Molecular analysis of the HPJ-JT syndrome and sporadic parathyroid carcinogenesis

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Chapter 1

Introduction and outline

Introduction

History

The parathyroid glands, the last major organ to be discovered in humans, were first recognized by Virchow (1863); however, it was Ivar Sandström (1852-1889) who is generally acknowledged as the first to describe these glands in detail.[44] Sandström demonstrated that the glands were structures separate from the thyroid and gave these organs their name of glandula parathyreoidea. He reported the number and histology of these glands, but the function of these glands remained unknown until 1891, when von Recklingshausen[50] reported the association between bone disease and hyperparathyroidsim (HPT).

Parathyroid glands

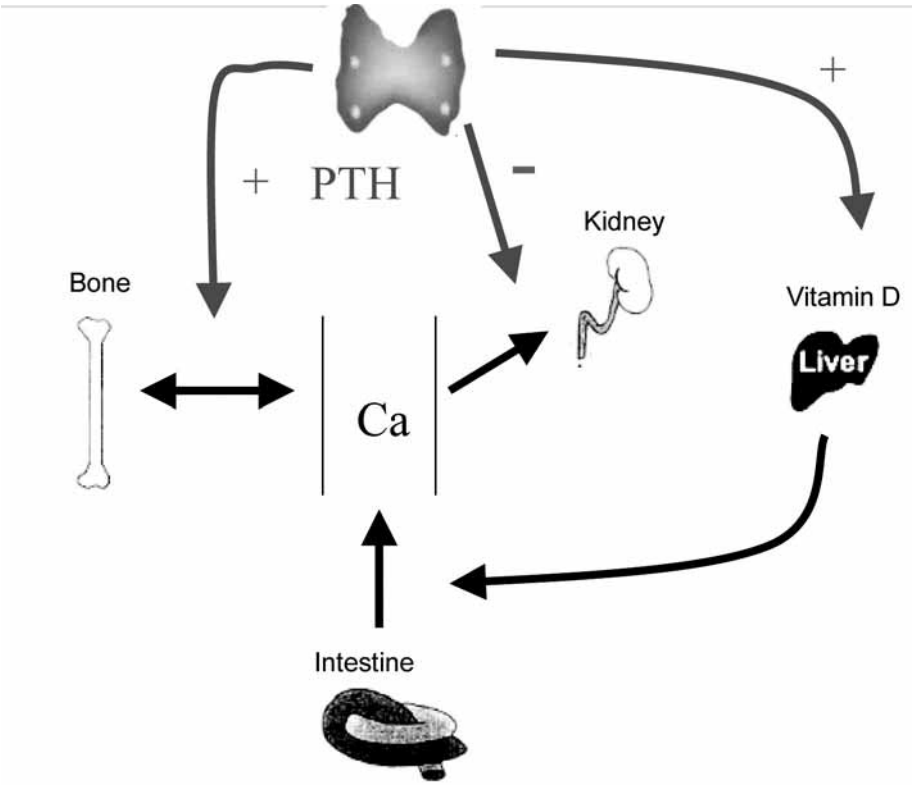
Normal gross anatomy and embryology
In the majority of cases, the parathyroid consists of four oval bone-shaped glands[25], two superior and two inferior. Five percent of people have supernumerary glands (defined as weight >5 mg and located apart from the other 4 glands).[16] The superior parathyroid gland arises from the fourth branchial (pharyngeal) pouch and descends into the neck with the thyroid gland. The inferior parathyroid glands, together with the thymus, are derived from the third branchial pouch.
The superior glands are most commonly localized in the fatty tissue on the middle third of the posterior lateral border of the thyroid gland, while the inferior glands are located on the lower thyroid poles close to the inferior thyroid artery.[5]
The mean weight of all four glands is approximately 120 mg in men and 130 mg in women.[16;25] Each gland has an average size of 4x3x1.5 mm, with the lower glands generally larger than the upper glands.[1] The colour varies from reddish brown to a yellow tan depending on the amount of stromal fat.
The arterial supply of the glands is derived from branches of the superior thyroid artery (upper parathyroid) and the inferior thyroid artery (lower parathyroid).
Venous drainage is achieved by the superior thyroid vene (upper parathyroid) and the inferior thyroid vene (lower parathyroid).[16]

Normal histology
The parathyroid glands are microscopically composed of three types of parenchymal cells interspersed with a varying amount of stroma surrounded by a thin connective tissue capsule. The parenchyma is composed of chief cells, oncocytic or oxyphilic cells and water clear cells.
Chief cells are small and regular cells with an amphophylic and relatively lucent cytoplasm. The nuclei are centrally located, with uniform chromatin and small inconspicuous nucleoli. They are often moulded and show overlap. These cells synthesize, transport, store, and secrete parathyroid hormone (PTH).[27;41]
Oncocytic or oxyphilic cells have a more abundant cytoplasm, which is deeply granular and acidophilic. These types of cells appear at puberty and increase in number as age progresses. The cells are often present in the form of clusters or nodular collections.
Water clear cells have an abundant and optically clear cytoplasm and sharply defined cell membranes. It is suggested that the water clear cells are inactive chief cells.[16]
The stromal component is composed of mature fat cells, blood vessels and a varying amount of connective tissue. Stromal fat cells begin to appear late in the first decade of life and increase throughout life, reaching a maximum in the third to fifth decades

of life.[16]
Parathyroid cells have a lifespan of approximately 20 yrs eventually undergoing apoptosis[52]. Mitoses are almost never seen in normal parathyroid cells.[40]

Physiology
Calcium plays a central role in a number of physiological processes that are essential for life including neuromuscular transmission, muscle contraction, cardiac automaticity, nerve function, cell division and movement and certain oxidative processes. Normal calcium concentrations are maintained as a result of tightly regulated ion transport by the kidneys, intestinal tract, and bone (see Figure 1). This is mediated by calcaemic hormones, in particular the parathyroid hormone (PTH) and the active form of Vitamin D.[24]

Figure 1



PTH is a linear polypeptide containing 84 amino acid residues, whose major function is to increase extracellular Ca^{2+} concentration. It is synthesized in the chief cells in parathyroid gland, in the form of a large precursor molecule: preproPTH, which is processed and shortened in the parathyroid cell. Once secreted, PTH has a half-life of approximately 2 minutes.
The primary function of PTH is to increase serum Ca^{2+} concentration and in this way maintain the extracellular fluid (ECF) calcium concentration within a narrow normal range. Secretion of PTH is regulated by extracellular calcium, via a G protein-coupled calcium-sensing receptor.[9]

The hormone stimulates calcium release from bone, reabsorption from the kidneys and uptake from the intestines.[12] The latter process is mediated by 1,25-dihydrocholecalciferol, which is the biological active form of Vitamin D3 (cholecalciferol). PTH is required to metabolise Vitamin D3, which is formed in the skin through the action of UV light, to 1,25-dihydrocholecalciferol in the liver.
A defect in the calcium sensing signalling cascade mentioned above can lead to hyperparathyroidism, characterized by inappropriately high levels of PTH in relation to extra cellular calcium levels and hyperplasia or increased cell proliferation.[10;11]

Hyperparathyroidism

Etiology
Increased cell proliferation manifests as hyperplastic or neoplastic parathyroid lesions. HPT may develop as a primary disorder, either idiopathic or familial, or as a secondary disorder in response to a biochemical imbalance, generally due to renal impairment. It may also arise in response to lithium treatment as a therapy for bipolar disorder. Secondary HPT may in turn progress to a tertiary disorder; the parathyroid hyperactivity becomes autonomous and is no longer responsive to physiological regulation. The mechanism and molecular pathway(s) underlying this phenomenon are unclear.

Parathyroid gland lesions
Primary hyperparathyroidism (PHPT) is caused by adenomas in 80% of the cases, hyperplasia in 20% and carcinoma in 1% of the cases
Hyperplasia is defined as an absolute increase in parathyroid parenchymal cell mass resulting from proliferation of chief cells, oncocytic cells and transitional oncocytic cells in multiple parathyroid glands in the absence of a known stimulus for PTH hypersecretion[15]
A parathyroid adenoma is a benign encapsulated neoplasm usually involving a single gland with an adjacent rim of normal glandular tissue. The presence of a microscopically normal second gland is thought to represent the best evidence that a given parathyroid lesion is an adenoma rather than hyperplasia.[15]
Carcinomas are malignant neoplasms derived from parathyroid parenchymal cells.[26]

Histology
Parathyroid tumours are genetically, clinically and histologically very heterogeneous lesions, which often makes the diagnosis difficult if not impossible.
Benign tumours (adenoma and hyperplasia) are treated with simple parathyroidectomy; however, there is an important distinction between adenoma and hyperplasia in that hyperplasia will recur or persist if only one gland has been removed. Intraoperatively, parathyroid carcinoma usually appears as a large, firm, whitish-gray tumour that commonly has invaded surrounding structures. Despite these defining characteristics, parathyroid carcinoma is often not recognized at the time of initial surgery.[43] In patients who undergo routine parathyroidectomy, as cancer is not suspected, 50% or more will develop local recurrence.[51] Furthermore, almost 90% of all patients with recurrent hyperparathyroidism will eventually die of the disease.[31] In contrast, patients where an adequate diagnosis was possible intraoperatively and treated by en bloc resection, local recurrence ranges from 10-33%, and long-term survival improves significantly.[31;53]
In summary, a quick (intra-operative) diagnosis of the three parathyroid tumours is essential as it has implications for (surgical) therapy.
However, intraoperative diagnosis is difficult, as there are almost no reliable differences between the tumours histologically. All three tumour types are characterized by the absence of intraparenchymatous fat and are composed of chief

cells, oncocytic cells or mixtures of these cell types. The only difference between adenoma and hyperplasia is the amount of affected glands and thus it is virtually impossible to differentiate between these two benign tumours purely on histological grounds.[19]
The distinction between parathyroid carcinoma and adenomas based on histology and morphology alone is also difficult. Some authors have claimed that trabecular growth, dense fibrous bands, spindle shape of tumour cells, mitotic figures and nuclear atypia[45] are helpful criteria to diagnose parathyroid carcinomas, but all these criteria can also be observed in benign parathyroid lesions.[7;34;46] Therefore, none of these characteristics are specific, although the presence of several in the same tumour increases the possibility of malignancy.[23] An unequivocal diagnosis of parathyroid carcinoma is only possible by demonstration of distant or local regional metastasis, characterized histologically by blood vessel invasion and/or capsular invasion.[42]
In conclusion, diagnostic accuracy of parathyroid tumours up until now has relied on multiple markers including the recognition of the constellation of macroscopic and microscopic features in combination with multidisciplinary correlation and not by histology alone. Based on recent insights, including work described in this thesis, histology might be supplemented by molecular investigations.

Primary hyperparathyroidism
PHPT is one of the most common endocrinopathies, with a prevalence of approximately 1-3 per 1000 individuals.[2] Sporadic PHPT is most common in post-menopausal women, with an estimated prevalence of 34 per 1000 individuals from this population subgroup.[33] The majority of tumours in primary hyperparathyroidism are sporadic. However, approximately 5% are associated with the autosomal dominant hereditary cancer syndromes Multiple Endocrine Neoplasia type 1 (MEN 1; OMIM #131100) and type 2A (MEN 2A; OMIM #171400), Hyperparathyroidism-Jaw Tumour Syndrome (HPT-JT, OMIM #145001), and Familial Isolated Hyperparathyroidism (FIHP, OMIM #145000).[35]
MEN1 syndrome is characterized by the occurrence of tumours of the parathyroids, pancreatic islet cells and anterior pituitary. PHPT represents the most common endocrinopathy in MEN1, reaching nearly 100% penetrance by age 40.[8] Parathyroid tumours occur in 95% of the MEN1 patients.[49]
The *MEN1* gene consists of 10 exons that encode a 610 amino acid protein, referred to as MENIN. MENIN appears to have a large number of potential functions through interactions with proteins that alter cell proliferation mechanisms.[49] The *MEN1* gene represents a tumour suppressor gene (TSG) and is located on chromosome 11q13. The majority of tumours (95%) show additional LOH consistent with Knudsen's two hit theory. MEN2 (OMIM 171400) is a rare autosomal dominant disorder of multiple endocrine neoplasms, including medullary thyroid carcinoma, pheochromocytoma, and parathyroid adenomas. Medullary thyroid carcinoma is the most prominent feature, as parathyroid tumours are found in 10-20% of affected family members.[30] MEN2 is caused by germline activating mutations of the *RET* proto-oncogene at 10q11.2[17;38].
HPT-JT (OMIM 145001) is an autosomal dominant syndrome characterised by parathyroid adenoma or carcinoma, ossifying fibroma of the mandible or maxilla, and renal lesions including Wilms tumour, renal cysts and tumours and uterine tumours.[14;22] About 80% of the patients present with hyperparathyroidism in late childhood or early adulthood[35]. The incidence of carcinoma in HPT-JT syndrome is reported to be 10-15%.[13;35] The high incidence of cystic change is another unique feature of parathyroid neoplasia in this syndrome.[34]
The gene causing HPT-JT is localized at chromosome 1q24-q32 and is known as the *HRPT2* gene (also known as Cdc73) and is thought to function as a tumour suppressor gene.[47]

A number of families with HPT alone (known as FIHP) have been described. A disease with an autosomal dominant pattern of inheritance, FIHP is known to be a genetically heterogeneous condition with germline mutations in *CASR* but also linkage to *MEN1*[48] and the *HRPT2* region.[37]

Sporadic parathyroid tumours

The etiology of sporadic HPT has long been unknown, until recently when several genetic mechanisms have been revealed that play a role in the development of sporadic parathyroid tumours. *CCND1* and *MEN1* have been established as having important roles in parathyroid tumourigenesis.
A translocation between *CCND1* and *PTH* resulting in the overexpression of *CCND1* has been found in a number of parathyroid adenomas. [6] Furthermore mutations in *MEN1* are reported in up to 30% of sporadic parathyroid adenomas.[18;28;36]

Chromosomal aberrations and genetic abnormalities in parathyroid tumours

Chromosomal losses and gains have been characterized in parathyroid tumours using comparative genomic hybridization and LOH studies. In general, parathyroid carcinomas show more chromosomal aberrations compared to adenomas (1.3x more losses and 3x more gains). In adenomas, more losses (2.7x) than gains have been found.
Regions frequently (in >10% of cases) lost in carcinomas are 1p, 13q, 6q, 9p, 4q, 18q and 2q. Regions frequently (in >10% of cases) gained in carcinomas are chromosomal regions xq, 1q, 16p, 9q, xp, 19q, 20q, 17q and 5q. Adenomas show frequently loss of chromosomal regions 11q, 11p, 15q, 1p, 13q and 22q. Gains are only seen in adenomas in chromosomal region 19p.[4;20;32;39]
Reports considering chromosomal changes in hyperplasia show conflicting results. Several studies using CGH[21] and LOH[29] report a relative lack of numerical chromosomal alterations (besides a gain of 12q in 11% of cases as reported by Imanishi et al). Other reported changes occurred in less than 10% of the cases, although Afonso et al[3] found by CGH analysis several regions with numerical changes.
Regions frequently lost in secondary hyperparathyroidism according this last study are 1p, 19p/q, 22p/q, 20q, 16q and 17p/q. Tertiary hyperplasia show in the same study losses in 1p, 20q, 12q, 19p/q and 22pq[3].
Gains are described in chromosomal region 6q, 13q, 5q, 4q and 12q in secondary hyperparathyroidism, tertiary HPT show gains in 4q and 6q. See Figure 2 for an overview.

FIGURE 2A

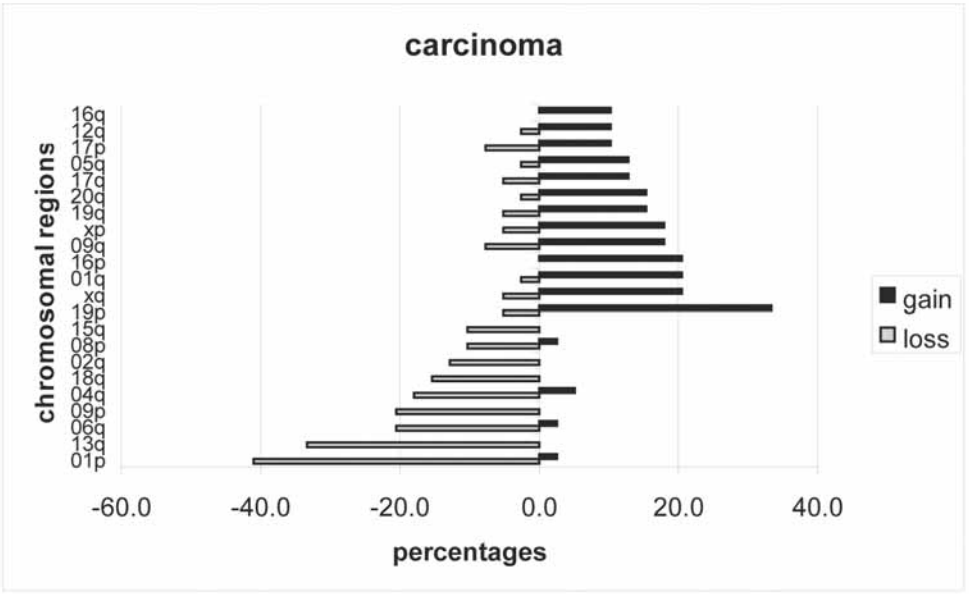


FIGURE 2B

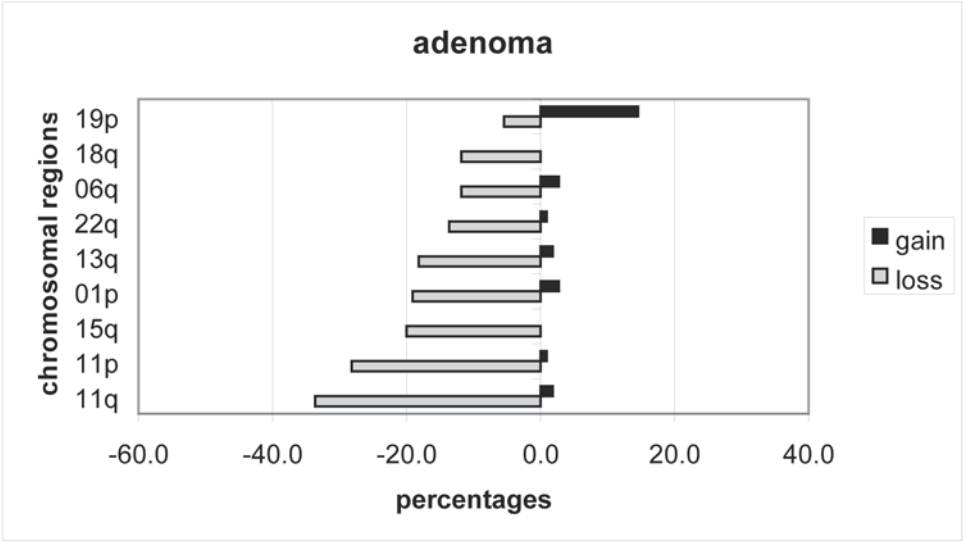


FIGURE 2C

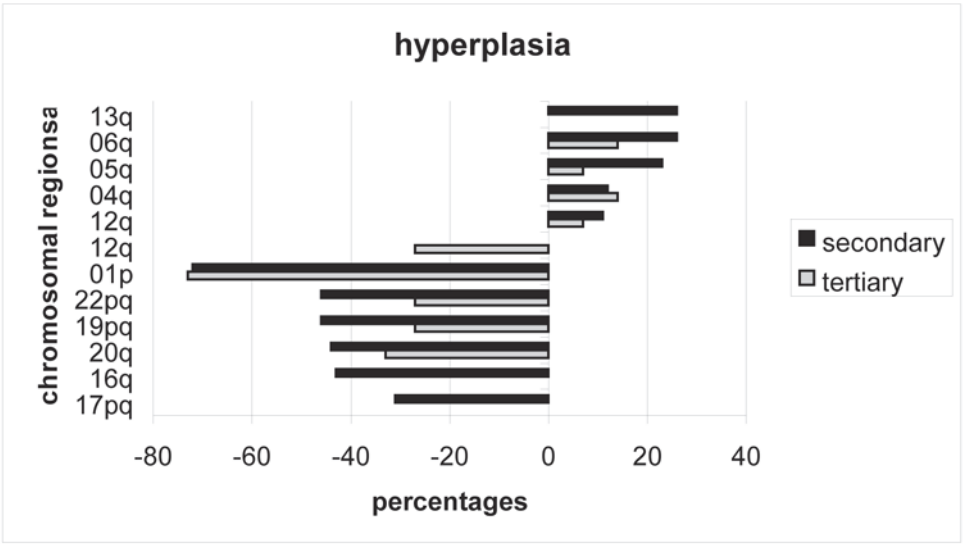


Figure 2 A, B and C depict the regions frequently (in >10% of cases) lost and gained in carcinomas (A) ,adenomas (B) and hyperplasia (C) found by CGH analysis.[3;4;20;32;39]. In C percentages of gains and losses are indicated in a similar way as in A/B.

Scope of this thesis

HPT-JT syndrome is a rare disease characterized by parathyroid tumours (with a high percentage of carcinomas), jaw and kidney tumours.
In this thesis, the clinical and genetic features of the HPT-JT syndrome and the relationship between the *HRPT2* gene and parathyroid tumours were investigated. Furthermore, we tried to gain insight in the molecular mechanisms of parathyroid tumourigenesis to improve the accuracy of diagnosis of these tumours.
Chapter 2 describes a clinical and histopathological study of a large kindred in which affected members presented with either parathyroid adenoma or carcinoma, although additional tumours were also found. Linkage analysis was performed to determine the genetics of this disease and the *HRPT2* region (locus associated with HPT-JT) was narrowed.
In ***chapter 3***, we refined the *HRPT2* region to 1q25-q32 by genotyping 26 affected kindreds. Furthermore, we report the identification of the gene responsible for the hyperparathyroidism–jaw tumour (HPT–JT) syndrome. The proposed role of *HRPT2* as a tumour suppressor was investigated by mutation screening in parathyroid adenomas with cystic features.
The *HRPT2* mutation status was determined in several types of parathyroid tumours in ***chapter 4*** including adenomas, carcinomas and hyperplasia both in a sporadic and familial context. Loss of heterozygosity analysis at 1q24-q32 was also performed on a subset of these tumours.
In ***chapter 5***, we hypothesize that loss of parafibromin, the protein product of the *HRPT2* gene, would distinguish carcinoma from benign tissue. We describe the immunohistochemical analysis of a newly generated antiparafibromin monoclonal antibody in mostly unequivocal carcinoma specimens, benign tumours en HPT-JT related tumours
In ***chapter 6***, morphological characteristics of primary parathyroid carcinomas and metastases were studied. Furthermore, immunohistochemical expression profiles were determined for parathyroid carcinomas, adenomas and hyperplasia using a tissue micro array. Loss of heterozygosity (LOH) of the chromosome 1q region containing the *HRPT2* gene and
chromosome 11q (*MEN1*) was determined in the carcinomas.
The aim of the study described in ***chapter 7*** was to further evaluate the role of *MEN1* and *HRPT2* mutations in sporadic formalin fixed paraffin embedded parathyroid tumours fulfilling histological criteria for malignancy. *HRPT2* and *MEN1* were analyzed by direct DNA sequencing in formalin fixed paraffin embedded parathyroid carcinoma tissue.
Chapter 8 describes a study based on microarray expression profiling of hereditary and sporadic benign and malignant parathyroid neoplasms to better define the molecular genetics of parathyroid tumours. A class discovery approach was used to identify distinct groups and gene sets able to distinguish between the groups. Several antibodies, selected based on the RNA profile, were analysed to discover potential useful markers for parathyroid carcinomas.
The aim of the study described in ***chapter 9*** was to find a method to rapidly screen parathyroid tumours for chromosomal aberrations. We applied a newly developed multiplex ligation-dependent probe amplification assay (MLPA) especially designed to detect genomic deletions and duplications in parathyroid neoplasms. Adenomas, carcinomas and normal tissue were analyzed.
Finally, ***chapter 10 and 11*** cover the concluding remarks, English summary and summary in Dutch, respectively.

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Chapter 2

A genotypic and histopathological study of a large Dutch kindred with hyperparathyroidism-jaw tumor syndrome.

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A Genotypic and Histopathological Study of a Large Dutch Kindred with Hyperparathyroidism-Jaw Tumor Syndrome*

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ABSTRACT

Familial primary hyperparathyroidism is the main feature of 2 familial endocrine neoplasia syndromes: multiple endocrine neoplasia type 1 (MEN 1) and hyperparathyroidism-jaw tumor syndrome (HPT-JT). The latter is a recently described syndrome that has been associated with ossifying fibroma of the jaw and various types of renal lesions, including benign cysts, Wilms' tumor, and hamartomas. To further illustrate the natural history of this syndrome, we describe a large, previously unreported Dutch kindred in which 13 affected members presented with either parathyroid adenoma or carcinoma; in 5 affected individuals, cystic kidney disease was found. Additionally, pancreatic adenocarcinoma, renal cortical adenoma, papillary renal cell carcinoma, testicular mixed germcell tumor with major seminoma component, and Hürthle cell thyroid adenoma were also identified. Linkage analysis of the family using *MEN1*-linked microsatellite markers and mutation analysis excluded the involvement of the *MEN1* gene. Using markers from the *HPT-JT* region in 1q25–31, cosegregation with the disease was found, with a maximum logarithm of odds score of 2.41 obtained for 6 markers using the most conservative calculation. Meiotic telomeric recombination between D1S413 and D1S477 was identified in 3 affected individuals, and when combined with previous reports, delineated the *HPT-JT* region to 14 centimorgan. Combined comparative genomic hybridization and loss of heterozygosity data revealed complex genetic abnormalities in the tumors, suggesting different possible genetic mechanisms for the disease.

In conclusion, we report a family with hyperparathyroidism linked to chromosome 1q, and exhibiting several types of renal and endocrine tumors that have not been previously described. (*J Clin Endocrinol Metab* **85:** 1449–1454, 2000)

FAMILIAL PRIMARY hyperparathyroidism (HPT) occurs in the context of familial isolated hyperparathyroidism or in families in which a hereditary tumor syndrome is coupled with primary hyperparathyroidism (1). In multiple endocrine neoplasia type 1 (MEN 1), primary hyperparathyroidism is associated with additional endocrine tumors of, *e.g.* pancreas, anterior pituitary, and stomach (2). The disease is associated with mutations inactivating the tumor suppressor gene *MEN1*, located on chromosome (chr.) 11q13 (3, 4). In MEN 2a hyperparathyroidism may occur together with medullary carcinoma of the thyroid or pheochromocytoma caused by germ-line activating mutations of the *RET* protooncogene on chr. 10q11 (5).

A distinct disorder, hereditary hyperparathyroidism-jaw tumor syndrome (HPT-JT), with an autosomal dominant mode of inheritance, has been described in which primary hyperparathyroidism caused by parathyroid adenoma is associated with ossifying fibroma of the jaw (6). In a number of families, parathyroid carcinoma has been noted (7–12). In addition, renal disease has also been described, including renal hamartomas, Wilms' tumor, polycystic kidney disease, and degenerative cysts (8, 11–14). The HPT-JT gene (*HRPT2*) has been mapped to the long arm of chr. 1q25-q31, later narrowed down to a 14.7-cm (centimorgan) region. Additionally, a 0.7-cm candidate region was recently suggested, based on shared haplotypes found in two Northern American families (15).

The nature of the *HRPT2* gene is still unresolved, although [based on the presence or loss of the wild-type alleles in several renal hamartomas and some parathyroid tumors (13, 16)] the *HRPT2* gene is considered a putative tumor suppressor gene. However, loss of heterozygosity (LOH) in HPT-JT is not always as evident as in MEN 1, in which the majority of familial tumors show loss at 11q13. For example, LOH has only been demonstrated in parathyroid tumors from a subset of families (11, 13, 14), possibly suggesting an alternative mechanism for tumorigenesis of parathyroid tu-

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mors in HPT-JT patients. To further understand the natural history and genetic involvement of this relatively new syndrome, we have studied the clinical, histopathological, and genotypic characteristics of a large Dutch kindred in which primary hyperparathyroidism is associated with other neoplasia.

Subjects and Methods

The pedigree of the family with the clinical phenotypes is shown in Fig. 1. To date, 13 family members have been documented with primary hyperparathyroidism caused by parathyroid adenoma. Five individuals (III-2, III-5, III-8, III-10, and III-11) had multiple renal cysts. Four of them (III-2, III-5, III-10, and III-11) developed renal insufficiency requiring dialysis. The sex distribution of affected individuals is roughly equal.

Additional tumors were found in four patients. These were Hürthle cell adenoma of the thyroid gland with cystic changes (III-2 at age 44), clear cell adenocarcinoma of the pancreas (III-2 at age 48, Fig. 2), papillary renal cell carcinoma and multiple cortical renal cell adenomas in the right kidney (III-5 at age 54, Fig. 2), parathyroid adenoma with atypia (III-11 at age 32) and parathyroid carcinoma (III-11 at age 36, Fig. 2), and left testicular mixed germcell tumor with a major seminoma component (IV-4 at age 32). Clinically unaffected carriers with the disease haplotype that were screened by renal ultrasonography (III-21, IV-7, IV-9, IV-12, and IV-13) showed no apparent abnormalities. Also the renal ultrasonography of carriers IV-11 and IV-14 was normal. Current biochemical testing of Ca^{2+} and PTH of individuals III-4, III-21, IV-7, IV-8, IV-9, IV-11, IV-12, IV-13, and IV-14 was normal. Of the four individuals (IV-11, IV-12, IV-13, and IV-14) that were radiographically screened, only two (IV-13 and IV-14) were suspect for carrying a small jaw tumor; however, this has not been histologically documented.

Linkage analysis

Informed consent was obtained from all participating members of the family or their legal representatives.

High-molecular-weight DNA was isolated from leukocytes using standard methods. For two patients (III-2 and III-11) from whom a blood sample could not be obtained, constitutional DNA was extracted from nontumorous formalin-fixed paraffin-embedded tissue, as described (17). Eight microsatellite markers in and flanking the *MEN1* gene in 11q13, cen-D11S4945-PYGM-D11S4946-D11S4940-D11S4947-D11S1783-D11S4937-D11S4936-tel, were used (4, 18). The 3–7th markers mentioned are intragenic, the other three are flanking the *MEN1* locus. Eleven microsatellite markers from a 26-cm region in 1q25-q31, between D1S215 and D1S249, encompassing the *HPRT2* locus (12), cen D1S215, D1S466, D1S191, D1S254, D1S422, D1S428, D1S222, D1S412, D1S413, D1S477, D1S510 (Genome Database: www. gdb.org) were used for linkage.

Genotyping was carried out by two methods: one using radiolabeled markers and the other fluorescent markers. PCR reactions were performed in a total vol of 10 μl, containing 100 ng genomic tumor DNA, 50 mmol/L KCL, 10 mmol/L Tris-HCL (pH 8.3), 1,5 mmol/L MgCl2, 125 mmol/L of each deoxynucleotide triphosphate, 2 pmol of each oligodeoxynucleotide primer (one of which was end-labeled with ^{32}Phosphate in the case of radiolabeled markers), and 0.2 U DNA C polymerase (Dynazyme/*Taq* polymerase). Samples were amplified for 30 cycles (denaturation at 96 C for 30 sec, annealing at 55 C for 30 sec, and elongation at 72 C for 30 sec; and the products were run on 1% polyacrylamide gels.

