-RI).

LOH

Allelic deletions of chromosome 1 were assessed using the following microsatellite markers (1pter-1qter): *D1S243*, *D1S468*, *D1S244*, *D1S2667*, *D1S228*, *D1S2728*, *D1S507*, *D1S478*, *D1S513*, and *MYCL1* in 1p; and *D1S218*, *D1S215*, *D1S191*, *D1S222*, *D1S428*, *D1S412*, *D1S413*, *D1S477*, and *D1S423* in 1q^{14,29} (<http://www.genome.wi.mit.edu>; <http://gdbwww.gdb.org>; <http://www.ncbi.nlm.nih.gov/genemap99>). LOH at the *MEN1* locus, 11q13, was analyzed using *PYGM*, *D11S4946* (within *MEN1*), *D11S4940*, and *D11S449*.^{14,30}

Polymerase chain reactions (PCR) were used to amplify genomic DNA (50 ng) from matched pairs of blood and tumor DNA samples as previously described.¹⁴ LOH was determined visually and quantitatively by comparison of intensities of the two alleles in informative cases. LOH was confirmed if a reduction of >50% of the signal intensity was observed in the tumor DNA for one of the constitutional alleles, as previously described for this method.^{14,31}

MEN1 Mutation Analysis

Mutation analysis of the *MEN1* gene was performed on six tumors from three patients using methods previously described.¹⁶ The nine coding exons and flanking intronic sequences of *MEN1* were directly sequenced after PCR amplification of the tumor DNA samples (100 ng) as 14 different fragments of 200 to 300 bp each. Standard PCR conditions were used and the products were purified using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Cycle sequencing was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems,

Figure 1. Summary of DNA copy number changes detected by CGH in six of the nine paired parathyroid tumors analyzed from patients with multiglandular PHPT. One alteration detected in one tumor is represented by one **bar** next to the ideogram, with losses indicated to the **left** and gains to the **right**. Those chromosomes that are not illustrated did not exhibit DNA copy number changes, as detected by CGH.

Perkin-Elmer Corp., Foster City, CA) and the following thermocycling conditions: an initial denaturation at 94°C for 5 minutes, was followed by 25 cycles of denaturation at 96°C for 10 seconds, and annealing at 50°C for 5 seconds, and a final extension at 60°C for 4 minutes. The products were electrophoresed in 4.8% PAGE Plus gels (Amresco, Cleveland, OH) and analyzed on a 377XL automated DNA sequencer (Applied Biosystems, Perkin-Elmer Corp.).

Results

CGH Analysis of Sporadic Parathyroid Tumors in Patients with Multiglandular PHPT

In all four patients in whom copy number changes were detected by CGH, the changes differed in the two glands from the one patient. Patients M2 and M5 exhibited different DNA sequence copy number changes between each of the paired glands, while in patients M1 and M4 DNA sequence copy number changes were detected in one gland but not the other gland from the same patient.

Specifically, six of the nine (67%) parathyroid tumors analyzed (M1-LS, M2-RS, M2-LS, M4-RS, M5-LS, and M5-RS) were found to exhibit alterations detected by CGH, involving 1 to 9 regions. Of those six tumors showing DNA sequence copy number changes, there was an average of three aberrations per sample. The gains and losses found are detailed in Table 1. Further to this, subchromosomal regions with increased and decreased DNA sequence copy number changes are illustrated in Figure 1. Losses at chromosome 11 and 13q were the most frequently detected changes in the multiglandular sporadic parathyroid tumors, with loss occurring at these chromosomes in two of nine (22%) tumors. Loss at 1p,

Figure 2. Summary of allelic loss at chromosome 1p, 1q, and 11q13 in the paired parathyroid tumors from five patients with multiglandular PHPT. The microsatellite markers genotyped are listed in order from telomere to centromere for chromosome 1p and centromere to telomere for chromosomes 1q and 11q13. The location of the *MEN1* gene and the HPT-JT locus are indicated next to ideograms of chromosomes 1 and 11, respectively. Individual parathyroid tumors, along with the affected glands (LS, left superior; RS, right superior; LI, left inferior; RI, right inferior) are labeled at the **top** of the figure. At a given locus, tumor-specific allelic loss is indicated by a **black box**; retention of heterozygosity by an **open box**, and constitutional homozygosity (noninformative) by a **hatched box**.