With the fluorescent markers, the PCR products were pooled into three panels, according to the emission spectra of fluorescent dyes and the expected sizes of the amplified products. Electrophoresis was performed on 6% polyacrylamide gels, running on an ABI 377 laser-fluorescent sequencer (Perkin-Elmer Corp., Foster City, CA), and electrophoresis data were analyzed with the Genescan 3.1 computer software (Perkin-Elmer Corp.).

Two point logarithm of odds (lod) scores were generated using the LINKAGE (version 5.1) program adopting a conservative approach. Only patients with primary HPT were scored as affected, whereas other members at risk were considered as unknown. An autosomal dominant mode of inheritance and a penetrance of 0.90 were assumed.

Tumor analysis

Formalin-fixed, paraffin-embedded tumor tissue was obtained from 4 patients (III-2, III-5, III-11, and IV-1) and fresh frozen tumor tissue samples from 2 other patients (IV-11 and IV-14). Whenever possible, different tumor foci, as selected by a pathologist (H. Morreau), were microdissected from 10 10-μm-thick hematoxylin-stained sections

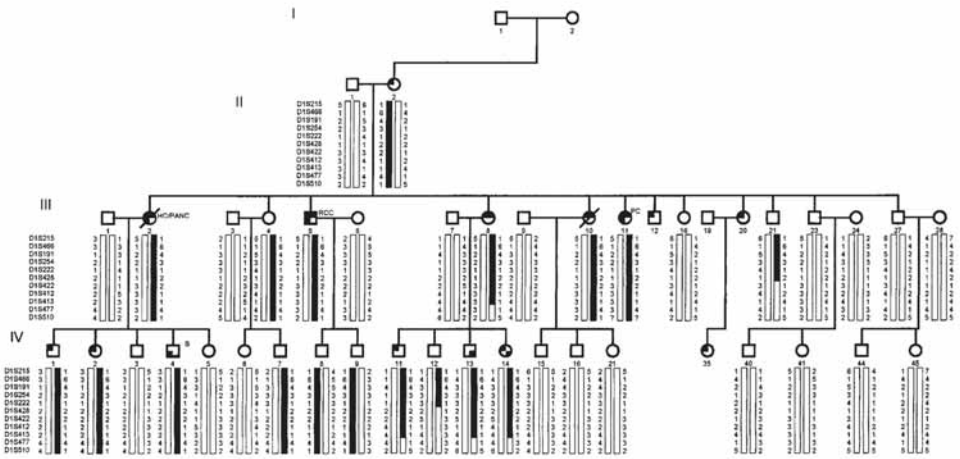


FIG. 1. Pedigrees and phenotypes of the family in this study. ◔, ◪, Hyperparathyroidism; ◔, ◪, renal cysts, ◔, ◪, jaw tumor; ○, □, unaffected; ◔, ◪, additional neoplasm; S, mixed germcell tumor with major seminoma component; RCC, renal cell carcinoma; PC, parathyroid carcinoma; HC, Hürthle cell adenoma of the thyroid. The affected haplotype is indicated by *black bars*.

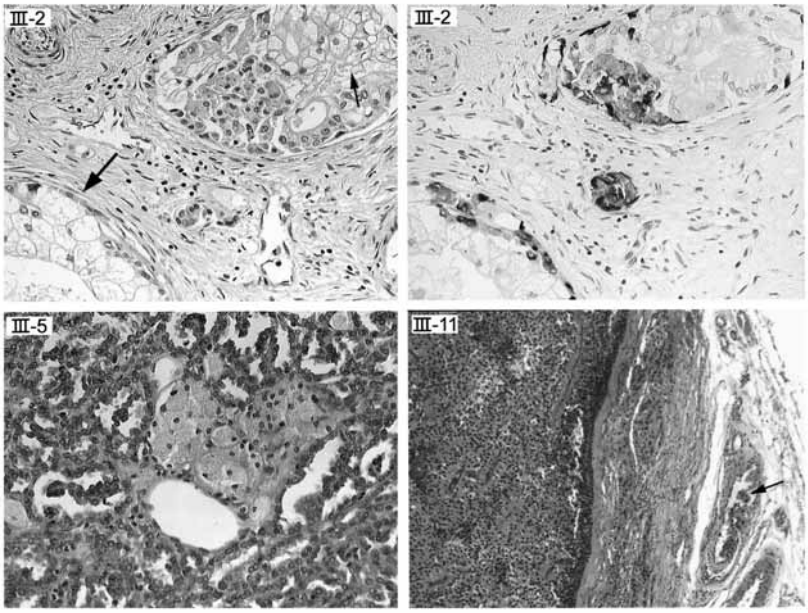


FIG. 2. Light microscopic pictures of three different tumors from individuals III-2, III-5, and III-11. III-2, a glandular area of the clear-cell adenocarcinoma of the pancreas (*top left*, *thick arrow*) is shown. In the surrounding preexistent pancreatic tissue, atypical ductal hyperplasia was present. Clear cells, similar to the infiltrating carcinoma cells, can be seen in islets of Langerhans (*top left*, *small arrow*). Positive staining with antibodies against neuron-specific enolase (NSE) is shown, indicating neuroendocrine features (*top right*). The islets stain positive also with antibodies against insulin and glucagon (data not shown). *Bottom left*, the papillary renal cell carcinoma from individual III-5 is shown; *bottom right*, the parathyroid carcinoma from individual III-11. The *arrow* indicates vasoinvasive growth of tumor cells.

mounted on glass-slides. Genomic DNA was extracted from the paraffin-embedded and fresh frozen specimens using standard methods.

Matched pairs of constitutional and tumor DNA were genotyped and analyzed for allelic imbalance, using markers as described under linkage analysis. For the chr.1q region, additional markers D1S2125, D1S1653, D1S408, D1S1614, D1S533, and D1S1660 were used. Allele status was identified on autoradiographic films and confirmed by digital images, which permitted computerized calculations of relative allele intensities. LOH was considered present when the signal intensity of one allele was reduced by more than 50%, in comparison with the corresponding allele in normal DNA.

Comparative genomic hybridization (CGH)

DNA from tumor samples was labeled with Fluorescein-12-deoxyuridine 5-triphosphate and DNA from the normal control with Lissamine-5-deoxyuridine 5-triphosphate (both from NEN Life Science Products, Boston, MA) by standard nick-translation.

The CGH was then performed according to the protocol described by Kallioniemi *et al.*(19), with a few modifications. Briefly, 200 ng of each labeled tumor and control DNA and 10 μg of human Cot-1 DNA (Life Technologies/BRL, Gaithersburg, MD) were dissolved in 10 μl hybridization buffer (50% formamide/2 $\times$ SSC/10% dextran-sulphate) and hybridized to normal male metaphase spreads at 37 C for 4 days. Post-hybridization washes were performed with 2 $\times$ SSC at 37 C (3 $\times$ 10 min) followed by 0.1 $\times$ SSC at 60 C (3 $\times$ 5 min). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole (0.5 μg/mL) in Vectashield antifade solution (Vector Laboratories, Inc., Burlingame, CA). Images were captured with a DM microscope (Leica Corp., Heidelberg, Germany) equipped with three single excitation filters, a multi-bandpass dichroic mirror, a multiband pass emission filter (P-1 filter set; Chroma Technology, Brattleborough, VT), and a cooled CCD camera (Photometrics Inc., Tucson, AZ). The green, red, and blue images were collected sequentially by changing the excitation filter. Images were analyzed using the QUIPS XL software from Vysis (Downers Grove, IL).

Losses of DNA sequences were defined as chromosomal regions where the average green-to-red ratio and its 95% confidence interval were below 0.8, whereas gains were above 1.2. These threshold values were based on measurements from a series of normal controls

Mutation analysis of the MEN1 gene

Mutation analysis was performed using single-stranded conformational polymorphism analysis and direct sequencing of all 10 exons and flanking intronic sequences after amplification of genomic DNA, as described (20).

Results

No germline mutations in the *MEN1* gene were detected. Furthermore, no linkage could be demonstrated for markers chosen in the 11q13 region. No LOH for markers at 11q13 was identified in eight tumors tested (data not shown).

Analysis of 11 markers indicated that primary HPT in this kindred was linked to the 1q25–31 *HPT-JT* region. The maximum 2-point lod score of 2.41 was obtained with markers

TABLE 1. Lod scores for linkage to chromosome 1q21–q32 markers in the family

Locus	Recombination fraction (θ)					
	0.000	0.010	0.05	0.100	0.200	0.300
D1S215	1.202	1.18	1.091	0.974	0.719	0.438
D1S466	2.405	2.362	2.182	1.948	1.437	0.865
D1S191	2.405	2.362	2.182	1.948	1.437	0.865
D1S254	2.405	2.363	2.182	1.948	1.437	0.865
D1S222	1.202	1.18	1.091	0.974	0.719	0.438
D1S428	1.202	1.18	1.091	0.974	0.719	0.438
D1S422	2.405	2.363	2.182	1.948	1.437	0.865
D1S412	2.405	2.363	2.182	1.948	1.437	0.865
D1S413	2.405	2.363	2.182	1.948	1.437	0.865
D1S477	−5.423	−1.614	−0.370	0.0047	0.245	0.224
D1S510	−5.492	−1.619	−0.372	−0.420	0.245	0.182
428 + 422	2.405	2.362	2.182	1.948	1.437	0.865
412 + 413	2.405	2.362	2.182	1.948	1.437	0.865

TABLE 2. LOH on chromosome 1q in different tumors from obligate gene-carriers

III-2	III-5		III-11		IV-1	IV-11	IV-14	Case no.
Pancreatic carcinoma	Renal cortical adenoma	Papillary renal cell carcinoma	Parathyroid adenoma	Parathyroid carcinoma	Parathyroid adenoma	Parathyroid adenoma	Parathyroid adenoma	pathology
								Locus
LOH	R	R	–	R	R	–	–	D1S2125
LOH	R	R	R	–	R	–	–	D1S1653
–	R	R	R	–	R	–	–	D1S215
–	–	–	–	–	–	R	R	D1S254
LOH	–	–	–	–	–	–	–	D1S428
LOH	R	R	–	–	–	R	R	D1S422
LOH	R	LOH d	R	R	–	R	R	D1S408
LOH	–	–	–	LOH w	R	R	R	D1S412
LOH	–	–	R	LOH d	R	R	R	D1S1614
–	–	–	–	–	R	R	R	D1S533
LOH	–	–	–	–	R	R	R	D1S413
–	–	–	–	–	R	R	R	D1S1660
–	–	–	–	–	–	–	–	D1S477
–	–	–	–	–	–	–	–	D1S510

R, Retention; w, wild-type allele; d, defective allele; –, not informative.

D1S466, D1S191, D1S254, D1S422, D1S412, and D1S413 (Table 1). Considering that the most conservative approach was used in our calculation, *i.e.* all individuals at risk were labeled as unknown, the lod score obtained is the maximum possible in this family. All affected individuals carried the disease haplotype (Fig. 1).

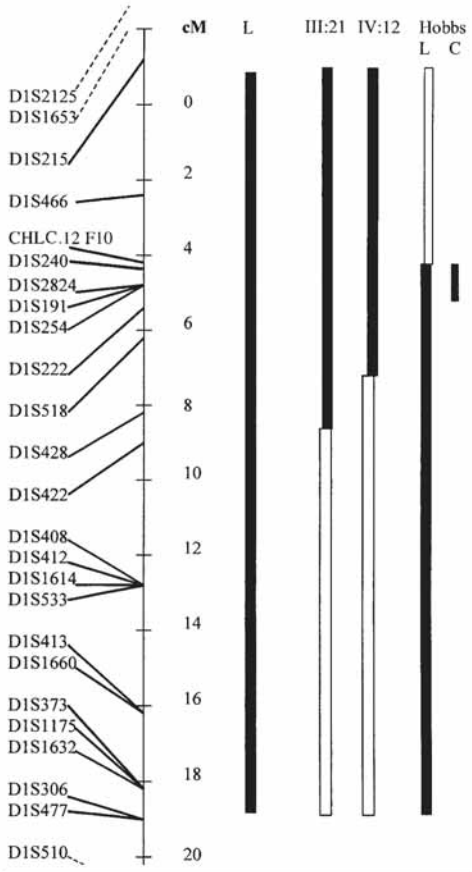
Meiotic recombination of the mutated chromosome was seen in patient III-8 (between D1S413 and D1S477), and the same telomeric recombination was transmitted to her offspring IV-11, IV-13, and IV-14 (Fig. 1). Her clinically unaffected son (IV-12) exhibited a recombination between D1S222 and D1S428. The unaffected male III-21 (at age 50) exhibited a recombination between D1S428 and D1S422.

LOH studies (Table 2) with markers from the HPT-JT genomic region identified no LOH at chr. 1q21–32 in any of the parathyroid adenomas or in a renal cell cortical adenoma studied. Only different foci of the pancreatic adenocarcinoma (III-2), the papillary renal cell carcinoma (III-5), and the parathyroid carcinoma (III-11) exhibited LOH in the chr.1q region, involving either the defective or wild-type chromosome (Table 2). CGH of fresh-frozen tissue from two parathyroid adenomas of the family (IV-11 and IV-14) revealed amplification of chr.16 for the tumor from IV-11 and deletion of chr.13q, as well as amplification of chr.1q and chr.17p for the tumor from IV-14.

Discussion

The familial inheritance of parathyroid adenoma/carcinoma and cystic kidney disease, its genetic exclusion of MEN 1, and the lod score of 2.41 with 6 markers at 1q25–31 strongly point to the diagnosis of HPT-JT syndrome. The latter is relatively new, and the spectrum of its clinical features is far from fully understood, as exemplified in this family. Several types of tumors occurring in this family (including pancreatic adenocarcinoma, renal cortical adenoma, papillary renal cell carcinoma, testicular mixed germcell tumor with major seminoma component, and Hürthle cell thyroid adenoma) have not been previously described in this syndrome. Although coincidence can not be ruled out, their occurrence in affected patients with parathyroid tumors and their multiplicity (*e.g.* renal cortical adenoma) indicate their association with the syndrome. Some of these tumors have been associated with or reported in other familial neoplasia syndromes. For example, papillary renal cell carcinoma is the main feature of hereditary papillary renal carcinoma syndrome involving chr.7; and in one such family, two cases of pancreatic car-

TABLE 3. Diagram illustrating recombination events defining the *HRPT2* region.



A scale and the approximate locations of markers on chromosome 1q are given on the left. The first vertical bar represents the recombination data of the family obtained by linkage analysis of affected individuals. The second and third bars denote the recombination data of two (yet) clinically unaffected individuals (III-21, IV-12). The fourth and fifth bars illustrate the 14.7 and 0.7 cM proposed candidate region for *HRPT2*, as described by Hobbs *et al.* (15).

cM, Centimorgan; L, linkage data; C, proposed candidate region.

cinoma have been described (21, 22). Of interest is that a subset of papillary renal cell carcinomas is known to carry a t(X;1)(p11;q21) as the sole cytogenetic abnormality present (23, 24). The breakpoint locus at 1q, however, does not seem to be located within the *HRPT2* region. Additionally, renal cortical adenomas have been described in von Hippel-Lindau syndrome (25); and testicular cancer, in familial testicular cancer syndrome (26).

The *HRPT2* region has been narrowed to 14.7 cm by the identification of recombinations in affected cases (15). A 0.7-cm candidate region was proposed, based on shared haplotypes in two Northern American families, but this data warrants confirmation. In the present studies, a number of recombinants have been identified. Three clinically affected cases (III-8, IV-11, and IV-14) carry a telomeric recombination between D1S413 and D1S477 which is the closest telomeric border found in affected cases. Therefore, when combining previously published data, the HPT-JT locus can be delineated to 14 cm bordered by marker CHLC.12F10 (15) and D1S1632 (present study) (Table 3). Interestingly, two critical re-combinants were identified in two members who remain disease-free to date. The first one is in a 50-yr-old man (III-21) who carries a telomeric recombination between markers D1S428 and D1S422, and the second is in a 34-yr-old man (IV-12) with recombination between D1S222 and D1S428. However, reduced penetrance is not uncommon in HPT-JT (9, 11), as evidenced by six other diseased haplotype carriers: III-4 (aged 62), IV-4 (aged 30), IV-7 (aged 24), IV-8 (aged 32), IV-9 (aged 30), and IV-13 (aged 33). As such, we are treating the two disease-free recombinants with great caution, although they may potentially further narrow the region. The *HRPT2* gene has been proposed as a putative tumor suppressor gene based on LOH involving the wild-type alleles in a subset of 1q-linked tumors (11, 13, 16). However, in the present study, we were unable to demonstrate consistent LOH of 1q21–32 in the tumors analyzed. This is in keeping with other published data that indicates that an imbalance at 1q is not always found in HPT-JT-related tumors (13, 14). It may be that the LOH detected in our study on chr.1 is a late somatic event during tumorigenesis, because only the malignant tumors exhibited this pattern. In addition, our CGH results, which showed amplification of chr.1q in one of the two parathyroid adenomas, are consistent with the CGH results of two other 1q-linked parathyroid tumors (16). Taken together, these complex data may suggest a different scenario: the involvement of an oncogene in which loss of the wild-type copy was a secondary event and followed by duplication of the mutated copy. A similar mechanism has been found in hereditary papillary renal cell carcinomas involving the *MET* locus on chr. 7 (21, 22, 27). Finally, the gain at chr. 16q and the loss at chr.13q by CGH analysis in the parathyroid adenomas in our family have also been described by others (16), suggesting a role for these regions in parathyroid tumorigenesis and progression. Future identification of the *HRPT2* gene will lead to a better understanding of the mechanisms causing this interesting disease to have a wide spectrum of clinical features.

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Chapter 3

HRPT2, encoding parafibromin, is mutated in hyperparathyroidism-jaw tumor syndrome.

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letter

HRPT2, encoding parafibromin, is mutated in hyperparathyroidism–jaw tumor syndrome

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We report here the identification of a gene associated with the hyperparathyroidism–jaw tumor (HPT–JT) syndrome. A single locus associated with HPT–JT (*HRPT2*) was previously mapped to chromosomal region 1q25–q32. We refined this region to a critical interval of 12 cM by genotyping in 26 affected kindreds. Using a positional candidate approach, we identified thirteen different heterozygous, germline, inactivating mutations in a single gene in fourteen families with HPT–JT. The proposed role of *HRPT2* as a tumor suppressor was supported by mutation screening in 48 parathyroid adenomas with cystic features, which identified three somatic inactivating mutations, all located in exon 1. None of these mutations were detected in normal controls, and all were predicted to cause deficient or impaired protein function. *HRPT2* is a ubiquitously expressed, evolutionarily conserved gene encoding a predicted protein of 531 amino acids, for which we propose the name parafibromin. Our findings suggest that *HRPT2* is a tumor-suppressor gene, the inactivation of which is directly involved in predisposition to HPT–JT and in development of some sporadic parathyroid tumors.

Parathyroid tumors affect 1 in 1,000 individuals in the general population in whom the resulting primary hyperparathyroidism (1°HPT) occurs as the only clinical feature or as part of a complex syndrome. The HPT–JT syndrome (OMIM *145001) is an autosomal dominant, multiple neoplasia syndrome primarily characterized by hyperparathyroidism due to parathyroid tumors[1,2]. Thirty percent of individuals with HPT–JT may also develop ossifying fibromas, primarily of the mandible and maxilla, which are distinct from the 'brown' tumors associated with severe hyperparathyroidism[1,3–5]. Kidney lesions may also occur in HPT–JT as bilateral cysts, renal hamartomas or Wilms tumors[2,5–7]. Linkage analysis previously assigned the locus associated with HPT–JT (*HRPT2*) to a region of roughly 15 cM within 1q24–q32 (refs 5,6,8). Some parathyroid carcinomas and renal hamartomas from individuals with HPT–JT, as well as some sporadic parathyroid adenomas, show somatic 1q loss of heterozygosity (LOH), in agreement with the inactivation of a tumor-suppressor gene in the region[6,7,9–11].

To facilitate the identification of the gene associated with HPT–JT, we studied a total of 26 kindreds, sixteen of whom have been described elsewhere and most of whom showed linkage to 1q24–q32 (refs 2,5–7,10,12–16). Twenty-four kindreds were affected with HPT–JT, and two were affected with familial isolated hyperparathyroidism (FIHP). The latter had a familial occurrence only of 1°HPT and showed linkage to 1q24–q32, but not to *MEN1*. (multiple endocrine neoplasia I; ref. 9). We genotyped 26 microsatellite markers within 1q24–q32. Key recombinants further narrowed the candidate interval to 12 cM flanked by *D1S238* and *D1S477* (Fig. 1*a*). Using existing transcript mapping information from this region[17] and the UCSC draft human genome sequence browser, we identified 67 potential candidate genes including known genes, full-length cDNAs with no homologies to known genes, spliced expressed sequence tags (ESTs) and predicted genes. Fig. 1*b* shows a partial transcript map of the critical candidate region highlighting the initial set of candidate genes selected for mutational screening. We carried out mutational analysis using double-stranded DNA sequencing on a panel of 26 lymphocyte DNA samples, each representing one affected individual from each kindred.

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After analysis of sequence data from the initial set of prioritized candidate genes, only the C1orf28 gene (Unigene cluster Hs.5722) had probable disease-causing mutations. Unigene cluster Hs.5722 is represented in cDNA libraries from all tissues of interest (parathyroid, kidney and bone), but is probably ubiquitously expressed. The C1orf28 gene consists of 17 exons, all of which are coding (Fig. 1*c*). Alignment of EST and full-length cDNA sequences from Unigene cluster Hs.5722 identified an open reading frame of 1,596 nucleotides encoding a protein of 531 amino acids. The combined coding sequence data suggested a full-length message of approximately 2.7 kb for this transcript, which correlates well with the major band of roughly 2.7 kb that we detected by northern-blot analysis using the C1orf28 coding region as a probe (Fig. 1*d*). Other bands present on the northern blot could represent alternative forms of the protein and are currently under investigation. Following the OMIM nomenclature for *145001, we refer to this gene as *HRPT2*.

We identified a total of 13 heterozygous germline mutations in *HRPT2* in 14 of the 26 index cases screened (Table 1). Each germline mutation in *HRPT2* is expected to lead to impaired protein function owing to truncation or premature stops, further supporting their pathogenetic importance. Inheritance of five different mutations is detailed in Fig. 2. One frameshift mutation, an insertion of 2 bp in exon 7 (Fig. 2*d*), was found in individuals of two independently identified, seemingly unrelated kindreds (kindred-01 and kindred-33) who were later found to share an identical disease haplotype through the entire 26-marker interval, suggesting that these individuals have a common ancestor. In all 14 affected families, all affected and some currently unaffected members of the 14 families harbored mutations. None of the mutations were present in 150 normal diploid control individuals.

HRPT2 and its encoded protein are evolutionarily conserved, as we found potentially orthologous sequences in mouse, *Drosophila melanogaster* and *Caenorhabditis elegans* in the National Center for Biotechnology Information non-redundant nucleotide database using the BLAST algorithm. We propose the name parafibromin for the encoded protein, owing to its involvement in the development of parathyroid tumors and ossifying jaw fibromas. Human parafibromin shares 54% identity and 67% similarity with the *D. melanogaster* ortholog and 25% identity and 45% similarity with the *C. elegans* ortholog (see Web Fig. A online).

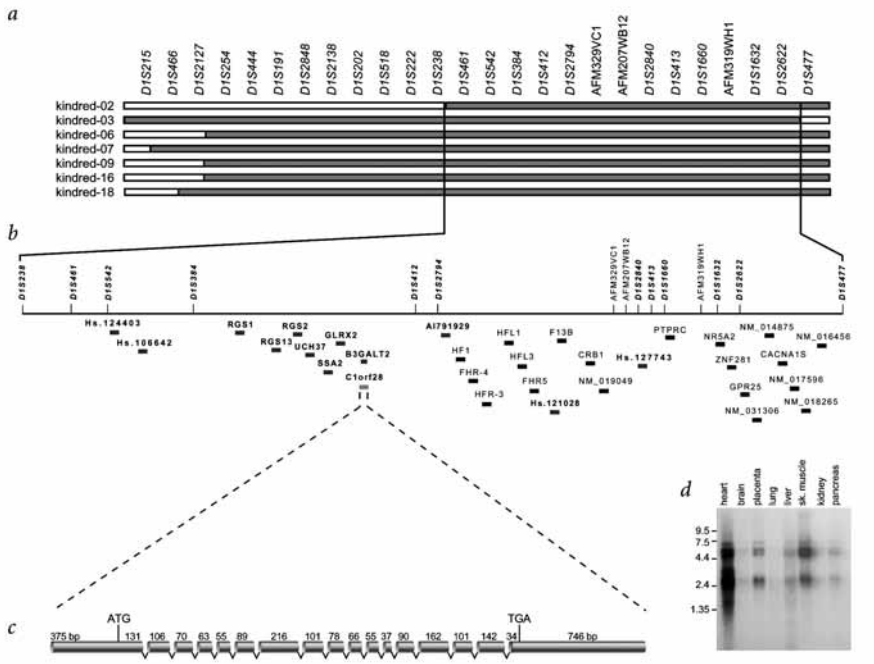

Fig. 1 Genetic analysis of kindreds affected with HPT–JT and partial transcript map of the critical region. ***a***, Recombination map spanning 26 microsatellite markers from the 1q24–q32 genetic interval showing key recombination events. Marker regions shaded in red represent regions shared among affected individuals within families. ***b***, A partial transcript map of the critical region defined by recombinants. Genes highlighted in blue were initially prioritized for mutational analysis. C1orf28 is labeled in red as the gene of interest (*HRPT2*). Known genes and full-length cDNAs are shown with GenBank accession numbers, and ESTs are shown with Unigene cluster ID numbers. ***c***, Genomic structure of *HRPT2*. Sizes of exons are given in base pairs (bp). ATG and TGA represent the initiation codon and termination codon, respectively, of *HRPT2*. ***d***, Northern-blot analysis using C1orf28 (*HRPT2*) as a probe. C1orf28 (*HRPT2*) was expressed at varying levels in all tissues examined. Molecular weight markers are given in kb.

Table 1 • Germline and somatic mutations detected in *HRPT2*

Kindred or tumor sample	Clinical data (number) HPT	PTC	JT	KL	Mutation in *HRPT2* Location	Nucleotide change	Coding change	Type
Mutations in kindreds affected with HPT–JT								
kindred-05	4	0	2	4	exon 1	3G→A	Met1Ileu	germline
kindred-15	7	0	3	1	exon 1	25C→T	Arg9X	germline
kindred-16	7	2	5	0	exon 1	41-bp duplication/insertion	frameshift	germline
kindred-24	2	0	1	1	exon 1	34delAACATCC	frameshift	germline
kindred-12	4	0	3	0	exon 1	30delG	frameshift	germline
kindred-19	2	1	2	0	exon 1	39delC	frameshift	germline
kindred-10	6	1	5	4	exon 2	165C-G	Tyr55X	germline
kindred-20	7	1	2	1	exon 3	*306delGTgtgagtacttttt	frameshift / splice mutation	germline
kindred-09	10	3	0	0	exon 4	356delA	frameshift	germline
kindred-22	3	0	2	1	exon 5	406A→T	Lys136X	germline
kindred-07	4	0	2	2	exon 7	636delT	frameshift	germline
kindred-01	5	3	0	3	exon 7	679insAG	frameshift	germline
kindred-33	2	0	1	0	exon 7	679insAG	frameshift	germline
kindred-11	3	0	2	1	exon 14	1238delA	frameshift	germline
Mutations in parathyroid tumors								
tumor sample 9	sporadic				exon 1	126del24	frameshift / splice mutation	somatic
tumor sample 10	FIHP-1q linked				exon 1	128G→A	Trp43X	somatic
tumor sample 31	sporadic				exon 1	53delT	frameshift	somatic

Nucleotide and amino-acid positions start from initiation codon. *Lower case nucleotides represent intronic sequences. HPT, hyperparathyroidism; PTC, parathyroid carcinoma; JT, jaw tumor; KL, kidney lesion.

There were no homologies to known protein domains, but moderate identity (32%) and similarity (54%) to a protein of *Saccharomyces cerevisiae* known as Cdc73p, which is an accessory factor associated with an alternative RNA polymerase II important in transcriptional initiation and elongation in yeast[18–20].

To evaluate further the probable tumor-suppressor effect of *HRPT2*, we analyzed LOH and screened for mutations in *HRPT2* in tumor and normal DNA from 48 individuals with parathyroid adenomas with cystic features (47 sporadic and 1 familial). Six sporadic tumor samples (12.5%) showed LOH at 1q without involvement of 11q13, two with LOH at 1q encompassing the *HRPT2* locus. We identified a total of three inactivating mutations in *HRPT2*, two in sporadic tumors and one in a tumor from a 1q-linked FIHP kindred. Details on mutations and LOH at 1q for the three tumor samples can be found in Table 1 (also see Web Fig. B online). None of the mutations were detected in adjacent normal tissue or in 150 normal diploid control individuals, suggesting that they were pathogenic for these tumors.

LOH at 1q has been previously reported in tumors from kindreds affected with HPT–JT in whom we identified germline mutations in this study, including kindred-07, kindred-09, kindred-10 and kindred-20, suggesting that biallelic inactivation of *HRPT2* is associated with HPT–JT[6,10,15]. These findings are in agreement with inactivation of a tumor-suppressor gene in the region. But the frequency of demonstrated LOH at 1q in parathyroid tumors related to HPT–JT is relatively low, especially compared with LOH of *MEN1*, which is inactivated in more than 70% of the associated parathyroid tumors[21–23]. Our demonstration of a somatic inactivating mutation in a parathyroid adenoma from a kindred affected with FIHP that showed linkage to 1q indicates that small mutations in *HRPT2* could be one explanation for the relative lack of LOH at 1q in parathyroid tumors related to HPT–JT. Other possible mechanisms for inactivation of *HRPT2* include hypermethylation and regulatory inactivation.

The identification of deleterious disease-associated germline mutations in *HRPT2* in 14 kindreds affected with HPT–JT indicates that this gene is directly associated with the pathogenesis of the HPT–JT syndrome. Somatic inactivating mutations in parathyroid tumors suggest an important role for *HRPT2* in parathyroid tumorigenesis. With the identification of *HRPT2*, the primary gene involved in each of the complex syndromes associated with 1°HPT is now known[24]. This finding is expected to be of clinical relevance for early risk assessment in individuals from families with HPT–JT and possibly a subset of individuals with FIHP. As parathyroid tumors are malignant at a higher frequency in HPT–JT than in MEN1 and MEN2, mutations in *HRPT2* are probably an important precursor for increased risk of parathyroid carcinoma. Further experimentation is also warranted to determine the actual role of parafibromin in normal cellular function and the exact mechanisms by which abnormal parafibromin leads to the development of tumors. In conclusion, the identification of *HRPT2* and further analysis of parafibromin may ultimately contribute to understanding parathyroid tumor development, and eventually to the development of novel therapies for 1°HPT and, possibly, other neoplasms.

Note added in proof: Recent analyses of additional members of kindred 08, an FIHP kindred, has identified a germline L64P mutation in exon 2 of HRPT2.

Methods

Linkage analysis and recombination mapping. All samples used in this study were collected with proper consent and approved for study by institutional review boards and ethics committees at all affiliated institutions. We genotyped genomic DNA samples using 26 short tandem-repeat microsatellite markers in the region of interest of chromosome 1. Primer sequences for the microsatellite repeat markers used in this study are available upon request. PCR reactions were set up using a TECAN Genesis200 robot. PCR amplification was done in 15-µl reactions using GeneAmp 9600 thermocyclers (PE/Applied Biosystems). Depending on the PCR yield, we pooled 5–15 µl of PCR product from up to 12 individual markers of appropriate size and fluorescent label. We separated PCR products using the ABI 377 DNA sequencer (PE/Applied Biosystems), which allows multiple fluorescently-labeled markers to be co-electrophoresed in a single lane. We used the ROX 400 size standard as an internal size-standard in each lane (PE/Applied Biosystems). We calculated allele sizes using the local southern algorithm available in the GENESCAN software program (PE/Applied Biosystems). Allele calling and binning was done using the GENOTYPER software (PE/Applied Biosystems). We included a control individual (CEPH 1347-02) in the genotyping analysis for quality control.

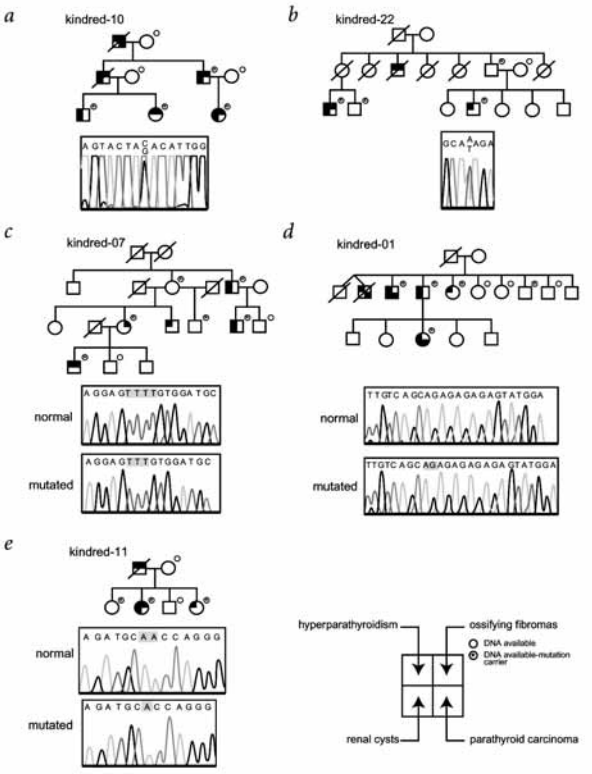

Fig. 2 Mutations in kindreds affected with HPT–JT. Shaded upper left quadrant represents hyperparathyroidism, upper right quadrant represents ossifying fibroma of the jaw, lower left quadrant represents renal cysts or other kidney tumors, and lower right quadrant represents parathyroid carcinoma. A line drawn through a symbol represents a deceased individual. Completely open symbols represent individuals who are currently unaffected. Small superscript circles to the upper right of family member symbols represent those individuals for whom DNA was available for mutational analysis. Small superscript circles with an asterisk (*) in the middle represent those individuals who are confirmed mutation carriers. ***a***, Kindred-10 and chromatogram showing the heterozygous 165C→G nonsense mutation in exon 2. ***b***, Kindred-22 and chromatogram showing the heterozygous 406A→T nonsense mutation in exon 5. ***c***, Kindred-07 and chromatograms showing the normal allele and corresponding 636delT mutated allele in exon 7. ***d***, Kindred-01 and chromatograms showing the normal allele and corresponding 679insAG mutated allele in exon 7. ***e***, Kindred-11 and chromatograms showing the normal allele and corresponding 1238delA mutated allele in exon 14. For *c*–*e*, PCR products from single affected individuals carrying mutations were subcloned and subsequently sequenced to obtain sequences for both the mutated and normal alleles from the same individual.

We created the genetic map of the typed markers using the GAS package version 2.3, applying the genetic model previously used[5]. We identified critical recombinants by constructing affected haplotypes using Genehunter[25], after inspecting the pedigrees to check that the program had enough information for this task.

PCR amplification and sequencing of germline mutations. We determined the genomic structures for candidate genes and designed primers from intronic sequence flanking coding exons (primer sequences are available upon request). We added M13 tails to all PCR primers for subsequent sequence analysis. We carried out PCR reactions for individual exons in 50-µl reaction volumes containing 20 ng of genomic DNA, PCR buffer (Invitrogen Life Technologies), 2.25 mM Mg^{++}, 250 nM dNTPs, 10 pmol forward/reverse primer mix, 0.06 unit Platinum Taq DNA polymerase (Invitrogen Life Technologies) and 0.06 unit AmpliTaq Gold (PE Biosystems). PCR cycles consisted of an initial denaturation at 94 °C for 12 min; 10 cycles of 94 °C for 20 s, annealing for 20 s and 72 °C for 20 s; then 25 cycles of 89 °C for 20 s, annealing for 20 s and 72 °C for 20 s; and a final extension at 72 °C for 10 min. Annealing temperatures were optimized for all primer sets, and this information is available upon request. We analyzed a 5 µl aliquot of PCR product from each reaction on 2% agarose gels to determine robustness of amplification. PCR amplicons were purified using the QiaQuick PCR purification kit on the BioRobot 8000 Automated Nucleic Acid Purification and Liquid Handling system (Qiagen). We carried out double-stranded sequencing using quarter-volume cycle-sequencing reactions prepared in 96-well format using standard M13 forward or reverse primers with the BigDye Terminator Chemistry (PE/Applied Biosystems). After Sephadex purification, we separated sequence products on a 3700 Capillary DNA Analyzer (PE/Applied Biosystems) using manufacturer's protocols. We aligned and analyzed sequence chromatograms using Sequencher version 4.1 (Gene Codes). For PCR products containing potential frameshift mutations, we subcloned PCR products from affected individuals using the TOPO TA cloning system (Invitrogen Life Technologies) according to the manufacturer's recommendations. Positively selected subclones were grown in 3 ml of Luria–Bertani broth supplemented with the appropriate antibiotic selection. We prepared DNA from subclones using the Qiagen Miniprep Plasmid Purification System. We sequenced plasmid DNA with the standard T7 and M13 reverse primers using BigDye Terminator Chemistry (PE/Applied Biosystems). We separated and analyzed samples as described above.

Northern-blot analysis. To determine the transcript size(s) and tissue distribution pattern of *HRPT2*, we hybridized PCR products spanning the coding region of the *HRPT2* mRNA sequence to a commercially available multiple-tissue northern blot, MTN1 (Clontech). The probes were labeled with α-^{32}P-dCTP by random priming (Stratagene) following the manufacturer's recommendations. We carried out hybridization at 42 °C overnight in Hybrisol 1 hybridization buffer (Intergen) followed by stringent washing. We then subjected filters to autoradiography.

Detection of mutations in *HRPT2* and LOH in tumors. Parathyroid tumors and matched leukocytes were obtained with informed consent in direct connection to surgery at the Karolinska Hospital. Tumor samples 1–9 and 11–48 were from individuals with a sporadic form of the disease and no personal or family history of MEN1, HPT–JT or other familial forms of 1°HPT. Tumor sample 10 was from a member of kindred-06, who was affected with FIHP linked to 1q[9]. Following the procedure and criteria previously described for tumor cases 1–30 (ref. 11), we confirmed that tumor samples 31–48 collected here were cystic parathyroid adenomas. By histopathological examination of representative sections from the frozen tumors, we confirmed that all samples used contained a sufficient proportion of tumor cells for DNA analyses (that is, >70%).

letter

We screened *HRPT2* for mutations by sequencing the coding region in all 48 tumors (primers and conditions are available on request). We designed the primers to detect mutations in the coding region as well as at the exon–intron junctions. We carried out cycle-sequencing reactions using the BigDye Terminating cycle sequencing kit (Perkin Elmer). Reactions were separated either using an ABI 377 automated sequencer or a 3700 Capillary DNA Analyzer (PE Applied Biosystems).

We genotyped 48 matched blood and tumor DNA samples using seven microsatellite markers located within the critical region encompassing *HRPT2* (Fig. 1). The typed loci included cen–*D1S222–D1S461–D1S542–HRPT2–D1S412–D1S2794–D1S2840–D1S2622*–tel. In addition, we genotyped tumor samples 31–48 for three microsatellites at the *MEN1* locus in 11q13: cen–(*D11S4946/MEN1*)–*D11S493–D11S4937*–tel. For tumor samples 1–30, LOH analyses of 11q13 have been previously published[11]. We analyzed the markers using fluorescence detection, and determined the LOH status both visually and by calculating the peak ratios between the constitutional and tumor alleles.

Accession numbers. C1orf28, AF312865; Cdc73p, NP_013522.

Note: Supplementary information is available on the Nature Genetics website.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Chapter 4

HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours.

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ORIGINAL ARTICLE

HRPT2 mutations are associated with malignancy in sporadic parathyroid tumours

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Background: Hyperparathyroidism is a common endocrinopathy characterised by the formation of parathyroid tumours. In this study, we determine the role of the recently identified gene, *HRPT2*, in parathyroid tumorigenesis.
Methods: Mutation analysis of *HRPT2* was undertaken in 60 parathyroid tumours: five HPT-JT, three FIHP, three MEN 1, one MEN 2A, 25 sporadic adenomas, 17 hyperplastic glands, two lithium associated tumours, and four sporadic carcinomas. Loss of heterozygosity at 1q24-32 was performed on a subset of these tumours.
Results: *HRPT2* somatic mutations were detected in four of four sporadic parathyroid carcinoma samples, and germline mutations were found in five of five HPT-JT parathyroid tumours (two families) and two parathyroid tumours from one FIHP family. One HPT-JT tumour with germline mutation also harboured a somatic mutation. In total, seven novel and one previously reported mutation were identified. "Two-hits" (double mutations or one mutation and loss of heterozygosity at 1q24-32) affecting *HRPT2* were found in two sporadic carcinomas, two HPT-JT-related and two FIHP related tumours.
Conclusions: The results in this study support the role of *HRPT2* as a tumour suppressor gene in sporadic parathyroid carcinoma, and provide further evidence for *HRPT2* as the causative gene in HPT-JT, and a subset of FIHP. In light of the strong association between mutations of *HRPT2* and sporadic parathyroid carcinoma demonstrated in this study, it is hypothesised that *HRPT2* mutation is an early event that may lead to parathyroid malignancy and suggest intragenic mutation of *HRPT2* as a marker of malignant potential in both familial and sporadic parathyroid tumours.

Hyperparathyroidism (HPT) is one of the most common endocrinopathies, believed to affect approximately three individuals per 1000 adults.[1] HPT is characterised by the formation of parathyroid tumours and, if left untreated, patients develop bone disease, renal stones, and neuromuscular dysfunction. The majority of tumours are sporadic, but approximately 5% are associated with the autosomal dominant hereditary cancer syndromes multiple endocrine neoplasia type 1 and 2A (MEN 1 and 2A), familial isolated hyperparathyroidism (FIHP), and hyperparathyroidism-jaw tumour syndrome (HPT-JT).[2]

Sporadic HPT may occur as primary, secondary, or tertiary disease. Primary HPT can be attributed to a single adenoma in 80–85% of cases, multiglandular hyperplasia in 15–20% of cases, and carcinoma in less than 1% of cases.[3] Secondary HPT arises in response to 1,25-dihydroxyvitamin D_3 deficiency, hyperphosphatemia, or hypocalcemia, due to renal failure, and presents as multiglandular hyperplasia. Tertiary HPT is defined as autonomous parathyroid hyperfunction in a patient with a previously well documented history of secondary HPT. Its presentation has been described as nodular hyperplasia or multiple adenoma. HPT may also arise in response to lithium treatment as a therapy for bipolar disorder.[4]

Our understanding of the molecular basis of parathyroid tumourigenesis has increased over the past 10 years. It has been clearly established that increased expression of the oncogene *cyclin D1* (formerly named *PRAD1*) is associated with the formation of parathyroid hyperplastic glands, adenomas and carcinomas.[5–7] The MEN 1 gene, *MEN1*, located at 11q13,[8 9] has also been shown to play a major role in both familial[10–12] and sporadic[13–16] parathyroid tumourigenesis, functioning as a tumour suppressor. However the genetic basis of many sporadic and familial parathyroid tumours is still unknown. Parathyroid carcinoma, for example, is not a feature of MEN 1, and neither loss of heterozygosity (LOH) at 11q13, nor mutations in *MEN1* have been reported.[17 18] The tumour suppressor gene, *Retinoblastoma (Rb1)*, located at 13q14, was suggested to have a primary role in parathyroid carcinogenesis following the finding of allelic loss of *Rb1* in the majority of parathyroid carcinomas but rarely in benign parathyroid tumours.[19 20] However, subsequent immunohistochemical studies found decreased Rb1 expression to be unreliable as a marker of malignancy.[21–23]

Recently, germline mutations in a newly identified gene, *HRPT2* (AF312865, Hs.5722), previously identified as *Chromosome 1 open reading frame 28*[24] have been identified in 14 of 24 HPT-JT kindreds and one of two FIHP kindreds.[25] *HRPT2* maps to 1q25, consists of 17 exons, containing 1596 nucleotides and encodes a 531 amino acid protein termed parafibromin with unknown function.[25] Patients with HPT-JT present in childhood or early adulthood with HPT, and are also at risk of developing fibro-osseous jaw tumours and renal lesions.[26 27] While sporadic parathyroid carcinoma is rare, the incidence of parathyroid carcinoma is reported to be

Abbreviations: FIHP, familial isolated hyperparathyroidism; HPT, hyperparathyroidism; HPT-JT, hyperparathyroidism-jaw tumour syndrome; LOH, loss of heterozygosity; MEN, multiple endocrine neoplasia

15% in HPT-JT.[2] The majority of parathyroid tumours in these patients are aggressive, occasionally recurrent adenomas, notable also for their cystic histology.[28] LOH studies at 1q24-32 have identified allelic loss in some, but not all, HPT-JT associated tumours, suggestive of a tumour suppressor role for *HRPT2*.[29 30] Similar loss of the *HRPT2* locus in tumours from members of a FIHP family has been reported.[31] Studies in sporadic parathyroid tumours report that 9–13% of adenomas have LOH at 1q24-32.[18 32 33] In addition to germline *HRPT2* mutations in HPT-JT and FIHP kindreds, Carpten *et al* also detected somatic *HRPT2* mutations in two of 47 sporadic cystic parathyroid adenomas.[25] One of these was also found to have LOH at 1q24-32.[25] LOH at this locus has not been identified in lithium-associated parathyroid tumours[34] or secondary HPT.[33]

To determine the role of *HRPT2* in parathyroid tumourigenesis, mutation analysis was undertaken in both sporadic and familial parathyroid neoplasms. A subset of tumours was also assessed for LOH at the *HRPT2* locus, 1q24-32.

MATERIALS AND METHODS

Subjects and samples

Sixty parathyroid samples were obtained from subjects (31 female and 29 male) who underwent parathyroidectomy for hyperparathyroidism at Royal North Shore Hospital, Sydney, Australia; Leiden University Medical Centre, Leiden, Netherlands; or Martin Luther University, Halle-Wittenberg, Germany. Peripheral blood samples were also available from 42 of these patients. Patients gave informed consent according to protocols approved by each centre's human ethics committee.

The tumours were classified as familial or sporadic, including lithium-associated. Further classification of adenoma, hyperplasia (secondary or tertiary), or carcinoma was established according to detailed WHO guidelines.[35] Twelve familial tumour specimens were collected. Three were from two FIHP families, five from two HPT-JT families, three from three MEN 1 families and one from an MEN 2A patient. Orthopentography of the jaw and renal ultrasound were performed on all affected members of the FIHP families. No case of jaw or renal tumour was found. Twenty-five sporadic adenomas, two lithium-associated tumours, 11 secondary and six tertiary hyperplastic glands, as well as four parathyroid carcinomas were also collected. The percentage of neoplastic tissue in each sample was assessed histologically from a paraffin embedded representative piece of tissue. All samples were composed of at least 70% neoplastic cells.

A panel of 65 anonymised peripheral blood samples collected from healthy volunteers constituted the normal germline DNA panel.

DNA/RNA preparation

Parathyroid tissue was frozen in liquid nitrogen immediately after surgical removal and stored at −70℃ or below. Peripheral blood was collected into EDTA anti-coagulant tubes and stored at −70℃. DNA was extracted from the frozen tissue and peripheral blood leucocytes according to standard procedures. RNA was extracted from frozen tissue using TRI Reagent (Sigma-Aldrich Corporation, St Louis, MO) according to the manufacturer's protocol.

LOH studies

Where DNA was available from matched tumour and blood samples, allelic deletion of the chromosome 1q24-32 region flanking *HRPT2* was assessed using a selection of the following microsatellite markers: centromeric-*D1S218-D1S238-D1S422-D1S2625-D1S081-HRPT2D1S533-D1S2757-D1S2794-D1S477*-telomeric.[25 36] PCR was performed in a 7.5 µl reaction volume containing 0.17 µM each of HEX-labelled forward and unlabelled reverse primer (Invitrogen, Life Technologies, Carlsbad, CA), 4 mM $MgCl_2$, 0.3 units AmpliTaq Gold polymerase and 1 × Buffer II (Applied Biosystems, Foster City, CA), 250 uM dNTPs (Invitrogen, Life Technologies), and 15 ng of genomic DNA. Amplification was performed in a DNA Engine Tetrad (MJ Research, Incline Village, NV) with an initial denaturation of 95℃ for 10 min, followed by 10 cycles of 94℃ for 15 seconds, 55℃ for 15 seconds, 72℃ for 30 seconds, and 20 cycles of 89℃ for 15 seconds, 55℃ for 15 seconds, 72℃ for 30 seconds, and a final extension at 72℃ for 10 min. The resulting microsatellite PCR products for each specimen were then pooled, and 1 µL was added to 10 µl of Hi-Di formamide (Applied Biosystems) and 0.2 µl of ROX 400HD size standard (Applied Biosystems), denatured at 95℃ for 5 min and loaded into an ABI Prism 3700 Genetic Analyser (Applied Biosystems). Assessment of LOH was performed using Genescan v. 3.7 and Genotyper v. 3.7 software (Applied Biosystems). LOH was defined according to the following formula: LOH index = (T2/T1)/(N2/N1), where T was the tumour sample, N was the matched normal sample, 1 and 2 were the intensities of smaller and larger alleles, respectively. If the ratio was <0.5 or >1.5, the result was determined to be LOH.[33]

HRPT2 mutation analysis

Initially, normal thyroid tissue RNA was sequenced in 4 overlapping segments between 5'UTR and 3'UTR to clarify the intron/exon boundaries and confirm the published sequence of the recently identified *HRPT2* gene.[25] cDNA was synthesized using the SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies) and amplified in a 20 µL reaction containing 200 ng of cDNA, 200 uM dNTPs (Invitrogen, Life Technologies), 1.5–4 mM $MgCl_2$, 1 µM of each primer, 1 unit of Platinum *Taq* DNA Polymerase and 1 × PCR buffer (Invitrogen, Life Technologies). Following an initial denaturation at 94℃ for 5 minutes, 35 cycles of 15 second steps at 94℃, 55–60℃ (depending on the primer set) and 72℃ were performed in a DNA Engine Dyad (MJ Research), finishing with an extension at 72℃ for 10 minutes. The PCR products were purified using the QIAquick PCR Purification kit (QIAGEN Pty Ltd, Clifton Hill, Vic, Australia), cycle sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and sequenced on an ABI PRISM 3700 Genetic Analyser, according to the manufacturer's recommended protocol. The organisation of the gene is detailed in table 1 and primer sequences are available on request.

Sequencing of *HRPT2* was then performed for all tumour samples. All 17 exons and the intron-exon boundaries were amplified in 15 separate reactions for each sample. PCR was performed in 40 µL reactions containing 75 ng of template DNA, 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM each primer, 1.5 units of *Taq* DNA Polymerase, and 1 × reaction buffer (Invitrogen, Life Technologies). Following an initial denaturation at 94℃ for 5 minutes, 35 cycles of 30 second steps at 94℃, 55℃, and 45 seconds at 72℃ were performed, finishing with an extension at 72℃ for 10 minutes in a DNA Engine Tetrad. Primer sequences are available on request. The PCR products were purified through a Multiscreen PCR Filter plate (Millipore Corp., Billerica, MA) and sequenced as above.

Where matched blood was available, the status of mutations identified in the tumours were determined as either somatic or germline by sequencing of the germline DNA.

RNA was available from three tumours to test whether there were aberrant splicing products of the gene. cDNA was synthesised, amplified and sequenced as above.

Table 1 *HRPT2* (Unigene cluster HS.5722) exon/intron organisation

Exon							Intron	
No	Bp	Nucleotides	Amino acids	Genomic sequence no*	3'splice acceptor region	5'splice donor region	No	Kb
1	131	1–131	1–44	2583–2713	aggggggggaag/ATG	TGGGG/gtaag	1	2.8
2	106	132–237	45–79	5494–5599	atttcag/GACT	AGCT/gtaag	2	5.0
3	70	238–307	80–102	10,556–10,625	gttttag/ACTG	GCGT/gtgag	3	5.1
4	63	308–370	103–123	15,773–15,835	cttcag/CAAC	CAAG/gtatg	4	0.1
5	53	371–423	124–141	15,919–15,971	cttttatag/TCAA	ATTGAG/gtaaa	5	2.5
6	89	424–512	142–170	18,467–18,555	atttccag/GATG	ATTAG/gtaag	6	3.7
7	217	513–729	171–243	22,232–22,448	cttttag/GTCT	GGAAAG/gtaatt	7	5.8
8	99	730–828	244–276	28,249–28,347	attttag/AATTTTT	TGTG/gtaag	8	2.4
9	79	829–907	277–302	30,686–30,764	attttttttacag/GATC	GAAG/gcaagt	9	2.0
10	65	908–972	303–324	32,762–32,826	cttttttttcgtag/AAAC	AACG/gtaag	10	51.3
11	58	973–1030	325–343	84,177–84,234	ttttaaag/GAGGT**G**‡C	CCAG/gtag	11	8.2
12	36	1031–1066	344–355	92,447–92,482	tcacag/TTTCT	GAAAG/gtga	12	0.3
13	88	1067–1154	356–385	92,772–92,859	ctttatag/GATC	TGAA/gtaag	13	20.5
14	162	1155–1316	385–438	113,375–113,536	gttttttcaaag/ATTT	ACTG/gtaag	14	3.1
15	101	1317–1417	439–472	116,638–116,738	cttgtag/GGAC	TAAAA/gtaag	15	13.4†
16	142	1418–1559	473–519	9248–9389	tatag/TTAA	ACAG/gtaat	16	0.8
17	37	1560–1596	520–531	10,194–10,230	attttttcag/GTAC	CTGA/attattt	3'UTR	0.7

*The genomic sequence no. is taken from clones RP11-239J11 (AL390863/AF312865) and RP11-185C19 (AL139133/AF312865).
†Position of overlap of these clones.
‡Base found to be discrepant with the published sequence is in bold.

Polymorphism scanning of the normal DNA panel

All amplifications were performed with 1 µM of each primer (Invitrogen, Life Technologies), 200 uM dNTPs (Invitrogen, Life Technologies), 2 units of AmpliTaq Gold polymerase (Applied Biosystems), 1 × Buffer II (Applied Biosystems), 1.5–4 mM $MgCl_2$, and cycling conditions as stated for PCR of cDNA above, with an annealing temperature of 55℃, unless otherwise stated. Primer sequences are available on request.

*Rsa*1 digest for detection of c.33C→T (Y11Y)

DNA was amplified in a 20 µL volume with exon 1 primers and 1 unit of AmpliTaq Gold polymerase. Four microlitres of the resulting 228 bp amplicon was digested with 1 unit of restriction endonuclease *Rsa*I (Fermentas AB, Vilnius, Lithuania) in 1 × Buffer Y+/Tango (Fermentas AB) with a final volume of 8 µL, and incubated at 37℃ for 8 hours. One microlitre of the resulting digest was mixed with 1 µL of loading dye (5 g/L Bromophenol Blue, 12.5 mM EDTA, 50% formamide) and electrophoresed in a Corbett Research GelScan 2000 Real-time Electrophoresis System (Corbett Research, Mortlake, NSW, Australia) through a 5% polyacrylamide, 0.6 × TBE gel under suggested non-denaturing conditions. Wild type amplicons were distinguished by the presence of bands at 109 bp and 119 bp. Heterozygotes contained an additional band at 228 bp.

dHPLC detection of polymorphisms

DNA was amplified in a 50 µL volume using exon 2, 7, or 13 primers and following amplification the resulting products underwent enhancement of heteroduplex formation. This involved a denaturation at 94℃ for 5 min immediately followed by a slow ramp over 35 cycles, starting at 94℃ and decreasing 2℃/1 min cycle, finishing with 1 min incubation at 25℃. Five microlitres of amplicon was then injected into the flowpath of a DNASep-MD cartridge in the WAVE-MD Mutation Detection System Model 2000 denaturing HPLC (Transgenomic, Omaha, NE) under WAVE-MD Standard Method gradient conditions and WAVEMAKER (Transgenomic) predicted temperatures for each amplicon. Eluted fragments were detected by the system's UV detector and analysed as chromatograms. Wild type amplicons eluted as a single homoduplex peak whereas heterozygous samples presented with up to four peaks representing homo- and heteroduplexes. For the IVS2+28C→T variant, homozygotes for either the C or T allele were mixed with an equal aliquot of an exon 2 amplicon homozygous for the C allele, denatured, slowly reannealed and re-injected into the dHPLC to determine which allele was present.

Statistical analysis

The χ^2 contingency test was performed to compare the occurrence of mutations between four cases of sporadic carcinomas and 44 cases of sporadic non-carcinomas. A p value less than 0.05 is considered as significant.

RESULTS

HRPT2 mutation analysis

HRPT2 mutations were detected in the DNA from four of four sporadic parathyroid carcinoma samples, five of five HPT-JT parathyroid tumours (two families) and parathyroid tumours from one of two FIHP families (fig 1). No mutations were detected in any of the other 49 tumours sequenced. Eight different *HRPT2* mutations were detected. With the exception of L64P, that has been previously published,[23] all the mutations identified were novel (fig 2). One of the mutations (c.76delA) was detected both in a sporadic carcinoma and in a HPT-JT family. Where available, matched constitutive DNA was analysed to determine the germline versus somatic nature of these mutations (fig 1). HPT-JT tumour #1613 harboured both a germline and a somatic *HRPT2* mutation, and two somatic *HRPT2* mutations were detected in carcinoma #2077.

LOH studies

LOH analysis of 1q24-32 was performed on 42 of the 60 tumours. All samples tested were informative for at least one of the markers (data not shown). LOH was detected in six samples: one sporadic carcinoma (#10977), one HPT-JT tumour (#1765), two FIHP tumours (#4 and #54) and two sporadic adenomas (#9 and #101) (fig 1). LOH at 1q24-32 has been previously reported in two of these samples (#9[24] and #4[31]). LOH was confirmed in sporadic carcinoma sample #10977, HPT-JT sample #1765 and FIHP samples #4 and #54 by the presence of only the mutant allele in tumour DNA sequence. Retention of heterozygosity was demonstrated in the remaining 18 tumours not assessed for LOH by the

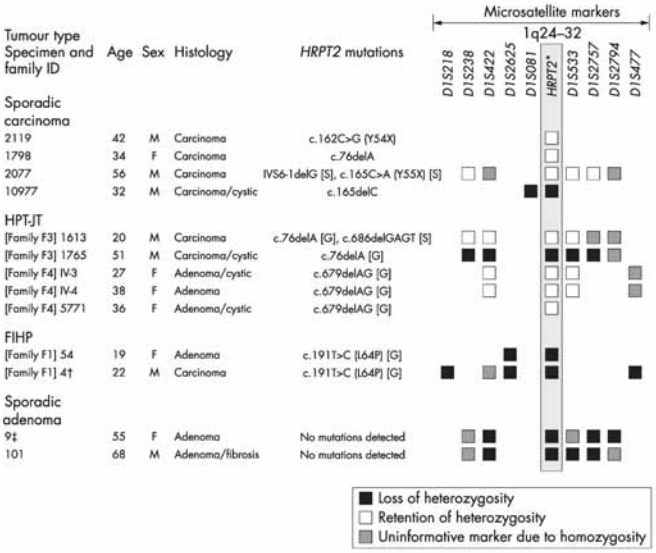

Figure 1 Mutations detected within *HRPT2*, and LOH analysis of flanking markers. "Age" is the age in years at the time of parathyroidectomy. *HRPT2* mutations detected are listed as nucleotide changes and amino acid changes where relevant. Where two mutations were detected in the same patient, both are listed. Germline mutations are denoted "[G]", and somatic mutations are denoted "[S]". For the microsatellite markers, see key in figure. Blank spaces indicate LOH testing not performed (germline DNA not available). *The approximate location of *HRPT2* within the 1q24-32 region. The results in this shaded column represent retention or loss of heterozygosity at the *HRPT2* locus by combined microsatellite and intragenic mutation results. †*D1S218* and *D1S477* have been reported previously.[31] ‡Previously reported as having LOH at 1q and 1p and CGH loss at 1q31-qter.[34]

presence of a heterozygous mutation or polymorphism in *HRPT2*.

Polymorphisms

Five intronic and one exonic sequence variants, five of which were also identified in a screen of 65 normal subjects, were identified in parathyroid lesions, and where available, matched constitutive DNA (table 2). An additional variant, not detected in the tumours, was found in the normal panel (fig 3). Twenty six of the 54 parathyroid tumour samples not displaying LOH were heterozygous for the common polymorphism, IVS2+28C→T, confirming retention of heterozygosity for *HRPT2*. A dinucleotide repeat variant in intron 7, IVS7+33$(GA)_8$ (wild type IVS7+33 $(GA)_9$) was found in five

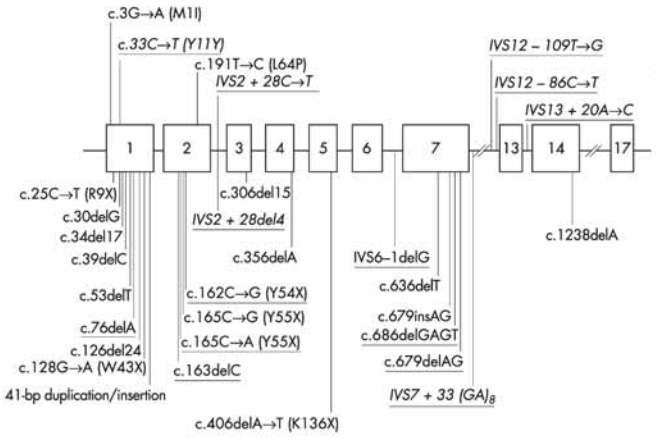

Figure 2 Distribution of *HRPT2* mutations and polymorphisms. Exons 8–12 inclusive, 15, and 16, have not been shown for clarity. Underlined variants are from this study, others are from Carpten *et al*.[25] Variants predicted to be polymorphisms are italicised.

Table 2 *HRPT2* predicted polymorphisms found in this study

Polymorphism	Heterozygote frequency in tumour DNA* (allele frequency)	Classification of tumours harbouring a polymorphism	Heterozygote frequency in germline DNA from a normal panel (allele frequency)
Y11Y (c.33C→T)	1 in 56 (0.01)	1 sporadic adenoma [G]	0 in 65
IVS2+28delCCTA	2 in 56 (0.02)	1 secondary hyperplasia [G] 1 tertiary hyperplasia [?]	5 in 65 (0.04)
IVS2+28C→T	26 in 56 (Heterozygous) (0.30) 4 in 56 (Homozygous for T)	all tumour types [G]	33 in 65 (Heterozygous) (0.32) 4 in 65 (Homozygous for T)
IVS7+33(GA)$_8$	4 in 56 (0.04)	1 lithium [G] 1 secondary hyperplasia [G] 1 MEN 1 [?] 1 FIHP† (2 affected family members [G])	1 in 65 (0.01)
IVS12-86C→T	3 in 56 (0.03)	1 sporadic adenoma [?] 2 secondary hyperplasia [?]	3 in 65 (0.02)
IVS12-109T→G	5 in 56 (0.05)	1 secondary hyperplasia [G] 1 tertiary hyperplasia [?] 3 sporadic adenomas [?]	6 in 65 (0.05)
IVS13+20A→C	0 in 56		1 in 65 (0.01)

*Only 1 sample from each family was included in the heterozygote frequency estimation.
[G]Present also in germline.
[?]Germline status not ascertained.
†The variant IVS7+33(GA)$_8$ was found in tumour and in germline in two affected members of the same family. Both tumours demonstrated LOH at 1q24-32 and the polymorphism was found on the mutated allele in tumour DNA.

tumours. We were unable to detect aberrant splicing in transcripts generated from three of these tumours (#4, #54, and #76).