12p, 12q, 15q, 18, 21q, and 22q was detected only once, while gains of chromosomes 5, 6, 7, 12, 16, and 19p were also detected once. The remaining three parathyroid tumors (M1-RI, M3-RS, and M4-LS) did not exhibit any alterations detectable by CGH.

LOH Analysis of Sporadic Parathyroid Tumors in Patients with Multiglandular PHPT

LOH analyses of chromosomes 1 and 11 were performed on 10 parathyroid samples from five patients with multiglandular sporadic PHPT. The results of these cases are summarized in Table 1 and Figure 2. Different LOH changes in the paired parathyroid tumors of three patients were detected. One patient (M5) exhibited LOH at 11q13 with retention of heterozygosity at 1p and 1q in the left superior gland, while the right superior gland showed LOH at 1p with retention of heterozygosity at 1q and 11q13, as seen in Figure 3. The other two patients (M2 and M4) exhibited LOH at 11q13 in one parathyroid tumor but the paired tumors from the same individuals exhibited retention of heterozygosity at 11q13 as well as 1p and 1q. All together, LOH was detected at 1p in 1 of 10 (10%) informative parathyroid tumors (M5-RS) and at 11q13 in 3 of 10 (30%) informative parathyroid tumors (M2-LS, M4-RS, and M5-LS). No allelic loss was detected in any of the 10 informative parathyroid tumors at 1q.

Figure 3. Representative PhosphorImage scans of DNA amplified from the paired parathyroid tumors from M5 showing differential LOH in the separate parathyroid tumors. At each represented locus, the leukocytes (N) are heterozygous and the tumor DNA, from the right superior (RS) or left superior (LS) parathyroid gland show either retention of heterozygosity or LOH. The figure demonstrates: **i**, LOH at 1p (*MYCL1*) in the RS parathyroid gland (upper allele), while the LS parathyroid gland has retention of heterozygosity; **ii**, retention of heterozygosity in both glands (RS and LS) at 1q (*D1S222*); and **iii**, LOH at 11q13 (*D11S449*) in the LS gland (upper allele), while the RS gland has retention of heterozygosity.

MEN1 Mutation Analysis of Sporadic Parathyroid Tumors in Patients with Multiglandular PHPT

The three parathyroid tumors (M2-LS, M4-RS, and M5-LS) in which LOH at 11q13 was detected were screened for *MEN1* mutations. Two of these tumors, M2-LS and M5-LS, also exhibited decreased DNA sequence copy number at chromosome 11 as detected by CGH. Although LOH at 11q13 was observed in M4-RS, no loss in this sample was detected by CGH analysis. The lower resolution of CGH could explain this result, as the region of loss detected by LOH may be too small to be detected by CGH. All three parathyroid tumors (M2-LS, M4-RS, and M5-LS) were found to contain somatic *MEN1* mutations, as shown in Table 1. In each patient in whom a mutation in *MEN1* was detected, the second gland from the same patient was not found to contain the same mutation. Wild-type sequence was present in the constitutional DNA of each of the patients M2, M4, and M5 thus confirming the nucleotide alterations as somatic.

In M2-LS, a single base deletion was detected in exon 10 (c.1539delG) of the remaining allele of the *MEN1* gene, whereas in M4-RS a single base insertion was detected in exon 5 (c.911-912insT) of *MEN1*, both mutations resulting in a frame-shift (Table 1). Sequencing of M5-LS detected two nucleotide changes, both within exon 2. One of the nucleotide changes resulted in a frameshift because of a single base deletion (c.318delG), whereas the other change is a substitution (c.311C>A) that does not result in an amino acid change.