Statistical analysis

A χ^2 contingency test showed that the occurrence of mutation in sporadic parathyroid carcinomas is highly significant compared with sporadic non-carcinomas ($\chi^2 = 48$; $p<0.001$).

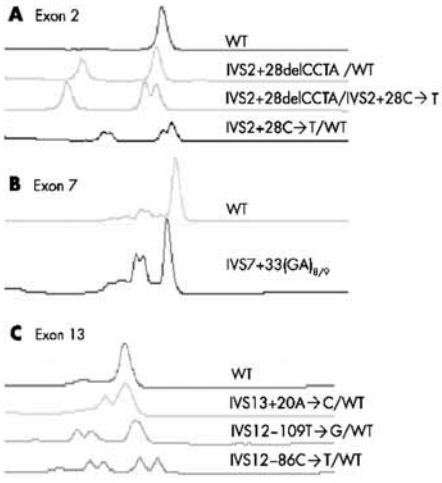

Figure 3 Denaturing HPLC chromatograms of HRPT2 polymorphisms. The chromatograms plot the absorbance at 260 nm in mVolts v elution time in minutes for the various amplicons and show the different profiles that distinguish between the wild type (WT) and various polymorphisms for each amplicon. Wild type amplicons elute as a single homoduplex peak, whereas samples heterozygous for a polymorphism present with up to four peaks, representing homo- and heteroduplexes. Samples harbouring both polymorphisms in intron 2 (A) are also distinguishable from those heterozygous for a single intron 2 polymorphism.

DISCUSSION

Here we report the first finding of *HRPT2* mutations in 100% (four of four) of sporadic parathyroid carcinomas studied (fig 1). All are predicted to prematurely truncate the parafibromin protein. No *HRPT2* mutations were found in any of the 44 sporadic, non-carcinoma samples in this study. These results demonstrate a strong association between intragenic mutation of *HRPT2* and malignancy in parathyroid tumours ($p<0.001$). Furthermore, our findings of two somatic mutations as well as one mutation with LOH at 1q24-32 in two of four carcinomas are consistent with Knudson's "two-hit" hypothesis, and suggest the role for *HRPT2* as a tumour suppressor gene in sporadic parathyroid tumorigenesis.

Our finding of germline mutations in two of two HPT-JT families and confirmation of a germline mutation in one of two FIHP families support the conclusions of Carpten *et al*,[25] that *HRPT2* is the causative gene in HPT-JT and in a subset of FIHP (fig 1). Of additional significance was our finding of the "second-hit" in four of the seven samples with germline mutations. Three familial tumour samples demonstrated loss of the wild-type allele, and one tumour contained a somatic frameshift mutation (fig 1). These results provide evidence of a role for *HRPT2* as a tumour suppressor gene in familial as well as sporadic parathyroid tumorigenesis.

The two germline mutations in the HPT-JT families are, like those found in the sporadic carcinomas, predicted to prematurely truncate the protein, whereas the FIHP mutation is a missense mutation of unknown consequence. Whether there is a phenotype/genotype correlation between mutation type and the presence of parathyroid disease alone, or in conjunction with jaw tumours or renal lesions (that is, FIHP *v* HPT-JT) will require a larger cohort of FIHP families with *HRPT2* mutations to establish. Similarly, it is uncertain whether the severity or number of "hits" affecting *HRPT2* determines the presentation of familial tumours as benign, cystic, or malignant. An alternative scenario may be that intragenic mutation of *HRPT2* is an early event in a subset of patients with parathyroid lesions, and that additional, yet unknown events are required for the progression to malignancy. It is noteworthy that *HRPT2* mutations have been previously detected in two of 47 (4%) sporadic cystic adenomas, and one (2%) had additional 1q24-32 LOH.[25] Given the strong association demonstrated between

intragenic mutation of *HRPT2* and malignancy in the current study, such a finding in a benign tumour might be considered a marker of malignant potential and long term monitoring is suggested for these patients.

Twenty four different *HRPT2* mutations have now been reported,[25] including seven in this paper (fig 2). Over 80% (20 of 24) of *HRPT2* mutations are located in exons 1, 2, or 7 or flanking intronic sequences. Exon 1 contains 42% (10 of 24) of all mutations detected to date, and exon 2 appears to contain a mutation hot spot at c.165C. Exon 7 is by far the largest exon, hinting at an important role for this exon, and to date, has been shown to harbour 21% (five of 24) of all *HRPT2* mutations detected. This exon contains a repeat element $(AG)_5$, and three different frameshift mutations altering this motif have been detected. This study also detected seven apparent polymorphisms. We found these, in particular, IVS2+28C→T, to be useful markers for exclusion of LOH at the *HRPT2* locus.

The consistent detection of LOH at 1q24-32 in 9–13% of sporadic adenomas[14 32 33] is interesting. Our finding of LOH in two of 25 (12.5%) sporadic adenomas is in agreement with these previous reports, as is our finding of no LOH in 17 hyperplasia or two lithium-associated tumours.[33 34] We have previously shown that extensive loss of both arms of chromosome 1 occurs in approximately 10% of sporadic adenomas,[14 32] including adenoma #9[34] from this study. We did not find a *HRPT2* mutation on the remaining allele of either of the sporadic adenomas with LOH at 1q24-32 in this study and suggest that the LOH at 1q24-32 in the majority of sporadic adenomas may in fact be targeted at a tumour suppressor gene elsewhere on chromosome 1. To date, there is no evidence that LOH at 1q24-32 alone confers a higher likelihood of malignancy.

In conclusion, the results in this study support a role for *HRPT2* as a tumour suppressor gene in agreement with Knudson's "two-hit" hypothesis in sporadic parathyroid carcinoma. These results also provide further evidence for *HRPT2* as the causative gene in HPT-JT, and a subset of FIHP. Efficient detection of *HRPT2* mutations should begin with exons 1, 2, and 7, which together harbour 80% of mutations found to date. In light of the strong association between mutations of *HRPT2* and sporadic parathyroid carcinoma demonstrated in this study, we hypothesise that *HRPT2* mutation is an early event that may lead to parathyroid malignancy and therefore suggest intragenic mutation of *HRPT2* as a marker of parathyroid malignant potential in both familial and sporadic tumours. Further studies will clarify the exact nature of the relationship between intragenic mutation of *HRPT2* and parathyroid malignancy.

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Chapter 5

Loss of parafibromin immuno-reactivity is a distinguishing feature of parathyroid carcinoma.

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Loss of Parafibromin Immunoreactivity Is a Distinguishing Feature of Parathyroid Carcinoma

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ABSTRACT

Purpose: **A reliable method for diagnosing parathyroid carcinoma has remained elusive over the years, resulting in its under-recognition and suboptimal therapy. Obtaining an accurate diagnosis has become an even more pressing matter with recent evidence that germline *HRPT2* gene mutations are found in patients with apparently sporadic parathyroid carcinoma. There is a high prevalence of *HRPT2* gene mutations and biallelic inactivation in parathyroid carcinoma. We hypothesize that loss of parafibromin, the protein product of the *HRPT2* gene, would distinguish carcinoma from benign tissue.**

Experimental Design: **We generated a novel antiparafibromin monoclonal antibody and performed immunostaining on 52 definite carcinoma specimens, 6 equivocal carcinoma specimens, 88 benign specimens, and 9 hyperparathyroidism-jaw tumor (HPT-JT) syndrome-related adenomas from patients with primary hyperparathyroidism from nine worldwide centers and one national database.**

Results: **We report that the loss of parafibromin nuclear immunoreactivity has 96% sensitivity [95% confidence interval (CI), 85–99%] and 99% specificity (95% CI, 92–100%) in diagnosing definite carcinoma. Inter-observer agreement for evaluation of parafibromin loss was excellent, with unweighted kappa of 0.89 (95% CI, 0.79–0.98). Two equivocal carcinomas misclassified as adenomas were highlighted by parafibromin immunostaining. One of these tumors has since recurred, satisfying criteria for a definite carcinoma. Similarly, eight of nine HPT-JT syndrome-related adenomas showed absent nuclear immunoreactivity.**

Conclusions: **Parafibromin is a promising molecular marker for diagnosing parathyroid carcinoma. The similar loss of parafibromin immunoreactivity in HPT-JT syndrome-related adenomas suggests that this is a pivotal step in parathyroid tumorigenesis.**

INTRODUCTION

Primary hyperparathyroidism is a common disorder, diagnosed with increasing frequency (1). The prevalence of parathyroid carcinoma has ranged in various studies from <1 to 5% of cases of primary hyperparathyroidism (1, 2) with especially high rates reported in Japan (3) and Italy (4). The causes of this variation are unclear but may reflect a true geographical difference, referral bias, or differences in the histologic criteria used. Apart from the presence of local invasiveness or metastasis, there is no definitive standard for pathologic diagnosis of parathyroid carcinoma; in the absence of these features, such a diagnosis may have subjective elements (1, 2, 5). As a result of this difficulty in recognition, up to 86% of cases are not initially recognized intra-operatively even in expert institutions and receive inadequate surgical resection (6). The diagnosis of carcinoma is often retrospectively made after relapse (2, 7), when treatment options are limited. In one series, half of all recurrent or metastatic carcinomas were initially diagnosed as benign (8). Indeed, it has been concluded by some authors that systematic diagnosis of parathyroid carcinoma may rest upon ongoing postoperative follow-up of patients who have undergone resection of apparently benign adenomas (9, 10). About 20% of patients with an apparently sporadic carcinoma may be manifesting a forme fruste of hyperparathyroidism-jaw tumor (HPT-JT) syndrome (11), a hereditary multitumor syndrome characterized by *HRPT2* gene mutations (12). In this light, an accurate diagnosis is even more critical. Timely diagnosis would allow definitive surgery and targeted genetic screening of individuals and their families (13). From a clinical viewpoint, it is unsatisfactory to rely on tumor extension for diagnosis, as early recognition and treatment by en-bloc resection are the main determinants of prognosis (2, 3, 6, 14).

The*HRPT2* gene is a recently identified tumor suppressor gene in parathyroid carcinoma, encoding a novel protein product named parafibromin (15). Somatic *HRPT2* gene mutations occur

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in 66 to 100% of sporadic parathyroid carcinomas, and biallelic inactivation is present in the majority of these cases (11, 16). We thus hypothesized that loss of parafibromin immunoreactivity would distinguish parathyroid carcinoma from benign pathologies of primary hyperparathyroidism.

Studies of Diagnostic Markers. The quest for a definitive method for diagnosis started in earnest 30 years ago, when initial histopathologic guidelines for the diagnosis of carcinoma described uniform sheets of cells arranged in a lobulated fashion separated by fibrous trabeculae, capsular or vascular invasion, and the presence of mitotic figures (17). Unfortunately, mitotic features, fibrous bands, and uniform sheets of cells were found not to be pathognomonic for parathyroid carcinoma (5, 14). As a result of the limited applicability of these guidelines, many adjunct investigations for parathyroid carcinoma have been studied, including electron microscopy, immunohistochemistry, DNA flow cytometry, and *in situ* hybridization (2). Immunohistochemical markers that have been studied include retinoblastoma tumor suppressor gene protein (pRb), calcium-sensing, Ki-67, cytokeratin-14, p27, mdm2, Bcl-2, cyclin D1, p53, and p21 (5, 18–24). However, many markers have not been shown to be useful in this regard (19).

The most extensively studied marker to date is the retinoblastoma tumor suppressor gene *RB* and the *RB* protein (pRb). Contrary to initial reports, recent evidence suggests that loss of heterozygosity is not specific to parathyroid carcinoma. Studies of pRb immunostaining have also yielded conflicting results. Some studies have reported that it may be a helpful marker (25, 26), but several other studies have contradicted these findings (22, 23). A recent review of *RB* gene abnormalities in parathyroid carcinoma concluded that no definitive conclusion could be drawn with regards to pRb staining (26).

Other markers studied include cell cycle-associated antigens. Erickson *et al.* (21) and Stojadinovic *et al.* (19) report that although carcinomas, relative to adenomas, have a lower percentage of p27-positive nuclei, there is a considerable overlap in staining percentages. Stojadinovic *et al.* also report that a multiple-marker phenotype including p27, Bcl-2, Ki-67, and mdm2 was useful in defining a subset of benign tumors but that carcinoma displayed a complex range of multi-marker phenotypes, some of which were not specific. Furthermore, DNA cytometry was shown to be of prognostic but not diagnostic use. It distinguishes a subset of parathyroid carcinomas that are aneuploid and that may behave in a more aggressive fashion (27–29) but is not specific (2, 3, 30, 31) or sensitive (27–30).

In summary, despite many studies over the years, diagnosing parathyroid carcinoma remains a major challenge for the expert pathologist. As there is a high prevalence of *HRPT2* gene mutations in parathyroid carcinoma (11, 16), we investigated parafibromin immunoreactivity as a means of differentiating parathyroid cancer from benign tissue, including adenomas, hyperplasias, and multiple endocrine neoplasia types 1 (MEN1)-associated tumors. As the prevalence of parathyroid carcinoma varies geographically for uncertain reasons (2), we selected a geographically diverse, multi-center approach, with a variety of pathologies that may be encountered in the evaluation of primary hyperparathyroidism. As histologic criteria varies because of the lack of standard guidelines, gold-standard criteria of invasion or metastasis was imposed on case selection for standardization. We additionally stained adenomas from patients from HPT-JT syndrome, in view of a common expression profiling signature distinguishing these adenomas and carcinomas from other types of benign tissue that we recently reported (32).

MATERIALS AND METHODS

Patient Samples. We conducted a multi-center, retrospective study involving anonymized formalin-fixed, paraffin-embedded parathyroid specimens from primary hyperparathyroidism cases. The participating centers were Ohio State University, Leiden University Medical Center (Netherlands), Northwestern University, University of Chicago, Seinäjoki Central Hospital (Finland), Singapore General Hospital (Singapore), National University Hospital (Singapore), Shared Pathology Informatics Network (West Michigan), and University of Tasmania (Australia). The study was reviewed and approved by the Van Andel Institute Institutional Review Board.

A total of 160 specimens were examined. One hundred and twenty-three full sections were studied, including sporadic primary carcinomas ($n = 19$), sporadic metastatic tissue ($n = 1$), sporadic equivocal carcinomas ($n = 2$), sporadic adenomas ($n = 50$), sporadic primary hyperplasia ($n = 25$), MEN1-associated tumors ($n = 13$), HPT-JT primary carcinoma ($n = 1$), HPT-JT adenomas ($n = 7$), and normal tissue ($n = 5$). A tissue array containing an additional 37 specimens obtained from the Dutch National Pathology Database (PALGA) was also studied: sporadic primary carcinomas ($n = 23$), sporadic metastatic tissue ($n = 7$), sporadic equivocal carcinomas ($n = 3$), HPT-JT primary carcinoma ($n = 1$), HPT-JT adenomas ($n = 2$), and normal tissue ($n = 1$). Triplicate tissue cores with a diameter of 0.6 mm, as selected by a pathologist (H. M.), were taken from each specimen (Beecher Instruments, Silver Spring, MD) wherever possible, and a standard procedure was used to array tissue cores on a recipient paraffin block (33). The full sections of all arrayed specimens were examined (H. M.) to ensure concordance with criteria. In all cases (both full sections and arrays), lesions were diagnosed as definite carcinomas only if vascular invasion, invasion of surrounding tissue, or distant metastasis were evident. Equivocal carcinomas were defined as tumors exhibiting histopathologic features of carcinoma without the presence of vascular invasion, invasion of surrounding tissue, or distant metastasis (22, 28). Clinicopathologic data for the definite and equivocal carcinoma cases, following reclassification as described below, are shown in Table 1. In summary, 52 definite carcinoma specimens were obtained from 48 patients; multiple specimens from single patients were obtained at separate clinical events. 49 of 52 definite carcinoma specimens had surrounding tissue or vascular invasion on histopathologic examination. For 25 specimens from 21 patients with available data, 19 specimens were from 15 patients who initially or eventually developed metastases, 5 specimens were from 5 patients who relapsed locally, and only 1 patient, who was followed up for 1 year, did not have local or systemic relapse during follow-up. Twenty-eight arrayed definite carcinoma specimens had been further characterized previously (5): 10 of 28 were cystic; 4 of 28 trabecular; 19 of 28 had fibrotic bands; 8 of 28 had >1 of 10 mitoses/high power field; 14 of 28 showed positive cyclin D1

Table 1 Clinicopathologic data and evaluation of immunostaining for carcinoma specimens

Patient	Histo-pathological assessment of carcinoma	Tumor location	Presence of local or vascular invasion at initial surgery	Presence of metastasis at initial surgery	Clinical progression during follow-up	Presence of metastasis or relapse during clinical history	Parafibromin immunoreactivity
1	Definite	Primary, local	Y	N	Local relapse	Y	Diffuse staining/intensity 3
2	Definite	Primary, local	N	N	Metastasis	Y	Diffuse loss
3	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
4	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
5	Definite	Primary, local	Y	N	Local relapse	Y	Focal loss
6	Definite	Primary, local	Y	N	Local relapse	Y	Focal loss
7	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Focal loss
8	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
9	Definite	Metastasis, initial	N	Y	Metastasis	Y	Diffuse loss
10	Definite	Relapse, local	Y	N	Local relapse	Y	Diffuse loss
11	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Diffuse loss
12	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Focal loss
12	Definite	Metastasis, relapse	Y	Y	Metastasis	Y	Diffuse loss
12	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Diffuse loss
13	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
14	Definite	Metastasis, relapse	Y	Y	Metastasis	Y	Diffuse loss
15	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Focal loss
16	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
17	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Focal loss
18	Definite	Primary, local	Y	N	No relapse	N	Focal loss
19	Definite	Primary, local	Y	N	Metastasis	Y	Diffuse staining/intensity 2
20	Definite	Primary, local	N	N	Local relapse	Y	Diffuse loss
21	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
22	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
23	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
24	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
25	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
26	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
27	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
28	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
29	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
30	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
31	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
32	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
33	Definite	Primary, local	Y	N	Metastasis	Y	Focal loss
33	Definite	Metastasis, relapse	Y	Y	Metastasis	Y	Focal loss
34	Definite	Metastasis, initial	Y	Y	Metastasis	Y	Diffuse loss
34	Definite	Metastasis, relapse	Y	Y	Metastasis	Y	Diffuse loss
35	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
36	Definite	Metastasis, initial	Y	Y	Unknown	Y	Diffuse loss
37	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
38	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
39	Definite	Primary, local	Y	N	Metastasis	Y	Focal loss
40	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
41	Definite	Metastasis, initial	Y	Y	Unknown	Y	Diffuse loss
42	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
43	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
44	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
45	Definite	Metastasis, initial	Y	Y	Unknown	Y	Diffuse loss
46	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
47	Definite	Primary, local	Y	N	Unknown	Unknown	Focal loss
48	Definite	Primary, local	Y	N	Unknown	Unknown	Diffuse loss
49	Equivocal	Local	N	N	No relapse	N	Diffuse staining/intensity 3
50	Equivocal	Local	N	N	No relapse	N	Diffuse staining/intensity 3
51	Equivocal	Local	N	N	No relapse	N	Diffuse staining/intensity 1
52	Equivocal	Local	N	N	Unknown	Unknown	Focal loss
53	Equivocal	Local	N	N	Unknown	Unknown	Diffuse loss
54	Equivocal	Local	N	N	No relapse	N	Focal loss

staining; 20 of 27 showed positive calcium-sensing receptor staining; 14 of 22 had loss of heterozygosity of chromosome 1q (*HRPT2* gene loci); 13 of 22 had loss of heterozygosity at chromosome 11q (*MEN1* gene loci); Ki-67 index ranged from 0.1 to 27.5. All HPT-JT adenomas had been sequenced previously (15, 33) and confirmed to have *HRPT2* gene mutations. All MEN1 tumors had been confirmed previously to have *MEN1* gene mutations.

Monoclonal Antibody Generation and Validation. We first generated a novel murine monoclonal antibody to parafibromin targeting the peptide RRPDRKDLLGYLNC, corresponding to amino acid positions 87 to 100. BALB/c mice were immunized with intraperitoneally administered keyhole limpet hemocyanin-conjugated synthetic peptides in complete Freund's adjuvant, followed by two additional injections in incomplete Freund's adjuvant at an interval of 2 weeks. After a month, injections without adjuvant were administered both intravenously and intraperitoneally. Spleen cells were fused with P3X63AF8/653 myeloma cells 3 days after the final injection. An immunofluorescence assay was used to screen positive hybridomas with COS7 cells transfected with a green fluorescent protein (GFP)-HRPT2 DNA fusion construct, validated by ELISA, subcloned to establish stable monoclonal antibody-secreting hybridomas, and revalidated by ELISA. The antibody was purified by fast performance liquid chromatography in a protein G affinity column.

Indirect immunofluorescence was used to show antibody specificity (Fig. 1). HEK293 cells transfected with a GFP-HRPT2 DNA fusion construct and control cells transfected with GFP-empty vector were fixed by immersion in cold acetone/methanol (1:1) for 10 minutes and rehydrated through 70% ethanol, 50% ethanol, and PBS. Anti-parafibromin antibody was applied and washed off, followed by Rhodamine Red-conjugated goat antimouse IgG (Jackson ImmunoResearch Lab, West Grove, PA) at 1:100 dilution for 1.5 hours at 37°C. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. In addition, Western blotting was done on COS7 cells transfected with empty pcDNA3 vector and pcDNA3-HRPT2 (Fig. 2). To further characterize parafibromin immunoreactivity, we did immunohistochemical studies in a variety of formalin-fixed paraffin-embedded human tissues, including lung, kidney, testis, thyroid, adrenal, thymus, and lymph nodes.

Immunohistochemistry. Immunohistochemistry was done with standard procedures: deparaffinized 5-μm sections were steamed in citrate buffer (pH 6) for 30 minutes. Sections were incubated in succession with 0.3% hydrogen peroxide in water for 30 minutes; 5% goat serum for 30 minutes; Avidin D solution for 15 minutes (SP2001, Vector Labs, Burlingame, CA); biotin solution for 15 minutes (SP2001, Vector Labs); primary antibody diluted in diluting buffer (M35, Biomeda Corp., Foster City, CA) at 10 μg/mL for 1 hour; biotinylated goat antimouse antibody (BA-9200, Vector Labs) at 6 μg/mL for 1 hour; streptavidin-biotinylated horseradish peroxidase complex (Vectastain Elite kit, PK-6100, Vector Labs) for 30 minutes; diaminobenzidine tetrahydrochloride for 5 minutes and counterstained in hematoxylin for 3 seconds. Sections were washed in 2 cycles of PBS (pH 7.4) between each step. Positive controls were transfected COS7 cells expressing parafibromin. Negative controls included experiments omitting primary antibody and experiments with primary antibody preabsorbed with a 20-fold excess of immunizing peptide. A random selection of duplicate slides were stained by a Dako LV-1 automated immunostainer (Dako, Carpinteria, CA) by a separate author (C.M.) in another laboratory.

Pathologic Evaluation. Slide sections were examined independently by two blinded investigators (C. M. and J. R.). The tissue array was examined unblinded by two investigators, who reached a common agreement. The staining pattern of each specimen was classified in three categories: diffuse positive, focal loss, or diffuse loss. Diffuse positive staining was defined as staining of all parathyroid tissue nuclei; heterogeneity of staining without loss was included in this category. Focal loss was defined as the absence of nuclear staining in variably sized regions. Diffuse loss was defined as the absence of nuclear staining in all tumor tissue. Where staining was diffuse, the overall staining intensity was evaluated on a semi-quantitative

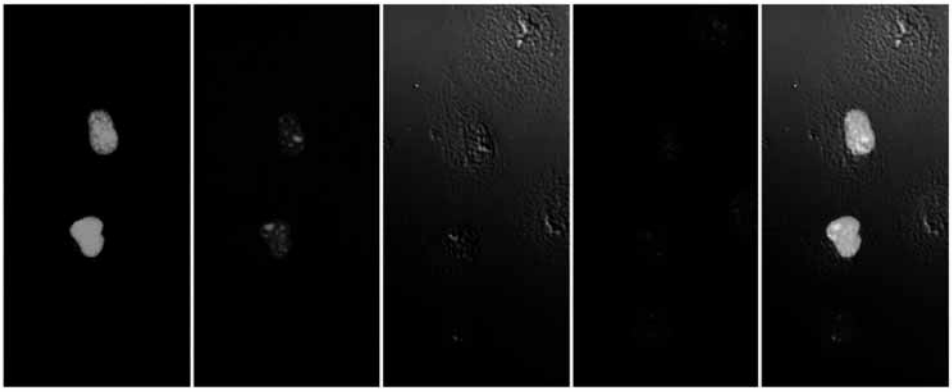

Fig. 1 Confocal images demonstrating co-localization of the GFP-parafibromin fusion protein with anti-parafibromin antibody within the nuclei of transfected HEK293 cells. *From left*, GFP-parafibromin fusion protein expression (*green*); anti-parafibromin monoclonal antibody binding, as detected by secondary Rhodamine-Red goat antimouse antibody (*red*); Nomarski image of cells; 4′,6-diamidino-2-phenylindole staining of nuclei (*blue*); superimposition of all images demonstrating co-localization within nuclei. All images captured with a Zeiss LSM510 META laser-scanning confocal microscope.

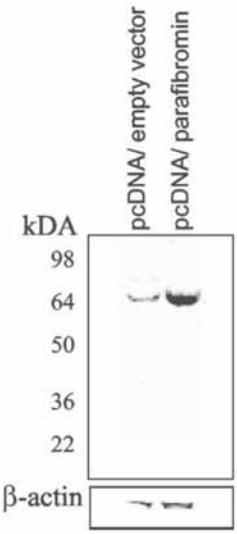

Fig. 2 Western blot showing an increase in parafibromin expression in COS7 cells transfected with pcDNA3-HRPT2, compared with cells transfected with empty vector, as detected by anti-parafibromin antibody. β-Actin is shown as control.

scale of 1 to 3, with 1 representing weak staining, 2 moderate staining, and 3 strong staining. A third pathologist (H. M.) provided the tie-breaking assessment, where there was observer disagreement. Calculation of sensitivity and specificity were based on the differentiation of carcinomas from the sporadic benign proliferations of adenoma and hyperplasia. All statistical calculations took into account pathologic reclassification of specimens. Unweighted kappa statistics were used to calculate the inter-observer agreement as to the presence of loss of immunoreactivity, and the calculations were restricted to the full tissue sections that were examined unblinded. Evaluation of all full sections (n = 123), including MEN1-related tumors, HPT-JT adenomas, and normal tissue were included in this calculation. Confidence intervals for sensitivity and specificity were calculated by the efficient score method with continuity correction described by Newcombe (35). H&E stained slides of the benign specimens that at least one pathologist assessed as having loss (n = 6) were re-evaluated by two independent pathologists.

Sequence Analysis. The ScanProSite program (36) was used to search the PROSITE Release 18.26 database.[13]

[13] Swiss Institute of Bioinformatics server, Geneva, Switzerland; http://www.expasy.ch.

RESULTS

Antibody Characterization. The anti-parafibromin monoclonal antibody co-localized with GFP-parafibromin fusion protein in the nuclei of transfected HEK293 cells (Fig. 1). Control cells transfected with GFP-empty vector did not show staining (data not shown). Western blotting done on transfected COS7 cells and empty vector control showed increased intensity of a single crisp band with the expected molecular mass (Fig. 2). We examined a range of human tissues to characterize its range of immunoreactivity and observed that parafibromin immunoreactivity was present in all organs but was cell-type specific (data not shown). Parafibromin was localized to the nucleus in all tissue examined. Controls with antibody pre-absorbed with a 20-fold excess of immunizing peptide did not show any immunoreactivity.

Sequence Analysis. Analysis of the peptide sequence of parafibromin showed the presence of bipartite nuclear localization signal domains at residues 76 to 92 and 393 to 409.

Parathyroid Tissue Immunohistochemistry. We report that parathyroid carcinoma may be distinguished from other benign pathologies by the loss of parafibromin nuclear immunoreactivity (Fig. 3). Table 1 shows individual case evaluation alongside clinicopathologic data for carcinoma cases, and Table 2 shows a summary of the evaluation of all specimens. Eleven benign cases from the adenoma, hyperplasia, and MEN1 tumor groups displayed heterogeneity of staining without absence of parafibromin immunoreactivity, and these were classified as diffusely positive. Loss of parafibromin immunoreactivity for arrayed carcinoma specimens was independent of tissue architecture, frequency of mitoses, Ki-67 index, loss of heterozygosity, cyclin D1 and calcium-sensing receptor immunostaining results. The assay had a calculated sensitivity of 96% [95% confidence interval (CI), 85–99%] and specificity of 99% (95% CI, 92–100%) for differentiating parathyroid carcinoma from sporadic benign proliferations (Table 3). Table 3 also shows the calculated positive predictive values and negative predictive values with confidence intervals for common estimates of prevalence (1% in countries of low reported prevalence, ref. 1; 5% in countries of high reported prevalence, such as Japan and Italy, ref. 3, 4). The data showing inter-observer variation is presented in Table 4. There was exceptional inter-observer agreement with regards to the blinded assessment of any immunoreactivity loss, with an unweighted kappa statistic of 0.89 (95% CI, 0.79–0.98). Agreement with regards to staining pattern was also excellent, with an unweighted kappa statistic of 0.77 (95% CI, 0.65–0.89). The H&E-stained sections of benign specimens that had at least one investigator assess as having a loss of parafibromin immunoreactivity (n = 6) were re-evaluated by two pathologists. Two adenomas from separate institutions received revised diagnoses of equivocal, but highly probable, carcinoma based on severe architectural atypia, nuclear atypia, and abundant mitotic figures. Neither case had a family history of parathyroid disease. One of these two cases (Patient 20) had a local relapse on follow-up, and was re-classified as a definite carcinoma. Another specimen, diagnosed previously as met-

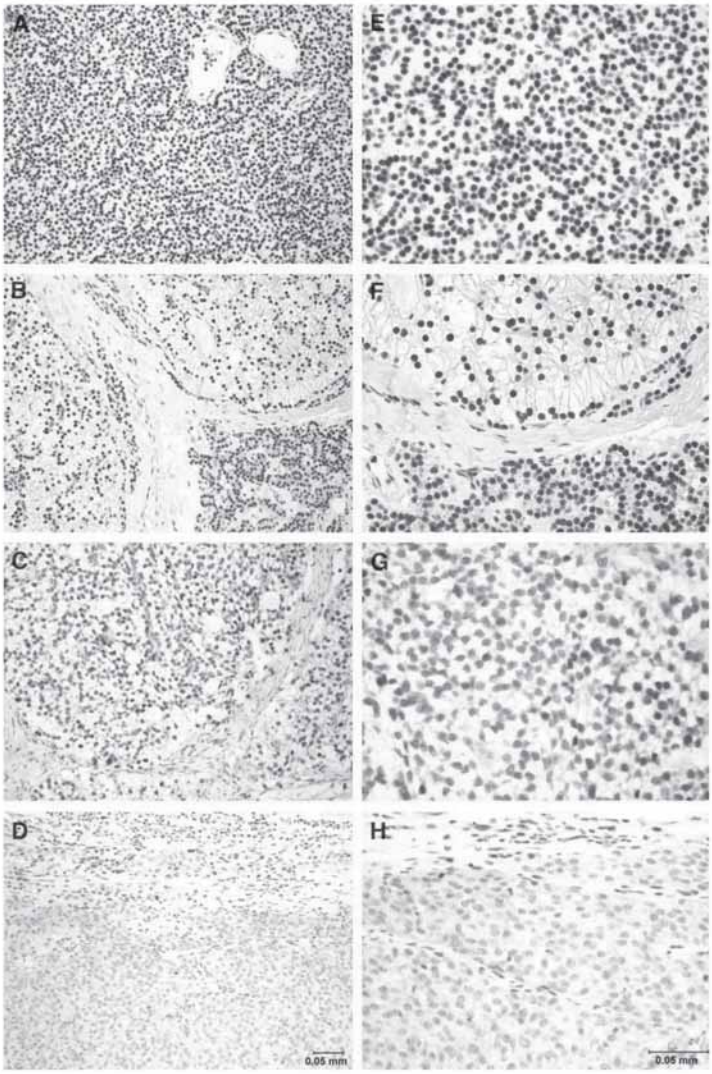

Fig.3 Immunohistochemical staining representing the various staining patterns manifested in the different pathologies through parafibromin immunostaining. *A-D*, magnification, 200×. *A*, diffuse staining (primary parathyroid hyperplasia); *B*, diffuse staining (sporadic adenoma with a rim of normal tissue); *C*, focal loss (parathyroid carcinoma); and *D*, diffuse loss (parathyroid carcinoma). *E-H*, higher magnifications of the respective parathyroid pathologies at a magnification of 400×. All images were taken with a Spot Insight Camera on a Nikon Eclipse E600.

astatic carcinoma, showed diffuse nuclear immunoreactivity and was shown by parathyroid hormone immunostaining (37) to be of non-parathyroid origin. All but one HPT-JT adenomas exhibited loss of parafibromin immunoreactivity; the remaining one was evaluated as diffusely weak staining by one investigator and by the other as diffuse loss. It is important to note that reclassification has been incorporated into all tables and statistical calculations.

DISCUSSION

This is the first study on parafibromin, the protein product of the *HRPT2* tumor suppressor gene. Our results localize hu-

Table 2 Summary of specimen evaluation

Pathology	Total (N = 159)	Loss of immunoreactivity		Diffuse staining		
		Diffuse loss (%)	Focal loss (%)	1 (%)	2 (%)	3 (%)
Definite carcinoma (sections)	22	9 (41)	11 (50)	0 (0)	1 (5)	1 (5)
Definite carcinoma (array)	30	17 (57)	13 (43)	0 (0)	0 (0)	0 (0)
Equivocal carcinoma (sections)	3	0 (0)	0 (0)	1 (33)	0 (0)	2 (67)
Equivocal carcinoma (array)	3	1 (33)	2 (67)	0 (0)	0 (0)	0 (0)
HPT-JT adenomas (sections)	7	4 (57)	2 (29)	1 (14)	0 (0)	0 (0)
HPT-JT adenomas (array)	2	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Sporadic adenomas	48	0 (0)	0 (0)	0 (0)	17 (35)	31 (65)
Sporadic primary hyperplasias	25	0 (0)	1 (4)	2 (8)	7 (28)	15 (60)
MEN1-related tumors	13	0 (0)	0 (0)	0 (0)	2 (15)	11 (73)
Normal tissue	6	0 (0)	0 (0)	0 (0)	1 (17)	5 (83)

man parafibromin to the nucleus, which is consistent with peptide sequence analysis, cellular fractionation studies,[14] and immunohistochemistry in a variety of tissues. Parafibromin shares 32% identity with yeast protein cdc73p (15), which is also a nuclear protein and part of the Paf1 complex mediating cell cycle regulation and transcription (38). Its function in humans is currently unknown.

Pathologic Assessment. This study shows that parafibromin immunoreactivity is a promising adjunct for differentiating carcinoma from benign tissue. Parathyroid carcinoma is often not recognized, even after histologic examination (2, 7). With the approximately 20% (3 of 15) possibility that apparently sporadic parathyroid carcinoma may be a manifestation of hereditary HPT-JT syndrome (11), making an accurate diagnosis is now paramount. To our knowledge, no other immunohistochemical marker for parathyroid carcinoma has been previously assessed in a study of similar size or geographical diversity. The loss of parafibromin immunoreactivity was true regardless of architecture, presence of mitotic figures, loss of heterozygosity, or immunostaining for Ki67, *CASR*, and cyclin D1. In addition, this study successfully detected the misclassification of two equivocal carcinomas among 50 adenomas, one of which subsequently recurred locally, demonstrating that parathyroid malignancy is often under-recognized.

In the assessment of the value of a diagnostic assay, the positive predictive values and negative predictive value are the most relevant clinically (39). These depend on the prevalence of a disease within a certain population, as well as the sensitivity and specificity. With an estimated prevalence of 1% of primary hyperparathyroidism cases, parafibromin immunostaining has a positive predictive value of 49% (95% CI, 10–100%) and an negative predictive value of 100% (95% CI, 100–100%, with rounding). In countries with a prevalence of 5%, such as Japan and Italy (3, 4), the positive predictive value and negative predictive value would be 83% (95% CI, 36–100%) and 99% (95% CI, 99–100%), respectively. Should these values be validated in additional studies, parafibromin immunostaining is likely to be a helpful diagnostic adjunct for the pathologist. Whereas definite carcinomas may be recognized on the definitive criteria of invasion or metastasis, tumors that have histopathologic features of malignancy but lack tumor extension represent challenging clinical and pathologic problems. Levin (40) distinguished between "typical" and "atypical adenomas." Others have chosen to label this group as "equivocal carcinomas" (22, 28). We prefer the terminology "equivocal carcinoma" in the research and clinical setting, as gold-standard pathologic criteria does not accommodate a localized parathyroid carcinoma (2, 27, 40). Considering such patients as having "equivocal carcinomas" is also logical clinically, because they are followed up in a similar fashion as patients with definite parathyroid carcinoma (27, 41). Thus, this terminology is more appropriate in view of the potential malignant behavior of these group of tumors, the under-recognition of carcinoma, and the fact that current gold-standard diagnostic criteria of malignancy of invasion or metastasis is limited to advanced disease. Our results support the view that this entity termed "equivocal carcinoma" is heterogenous (40). Of the five cases initially diagnosed as equivocal carcinomas, three displayed loss of parafibromin immunoreactivity. No cases showing parafibromin immunoreactivity relapsed. In addition, two cases initially diagnosed as adenomas were subsequently reclassified pathologically as equivocal carcinomas after parafibromin immunostaining and re-evaluation. One case relapsed locally on follow-up and was reclassified clinically as a definite carcinoma. The

Table 3 Calculated diagnostic value indices

Diagnostic indices	Value/%
Sensitivity (95% CI)	96 (86–99)
Specificity (95% CI)	99 (92–100)
Positive predictive value at 1% prevalence (95% CI)	49 (10–100)
Negative predictive value at 1% prevalence (95% CI)	100 (100–100)
Positive predictive value at 5% prevalence (95% CI)	83 (36–100)
Negative predictive value at 5% prevalence (95% CI)	100 (99–100)

Table 4 Results of blinded individual observer evaluation for all sections (N = 123)

Staining patterns	Pathologist 1		
	Diffusely positive	Focal loss	Diffuse loss
Pathologist 2			
Diffusely positive	92	1	1
Focal loss	3	8	1
Diffuse loss	0	5	12

[14] C. Zhang, D. Pappas, M-H. Tan, C. N. Qian, J. D. Chen, D. Kong, C. F. Gao, H. M. Koo, M. Weinreich, B. O. Williams, B. T. Teh, unpublished results.

classification of this case by parafibromin immunoreactivity shows a utility exceeding that of current gold-standard criteria, which is restricted to advanced disease. From a clinical point of view, such a utility is highly desirable, as current gold-standard criteria only define advanced disease, but surgical intervention is most appropriate with localized disease (2, 3, 6, 14). In addition, this result suggests that parafibromin loss occurs at an early stage.

Adenomas with HRPT2 mutations also shows diffuse loss of parafibromin immunoreactivity in our study. These occur at a very low frequency in sporadic parathyroid adenomas (15, 16). However, the loss of parafibromin immunoreactivity is useful in detecting these tumors. For patients with HPT-JT syndrome who are at high risk of carcinoma, deliberate total prophylactic parathyroidectomy has been considered (42). Whether radical surgery will benefit the rare patient who has had a somatic HRPT2 mutation detected in a parathyroid adenoma remains uncertain.

HRPT2 Mutation and Parafibromin Loss. Fifty of fifty-two definite parathyroid carcinomas displayed loss of parafibromin immunoreactivity. This is consistent with the high rate of somatic *HRPT2* gene mutations with biallelic inactivation reported in sporadic parathyroid carcinoma (11, 16). HPT-JT syndrome is characterized by *HRPT2* gene mutations, and patients with HPT-JT syndrome have a high risk of parathyroid carcinoma (15, 16). In conjunction with microarray studies showing a common gene signature for parathyroid carcinomas and HPT-JT-related adenomas, the loss of parafibromin immunoreactivity in both groups suggests that parafibromin downregulation is an early and pivotal step in parathyroid tumorigenesis. It was interesting that focal expression of parafibromin was retained in a small subset of parathyroid adenomas with documented *HRPT2* gene mutations. It was observed that in tumors with focal loss of parafibromin immunoreactivity, parafibromin expression was markedly higher near blood vessels and margins (both internal and external) such as fibrous septa and capsular tissue (data not shown). Whether the antibody was binding to wild-type or mutant parafibromin remains uncertain. A small subset of carcinomas display normal parafibromin expression. This may be because of alternative tumorigenic mechanisms, and there is evidence that at least one additional tumor suppressor gene may exist on chromosome 13q (25, 43–46). A recent study of two such candidate genes, *RB* and *BRCA2*, did not identify any mutations in seven specimens of parathyroid carcinoma (47). Parathyroid carcinoma is a rare manifestation in *MEN1* (48, 49), but our study showed normal parafibromin expression in *MEN1*-related benign tumors.

Study Limitations. The evaluation of immunohistochemically stained slides is inherently subjective, with considerable observer dependence. To address this, two observers independently evaluated the slides. The calculated inter-observer agreement was excellent for assessment of parafibromin loss, with a kappa statistic of 0.89. However, one limitation of our study was the risk of subconscious bias. Although the diagnoses were blinded, vascular or local tissue invasion may have been visible on inspection. However, we conclude that any biases are unlikely to be substantial as the majority of benign cases stain with either moderate or strong intensity, rather than with weak staining (Table 2). Additionally, adenomas with HPT-JT mutations were uniformly diagnosed with loss of parafibromin immunoreactivity, whereas adenomas without these mutations were not.

CONCLUSION

Parafibromin immunostaining is a promising adjunct for the diagnosis of parathyroid carcinoma, an often unrecognized entity that may be hereditary (11). This recognition of carcinoma is critical for genetic screening (13), and our results provide direct evidence that carcinoma may not be recognized during initial histopathologic evaluation. Because en-bloc resection constitutes definitive therapy for parathyroid carcinoma and 86% of carcinomas may not be detected intraoperatively (6), studying the intra-operative assessment of parafibromin immunoreactivity through ultrarapid immunostaining (50) would be logical. Finally, the shared loss of parafibromin between parathyroid carcinoma and HPT-JT-related adenomas, alongside evidence of a common gene expression signature (32), suggests novel tumorigenesis pathways mediated by the *HRPT2* gene.

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The authors dedicate this report to the late Dr. J. J. Shepherd of the University of Tasmania.

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Chapter 6

Differential expression of the calcium sensing receptor and combined loss of chromosomes 1q and 11q in parathyroid carcinoma.

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Original Paper

Differential expression of the calcium sensing receptor and combined loss of chromosomes 1q and 11q in parathyroid carcinoma

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Abstract

Malignant transformation of parathyroid tumours is rare. Nevertheless, this small subset of malignant tumours often creates diagnostic and therapeutic problems. In this work, the morphological characteristics of 26 primary parathyroid carcinomas and seven metastases have been studied. Furthermore, immunohistochemical expression profiles for the calcium sensing receptor (CASR), cyclin D1 (CCND1), and Ki-67 were determined for parathyroid carcinomas and compared with adenomas and hyperplasias using a tissue microarray. Loss of heterozygosity (LOH) of the chromosome 1q region containing the *HRPT2* gene and chromosome 11q (*MEN1*) was determined in the carcinomas. In contrast to the adenomas and hyperplasias, 31% of carcinomas demonstrated down-regulation of CASR. A significant correlation was found between CASR expression and the Ki-67 proliferation index. Chromosome 1q and chromosome 11q LOH were found in 12 of 22 (55%) and 11 of 22 (50%) carcinomas tested, respectively. Combined 1q and 11q LOH was seen in 8 of 22 (36%) carcinomas, in contrast to the low percentage of LOH reported in both regions in adenomas. In conclusion, this study demonstrates that combined 1q and 11q LOH in parathyroid tumours is suggestive of malignant behaviour. Strong down-regulation of the CASR protein is seen in a proportion of parathyroid carcinomas with a high proliferation index. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: parathyroid; parathyroid carcinoma; parathyroid adenoma; immunohistochemistry; tissue micro-array; LOH; CASR

Introduction

Parathyroid hyperfunction concomitant with parathyroid tumourigenesis is one of the most common endocrinopathies, with a prevalence of about 1 per 1000 individuals. Although parathyroid carcinoma is rare (less than 1%) in patients with hyperparathyroidism, it creates diagnostic and therapeutic problems, partly due to its heterogeneous morphological appearance. Classically, the presence of fibrous bands and mitotic activity are considered to be histological features of malignancy. However, a definitive diagnosis of parathyroid carcinoma can sometimes not be established, based on morphology alone, in the absence of invasion of adjacent structures, vaso-invasive growth, and/or metastasis. Prognosis is variable, and early diagnosis and radical surgery seem to be favourable prognostic factors.

Little is known about the pathways involved in parathyroid tumourigenesis. The multiple endocrine neoplasia tumour suppressor gene (*MEN1*) is mutated in 30% of sporadic parathyroid tumours (mostly adenomas). Furthermore, overexpression of the cyclin D1 oncogene (CCND1) seems to be involved in parathyroid tumourigenesis. Analysis of RB and P53 showed varying results or ruled out their importance [1].

An inherited form of parathyroid carcinoma occurs as part of a rare syndrome, the so-called hyperparathyroidism–jaw tumour syndrome (HPT–JT: OMIM; 145 001), a syndrome characterized by primary hyperparathyroidism due to neoplastic transformation of parathyroid tissue in combination with rare tumours such as ossifying fibromas of the maxilla or mandible, bilateral renal cysts, hamartomas, and Wilms' tumours [2–8]. In HPT–JT, 10% of all parathyroid tumours are or become malignant [4–16]. Mutations in the *HRPT2* gene at chromosome1q25–32 are responsible for HPT–JT and are also found in a selection of patients with familial isolated hyperparathyroidism (FIHP) [6,17,18]. *HRPT2* mutations are found in a limited subset of sporadic parathyroid adenomas [18]. This gene is considered to be a tumour suppressor, according to LOH studies, although chromosome 1q21–32 LOH was found in only 17% (in two of the ten families) of the adenomas. In LOH studies performed on parathyroid carcinomas from proven *HRPT2*-linked patients [4,6,7,16,19], however, all of the tumours (5) showed chromosome 1q21–32 LOH.

The latter observation might indicate that chromosome 1q loss, probably in combination with mutation, plays an important role in parathyroid carcinoma formation.

As the diagnosis of parathyroid carcinoma is important but difficult, we tried to find specific features that characterize parathyroid malignancy and as such, can possibly serve as diagnostic markers of these tumours. We therefore studied the morphological characteristics of a series of parathyroid carcinomas in combination with immunohistochemistry (IHC) for the calcium sensing receptor (CASR), cyclin D1, and Ki-67 using a tissue micro-array (TMA). Comparison with a TMA from parathyroid adenomas and hyperplasias was performed. Furthermore, LOH analysis of the chromosome 1q region containing the *HRPT2* gene and of chromosome 11q (*MEN1*) was performed on the parathyroid carcinomas.

Materials and methods

Parathyroid carcinomas

Formalin-fixed, paraffin wax-embedded tumour tissue from 26 primary parathyroid carcinomas, six regional lymph node metastases, and one lung metastasis from 30 patients was obtained from different laboratories in The Netherlands using PALGA (Dutch National Information System for Pathology, Utrecht, The Netherlands). One parathyroid carcinoma (No 30) came from a documented HPT–JT family [16]. Thirty samples had undoubted features of carcinoma, namely the presence of vascular invasion and/or metastasis [20] based on evaluation of representative haematoxylin and eosin-stained slides from each tumour by a pathologist (HM) and the initial pathology report. Three cases were diagnosed as carcinomas based on their clinical presentation; definitive vascular invasion was not found in these cases. Each tumour was scored for the overall histological pattern (gross architecture), vascular invasion, cysts, and fibrous bands. Furthermore, variation in nuclear size, the presence of nucleoli, cytoplasmic characteristics, mitotic activity, haemosiderin deposition, and the presence or absence of inflammation were evaluated.

Available data from the pathology reports regarding tumour size, weight, and gross appearance were incomplete and have not been included except for additional information regarding the presence of cysts or vascular invasion. There were 15 males (age range 32–76 years, mean age 56.8 years) and 15 females (age range 30–83 years, mean age 59 years).

Parathyroid adenoma/hyperplasia

Lesions from 109 patients were used to construct a TMA (see below). These comprised 93 primary parathyroid adenomas (87 sporadic and five MEN1) and 26 hyperplasias [12 primary hyperparathyroidism (HPT), three secondary HPT, three tertiary HPT, and eight MEN1]. Of the 109 patients, 39 were males (age range 18–76 years, mean 41.4 years) and 70 females (age range 12–81 years, mean 56.5 years).

Tissue micro-array (TMA)

Whenever possible, triplicate tissue cores with a diameter of 0.6 mm, as selected by a pathologist (HM), were taken from each specimen (carcinoma, adenoma or hyperplasia) (Beecher Instruments, Silver Springs, MD, USA) and arrayed on a recipient paraffin block, using standard procedures [21]. Nine cores of nine normal parathyroid tissues were added to serve as internal controls.

Loss of heterozygosity (LOH) analysis

Eighteen primary parathyroid carcinomas, five metastases to regional lymph nodes, and one lung metastasis from 22 patients from whom both tumour and normal tissue were available were screened for LOH. Genomic DNA was isolated from the paraffin wax-embedded material using standard methods. Seven microsatellite markers located in the *HRPT2* region were selected: D1S428, D1S492, D1S384, D1S081*, D1S556*, D1S173*, D1S422 (markers with an asterisk were obtained through the HPT–JT linkage consortium). These markers covered a 10 Mb area at 1q21–1q41 (source UDB: <URL> http://bioinformatics.weizmann.ac.il/udb/).

D11S4940, D11S4946, and PYGM were the markers used for analysis of the *MEN1* region. Polymerase chain reactions (PCRs) were performed according to standard procedures. Fluorescence-labelled PCR products were electrophoresed using an ABI 310 automated sequencer (Applied Biosystems, Foster City, USA) and the results were analysed using the ABI prism GeneScan 3.1 program. As we used paraffin wax tissue blocks obtained from different hospitals and years, not all the markers gave interpretable results, even after repeated experiments.

The threshold for LOH, comparing normal and tumour DNA, was defined as 40% reduction of one allele, equating to a ratio of ≥ 1.7 or ≤ 0.59 [22]. The density of the tumour cells was greater than 80%. The threshold for retention ranged from 0.76 to 1.3. For so-called grey areas with ratios of 0.60–0.75 and 1.31–1.69, no definite decision was made. For HPT–JT case 30, LOH was concluded using HRPT2 mutation specific primers and therefore not counted.

Immunohistochemical analysis of cyclin D1, CASR, and Ki-67

Immunohistochemical staining was performed on 5 μm sections of the tissue array, using a paraffin sectioning aid system (Instrumedics Inc, Hackensack, NJ, USA). Sections were deparaffinized, treated with 0.3% H_2O_2 in methanol, and submitted to antigen retrieval by microwave oven treatment for 10 min in 10 mM citrate buffer (pH 6) for cyclin D1, CASR,

and Ki-67. Tissue sections were incubated overnight at room temperature with monoclonal mouse anti-human CASR (ADD antibody) [23] (1 : 16 000; NPS Pharmaceuticals, Inc, Salt lake City, UT, USA), cyclin D1 (1 : 500, clone DCS-6; Neomarkers, Fremont, CA, USA), and Ki-67 (1 : 300, clone MIB-1 [10,11]; DAKO, Denmark). The sections were then washed (3 × 5 min in PBS) and incubated (30 min) with biotinylated secondary antibody in 1% BSA in PBS and washed (3 × 5 min in PBS) and incubated (30 min) with HRP/streptavidin complex (SABC). Diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen, followed by counterstaining with haematoxylin. As a negative control, the primary antibody was omitted. Tonsil served as a positive control for cyclin D1 and Ki-67, and kidney tissue as a positive control for CASR. Expression was scored by light microscopy.

For cyclin D1, both the staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong intensity, as related to a positive internal control) and the percentage of positive tumour cells (0 = 0%; 1 = <10%; 2 = 10–30%; 3 = 30–50%; 4 = 50–80%; 5 = >80%) were evaluated and scores of the intensity and the percentage of positive cells were added. If 30% or more of the sample cells showed nuclear immunopositivity for cyclin D1 (and score >3), the sample was considered to be positive [24]. In cases classified as negative, the proportion of the stained cells never exceeded 10%. The calcium sensing receptor was evaluated for the presence or absence of clear and regular membranous staining [25]. Ki-67 staining was quantified by counting all the cells in a punch (>600), ensuring a total of at least 1000 cells as the punches were present on the tissue array in triplicate. The percentage of positive nuclei was calculated.

Statistical analysis

Summary statistics were obtained utilizing established methods: chi-square analysis [26] was undertaken using the SPSS statistical package (version 9.0.0) to determine any association between categorical variables (as shown in Table 1).

Results

Histological parameters of parathyroid carcinomas

We scored the histological pattern (partly shown in Table 1) at low microscopic magnification of 26 primary parathyroid carcinomas and seven metastases (the primary tumour was also analysed in three of these cases). One sample was not scored for some of the features, as it was only a small lymph node metastasis (No 19). There were two common patterns. The majority of the parathyroid carcinoma foci (primary tumours and metastases) had a sheet-like architecture (19/32, 59%), 6/32 (19%) had a nodular pattern, and the remaining 7/32 (22%) had other types of architecture. At higher magnification, 6/32 (19%) tumour foci showed a region with a trabecular tumour cell arrangement. There was no mitotic activity in 10/33 (30%) tumour foci, sporadic mitotic activity in 13/33 (39%), and >1/10 HPF in 10/33 (30%). In 23/33 (70%) of the tumour foci analysed, a region with fibrous bands was seen.

Cystic features were seen in 10/33 (30%) of the tumour foci. No variation in nuclear size was seen in 3/33 (9%); 13 (39%) showed little nuclear anisomorphism, 11/33 (33%) moderate differences, and 6/33 (18%) had very pleomorphic nuclei. In 9/33 foci (27%), we did not see nucleoli in nuclei; in 17/33 (52%) of foci, we found less than 50% of the nuclei with nucleoli; and in 7/33 (21%), we saw nucleoli in more than 50% of the nuclei. The majority (19/33, 61%) of tumour foci showed a granular cytoplasm reminiscent of chief cells; 4/33 (12%) consisted of oxyphilic cells; 2/33 (6%) of so-called water-clear cells; 5/33 (15%) had a mixture of water-clear and granular cytoplasm (transitional water-clear cells); 2/33 (6%) foci a mixture of oxyphilic and water-clear cells; and 1/33 (3%) a mixture of granular and oxyphilic cells (so-called transitional oxyphilic cells).

In 2/33 (6%) foci, we found signs of inflammation, and deposition of haemosiderin was found in 10/32 (31%) tumour foci.

Immunohistochemical analysis

Overexpression of cyclin D1 was identified in 17/30 (57%) parathyroid carcinomas (Table 2), 13/90 (14%) adenomas, and 10/23 (43%) hyperplasias. Nine/29 (31%) parathyroid carcinomas showed an irregular or absent staining pattern for CASR. In the 'adenoma/hyperplasia' TMA, only 1/104 of the interpretable tumours (an adenoma) showed such irregular or absent calcium sensing receptor staining. Seventeen of 30 (57%) carcinomas had Ki-67 nuclear positivity, a marker of proliferative activity, in 0–5% of cells; 11/30 (37%) had Ki-67 nuclear positivity in 5–20% of cells; and 2/30 (7%) of the samples had Ki-67 positivity in more than 20% of the cells. In the aforementioned adenoma/hyperplasia TMA, all of the 117 interpretable cases showed Ki-67 positivity in 0–5% of cells.

The statistical correlation between the determined parameters was determined using the chi-square test. A significant correlation was found between CASR expression and the proliferation (Ki-67) index ($p < 0.05$). A Ki-67 index ≥5% was present in eight of the nine samples that showed down-regulation of CASR. This observation is illustrated in Figure 1 with three tumours, one with normal CASR (case 18) and two with altered CASR staining (cases 4 and 23).

Significant correlations were found between cyclin D1 and Ki-67 ($p < 0.05$). In 12/18 (67%) carcinomas with overexpression of cyclin D1, the Ki-67 index was ≥5%, whereas only 3/12 (27%) parathyroid carcinomas with normal cyclin D1 expression had a Ki-67 index ≥5%.

Table 1. Overview of the histological, immunohistochemical, and genetic (LOH) data of 30 parathyroid carcinoma cases (33 tumour foci)

Patient	Sex	Diagnosis	Cyst	Fibrotic bands	Mitosis	Vaso-invasive	Age (years)	Pattern	Variation in nuclear size	Cytoplasm	Trabecular	Ki-67	CASR	Cyclin D1	LOH 1q	LOH 11q
1	F	Prim	Yes	Yes	Sporadic	Yes	30	Sheet	Little	Gran	Yes	5	0	5	No	No
2	F	Prim	No	No	Sporadic	Yes	83	Nod	Little	Gran	No	5.8	I	4	No	Yes
3	F	Prim	No	Yes	> 1/10 HPF	Yes	74	Other	Large	Oxy	No	10	I	3	Yes	Yes
4	M	Prim	No	Yes	> 1/10 HPF	Yes	55	Other	Moderate	Gran/eos	No	27.5	0	4	Yes	Yes
5	F	Prim	Yes	Yes	> 1/10 HPF	Yes	77	Sheet	Large	Gran	No	1	I	0	Yes	No
6	M	Prim	Yes	Yes	Sporadic	Yes	72	Other	Moderate	Gran	No	9.6	0	0	Yes	Yes
7	F	Prim	Yes	No	No	Yes	78	Sheet	No	clear	No	0.1	I	4	No	No
8	M	Prim	No	Yes	Sporadic	Yes	32	Sheet	Little	Gran	No	10	0	4	Yes	No
9	M	Prim	No	Yes	> 1/10 HPF	No	71	Sheet	Moderate	Gran	No	1.9	I	5	nd	nd
10	F	Prim	No	No	No	Yes	49	Sheet	No	Gran/cle	No	1.3	I	6	No	No
11	M	Prim	No	Yes	Sporadic	No	58	Nod	Little	Gran	No	2	I	0	No	No
12	M	Prim	No	Yes	Sporadic	Yes	49	Sheet	Moderate	Gran/cle	No	1.4	I	0	Yes	No
13	M	Prim	No	Yes	No	Yes	32	Sheet	Moderate	Gran/cle	No	5.6	I	4	nd	nd
14	M	Prim	No	Yes	No	Yes	76	Sheet	Moderate	Gran	No	0.6	I	0	nd	nd
15	F	Prim	No	Yes	Sporadic	Yes	74	Sheet	Little	Gran	No	10	I	0	Yes	Yes
15	F	Reg LN	No	Yes	Sporadic	Yes	75	Nod	Little	Gran	No	10	I	0	Yes	Yes
16	F	Prim	Yes	Yes	No	Yes	66	Other	Large	Oxy/cle	No	2.7	I	0	Yes	Yes
16	F	Reg LN	Yes	No	No	Yes	73	Sheet	Large	Oxy	No	2.8	I	0	Yes	Yes
16	F	Reg LN	Yes	No	Sporadic	Yes	73	Sheet	Moderate	Oxy	No	0.6	I	0	Yes	Yes
17	M	Lung MT	No	Yes	> 1/10 HPF	Yes	42	Sheet	Little	Gran	No	6.3	I	5	No	Yes
18	F	Prim	No	Yes	Sporadic	Yes	51	Sheet	Little	Gran/cle	Yes	3	I	0	Yes	Yes
19	F	Reg LN	No	No	> 1/10 HPF	Yes	30	nd	Moderate	Clear	nd	35	0	7	nd	nd
20	F	Prim	No	Yes	> 1/10 HPF	Yes	52	Sheet	Moderate	Gran	Yes	3.3	I	4	No	Yes
21	M	Prim	Yes	No	No	Yes	54	Other	Large	Oxy/cle	Yes	5.5	I	4	Yes	Yes
22	F	Prim	No	No	> 1/10 HPF	Yes	37	Sheet	Moderate	Gran	No	0.6	I	4	nd	nd
23	M	Reg LN	No	No	> 1/10 HPF	Yes	41	Nod	Little	Gran	No	9.8	0	4	No	No
24	F	Prim	No	Yes	No	Yes	62	Nod	Little	Gran	No	2.4	0	0	No	No
25	M	Prim	No	Yes	No	No	39	Sheet	Little	Gran	Yes	1.5	I	4	Yes	Yes
26	F	Prim	Yes	Yes	Sporadic	Yes	80	Other	Large	Gran	No	5	nd	4	nd	nd
27	M	Reg LN	No	Yes	> 1/10 HPF	Yes	55	Sheet	Little	Gran	No	13.6	0	0	Yes	No
28	M	Prim	Yes	No	Sporadic	Yes	65	Sheet	Little	Gran	No	0.3	I	0	nd	nd
29	M	Prim	No	Yes	No	Yes	63	Nod	No	Gran/cle	No	0.2	I	0	nd	nd
30	F	Prim	No	Yes	Sporadic	Yes	36	Other	Moderate	Oxy	Yes	15	0	2	No	No

nd = no data; prim = primary tumour; lung MT = lung metastasis; reg LN = regional lymph node metastasis; nod = nodular; gran = granular; cle = clear; oxy = oxyphilic. Ki-67: percentage of positive nuclei. CASR: I = normal staining; 0 = absent or faint staining. Cyclin D1: sum of score of intensity and percentage of positive cells (see the materials and methods section).

Table 2. Percentage of parathyroid hyperplasias, adenomas, and carcinomas that showed overexpression (cyclin D1, Ki-67) or loss of expression/altered staining (CASR). Significance: *p* value of chi-square test comparing the different groups

Marker	Hyperplasian $n = 26$	Adenoma $n = 93$	Carcinoma $n = 30$	Significance
CASR	0	1	31	$p < 0.05$
Cyclin D1	43	14	57	$p < 0.05$
Ki-67	0	0	44	$p < 0.05$

As expected, the absence of mitosis was correlated with a low Ki-67 index (<5%) in 8/10 (80%) samples.