Discussion

The development of apparently sporadic multiple tumors, involving the same organ, is not uncommon and has been shown to occur in the parathyroid glands³⁻⁶ as well as in a number of other organs, including the breast³² and adrenal gland.³³ In patients with PHPT, there have been no specific studies that have focused on determining whether independent genetic events are responsible

for the development of multiple parathyroid tumors within the same individual. In this study, two parathyroid tumors removed from each of five patients with PHPT were analyzed. Within the limits of the resolution of the methods used in this analysis and at the loci studied, different changes were found in the two tumors from the same patient, in which changes were detected.

Results obtained from CGH analysis, showed that none of the paired tumors removed from the same patient were found to exhibit any of the same DNA sequence copy number changes. Suggesting that at the gross chromosomal level, independent somatic genetic events are associated with the development of multiple parathyroid tumors. Six of nine (67%) sporadic parathyroid tumors from multiglandular PHPT patients were found by CGH analyses to contain DNA sequence copy number changes. This is comparable to previous studies of sporadic parathyroid tumors in which 69 to 93% of tumors were found to contain DNA sequence copy number alterations.^{10,11,13,34,35} The analyses revealed frequent loss of chromosome 11 (22%) and 13q (22%), losses of which have previously been shown to occur at increased frequency in sporadic parathyroid tumors.^{10,11,13,34,35}

LOH analysis of regions at 1p, 1q, and 11q13, intervals previously shown to be lost in both familial and sporadic parathyroid tumor development, were performed. Three of 10 tumors (30%) exhibited LOH at the *MEN1* locus and were also found to contain a *MEN1* mutation in the remaining allele. The three mutations identified result in the generation of a frameshift predicted to prematurely truncate menin (the MEN 1 protein). The incidence of somatic *MEN1* mutations arising in this group of sporadic parathyroid tumors from multiglandular PHPT patients fits within the range previously reported in sporadic parathyroid tumors (7 to 27%).^{14,17-22} These findings suggest that *MEN1* gene involvement is associated with the pathogenesis of a subset of parathyroid tumors in multiglandular PHPT patients, however *MEN1* involvement in these patients does not encompass all tumors within the same patient.

In this study, LOH analyses of two regions on chromosome 1 at 1p34.3-pter and 1q21-qter (encompassing the HPT-JT locus at 1q21-q32) detected allelic loss in only 1 tumor of the 10 (10%) sporadic parathyroid tumors analyzed. This tumor, M5-RS, exhibited allelic loss at 1p only. The other parathyroid tumor from this patient, M5-LS, was found to exhibit LOH at 11q13 and also contained a *MEN1* mutation in the remaining allele, which was not present in M5-RS. The incidence of LOH at 1p arising in this group of sporadic parathyroid tumors from multiglandular PHPT patients fits within the range previously reported in sporadic parathyroid tumors (0 to 44%).^{9,14,31,36} Of interest, in one patient (M3), genetic alterations were not detected, at any of the loci analyzed, in either of the two glands studied. Although there may have been genetic alterations at other loci not analyzed in this study, this does raise the possibility that the development of multiple parathyroid tumors may not always show different genetic alterations.

Although there is evidence for apparently sporadic bilateral tumors arising in other organs, such as breast³² and adrenal gland,³³ it would seem relatively unlikely, but

not impossible, that different primary genetic events would arise in the same individual entirely by chance. It may seem more likely that the development of multiple parathyroid tumors, in patients with PHPT, has arisen because of the contribution of other unknown genetic, epigenetic, physiological, or environmental factors. These underlying factors may provide a background of increased cell proliferation, thereby increasing the likelihood of later tumorigenic somatic events. The later somatic events may differ in the different parathyroid glands within the same individual, as indicated by the results of this study.

Genetic events that may explain development of multiple parathyroid tumors in these individuals, include germline mutation(s), in an unknown low-penetrance gene(s). It is possible that one or more germline mutations, in an unknown low-penetrance gene(s), are responsible for the development of multiple parathyroid tumors in a proportion of PHPT patients. Such germline mutation(s) may provide the basis for increased parathyroid cell proliferation and hence an increased risk of additional somatic events arising. Somatic genetic differences have previously been reported between the bilateral tumors of patients with a germline mutation in a highly penetrant gene. An example of this was reported in a MEN 2A patient with a germline mutation in the *RET* proto-oncogene and bilateral pheochromocytomas in which the right adrenal gland exhibited LOH at 1p, while the left adrenal gland exhibited retention of heterozygosity at 1p.³⁷ The presence of a germline mutation(s) in a low-penetrance gene(s) would explain the absence of an obvious family history in these apparently sporadic PHPT patients.