LOH analysis at the MEN1/HRPT2 loci

LOH of chromosome 1q was found in 12/22 (55%) cases analysed and 11/22 (50%) showed loss of chromosome 11q (Figure 2). The LOH pattern identified was identical in different regions of primary tumours (eg cases 10, 20, and 25) or in comparison between primary tumours and metastases (cases 15 and 16, Figure 2). Eight of 22 (36%) cases showed LOH of both chromosome 1q and 11q, whereas LOH of either chromosome 1q or 11q occurred in four (16%) and three samples (12%), respectively. Seven out of 22 (32%) cases tested showed LOH at neither chromosome 11q nor chromosome 1q using the markers investigated.

No chromosomal boundary of LOH could be identified with the markers used.

Four of the six parathyroid carcinomas with cystic features in our series in which LOH status was determined showed LOH of chromosome 1q (67%) and three of these four showed both chromosome 1q and chromosome 11q loss. In tumours without cystic features, we found LOH at chromosome 1q in 8/16 and in five of these eight, loss of both chromosome 1q and chromosome 11q.

Discussion

In sporadic parathyroid adenomas, both mutations in the *HRPT2* gene [18] and LOH of chromosome 1q are uncommon [27–32], being present in 2–4% and 0–9% of cases, respectively. In HPT–JT-related tumours, the percentage of LOH of chromosome 1q in adenomas is relatively low (17%) compared with that in carcinomas (100%). The incidence of carcinoma in HPT–JT syndrome is approximately 10%, whereas it is less than 1% in sporadic primary HPT. Taken together, this suggests an important role for the *HRPT2* gene in parathyroid carcinoma tumourigenesis. The high percentage of LOH at 1q that we found in our set of sporadic parathyroid carcinomas seems to support this hypothesis and might underscore the important role of (LOH of) *HRPT2* in the development of sporadic parathyroid carcinoma. To our knowledge, no LOH data are available for the 1q region in parathyroid carcinoma and the reported percentage of LOH of chromosome 1q in adenomas is low (8%) [28].

LOH of chromosome 11q13 in 50% of the parathyroid carcinomas is higher than reported for parathyroid adenomas (with LOH of chromosome 11q13 in 30% of cases) [25,29,32]. This finding might also suggest involvement of the *MEN1* gene in the tumourigenesis of some parathyroid carcinomas.

A striking feature of our cohort of parathyroid carcinomas is the combination of chromosome 1q21–32 and 11q13 loss, both of which were lost in 36% of cases. To our knowledge, this is the first time that this combination of loss has been described in parathyroid carcinoma, whereas in parathyroid adenoma such an event appears to be rare [27,32]. Therefore it might be concluded that the combined 1q/11q loss can be used as a supportive criterion in the diagnosis of parathyroid carcinoma. The findings also seem to suggest that inactivation of *HRPT2* on chromosome 1q21–32 may function either independently or in concert with *MEN1* gene inactivation to promote parathyroid carcinogenesis.

Immunohistochemistry for CASR showed decreased or absent expression in parathyroid carcinomas (31%), whereas expression was decreased in only one adenoma. So far, no mutations in the coding sequence of the *CASR* gene have been described [33–35]. In parathyroid adenomas, however, a decrease in the CASR mRNA level of 24–98% has been detected [36,37].

At the protein level, a reduction in the intensity of staining for the CASR protein (14–60%) has also been described [34,38]. Our results in parathyroid adenomas appear to be different from the results of the latter studies. Using expression arrays (Haven *et al*, manuscript in preparation), we clearly see that expression of CASR is greatly diminished in parathyroid carcinomas and to a lesser extent in adenomas, supporting our immunohistochemical data. The differences between these observations might also be explained by the use of different antibodies.

In our study, the Ki-67 proliferation index in parathyroid carcinomas is, as described previously, significantly higher in parathyroid carcinomas (>5%) than in adenomas/hyperplasias (<1%) [39–42].

It is striking that the altered expression of the CASR protein in 31% of the parathyroid carcinomas seems to occur concomitantly with a high Ki-67 proliferation index in these tumours. In contrast, in 119 parathyroid adenomas/hyperplasias in our TMA, a very low proliferation index (mean 0.4%) in combination with positive staining for the CASR receptor was seen.

The association between the proliferation index and a decrease in the expression of CASR has been previously reported for secondary hyperparathyroidism [43–45]. Ritter *et al* [43] showed in uraemic rats that parathyroid cell proliferation triggers a cascade of effects that leads (directly or indirectly) to down-regulation of CASR.

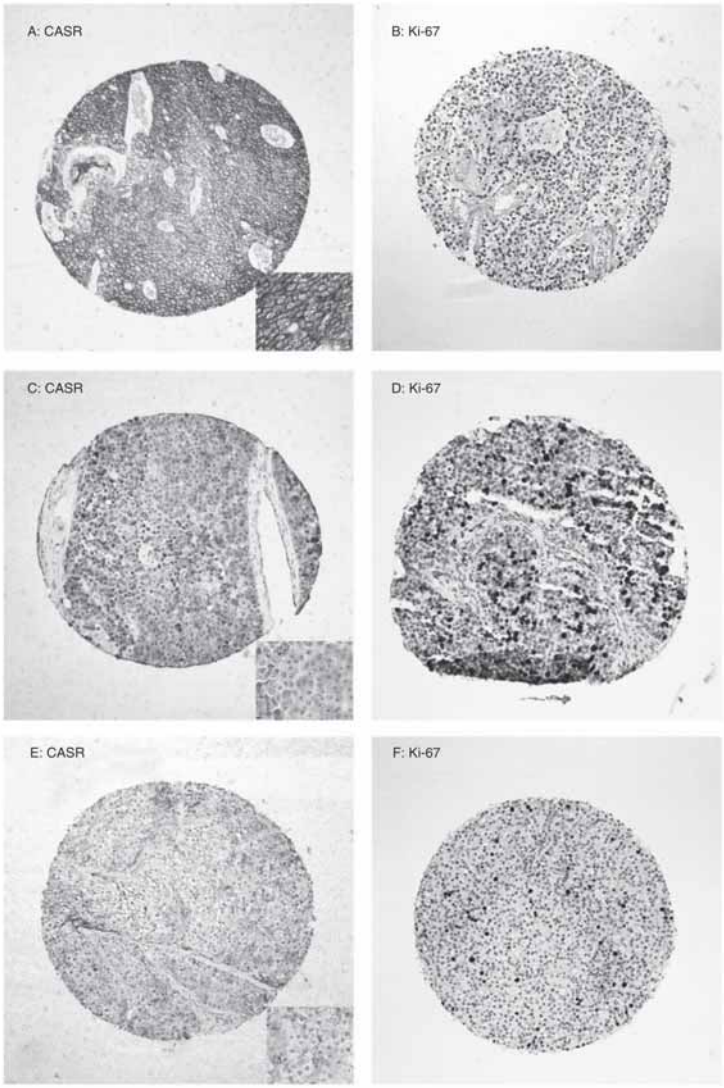
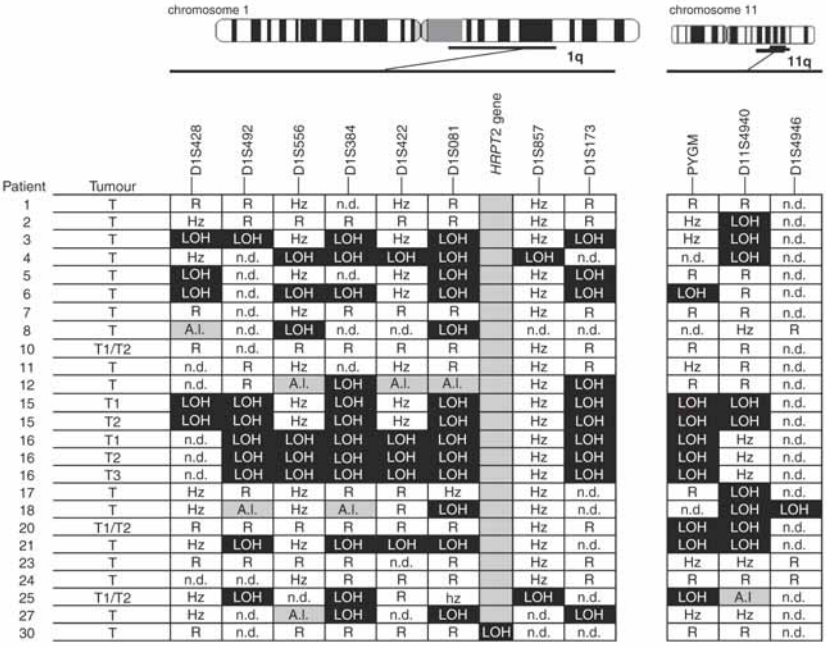


Figure 1. Immunohistochemical staining of a 5 μm section from a tissue micro-array showing the immunophenotype of three different parathyroid carcinomas: one (A, B) shows membranous staining from CASR (A) and no Ki-67 positivity (B); the second (C, D) shows no staining for CASR and strong Ki-67 positivity, whereas the third (E, F) shows diminished, but not absent, CASR staining, concomitant with moderate overexpression (≥5%) of Ki-67 (magnification ×100; inserts ×200)

The finding that cyclin D1 overexpression is more common in parathyroid carcinoma (55%) and hyperplasia (43%) than in adenomas (14%) is in agreement with previous studies [46–48], but contrasts with the results of Stojadinovic *et al*, who reported a lower percentage of overexpression in carcinomas than in adenomas [42]. The latter observation may be due to the different antibodies used or the small number of



Patient	Tumour	D1S428	D1S492	D1S556	D1S384	D1S422	D1S081	*HRPT2* gene	D1S857	D1S173	PYGM	D11S4940	D1S4946
1	T	R	R	Hz	n.d.	Hz	R		Hz	R	R	R	n.d.
2	T	Hz	R	R	R	R	R		Hz	R	Hz	LOH	n.d.
3	T	LOH	LOH	Hz	LOH	Hz	LOH		Hz	LOH	Hz	LOH	n.d.
4	T	Hz	n.d.	LOH	LOH	LOH	LOH		LOH	n.d.	n.d.	LOH	n.d.
5	T	LOH	n.d.	Hz	n.d.	Hz	LOH		Hz	LOH	R	R	n.d.
6	T	LOH	n.d.	LOH	LOH	Hz	LOH		Hz	LOH	LOH	R	n.d.
7	T	R	n.d.	Hz	R	R	R		Hz	R	R	R	n.d.
8	T	A.I.	n.d.	LOH	n.d.	n.d.	LOH		n.d.	n.d.	n.d.	Hz	R
10	T1/T2	R	n.d.	R	R	R	R		Hz	R	R	R	n.d.
11	T	n.d.	R	Hz	n.d.	Hz	R		Hz	R	Hz	R	n.d.
12	T	n.d.	R	A.I.	LOH	A.I.	A.I.		Hz	LOH	R	R	n.d.
15	T1	LOH	LOH	Hz	LOH	Hz	LOH		Hz	LOH	LOH	LOH	n.d.
15	T2	LOH	LOH	Hz	LOH	Hz	LOH		Hz	LOH	LOH	LOH	n.d.
16	T1	n.d.	LOH	LOH	LOH	LOH	LOH		Hz	LOH	LOH	Hz	n.d.
16	T2	n.d.	LOH	LOH	LOH	LOH	LOH		Hz	LOH	LOH	Hz	n.d.
16	T3	n.d.	LOH	LOH	LOH	LOH	LOH		Hz	LOH	LOH	Hz	n.d.
17	T	Hz	R	Hz	R	R	Hz		Hz	n.d.	R	LOH	n.d.
18	T	Hz	A.I.	Hz	A.I.	R	LOH		Hz	n.d.	n.d.	LOH	LOH
20	T1/T2	R	R	R	R	R	R		Hz	R	LOH	LOH	n.d.
21	T	Hz	LOH	Hz	LOH	LOH	LOH		Hz	n.d.	LOH	LOH	n.d.
23	T	R	R	R	R	n.d.	R		Hz	R	Hz	Hz	R
24	T	n.d.	n.d.	Hz	R	R	R		Hz	R	R	R	R
25	T1/T2	Hz	LOH	n.d.	LOH	R	hz		LOH	n.d.	LOH	A.I	n.d.
27	T	Hz	n.d.	A.I.	LOH	n.d.	LOH		n.d.	LOH	Hz	Hz	n.d.
30	T	R	n.d.	R	R	R	R	LOH	n.d.	n.d.	R	R	n.d.

Figure 2. Details of LOH analysis. LOH (shaded black): allele ratio ≥ 1.7 or ≤ 0.59. Retention (R): allele ratio between 0.76 and 1.3. Grey area (A.I.): allele ratio between 0.60–0.75 and 1.31–1.69. n.d. = no data; Hz = homozygous

samples included in the study ($n = 8$ in the study of Stojadinovic *et al*). Using expression arrays (Haven *et al*, manuscript in preparation), we clearly see the same trend that we now describe using immunohistochemistry.

No relationship could be found between the histological parameters and the genetic or immunohistochemical features of the parathyroid carcinomas, other than the recently described relationship between cystic appearance and chromosome 1q LOH. Cystic morphology is linked to HPT–JT tumours and chromosome 1q LOH. In sporadic cystic parathyroid adenomas, chromosome 1q LOH is present in 20% of the samples as opposed to 0–9% in tumours without cystic changes [49]. We saw the same trend in the parathyroid carcinomas, with 67% of the cystic parathyroid carcinomas showing chromosome 1q LOH compared with 50% of the parathyroid carcinomas without cysts.

In conclusion, our study suggests that the combined loss of chromosomes 1q and 11q in parathyroid tumours might be an indication of malignant behaviour. Loss of CASR protein expression in combination with an increased proliferation rate seems, in our hands, an almost typical feature of parathyroid carcinoma. Furthermore, TMA is a very powerful tool for the analysis of a large number of different tumours at once, the equal staining conditions making the comparison easy in such heterogeneous and cell-rich tumours as those from the parathyroid.

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Chapter 7

Identification of MEN1 and HRPT2 somatic mutations in paraffin-embedded (sporadic) parathyroid carcinomas.

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ORIGINAL ARTICLE

Identification of *MEN1* and *HRPT2* somatic mutations in paraffin-embedded (sporadic) parathyroid carcinomas

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Summary

Objective Parathyroid carcinoma remains difficult to diagnose. Recently, it has been shown that mutations in the *HRPT2* gene (encoding parafibromin) are associated with the development of parathyroid carcinoma. Although *MEN1* is not typically thought to be involved in carcinoma formation, parathyroid carcinoma may be an extremely rare feature of the multiple endocrine neoplasia type 1 (MEN1) syndrome. We recently concluded that loss of heterozygosity (LOH) of the *MEN1* gene is present in a relatively large number of parathyroid carcinomas, often in combination with LOH at the *HRPT2* locus. The aim of this study was to evaluate the role of *MEN1* and *HRPT2* mutations in sporadic parathyroid tumours fulfilling histological criteria for malignancy.

Patients and design Formalin-fixed, paraffin-embedded (FFPE) parathyroid carcinoma tissue from 28 cases identified in the period 1985–2000 in the Netherlands was studied. *HRPT2* (27/28 cases) and *MEN1* (23/28 cases) were analysed by direct sequencing.

Results Somatic *MEN1* mutations were found in three of 23 (13%) sporadic parathyroid carcinoma cases; these consisted of one missense and two frameshift mutations. One of the latter two cases displayed lymph-node and lung metastases during follow-up. Six *HRPT2* mutations were found in 4/27 cases (15%): five were truncating mutations and one was a missense mutation. Consistent with previously published reports, we found double mutations (2×) and germline mutations (2×) in apparently sporadic parathyroid carcinomas.

Conclusions These results suggest that not only *HRPT2* but also *MEN1* mutations may play a role in sporadic parathyroid cancer formation.

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Introduction

Primary parathyroid hyperparathyroidism (PHPT) has an incidence of one in 1000[1] and may result from a single parathyroid adenoma (80–85%) or from hyperplasia (15–20%), but rarely (less than 1%) from carcinomas.[2] Although parathyroid carcinomas are slow growing, they have a high propensity to recur locally and recurrent disease is difficult to eradicate. Parathyroid carcinoma is also difficult to diagnose because its histopathological features can overlap with those of adenoma.

Whereas PHPT is usually encountered as a nonfamilial disorder, in a minority of cases (5%) it is part of a hereditary syndrome; multiple endocrine neoplasia type 1 (MEN1; OMIM 131100) and type 2A (MEN2A; OMIM 171400), hyperparathyroidism–jaw tumour syndrome (HPT-JT; OMIM 607393) and familial isolated hyperparathyroidism (FIHP; OMIM 145000) are all hereditary.

MEN1, caused by mutations in the *MEN1* gene, is characterized by the occurrence of tumours of the parathyroid (in 95% of patients[3]), pancreatic islet cells and anterior pituitary. *MEN1* consists of 10 exons and encodes a 610-amino-acid protein menin. Menin appears to have a large number of potential functions through interactions with proteins that alter cell proliferation mechanisms.[3] *MEN1* represents a tumour suppressor gene (TSG) and is located on chromosome 11q13. The majority of tumours (95%) show additional loss of heterozygosity (LOH) consistent with Knudson's two-hit theory. The *MEN1* gene is also known to be mutated in a subset (20–30%) of sporadic parathyroid adenomas.[4–6] Two comparative genomic hybridization (CGH) studies investigating physical loss show that somatic loss of chromosome 11q is not a frequent feature in parathyroid carcinomas; however, these studies could not detect possible loss due to homologous recombination.[7,8] Although PHPT represents the most common endocrinopathy in MEN1, reaching nearly 100% penetrance by the age of 40,[9] parathyroid carcinoma is an extremely rare feature of the MEN1 syndrome. So far, only two cases[10,11] of parathyroid carcinomas in *MEN1* mutation carriers have been reported. Therefore, it is assumed that *MEN1* plays no role in the development of parathyroid carcinomas.[12,13] We recently found a considerable percentage of LOH in the chromosome 11q13 region in a cohort of 30 carcinomas by studying polymorphic markers.[14] This led to the idea that the role of *MEN1* mutations in the development of parathyroid carcinomas might be greater than previously believed.

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HPT-JT is an autosomal dominant disorder characterized by parathyroid tumours, ossifying fibromas of the mandible and maxilla, renal hamartomas and cystic kidney disease. The gene causing HPT-JT, localized at chromosome 1q24-q32, is known as the *HRPT2* gene (also known as *Cdc73*) and is thought to function as a TSG. *HRPT2* consists of 17 exons coding for the 531-amino-acid protein parafibromin. Subsequent investigations have revealed that mutations in *HRPT2* are present in 66–100% of sporadic carcinomas.[12,15] This is in contrast to sporadic adenomas, where these mutations are rarely found (1·8%).[12,16–19]

Parathyroid carcinoma is an uncommon cause of PHPT. However, the HPT-JT syndrome is associated with an increased risk of parathyroid carcinoma; 10–15% of the patients develop parathyroid carcinomas.[20]

FIHP is a diagnosis per exclusionem. Germline mutations in the *HRPT2* (5·3%), *MEN1* (17·6%) and *CASR* (11·8%) genes are reported, but the majority of FIHP cases have a still unrecognized cause.[21,22] It is unknown if these remaining cases have a distinct genetic basis.

In this study formalin-fixed paraffin-embedded tissue (FFPE) of parathyroid carcinoma was used for mutation analysis. In such archival tissue, DNA is fragmented, depending on the time of fixation and the length of storage as paraffin blocks. However, in the current study this gave us the opportunity to examine a relatively large number of cases, given the rarity of parathyroid carcinoma. In this cohort of parathyroid carcinomas,[14,23,24] we have identified somatic mutations in *HRPT2* and *MEN1*.

Materials and methods

Clinical data and tumour samples

We recently studied 30 parathyroid carcinoma cases from the Netherlands diagnosed in the period 1985–2000.[14] One of these cases (case 30[16]) came from a documented HPT-JT family in which the germline mutation was identified. From the remaining, apparently sporadic, carcinomas we could further study FFPE tumour tissue of 28 cases (24 sporadic primary parathyroid carcinomas, three regional lymph-node metastases, one lung metastasis; 13 females and 15 males in total). Case 22 was not tested for *HRPT2* mutations, and cases 1, 16, 17, 27 and 29 were not tested for *MEN1*, mostly because of limited availability of tissue. Carcinoma features primarily included the presence of vasoinvasion, with or without invasion into the capsule and/or distant metastasis. Three equivocal cases (9, 11 and 25) were diagnosed as carcinomas based on their clinical presentation; vasoinvasion was not found in the histological slides of these cases.[14]

HRPT2 *and* MEN1 *mutation analysis*

DNA extracted from FFPE material was used for polymerase chain reaction (PCR) amplification as described previously.[25] Primers for *HRPT2* and *MEN1* that would specifically amplify and sequence the degraded DNA from FFPE tissue were designed (Table 1). To sequence *HRPT2*, we used 24 primer pairs to cover the 17 exons of *HRPT2* and 15 primer pairs to cover the 10 exons of *MEN1*. Some of the products failed to amplify from FFPE DNA. This is because of the limited fragment size that can be amplified from FFPE tissue, in which combinations of repetitive sequences, low genomic complexity or either low or high GC content impair possibilities for primer design.

Immunohistochemical staining of parafibromin

Immunohistochemical staining of parafibromin was described for 26/28 cases.[24]

Results

HRPT2 *mutation analysis*

Twenty-seven of 28 FFPE parathyroid carcinomas were screened for *HRPT2* gene mutations. In 24 of the samples, the sequences of > 75% of the *HRPT2* gene could be analysed in the fragmented DNA, except in case 25 (66% of the sequence analysable) and cases 16 and 26 (69% analysable; in the latter case, two mutations were identified). Exons 15 (101 bp, GC content 29%) and 17 (33 bp, GC content 37%), together comprising 8% of the complete *HRPT2* gene, could not be sequenced in any of the samples. However, exons 1, 2 and 7, known to harbour 85% of all somatic *HRPT2* mutations, were sequenced completely in all samples.

Six mutations were found in four cases (15%, Table 2). Case 1 harboured a germline mutation in exon 2 (c.176C>T), resulting in a change from a serine (an aliphatic amino acid) into a phenylalanine (an aromatic amino acid). Further studies are required to determine a possible pathogenic effect of this mutation.

Case 8, with a frameshift mutation in exon 2 (c.165delC), was described previously in a paper by Howell *et al.*[18] In cases 23 and 26, a somatic mutation in exon 7, c.128G>A, resulting in the formation of a stop codon, was identified. This mutation has also been found in other studies.[18] In both cases, additional frameshift mutations were found in exon 8: c.692_693insT (germline) and c.693_694insG for cases 23 and 26, respectively.

In 17 of 27 tumours, a previously described polymorphism (IVS12–86C>T)[18] was identified.

MEN1 *mutation analysis*

Twenty-three of the 28 parathyroid carcinomas were screened for mutations in the *MEN1* gene. In most cases, more than 70% of *MEN1* could be analysed. The exceptions were cases 22, 23, 24 and 26, for which 45%, 51% 61% and 62% were analysed, respectively.

The first part of exon 2 (63 bp) and exon 10 (187 bp), could not be reliably sequenced in any of the samples. Together, these products cover 19% of the coding exons.

Three somatic mutations were found (see Table 2 and Fig. 1), consisting of an (unreported) missense mutation in exon 3 (patient 6: c.646G>T), changing a valine (an aliphatic amino acid) into a phenylalanine (an aromatic amino acid) and two as yet unreported frameshift mutations (patient 18: exon 9, c.1271delG and patient 20: exon 2, c.167_170del4).

Three polymorphisms in the *MEN1* gene were identified: nucleotide substitutions in exon 4 (c.710G>A), resulting in the change of CGC (arginine) into CAC (histidine), and in exon 9 (c.1269C>T),

Table 1. Primer pair choice for FFPE genomic sequencing of *HRPT2* and *MEN1* (5′–3′), with product size in base pairs

Exon	Product size (bp)	Primer sequence
HRPT2 mutations		
1A	173	CGAGGCGACAAGAGAAGAAG
		CAGGAGAACTCCCCGAAGAT
1B	160	ATTGTGGTGAAGGGAGACGA
		GGGAGGGGTTAAGAAAGAGG
2A	166	TGAATCCAGCCTGAAGAGTTG
		CACGTCGGACATAAACAGGA
2B	172	GAAGGCCAACCCAGAGAGTA
		AGGCCAGACCCTGTCTCTTA
3	162	AGTTGTGTATCATTGTTATTCATTTCA
		TGTCTGTTTAAGACTGGGAACAA
4	199	AAAAACCTAAAGCATTTCACTTGT
		GTTTTGGAATGGGCTTCTGA
5	199	CAGAAGCCCATTCCAAAACT
		TCCTCAGGTTACTGCAATCAAA
6A	195	TTGGCCTAAAGACACTGATACC
		CCTTCTTTGTGACCCTCCAA
6B	192	TGCGCCTTGATAAAGAGAGA
		GGCATAAAATGAATCCAAGAGG
7A	195	GGAATGCCTGCTGTGAAAAT
		CGGGTCACATCTACCTCAGC
7B	176	TGACATAACTGCCCTTAAACAGA
		TGAAACTTCCACCTAAAAGCAA
8	236	TGTAGTAGGGAAGAATCGATAGTAAGA
		AATCTACTGTAAAGCAGTAAAGCATT
9	225	GGTCATGCTACTGCACTCCA
		GCCACACTGCCTCTCAAGTT
10	221	GGCTTTGTATATTATTGAACCATCA
		TCCCTGGAACAAAAGAACAT
11A	171	CAGTGGAGTAACCAACTGAGTGAG
		GGGCTGCAGGAGTCTGAGT
11B	205	TTTAAAGGAGGGTGCATCTG
		CGACAGTCTTCAAAGAAACATGA
12	216	GGTTTTTATGACACAGAGTTGTG
		TGTGGCTTGGGCACTAATAA
13A	207	GCCCAAGCCACACTGATTAT
		GAGGTGGTAGCTGCAGGAAT
13B	219	TGTCTTTATAGGATCTCGAACACC
		GCCTATAGCACAGAAACCGAAA
14A	206	CCATTTTCATCACGTGGAAT
		GCATAAGTTTAAGGGGCTGGT
14B	176	GAAGAAAAGACCAGATGCAACC
		CGTCATCAACGGCAATAACA
15	178	CCCCCACCCACTTTTCTACT
		CACATCATATGCGCAGAACT
16	198	TGATAACTTCTCTCCACCCTCTC
		CACAAGCATATTTTAGAATCGGAAT
17	161	ATTTGGCTCCTCCATTTCTG
		GCCAAAAAGTTTGCTTATATGGAT
MEN1 mutations		
2A	63	GGGCGGGTGGAACCTTAG
		ACCAAGGAAAGGAGCACCAG
2B	152	CCTGTTTGCTGCCGAGCTGG
		GGCGGCGATGATAGACAGGTC
2C	83	CTGGCGGCCTCACCTACTTTC
		GGAGACCTTCTTCACCAGCTCAC
2D	163	GCCGTCGACCTGTCCCTCTATC
		CATGGATAAGATTCCCACCTACTGG
3A	77	GCACAGAGGACCCTCTTTCATTAC
		CTTGCCGTGCCAGGTGAC
3B	132	CTCGCCCTGTCTGAGGATCATG
		TGGGTGGCTTGGGCTACTACAG
4	129	GGGCCATCATGAGACATAATG
		CTGCCCCATTGGCTCAG
5	41	CCTGTTCCGTGGCTCATAACTC
		CTAGGAAAGGATCATAATTCAGGC
6	88	GGGTGGCAGCCTGAATTATG
		CTCAGCCACTGTTAGGGTCTCC
7	137	ATCCTCTGCCTCACCTCCAT
		AGGGTGGTTGGAAACTGATG
8	136	GTGAGACCCCTTCAGACCCTAC
		TGGGAGGCTGGACACAGG
9	165	ATCTGTGCCCTCCCTTCC
		CACCTGTAGTGCCCAGACCT
10A	103	CGGCAACCTTGCTCTCACC
		CCAGGCCCTTGTCCAGTG
10B	184	GGGAGTCCAAGCCAGAGGAG
		GCCCTTCATCTTCTCACTCTGG
10C	195	GAGGGTCCAGTGCTCACTTT
		GGTCCGAAGTCCCCAGTAGT

A, B and C indicate division of relatively large exons.

Table 2. Overview of *HRPT2* and *MEN1* mutations found in apparently sporadic carcinomas. Patient numbers refer to Haven *et al.*[14]

Case	Exon	Nucleotide change	Amino acid change	Somatic/ germline
HRPT2 mutations				
1	Exon 2	c.176C>T	p.Ser59Phe	Germline
8	Exon 2	c.165delC	p.Tyr55fsX	Somatic
23	Exon 7	c.128G>A	p.Trp42fsX	Somatic
23	Exon 8	c.692_693insT	p.Trp230LeufsX38	Germline
26	Exon 7	c.128G>A	p.Trp42fsX	Somatic
26	Exon 8	c.693_694insG	p.Arg231GlufsX37	ND
MEN1 mutations				
6	Exon 3	c.646G>T	p.Val215Phe	Somatic
18	Exon 9	c.1271delG	p.Gly423AlafsX25	Somatic
20	Exon 2	c.167_170del4	p.Thr55ThrfsX62	Somatic

ND, not determined.

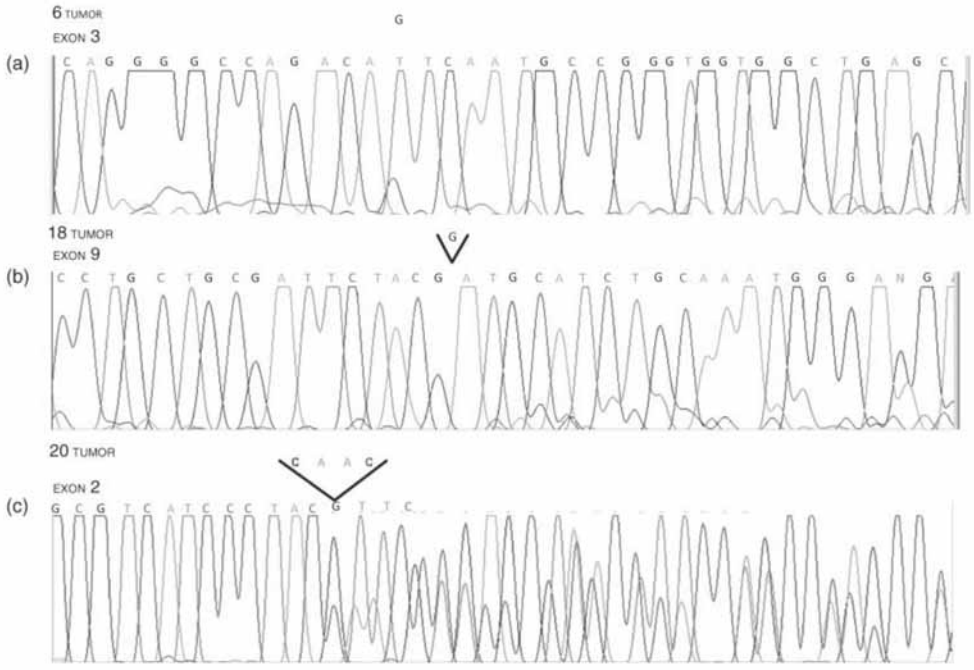

Fig. 1 Sequencing chromatograms of parathyroid carcinomas with somatic *MEN1* mutations: (a) a missense mutation in exon 3 (c.646G>T), resulting in the change of a valine into a phenylalanine (patient 6); (b) a 1-bp deletion in exon 9 (c.1271delG) (patient 18); (c) a 4-bp deletion in exon 2 (c.167_170del4) (patient 20).

resulting in the change of a GAC (asparagine) into GAT (asparagine), and in exon 9 an (c.1303G>A) ACG into ACA (threonine–threonine) polymorphism was found.

Histology of the three parathyroid carcinomas with *MEN1* mutations is shown in Fig. 2. Notably, the clinical history of patient 18 displayed lymph-node and lung metastases during follow-up. Matched constitutive DNA was analysed to determine the germline *vs.* somatic nature of these mutations; all mutations were found to be somatic.

Comparison of HRPT2, MEN1 *mutated and remaining parathyroid carcinomas*

We recently studied the expression profiles of benign and malignant parathyroid lesions with or without *MEN1* or *HRPT2* mutations.[23] A distinct profile was identified for a set of tumours consisting of HPT-JT benign and malignant tumours, including sporadic parathyroid carcinomas. The dominant profile in this subset of tumours appeared to be determined by the abrogation of *HRPT2* function. We identified several differentially expressed genes such as E-cadherin (*CDH1*), histone 1 H1c (*HIST1H1C*) and amyloid beta precursor protein 1 (*APPBP1*). Expression of these genes was confirmed in FFPE tumours using immunohistochemistry. In the current study we related the identified *MEN1* and *HRPT2* mutations with the previous results for the parathyroid carcinomas (Table 3), including clinical features, immunohistochemical staining results of Ki-67, calcium-sensing receptor (CASR), cyclin D1 (CCND1), HIST1H1C, APPBP1 and parafibromin[14,23,24] and LOH of *HRPT2* and *MEN1* based on sequence results. Furthermore, we compared the mutation group of tumours with the remaining set of parathyroid carcinomas (Table 4). Combining results in Tables 3 and 4 showed that the *HRPT2* mutated samples, although based on only a small number of samples, showed significantly more CASR downregulation and more CCND1 and APPBP1 overexpression compared with both *MEN1* mutated carcinomas and the remaining carcinomas.

Discussion

As discussed recently by Rubin and Silverberg,[26] the diagnosis of parathyroid carcinoma is difficult, based on clinical and histological grounds; lymph-node and/or distant metastasis denote evident malignancy. Fibrous septa in the tumour, mitotic figures, capsular and vasoinvasive growth remain unreliable features for diagnosis. To identify possible molecular tools to improve the diagnosis of parathyroid carcinomas, we studied a series of 28 cases of parathyroid carcinoma. This relatively large number of tumours could only be obtained by using FFPE tissue of parathyroid carcinomas diagnosed

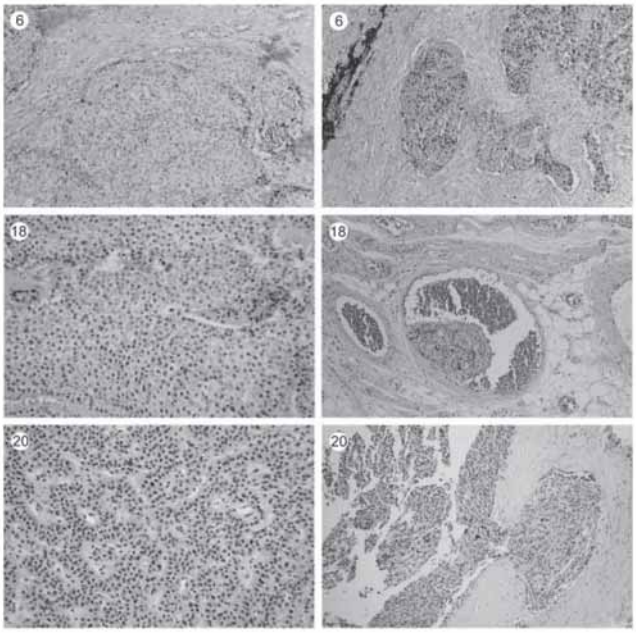

Fig. 2 Histology of the three sporadic carcinomas with a *MEN1* mutation. First column: a gross overview of the tumours (magnification × 100). Second column: a detail (magnification × 200) image that shows vasoinvasion (cases 6 and 18) and invasion of the capsule (case 20).

Table 3. Parathyroid carcinomas with *HRPT2* or *MEN1* mutations. Patient numbers (as used by Haven *et al.*[14]) and clinical data such as sex, age, diagnosis, metastasis and recurrence are depicted in rows. The presence of vasoinvasion and the mutation status of *HRPT2* and *MEN1* are also included. The results of immunohistochemical staining for Ki-67 (index), CASR (0, downregulation; 1, normal expression), CCND1 (0, normal expression; 1, upregulation), CDH1 (1, normal expression; 2, aberrant expression), APPBP1 (0, normal expression; 1, overexpression), HIST1H1C (0, normal expression; 1, overexpression) and parafibromin are shown

Patient number	1	8	23	26	6	18	20
Sex	F	M	M	F	M	F	F
Age (years)	30	32	41	80	72	51	52
Diagnosis	Primary	Primary	Reg.L.N.	Primary	Primary	Primary	Primary
Vasoinvasion	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Metastasis/recurrence	NK	Yes	Yes	NK	NK	Yes	NK
HRPT2	Mut	Mut	Mut2	Mut2	No	No	No
MEN1	ND	No	No	No	Mut	Mut	Mut
Ki-67	5	10	9·8	5	9·6	3	3·3
CASR	0	0	0	–	0	1	1
CCND1	1	1	1	1	0	0	1
CDH1	2	2	1	2	2	1	1
HIST1H1C	1	ND	1	0	1	0	1
APPBP1	1	1	1	1	1	0	1
Parafibromin	Global loss	Global loss	Global loss	Focal loss	Global loss	Global loss	Focal loss
seqLOH1q	No	Yes	No/No	No/No	ND	ND	ND
seqLOH11q	ND	ND	ND	ND	Yes	Yes	No

F, female; M, male; Primary, primary tumour; Reg.L.N., regional lymph node; –, no data; NK, not known; Mut, mutation; Mut2, double mutation; ND, not determined; seqLOH, intragenic loss of heterozygosity LOH based on sequencing results.

Table 4. Comparison of *HRPT2*/*MEN1* mutated and remaining parathyroid carcinomas. Depicted are the percentages of samples per group that show aberrations. The significant percentages ($P < 0{\cdot}05$) are shown in bold

		MEN1 mutated (%)	*HRPT2* mutated (%)	Remaining (%)
No. of samples		3	4	21
Ki-67	Average	5·3	7·5	4·8
CASR	Downregulation	33·0	**100·0**	14·0
CCND1	Overexpression	33·3	**100·0**	50·0
CDH1	Aberrant	33·3	75·0	28·0
APPBP1	Overexpression	66·6	**100·0**	50·0
HIST1H1C	Overexpression	33·3	66·6	38·0
Parafibromin	Global loss	66·0	75·0	50·0
Parafibromin	Focal loss	33·0	25·0	50·0

in several hospitals in the Netherlands over a period of 15 years. We designed relatively reliable PCRs taking the fragmented DNA in FFPE tissue into account as well as a critical level of DNA input.[27] The parathyroid tumours were revised and studied with regard to morphological and molecular features, including LOH of chromosomes 1q and 11q.[14] We report the presence of inactivating somatic mutations of both *MEN1* and *HRPT2* in sporadic parathyroid carcinomas.

Although *MEN1* mutations are frequently found in familial (95%) and also sporadic adenomas (20–30%), the contribution of germline *MEN1* mutations to the development of parathyroid carcinomas was previously shown in only two MEN1 syndrome cases. These two parathyroid carcinomas exhibited a concurrent parathyroid adenoma. The number of parathyroid carcinomas reported in patients suffering from the MEN1 syndrome is thus almost negligible in relation to the prevalence of PHPT in this syndrome. We have found a somatic *MEN1* mutation in 13% of the sporadic parathyroid carcinomas, suggesting that the prevalence of *MEN1* mutated carcinomas as a cause of sporadic PHPT appears to be higher than originally thought. As the prevalence of *MEN1* mutations in sporadic adenomas is higher (30%) than now found in carcinomas (13%), this may indicate that, in time, an adenoma can progress into a carcinoma. The difference in prevalence of parathyroid carcinomas in MEN1 and sporadic PHPT with somatic *MEN1* mutation may be explained by the regular screening of patients with MEN1 syndrome. Hyperfunctioning parathyroid glands are thus detected at an early stage and removed before they can progress into carcinoma.

Mutations and subsequent abrogation of *HRPT2* TSG function have recently been associated with sporadic parathyroid carcinomas or with parathyroid carcinomas in the context of HPT-JT syndrome.[15,18] Subsequent immunohistochemical analysis often detected global loss of parafibromin encoded by *HRPT2* in parathyroid carcinomas.[24,28]

We detected six inactivating *HRPT2* gene mutations in only four cases (15%) of our series of parathyroid carcinomas. At least two *HRPT2* mutations are of germline origin, possibly illustrating the incomplete penetrance of the HPT-JT syndrome, which was also reported by others.[12,15] The overall frequency of *HRPT2* mutations detected in this study is substantially lower than that recently described in parathyroid carcinomas (4/4, 6/7 and 10/15),[12,15,18] but it is still higher than found in parathyroid adenomas (1·8%).[12,16–19] Although exons 1, 2 and 7,[12,15–17] which harbour 85% of all known mutations, were completely screened in all cases, the low mutation frequency could be explained in part by the fact that not all exons could be completely screened because of the nature of the FFPE tissue. Another possible explanation could be the existence of large somatic or germline genomic deletions that were not studied. The latter case, with, for example, a founder mutation in the Dutch population, might explain these results. Finally, we did not address gene silencing of *HRPT2* due to promoter methylation. Part of the apparently sporadic parathyroid carcinoma cases could in fact be familial cases suffering from FIHP. A proportion of FIHP families with parathyroid carcinomas and/or cystic adenomas are found to carry *HRPT2* mutations;[22] however, in the majority of tumours (65%) in an FIHP context, the cause is unknown and may be the result of mutations in a yet unknown (*HRPT1*) gene.[21,22]

Importantly, the selection of parathyroid carcinomas is different compared to the previous studies by Howell,[18] Shattuck,[15] Cetani[12] and co-workers, in which only cases with metastases or recurrence were included. It might be speculated that parathyroid tumours fulfilling only the classic histological features (vasoinvasive growth, fibrous bands, etc.), but without signs of recurrence or metastasis, should be considered as less aggressive carcinomas, in contrast to unequivocal carcinomas with *HRPT2* mutations. Our series of 28 parathyroid carcinomas fulfil the histological criteria for malignancy; however, we do not have complete follow-up to address the above hypothesis. Only one out of four *HRPT2* mutated cases had documented lymph-node metastases. However, in five metastasized parathyroid carcinomas (four with regional lymph nodes and one with a lung metastasis), no *HRPT2* mutations were identified. Notably, the tumours in this study were previously analysed for parafibromin immunoreactivity and all showed global or focal loss of staining,[24] indicative of the diagnosis of parathyroid carcinoma. *HRPT2* mutated parathyroid carcinomas might be different from *MEN1* mutated or remaining tumours. This seems to be supported by the differential immunohistochemical expression of molecules such as CASR, CCND1 and APPBP1. The latter were recently identified as part of a distinct cDNA expression profile in *HRPT2* mutated benign and malignant parathyroid tumours.[23]

In conclusion, we have successfully identified inactivating *HRPT2* somatic mutations in archival sporadic parathyroid carcinomas. Additionally, we report for the first time the presence of *MEN1* somatic mutations in these tumours, suggesting that *MEN1* mutations play a role in the development of parathyroid carcinomas.

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Chapter 8

Gene expression of parathyroid tumors and identification of the potential malignant phenotype.

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Gene Expression of Parathyroid Tumors: Molecular Subclassification and Identification of the Potential Malignant Phenotype

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ABSTRACT

Parathyroid tumors are heterogeneous, and diagnosis is often difficult using histologic and clinical features.

We have undertaken expression profiling of 53 hereditary and sporadic parathyroid tumors to better define the molecular genetics of parathyroid tumors. A class discovery approach identified three distinct groups: (1) predominantly hyperplasia cluster, (2) *HRPT2*/carcinoma cluster consisting of sporadic carcinomas and benign and malignant tumors from Hyperparathyroidism-Jaw Tumor Syndrome patients, and (3) adenoma cluster consisting mainly of primary adenoma and MEN 1 tumors. Gene sets able to distinguish between the groups were identified and may serve as diagnostic biomarkers. We demonstrated, by both gene and protein expression, that *Histone 1 Family 2, amyloid β precursor protein*, and *E-cadherin* are useful markers for parathyroid carcinoma and suggest that the presence of a *HRPT2* mutation, whether germ-line or somatic, strongly influences the expression pattern of these 3 genes. Cluster 2, characterized by *HRPT2* mutations, was the most striking, suggesting that parathyroid tumors with somatic *HRPT2* mutation or tumors developing on a background of germ-line *HRPT2* mutation follow pathways distinct from those involved in mutant MEN 1-related parathyroid tumors. Furthermore, our findings likely preclude an adenoma to carcinoma progression model for parathyroid tumorigenesis outside of the presence of either a germ-line or somatic *HRPT2* mutation. These findings provide insights into the molecular pathways involved in parathyroid tumorigenesis and will contribute to a better understanding, diagnosis, and treatment of parathyroid tumors.

INTRODUCTION

Hyperparathyroidism is a common endocrinopathy, believed to affect ~3 in 1,000 adults (1). It is characterized by calcium-insensitive hypersecretion of parathyroid hormone and increased parathyroid cell proliferation. Hyperparathyroidism may be classified as a primary, secondary, or tertiary disorder. Primary hyperparathyroidism is caused by an inherently abnormal or excessive growth of the parathyroid glands. Secondary hyperparathyroidism (hyperplasia) develops in response to chronic depression of serum calcium levels, generally due to renal impairment. In a minority of patients, this parathyroid hyperactivity becomes autonomous, resulting in tertiary hyperparathyroidism. Hyperparathyroidism may also arise in response to lithium treatment as a therapy for bipolar disorder.

The majority of tumors in primary hyperparathyroidism are sporadic; however, ~5% are associated with the autosomal dominant hereditary cancer syndromes Multiple Endocrine Neoplasia types 1 (MEN 1; OMIM #131100) and 2A (MEN 2A; OMIM #171400), Hyperparathyroidism-Jaw Tumor Syndrome (OMIM #145001), and Familial Isolated Hyperparathyroidism (OMIM #145000).

Histologically, primary hyperparathyroidism can be attributed to a single adenoma in 80% to 85% of cases, hyperplasia in 15% to 20% of cases, and carcinoma in <1% of cases (2). However, parathyroid tumors are heterogeneous, and the differences between histologic types are subtle, confounding the classification. Hyperplasia is defined as an enlargement of more than two glands, whereas adenoma is traditionally considered to be a single gland disorder. However, double or multiple adenomas have been reported in patients with primary hyperparathyroidism including MEN 1 (3), additionally confounding the distinction between adenoma and hyperplasia. Studies of clonality have not been able to clearly distinguish between these tumor types (4), and the diagnosis of parathyroid tumor subtypes remains challenging.

The molecular events involved in the formation of parathyroid lesions are poorly understood. Two genes, *cyclin D1* (*CCND1*) and *MEN1*, have been established as having major roles in parathyroid tumorigenesis. The tumor suppressor gene *MEN1* is involved in the formation of sporadic as well as familial MEN 1 tumors. Recently, we have shown that the putative tumor suppressor gene *HRPT2* is mutated in sporadic parathyroid carcinoma (5) and a small subset of cystic parathyroid adenomas (6), likely playing an important role in the development of these tumors.

Both the *calcium sensing receptor* (*CaSR*) and *vitamin D receptor* (*VDR*) are also known to play a role in parathyroid tumorigenesis (7).

To additionally elucidate the underlying molecular mechanisms involved in the formation of parathyroid lesions and, thus, improve clinical diagnosis and management of these patients, we have undertaken gene expression profiling. We report results for 53 parathyroid tumors belonging to 11 different clinical entities: normal tissue, MEN 1, MEN 2A, Hyperparathyroidism-Jaw Tumor Syndrome, familial isolated hyperparathyroidism, primary hyperparathyroidism, secondary hyperparathyroidism, tertiary hyperparathyroidism, carcinoma, adenoma, and a lithium-associated tumor.

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Note: C. J. Haven and V. M. Howell contributed equally to this work. A. Nelson is currently at the Garvan Institute of Medical Research, Darlinghurst, Sydney, Australia. Supplementary data regarding the Gene-Rave analysis, including details of normalization, treatment of missing spots, pairwise class discrimination error matrices, and histograms of cross-validation error rates are available to download from http://www.kolling.usyd.edu.au or http://www.bioinformatics.csiro.au. Supplementary Tables S1-S6 can be found at Cancer Research Online (http://cancerres.aacrjournals.org).

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MATERIALS AND METHODS

Gene Expression Profiling

Tumor Samples. A total of 53 parathyroid tumors and 16 normal specimens of parathyroid tissue were obtained from the Leiden University Medical Center, Royal North Shore Hospital, and Martin Luther University. Normal parathyroid tissue was pooled from excess cell washings after routine parathyroid autotransplantation during thyroidectomy. Approval for this study was obtained from the Human Research Ethics Committees of the participating institutions and the Van Andel Research Institute. Tumors were snap-frozen in liquid nitrogen immediately after surgery and stored at −80°C until use. Patient data are summarized in Supplementary Data Table S1. Histologic classification was established according to WHO published guidelines (8).

Fifteen familial tumor specimens were collected. Five were from two Hyperparathyroidism-Jaw Tumor Syndrome families. Two of these 5 tumors were classified as carcinoma and 3 as adenoma. All harbored germ-line *HRPT2* mutations. Five tumors (2 single adenomas and 3 multigland hyperplasia: Table S1) were from MEN 1 families and had documented *MEN1* mutations. One tumor was from a clinically diagnosed MEN 2A patient, and 4 were from familial isolated hyperparathyroidism families. No additional tumors were found in the familial isolated hyperparathyroidism families. Two familial isolated hyperparathyroidism patients harbored a germ-line *HRPT2* mutation. Linkage to the *MEN1* locus 11q13 could not be excluded in the other two familial isolated hyperparathyroidism families, and no germ-line *MEN1* mutations have been detected. Of the sporadic tumors, 16 were classified as adenomas, 1 as lithium-associated, 5 as carcinoma, and 16 as hyperplasia. The hyperplasia specimens were additionally classified as primary ($n = 2$), secondary ($n = 9$), or tertiary ($n = 5$). The percentage of neoplastic tissue in each sample was assessed histologically to ensure the presence of at least 70% neoplastic cells. However, the possibility of contamination of parathyroid tissue with adjacent nonparathyroid tissue could not entirely be excluded due to wide resection of the carcinoma tumors.

Preparation of RNA. Total RNA was isolated from frozen tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified either by precipitation with 2.5 mol/L $LiCl_2$ or Qiagen RNeasy spin columns (Qiagen Inc., Valencia, CA) according to the manufacturer's protocols.

cDNA Microarray Fabrication and Experimental Procedure. Microarray slides spotted with 19,968 cDNA clones from the Research Genetics 40K Human Clone Set (Research Genetics Inc., Huntsville, AL) were fabricated at the Van Andel Research Institute as described previously (9), and microarray experiments were performed as described previously (10). Briefly, 25 to 50 μg of total RNA from parathyroid tissue and an equal quantity of Universal Human Reference total RNA (Stratagene, Cedar Creek, TX), were reverse transcribed using Superscript II (Invitrogen) and oligo $d(T)_{20}VN$ in the presence of Cy5-dCTP and Cy3-dCTP (Amersham Inc., Piscataway, NJ), respectively. After direct labeling, the two probes were hybridized for 20 hours at 50°C to a microarray slide. The slides were then washed, immediately dried, and scanned at 532 nm and 635 nm in a Scan Array Lite (Perkin-Elmer Life, Boston, MA). Reciprocal labeling was performed on 3 samples (Supplementary Table S1). All of the other samples were tested in singlicate.

Data Analysis. Images were analyzed using Genepix Pro 3.0 (Axon, Union City, CA). The ratio of Cy5 intensity to Cy3 intensity for each spot represented tumor RNA expression relative to the Universal Human Reference. The Genepix result files were individually normalized using Another Microarray Database based on spots with pixel correlation values >0.75. Flagged spots and spots with background-subtracted intensities <150 in either the Cy5 or Cy3 channel were excluded from cluster analysis.

To check for color bias and dye incorporation efficiency, the correlation coefficient (r) was determined for each reciprocal-labeled array against its matched standard-labeled array. In all three cases $r > 0.8$, the results were averaged, and these averages were used for additional analysis. In the pooled normal sample all of the genes for which two results were not available or where the difference between the results was outside the 95% confidence interval of the log-transformed ratio, results were excluded from additional analysis. For some analyses, the Cy5:Cy3 ratios for the remaining genes in the tumor arrays were divided by the averaged normal result so that the ratios now represented tumor expression relative to normal parathyroid tissue.

Gene expression values were visualized using CLUSTER and TREEVIEW software (11). Ratios were log-transformed and median-centered so that the median log-transformed ratio equalled zero. The gene expression ratios were median-polished across all of the samples. To test the influence of different gene sets on the unsupervised clustering, the filtering parameters (genes present: 80% to 100%; number of observations: 2 to 20; fold change: 2 to 4) in CLUSTER were varied to create distinct gene sets.

Statistical Classification and Supervised Cluster Analysis Using Cluster Identification Tool and Significance Analysis of Microarrays. Cluster Identification Tool software was used to find genes that were differentially expressed (using Student's *t* test) between the clusters (12). To find significantly discriminating genes, 1,000 t-statistics were calculated by randomly placing patients into two groups (for example, cluster 1 *versus* the remainder). A 99.9% significance threshold ($\alpha \leq 0.01$) was used to identify genes that could significantly distinguish between two clusters *versus* the random patient groupings. In addition, Significance Analysis of Microarrays was used to identify genes with statistically significant changes in expression by assimilating a set of gene-specific *t* tests (13).

Statistical Classification and Probability Scores in Clinical Diagnostic Categories by Penalized Logistic Regression and Significance Analysis of Microarrays. We used a Penalized Logistic Regression model (14) in a two-step approach to identify molecular classifiers for each cluster. The first step separated clusters 1 and 3 from cluster 2. In the second step (including only tumors that had a low probability of belonging to cluster 2), cluster 3 samples were separated from the remainder. Penalized Logistic Regression generated a probability score for each array, indicating the chance for a sample to be part of one of two *a priori* defined groups. These probabilities were computed from the logarithmic expression of a selected set of genes on an array. This selection was not based on a predictive measure of the performance of a gene but simply on it being measured reliably in all arrays. To decrease the set of genes, we used Significance Analysis of Microarrays to rank the genes. We performed a Penalized Logistic Regression analysis with different numbers of genes to find the gene set with the smallest number that could still predict robustly. An acceptable prediction was performed using a maximum of 1,460 genes (genes with results for all arrays) or a minimum of 50 genes for each step.

The model involved a penalty parameter. It was not automatically optimized with Akaike's information criterion but rather was set manually to a value that gave log-odds (base 10) in the approximate range of −3 to 3. To determine the variability of the estimated log-odds, 100 bootstrap samples were taken and means and SDs computed.

Statistical Classification Using Gene-Rave. The expression array data were independently analyzed to find small gene sets suitable as accurate molecular classifiers for the different clinical groups. For this analysis the array results were normalized using a series of strategies including loess fitting and spatial smoothing (15, 16). After normalization, flagged spots with $M > 5$, where $M = \log_2 (Cy5/Cy3)$, were removed. The data were then analyzed by pair-wise discrimination of clinical groups using Gene-Rave software. Only groups with array results for at least 5 tumors were analyzed, *i.e.,* Hyperparathyroidism-Jaw Tumor Syndrome, sporadic carcinoma, sporadic adenoma, hyperplasia, and MEN 1. Gene-Rave uses a Bayesian penalty term with an improper prior that puts a high weight on finding a small solution set of genes. Cross-validation and permutation testing are used to assess the prediction error rate (17). A canonical variate plot produced by the "profile analysis" algorithm was used to display the data in low dimensional space. This algorithm involves a factor analytical approach for the within-class covariance matrices specifically for use with large numbers of variables (genes).

Statistical Analyses. χ^2 analysis (SPSS version 10.0) was used to determine the significance of associations between RNA (as measured by expression microarray) and protein expression and correlations between expression profiles and cluster groups.

Immunohistochemical Analysis of Protein Expression

Samples. Paraffin-embedded blocks from 149 parathyroid tumors (16 of which also had cDNA microarrays performed) and 9 normal parathyroid tissues were used for the construction of a tissue microarray. Eighty-seven patients were diagnosed as having primary parathyroid adenomas (80 sporadic, 5 MEN 1-related, and 2 Hyperparathyroidism-Jaw Tumor Syndrome -related), and 26 were diagnosed with hyperplasia (12 with primary hyperparathyroid-

ism, 3 with secondary hyperparathyroidism, 3 with tertiary hyperparathyroidism, and 8 MEN 2A-related). In addition to these benign tumors, 36 tumors from 30 patients with parathyroid carcinoma were analyzed.

Immunohistochemical Procedures. A paraffin sectioning aid system (Instrumedics Inc., Hackensack, NJ) was used to facilitate cutting of 5-μm sections of the tissue microarray. The sections were deparaffinized, treated with 0.3% H_2O_2 in CH_3OH, and submitted to antigen retrieval (microwave oven treatment for 10 minutes in 10 mmol/L citrate buffer pH 6), except histone H1, for which no antigen retrieval was necessary. Tissue sections were incubated overnight at room temperature with mouse antihuman amyloid βA4 precursor protein (APP; dilution 1:80, clone 22C11, Boehringer, Ingelheim GmbH), H1 (dilution 1:6400, clone 1415-I, Neomarkers, Fremont, CA), cyclin D1 (CCND1; dilution 1:500, clone DCS-6, Neomarkers, Fremont, CA), and E-Cadherin (CDH1; dilution 1:500, clone HECD1, Zymed, Carlton, CT). Sections were subsequently washed (3 × 5 minutes in PBS) and incubated (30 minutes) with biotinylated secondary antibody in PBS/bovine serum albumen 1%, washed (3 × 5 minutes in PBS) and incubated (30 minutes) with horseradish peroxidase-streptavidin complex. Diaminobenzidine tetrahydrochloride was used as a chromogen followed by counterstaining with hematoxylin. The primary antibody was omitted as a negative control. Brain (sclerotic plaques) and cervix served as positive controls for APP and CDH1, respectively, and tonsil was used for CCND1 and H1. Expression was scored by light microscopy. The cutoff parameters used for classification of normal and overexpression are summarized in Table 1.

RESULTS AND DISCUSSION

Gene Expression Profiling

Unsupervised Clustering. Using expression microarray analysis of 11 clinical parathyroid entities, we have identified three broad and distinct tumor groupings based on unsupervised clustering according to gene expression profiles. Using different gene sets by varying the filtering parameters, these three clusters remained constant, although the relative positions of the clusters did vary. The dendrogram in Fig. 1 is derived from hierarchical clustering with a set of 6,150 genes. The composition of the three clusters reflected, in part, the histologic classification of the tumors. However, concordance with histologic classification was not complete. Cluster 1 was composed of predominantly hyperplastic specimens but also a lithium-associated tumor, a MEN 2A adenoma, and 3 sporadic adenomas. The pooled normal sample also clustered with this group.

The most robust cluster identified in this study was cluster 2, which was composed of the sporadic carcinomas, familial Hyperparathyroidism-Jaw Tumor Syndrome (both benign and malignant tumors), and 2 familial isolated hyperparathyroidism tumors. Two sporadic carcinomas in the small cluster 2 subgroup (#779g and #1798g) demonstrated expression of some thyroid-specific genes (including *metallothionein 1H, E,* and *G*), suggesting possible admix with nearby thyroid tissue that may have occurred due to extensive surgical clearance. This was not observed in other tumors in this cluster, and for this reason these 2 tumors were excluded from additional analyses. The small intracluster variance (V = 0.25) and the large distance from the other two clusters demonstrated the distinct nature of the gene expression profile

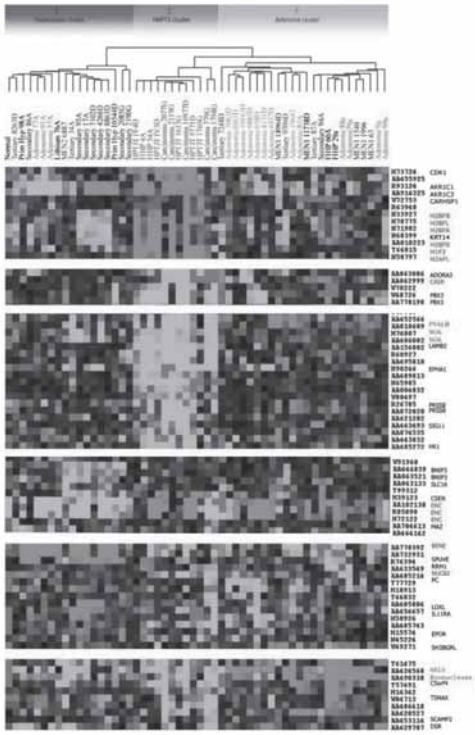

Fig. 1. Detailed sample dendrogram of unsupervised hierarchical clustering. *Red* and *green* indicate transcript expression levels respectively above and below the median (*black*) for each gene across all samples. *Grey squares* indicate no results.

of this cluster. These results, in combination with the knowledge that all of the tumors tested in this cluster (11 of 12) harbored *HRPT2* mutations (Supplementary Data Table S1), support a distinct molecular pathway of parathyroid tumorigenesis for sporadic carcinomas, familial Hyperparathyroidism-Jaw Tumor Syndrome and familial isolated hyperparathyroidism tumors with a *HRPT2* mutation, that is separate from that for other parathyroid tumors. Our results are striking in that we demonstrate that Hyperparathyroidism-Jaw Tumor Syndrome adenomas, otherwise indistinguishable from non-Hyperparathyroidism-Jaw Tumor Syndrome adenomas, exhibit a common microarray signature with parathyroid carcinomas, which have a high

Table 1 *Cut-off values*

Molecule	Method	Normal	Overexpression
E-cadherin	IHC	Membranous staining/no staining	Irregular membranous staining or depositions/droplets in cell
	MA	Fold change <2	Fold change >2
Amyloid βA4 precursor protein	IHC	Weak/moderate cytoplasmic staining	Strong cytoplasmic staining
	MA	Fold change <2	Fold change >2
Histone H1	IHC	≤60% of positive nuclei	≥60% of positive nuclei
H1F2	MA	Fold change <2	Fold change >2
Cyclin D1	IHC	<10% positive cells	>30% positive cells
	MA	Fold change <4	Fold change >4

NOTE. MA values are ratios of tumor to normal.
Abbreviations: IHC, immunohistochemistry; MA, cDNA microarray.

incidence of *HRPT2* mutations. These results suggest that *HRPT2* mediates novel and fundamental pathways of tumorigenesis and malignant transformation. This has also been supported by the multiple genetic changes identified by previous comparative genomic hybridization studies of parathyroid carcinoma (18). The increased incidence of parathyroid carcinoma in Hyperparathyroidism-Jaw Tumor Syndrome patients and our demonstration of a close relationship between sporadic carcinomas and familial Hyperparathyroidism-Jaw Tumor Syndrome tumors suggest that apparently benign tumors within this cluster may have the potential to progress to malignant tumors.

Cluster 3 contained the majority of the sporadic adenoma specimens and all of the MEN 1 and 2 familial isolated hyperparathyroidism tumors. Three of 5 tumors, clinically classified as tertiary hyperplasia, as well as 1 secondary hyperplasia, were found in this cluster. This clustering demonstrates at the molecular level the recognized clinical transition from secondary to tertiary or autonomous disease. Additional studies are warranted to confirm this observation and to identify gene sets that separate secondary from tertiary hyperplasia.