Alternatively, germline mosaicism may be responsible for the development of multiple parathyroid tumors within these patients, as has previously been reported in retinoblastoma patients, in which germline mosaicism has been reported in a larger proportion of bilateral cases compared with unilateral cases.³⁸ A mutation arising in a gene at some point during embryogenesis could result in germline mosaicism and the parathyroid glands may have developed from a mix of cells, both with or without a genetic defect. It is possible that a mutation, either in *MEN1* or another unknown gene associated with parathyroid tumorigenesis, may have occurred at some point during embryogenesis and is therefore present in some, but not all, parathyroid cells. Those cells containing this primary genetic mutation may have a proliferative advantage providing a background on which additional somatic events can occur.

Another possibility is that genetic alterations have affected the calcium-sensing abilities of the parathyroid cells, leading to increased parathyroid cell proliferation and an increased risk of somatic mutations. Increased parathyroid cell proliferation has been shown to occur as a result of an alteration in the calcium-sensing abilities of the parathyroid cells.³⁹ Mutations within the calcium-sensing receptor gene (*CaR*) on chromosome 3q, have been linked to two inherited conditions, familial hypocalciuric hypocalcemia and neonatal severe hyperparathyroidism.⁴⁰ Both of these familial conditions exhibit altered calcium homeostasis,⁴⁰ with enlarged parathyroid glands

occurring as a result of mutations in *CaR*.⁴⁰ Several studies have failed to identify somatic mutations within *CaR* in sporadic parathyroid tumors,^{41,42} however decreased expression of *CaR* mRNA and protein have been shown.^{43–45} It is possible however, that other loci associated with familial hypocalciuric hypocalcemia and neonatal severe hyperparathyroidism on chromosome 19^{46,47} could be involved in the pathogenesis of sporadic parathyroid tumors. It is of interest to note that chromosomal amplification at chromosome 19p has been detected frequently in sporadic parathyroid tumors^{11,13,34} and also in a parathyroid tumor in this study (M1-LS).

Epigenetic mechanisms, leading to changes in gene expression have been shown to be responsible for the development and/or progression of a number of human tumors, such as breast⁴⁸ and prostate⁴⁹ cancer. It is possible that DNA methylation or histone acetylation/deacetylation of genes known to be associated with parathyroid tumorigenesis, or as yet unidentified genes, may lead to increased parathyroid cell proliferation. This in turn may provide a background on which further somatic events may arise, leading to the development of multiple tumors in a subset of PHPT patients.

Physiological stimuli or unknown environmental factors could also be responsible for the sporadic development of multiple tumors in a small proportion of PHPT patients. Physiologically, PTH secretion is regulated by ionized calcium, phosphate, and the active metabolite of vitamin D, 1,25-dihydroxyvitamin D [$1,25(\text{OH})_2 \text{D}_3$], as reviewed by Juppner and colleagues.⁵⁰ Increased PTH secretion can lead to increased PTH gene expression and increased parathyroid cell proliferation. It is possible that decreased ionized calcium, decreased $1,25(\text{OH})_2 \text{D}_3$ and/or increased phosphate have led to increased parathyroid cell proliferation, even though it was not clinically evident. It is also possible that unknown environmental factors, such as smoking, irradiation, or the use of certain drugs, could also be an underlying cause in these patients, however epidemiological studies would need to be conducted to address these issues.

It is clear from these studies that independent genetic events are associated with the development of multiple parathyroid tumors in some patients with PHPT. In some patients, different genetic changes were detected in both glands, whereas in other patients, the genetic changes detected in one gland were absent from the other gland. Although it is possible that a distinct series of genetic events has arisen randomly in two separate glands within the same individual, it seems more likely that the development of these multiple tumors has arisen on a background of increased susceptibility to tumorigenesis because of unknown genetic, epigenetic, physiological, or environmental factors. Further studies are required to test these hypotheses.

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