All 5 of the MEN 1 tumors (2 single adenomas and 3 multigland hyperplasia) were also located in cluster 3, suggesting that familial MEN 1 tumors and a subset of sporadic adenomas may share a similar genetic pathway of tumorigenesis. This is supported by previous findings of frequent LOH at 11q13 and of *MEN1* mutations in 20% to 40% of sporadic adenomas in contrast to hyperplasia (19).

Two of the 4 familial isolated hyperparathyroidism tumors in this study also clustered with the sporadic adenomas and MEN 1 tumors in cluster 3. These tumors were from families where linkage to the *MEN1* region could not be excluded. The separation of these 2 familial isolated hyperparathyroidism tumors (cluster 3) from the 2 familial isolated hyperparathyroidism tumors harboring *HRPT2* mutations (cluster 2) again clearly indicates that different molecular pathways are involved in familial isolated hyperparathyroidism patients and adds additional weight to a subset of familial isolated hyperparathyroidism being variants of either Hyperparathyroidism-Jaw Tumor Syndrome or MEN 1 rather than a distinct entity. It is possible that those familial isolated hyperparathyroidism tumors displaying a *HRPT2*-like expression profile may benefit from more aggressive clinical management than those displaying a *MEN1*-like profile.

Genes with established roles in parathyroid tumorigenesis were evaluated by comparing the expression levels of these genes in tumors with the expression level in normal parathyroid tissue. The gene encoding parathyroid hormone was highly expressed resulting in image-saturation, thus data unable to be included in additional analysis. *VDR* was down-regulated in all of the groups. *CCND1, parvalbumin (PVALB),* and *CaSR* were differentially expressed with *CCND1* up-regulated in cluster 1 and 2 and *PVALB* down-regulated in cluster 2. *CaSR* was down-regulated in all of the clusters but significantly down-regulated in cluster 2 ($P < 0.05$). There was a trend toward down-regulation of *MEN1* in *MEN1*-related tumors (from patients with MEN 1 or with Familial isolated hyperparathyroidism where linkage to *MEN1* could not be excluded and from sporadic tumors with somatic *MEN1* mutations); however, this did not reach significance, possibly due to the small size of this group ($n = 9$). *TP53* was not differentially expressed in any of the clusters. *RET* and *HRPT2* were not represented on the arrays.

Molecular Classifiers

Cluster Identification Tool. Supervised clustering using Cluster Identification Tool identified 22, 329, and 17 genes differentially expressed between clusters 1, 2, and 3 respectively (Tables S2, S3 and S4, respectively, in the Supplementary Data) In cluster 1, 22 genes were differentially expressed. Of note, *ectodermal-neural cortex* (*ENC1*), *hypothetical protein MGC11034,* and *Homo sapiens clone MGC:16152* were down-regulated relative to the other two groups. Up-regulated were *BENE protein* (*BENE*), *membrane glycoprotein MRC OX-2* (*MOX2*), and *immunoglobulin superfamily member 4* (*IGSF4*).

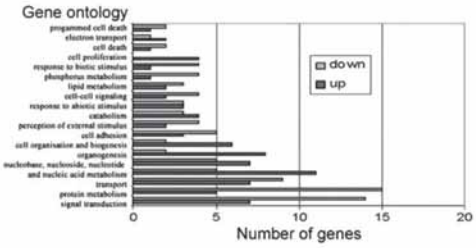

Fig. 2. Gene ontology for biological processes of 204 genes differentially expressed in cluster 2 (Hyperparathyroidism-Jaw Tumor Syndrome/sporadic carcinoma). Only categories with at least 3 genes over- or underexpressed are listed. The ontology information was compiled from GeneCards, Genatlas and DAVID.

In cluster 2, of the 329 genes identified by Cluster Identification Tool ($P < 0.05$), 204 were also identified as differentially expressed by Significance Analysis of Microarrays. This subset of genes was classified according to gene ontology information (Fig. 2), which indicated that major changes had occurred to genes involved in signal transduction, protein metabolism, transport, nucleic acid metabolism, organogenesis, cell organization, and cell adhesion. Examination of individual genes in this subset suggests important roles for *KIAA1376* (signal transduction), *Serum/glucocorticoid regulated kinase* (*SGK*; Phosphate metabolism, transport), *Cullin 5* (*CUL5*; cell death), and *Nucleobindin* (*NUCB2),* all of which are down-regulated. Up-regulated are *amyloid β precursor protein* (*APP*; programmed cell death), *E-cadherin* (*CDH1*; cell adhesion and signal transduction), *histone H1 family member2* (*H1F2*; cell organization and biogenesis), the *aldo-keto reductase family 1 C3* (*AKR1C3*; cell proliferation and lipid metabolism), *CD24* (signal transduction), *ubiquitin carboxyl-terminal esterase L1* (*UCHL1*; catabolism), *development and differentiation enhancing factor 1* (*DDEF1*), and *laminin β1* (*LAMB1*; cell adhesion).

In cluster 3, 17 genes were differentially expressed. *Aldehyde dehydrogenase 1 family, member A2* (*ALDH1A2*), and *metallocarboxypeptidase* (*CPX-1*) were down-regulated, and the *KIAA0435 gene product*, *ADP-ribosylation factor-like 5* (*ARL5*), and *exonuclease NEF-sp* were up-regulated relative to the other 2 clusters.

Statistical Classification and Probability Scores in Clinical Diagnostic Categories by Penalized Logistic Regression and Significance Analysis of Microarrays. Using Penalized Logistic Regression analysis, a two-step statistical model for classifying tumors was built (Fig. 3). With 50 genes (Supplementary Data Table S5), Penalized Logistic Regression separated cluster 2 tumors from the rest with a minimal log-odds separation of 1:1,000 between the 2 groups of tumors (Fig. 3*A*), confirming the results obtained by unsupervised hierarchical clustering (Fig. 1). A second step with a different set of 50 genes (Supplementary Data Table S6) separated cluster 3 tumors (predominantly adenomas) from the rest, which now consisted of all cluster 1 specimens (predominantly hyperplasia; Fig. 3*B*). This second-step classification was less pronounced (log-odds separation of 1:100), and larger SDs were apparent, again reflecting the results of hierarchical clustering. Cross-validation confirmed the above separation. The subgroupings observed under clusters 1 and 3 were not apparent in the Penalized Logistic Regression model.

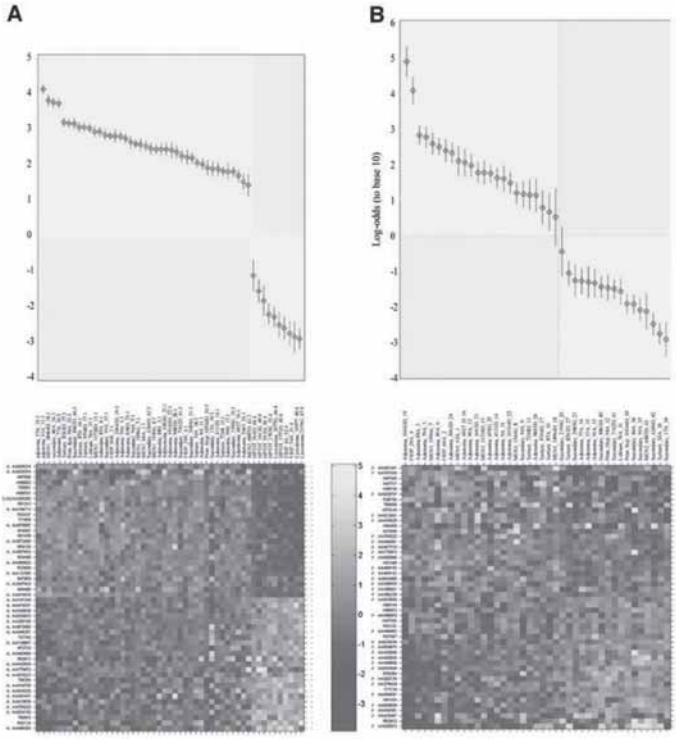

Fig. 3. Results of classification with a penalized logistic regression model, based on two sets of 50 most significant genes (according to Significance Analysis of Microarray) present in all arrays. *Top panel* depicts the results of 100 bootstrap replications: means ([circle]) ±1 SD (*lines*) of log-odds per array. The vertical scale is log-odds to base 10, where "0" represents equal odds, so that in *A*, "2" indicates a probability of 100 to 1 that the specimen is not a cluster 2 tumor, and "−3" alternately indicates a probability of 1000 to 1 that the specimen is a cluster 2 tumor. Outcomes in the green (*pink*) regions are correctly (incorrectly) classified. *A* represents step-1 differentiation between non-Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma tumors (clusters 1 and 3) and Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma tumors (cluster 2) using 50 genes. *B* represents step-2 differentiation between adenomas (cluster 3) and the remainder (cluster 1) using a different set of 50 genes. *Bottom panel* provides graphical presentation of the log expressions with the arrays in the same order as in the *top panel*. The genes have been ordered along the values of their coefficients in the model with *yellow squares* indicating the highest expression.

Statistical Classification Using Gene-Rave. Using a different approach to normalization and analysis of the array data, Gene-Rave confirmed separation of the tumors into the three main clusters as demonstrated by unsupervised clustering (Figs. 1 and 4). When testing for genes to additionally separate the Hyperparathyroidism-Jaw Tumor Syndrome (including the 2 familial isolated hyperparathyroidism specimens, #4A and #54A, with *HRPT2* mutations) and carcinoma groups, the cross-validation error rates for correct classifications were indistinguishable from the permuted versions (Supplementary Data Fig. 15). This indicated that these two classes were inseparable. The Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma, adenoma, hyperplasia, and MEN 1 groups were found to be separable, although the separation between adenoma and MEN 1 was small (Fig. 4). Two sporadic carcinomas (#779G and #1798G) were consistent outliers, as also noted in the unsupervised cluster analysis (Fig. 1) and were excluded from additional analysis. An advantage of Gene-Rave technology is that the gene sets able to discriminate between groups are typically very small. In this study, 9 genes [*UCHL1, CD24, ALDH1A2, PVALB, clone DKFZp434E03, Vascular Cell Adhesion Molecule 1*(*VCAM1*), *Testican-3, MOX2,* and *glutamate decarboxylase 1* (*GAD1*)] were able to discriminate between the four groups.

Immunohistochemical Analysis of Protein Expression

We selected four highly differentially expressed genes for which antibodies to their respective proteins were available, to compare transcript and protein overexpression. Three of these genes, *CDH1, H1F2*, and *APP* were highly overexpressed in cluster 2 compared with clusters 1 and 3. *CCND1* demonstrated differential expression between clusters 1 and 3, with higher expression in cluster 3.

Parameters were established for classification as normal or overexpression of each transcript and protein (Table 1), and examples of the differential expression assessed by immunohistochemical staining are shown in Fig. 5.

Comparing microarray expression with immunohistochemistry in 16 specimens represented on both the expression microarrays and tissue microarray, 94% correlation was found for H1, APP, and CCND1, and 75% for CDH1. To additionally evaluate the expression of these four proteins, all of the tissue microarray tumor specimens were grouped in accordance with the cluster sets created by unsupervised clustering (Fig. 1). For CDH1, H1, and APP, protein overexpression occurred predominantly in the carcinoma and Hyperparathyroidism-Jaw Tumor Syndrome familial tumors, resulting in a significant correlation between the immunohistochemistry and expression data ($P < 0.01$; Fig. 6).

Increased expression of CCND1 is known to play a primary role in the formation of parathyroid tumors. Overexpression of CCND1 was noted primarily in the hyperplasia and carcinoma/Hyperparathyroidism-Jaw Tumor Syndrome tumors, with only 12% of adenomas/MEN 1 tumors demonstrating overexpression by immunohistochemistry. This allowed moderate discrimination between adenoma and hyperplasia and also correlated with the expression microarray results.

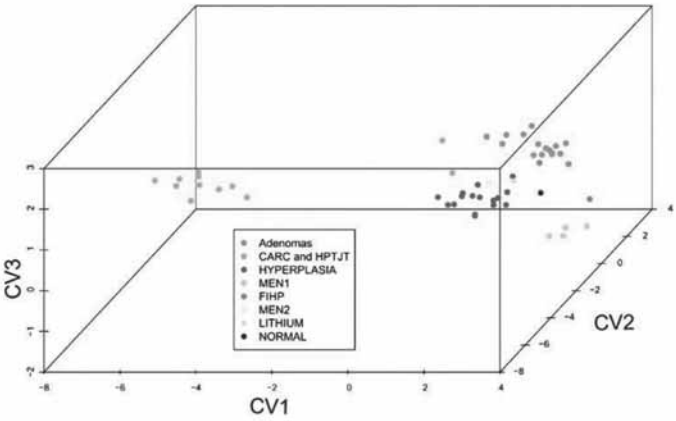

Fig. 4. Canonical variate plot of all arrays. The plot shows the first three canonical variates (*CV1–3*). CV1 consisted of 125 genes, CV2, 57 genes, and CV3, 63 genes. Among genes of significance in CV1–3 were *CDH1, APP, UCHL1, IGSF4, MOX2,* and *GAD1*. A large separation between the carcinoma/Hyperparathyroidism-Jaw Tumor Syndrome group and the rest of the tumors is evident. The 2 familial isolated hyperparathyroidism specimens with *HRPT2* mutations are included in the carcinoma and Hyperparathyroidism-Jaw Tumor Syndrome group that is depicted in green. The 2 (*green*) carcinomas distant from the main Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma cluster are the outliers #779G and #1798G. Clear separation of the adenoma (*red*), hyperplasia (*purple*), and MEN 1 (*light blue*) groups is also evident. Two MEN 1 tumors are overlaid in this analysis and appear as *one blue spot*. The pooled normal (*black*) is among between the adenoma, hyperplasia, and MEN 1 groups. The 2 familial isolated hyperparathyroidism tumors with linkage at 11q13 (*pink*) are located between the adenomas and MEN 1 tumors. The MEN 2A (*yellow*) and lithium-associated tumor (*gray*) are situated closest to the hyperplasia group.

These results are in agreement with previous immunohistochemistry studies in which overexpression of CCND1 has been observed in 50% to 91% of parathyroid carcinomas, up to 61% of sporadic parathyroid hyperplasia, and 18% to 40% of sporadic parathyroid adenomas (20, 21).

Up-regulation of two of four members of the histone family (*H1* and *H2*; Supplementary Data Table S5) was evident in cluster 2 from the microarray experiments, and H1 protein overexpression was confirmed in sporadic carcinomas by an increased percentage of nuclei expressing this histone. It has been postulated recently that H1 may regulate specific genome repair mechanisms in some species (22). Because DNA repair pathways are involved in DNA maintenance, they are likely to have relevance to tumorigenic processes. APP is a functional neuronal receptor, shown in this study to be up-regulated at both the RNA and protein level. Both transcript and protein were found overexpressed in cluster 2. The soluble N-terminal ectodomain (sAPP) is a product of the APP protein that can function as an epithelial growth factor in thyroid cells (23) and has been proposed recently to have a pro-proliferative role in some pancreatic cancer cell types.

CDH1 is a calcium-dependent cell-cell adhesion glycoprotein. Loss of function due to mutation or methylation is thought to contribute to cancer progression by increased proliferation, invasion, or metastasis. It has also been reported that increased parathyroid hormone or low extracellular calcium affects the expression and localization of this molecule (24). We found *CDH1* up-regulated at the mRNA level in cluster 2, with aberrant staining noted by, indicating loss of function in cell adhesion (25).

We also evaluated the diagnostic sensitivity of combined immunohistochemistry using these four proteins. If two or more of these four proteins are overexpressed in a single case, there is an 81% chance that it is a carcinoma, and a 2% and 11% chance that it is an adenoma

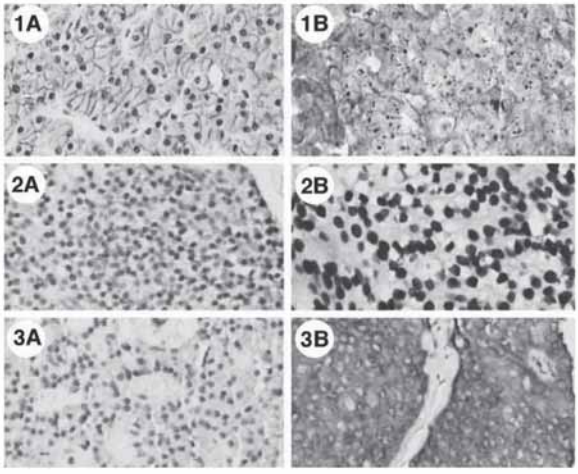

Fig. 5. Differential immunohistochemical staining at ×200 magnification between (*A*) adenomas and (*B*) carcinomas (overexpression) for E-cadherin (*1*), histone H1 (*2*), and amyloid βA4 precursor protein (*3*).

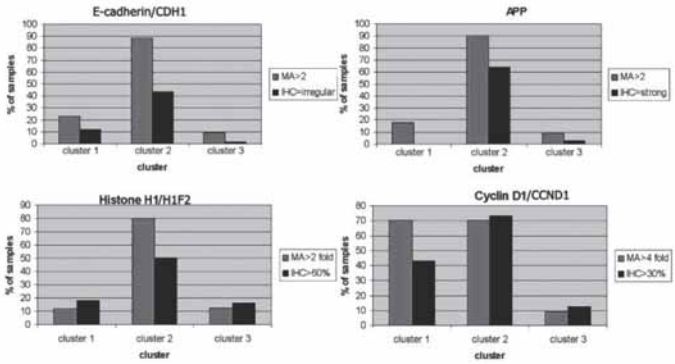

Fig. 6. Correlation between Microarray (*MA*; $n = 53$) and Immunohistochemistry (*IHC*; $n = 147$) data for the three different parathyroid subgroups as defined by unsupervised clustering of the microarray data. The percentage of samples displaying overexpression (as defined in Table 1) in each cluster group are graphed.

or hyperplasia, respectively ($P < 0.01$). We have demonstrated that *H1F2, APP,* and *CDH1*, identified from our microarray experiments, are also useful immunohistochemistry makers for the diagnosis of parathyroid carcinoma. Our results suggest that the presence of a *HRPT2* mutation, whether germ-line or somatic, strongly influences the expression pattern of these 3 genes, and we postulate that these 3 genes may have potential biological significance in parathyroid tumorigenesis.

In conclusion, we have identified three broad cluster groupings of parathyroid disease based on distinct molecular signatures. Cluster 2 was the most striking and included both benign and malignant tumors from patients with Hyperparathyroidism-Jaw Tumor Syndrome, as well as sporadic parathyroid carcinomas with somatic *HRPT2* mutation and a subset of tumors from patients with familial isolated hyperparathyroidism. This cluster is strongly suggestive that parathyroid tumors with somatic *HRPT2* mutation or tumors developing on a background of germ-line *HRPT2* mutation follow pathways distinct from those involved in mutant MEN 1-related parathyroid tumors. Furthermore, our findings likely preclude an adenoma to carcinoma progression model for parathyroid tumorigenesis outside of the presence of either a germ-line or somatic *HRPT2* mutation.

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Chapter 9

Multiplex ligation-dependent probe amplification analysis in parathyroid tumours

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manuscript

Abstract

Objective
The objective of the present study was to develop a genomic assay based on Multiplex Ligation-dependent Probe Amplification (MLPA) for the rapid characterisation of parathyroid carcinomas based on a combination of known chromosomal amplification and deletions.

Patients and design
Formalin-fixed, paraffin-embedded (FFPE) parathyroid tissues from 33 carcinoma cases and 16 adenoma cases identified in the period 1985-2003 in the Netherlands were studied. Histologically normal parathyroid tissues from 22 patients were taken from paraffin blocks and used together with a pool of 6 different normal colon appendices to serve as a reference. A MLPA probe kit was designed based on reported chromosomal amplification and deletions in parathyroid tumours.

Results
Chromosomal loss in carcinomas was found on chromosome 1p (27%), 3q (21%) and 13q (21%) but was even more prominently and significantly deleted in *HRPT2* mutated carcinomas as compared to adenomas and carcinomas without a *HRPT2* mutation. Chromosome 1p, 3q and 13q showed loss in 3/5, 3/5 (both 60%) and 5/5 (100%) of the HRPT2 mutated carcinomas, respectively.

Conclusion
These results suggest that loss of chromosome arms 1p, 3q and especially 13q play a role in *HRPT*2 driven tumorigenesis. Furthermore, MLPA is a useful tool to study parathyroid tumorigenesis because of the specificity/sensitivity and speed of the analysis.

Introduction

Hyperparathyroidism is a common endocrinopathy believed to affect three in 1,000 adults [1] and may result from a single parathyroid adenoma (80-85%) or from hyperplasia (15-20%) but rarely (less than 1%) from carcinomas.[24]
Although parathyroid carcinomas are mostly slow growing, they have a high propensity (50% or more) to recur locally when not recognized at the initial surgery and treated by a simple parathyroidectomy.[37] Importantly, the recurrent disease is difficult to eradicate and almost 90% of all patients with recurrent hyperparathyroidism will die of the disease.[20] In contrast, in patients where an adequate diagnosis was made intraoperatively and who were subsequently treated by en bloc resection, local recurrence ranges from 10-33%, and long term survival improves significantly.[20,38]
Intraoperatively, parathyroid carcinoma usually appears as a large, firm, whitish-grey tumor that has often invaded surrounding structures. Despite these defining characteristics, parathyroid carcinoma is often not recognized at the time of initial surgery. The distinction between parathyroid carcinomas and adenomas based on histology is also difficult since the histopathological features of parathyroid carcinoma and adenoma may overlap. Some authors have claimed that trabecular growth, dense fibrous bands, spindle shape of tumour cells, mitotic figures and nuclear atypia[30] are helpful criteria in diagnosing parathyroid carcinomas, but all these criteria can also be observed in benign parathyroid lesions.[5,23,33] An unequivocal diagnosis of parathyroid carcinoma is only possible by demonstration of distant or locoregional metastasis, as well as histologically by blood vessel invasion and/or capsular invasion.[29] This stresses the importance of adequate

diagnosis. Therefore, there is an ongoing search for markers to provide reproducible and both biologically and clinically meaningful predictions for the diagnosis of malignancy and/or aggressive tumour behaviour that is not based on subjective histological criteria to a large degree.
So far, a variety of methods for finding and detecting molecular markers have been used, like detection of loss of heterozygosity (LOH) by microsatellite repeat analysis, comparative genomic hybridisation (CGH) CGH, immunohistochemistry (IHC) and microarray expression analysis. Results from CGH, supported by LOH studies, suggested that in those carcinomas having a physical loss of regions on chromosomes 1p (41%) and 13q (26%), there is inactivation of possible tumour suppressor genes. Chromosomal gain and thus the existence of potential oncogenes in these tumours were found in regions 1q (21%), 9q (12%) and 19p (13%).
Both CGH and LOH analysis showed that loss of 11q is a frequent event in adenomas and also in combination with *MEN1* mutations (95% in familial MEN1 syndrome and 20-30% in sporadic adenomas). However, in a recently published paper, a high percentage (50%) of carcinomas with LOH of 11q was also detected, suggesting that it also plays a role in parathyroid carcinoma formation. [16]
Recently it was shown that *HRPT2* mutations are found in HPT-JT syndrome and in a substantial portion of sporadic parathyroid carcinomas, suggesting that this gene plays a pivotal role in malignant transformation of parathyroid tumours. Parafibromin encoded by *HRPT2* shows downregulation in such tumours. Furthermore, expression microarray analysis revealed that *HRPT2* mutated tumours have a unique and distinct expression profile as compared to other parathyroid tumour types. *LMNA, FGFR1, FGFR4, DDEF1, IGSF4, ITMB2, APP,* and *CDH1* are the genes that are significantly up or down regulated in the microarray analysis of a group of parathyroid carcinomas and tumours with *HRPT2* mutations. Other genes that are involved in parathyroid tumorigenensis are *CASR* and *CyclinD1 (CCND1*).
Overexpression of the cyclin D1 protein has been demonstrated in up to 40% of parathyroid adenomas, and overexpression of PRAD1/cyclin D1, following a rearrangement with the PTH gene, has been shown in a few cases.[3,18] Two recent publications showed evidence that parafibromin downregulation causes an increase in CCND1 protein levels [39,40]. Furthermore *CASR* germline mutations can cause familial hypocalciuric hypercalcemia or neonatal severe hyperparathyroidism when partially or markedly deficient[28]**.** Mutations are also found in families suffering from FIHP.[8] Also, CASR is considered to have a potentially important secondary role in the manifestations of sporadic parathyroid tumours [4], although up till now no mutations have been described in sporadic parathyroid tumours.
Multiplex ligation dependent probe amplification (MLPA) is a recently developed technique for the relative quantification of DNA sequences that can detect chromosomal deletions or amplifications.[31] The principle of MLPA relies on the hybridisation of sequence-specific oligonucleotides to genomic DNA, followed by ligation of the oligonucleotides and subsequent amplification of the probe. The relative peak heights or band intensities from each target indicate their initial concentration [32] and can be semi-quantitatively analysed. [25]MLPA has several advantages over currently used techniques. The first advantage is the amount of loci that can be analysed in one reaction. Furthermore, no (paired) normal tissue is needed. Finally, it is a sensitive and relatively fast technique; only a small amount of DNA is required (20 ng is sufficient for one reaction in which 40 loci are tested) and results are available within 2 days. The method is useful for archival, formalin-fixed, paraffin-embedded (FFPE) tissue as the probe target sequences are small (50-70bp).
The objective of the present study was to develop an MLPA based assay for the diagnosis of parathyroid carcinomas based on a combination of known chromosomal amplification and deletions.

Materials and methods

Samples
Formalin-fixed, paraffin-embedded tumour tissue from 28 primary parathyroid carcinomas, 4 regional lymph node metastases, and one lung metastasis taken from 30 patients was obtained from different laboratories in the Netherlands using PALGA (Dutch National Information System for Pathology, Utrecht, The Netherlands) and the archives of the Leiden University Medial Center. The samples were collected over the past 18 years (1985-2003). All but three of these samples were previously described[6]
Included were 30 samples with clear carcinoma features, i.e. presence of vasoinvasion and/or metastasis[11], based on evaluation of representative haematoxylin and eosin stained slides of each tumour by a pathologist (HM)) and the initial pathology report. Three cases (9,11,25) were diagnosed as carcinomas based on their clinical presentation; definitive vasoinvasion was not found in these cases.
Furthermore formalin-fixed, paraffin-embedded tumour tissues from 16 parathyroid adenoma samples taken from 16 patients were obtained from the archives of the LUMC. One adenoma (48) and 1 parathyroid carcinoma (30) came from a documented HPT-JT family.[17]
Normal parathyroid tissues from 22 patients were taken from paraffin blocks and used together with a pool of 6 different normal appendices to serve as a reference for the Multiplex Ligation-dependent Probe Amplification (MLPA).

DNA extraction
Genomic DNA from normal and tumor tissue was isolated from the paraffin-embedded material by taking tissue cores (diameter 0.6 mm) with a tissue microarrayer (Beecher) from tumor and normal areas selected on the basis of a hematoxylin and eosin-stained (HE) slide. Using a chelex extraction method, DNA was isolated from three punches, re-suspended in 96 ml of PK-1 lysis buffer (50 mM KCl, 10 mM Tris [pH 8.3], 2.5 mM MgCl2, 0.45% NP40, 0.45% Tween 20, 0.1 mg/ml gelatin) containing 5% Chelex beads (Biorad, Hercules, California, USA) and 5 ml of proteinase K (10 mg/ml), and incubated for 12 hours at 56° C. The suspension was incubated at 100° C for 10 minutes, centrifuged at 13,000 rpm for 10 minutes, and the supernatant containing the DNA was used for PCR reactions.

MLPA
MLPA has previously been described.[31] In brief, MLPA is based on the ligation of two DNA oligonucleotides that hybridize adjacently to a target DNA sequence. The ?rst oligonucleotide was synthesized with, on average, a 26 bp (min: 21 bp, max: 39 bp) target-speci?c part and a universal M13-forward tail. The second oligonucleotide was an M13-derived single-stranded DNA sequence containing, on average, a 42 bp (min: 31 bp, max: 50 bp) target speci?c-part, a stuffer sequence of variable length (130-480 base pairs) and an M13-reversed tail. Thus, a probe consists of 2 oligonucleotides of which the target-speci?c parts hybridize adjacently and ligate. The M13 forward and reversed tails are attached to all probes, and the different length of each probe made it possible to perform a single primer multiplex PCR.[25]
An MLPA kit was assembled by MRC-Holland (Amsterdam,The Netherlands). Details of MLPA can be found at http://www.mlpa.com. The MLPA kit was designed especially/ specifically to investigate parathyroid tumours and consisted of 42 probes of chromosomal regions (based on CGH analysis[2,14,21,26]) and genes (based on microarray[15] and mutation data [7,9]) frequently altered in parathyroid tumours. For three important genes, we took two (*MEN1*) or three (*CASR and HRPT2*) different probes.
Thirty-eight experiments were performed in triplicate or more, and ten were performed in duplicate.
After denaturing 15 to 250 ng of DNA for 5 minutes at 95°C, the probe mix containing all

the probe sets was added. After overnight hybridization at 60°C, the hybridized probes were ligated for 15 minutes at 54°C with a DNA ligase. An aliquot was taken out of the ligation mix and the ligated products were ampli?ed in a multiplex PCR reaction using forward and reverse M13 primers for 20 seconds at 95°C, 30 seconds at 60°C and 60 seconds at 72°C for 33 cycles in an Applied Biosystems® 9700 PCR machine. After PCR, 3 µl of the PCR products were mixed with one µL of 500 TAMRA (Applied Biosystems®) internal size marker and 20 µl deionised formamide and injected for 5 seconds in an ABI310® capillary ?lled with POP5 polymer. After a 30 minute run, the data were collected and analyzed with Genescan analysis and Genotyper software (Applied Biosystems®) (Figure 1). A Genotyper output ?le was generated combining probe set number, size and peak heights. This table was exported to a comparative access in-house adapted database where probe annotation is added to the data table. Subsequently, normalization and diagnosis of the pro?les were performed.

Data analysis for MLPA. Normalization.
The MLPA traces were analyzed using the MLPAanalyzer application (http://sourceforge.net/projects/mlpaanalyzer/). Peak heights were dependent on sample quality, DNA concentration, hybridization parameters and instrument settings. Peaks from different probe sets also differed in magnitude in a systematic way. To normalize the raw data, MLPAanalyzer performs the following steps:
1. Distinguishe focus probes and reference probes (5 loci usually unaltered in parathyroid tumours).
2. Select the reference probes from the control (non-tumour) samples. Performs steps 3 to 5 with this subset of data.
3. Within each sample divide all peak heights by the median peak height of the sample. This is to correct for the sample-to-sample variation.
4. Within each probe, divide all peak heights by the median peak height of the probe. This is to correct for systematic differences between probes. The results of 3 and 4 we call normalized peak heights.
5. Determine which of the (reference) probes are most stable. Subtract 1 from each normalized peak height and take the absolute value. Compute the median of these numbers for each probe. This is the median of the absolute deviations: MAD.
6. Select the 5 reference probes with the lowest MAD. These 5 reference probes are named calibration probes and are used to normalize the complete experiment as described in step 7 and 8.
7. Within each sample (parathyroid tumour and normal control samples), divide all peak heights by the median peak height of the 5 calibration probes of the sample of concern. This is to correct for the sample-to-sample variation.
8. Within each probe (focus and reference probes), take the median peak height of the control samples. Then, within each probe (focus and reference probes), divides all peak heights (parathyroid tumour and normal control samples) by the median peak height of the probe of concern. This is to correct for systematic differences between probes.

Data visualization and interpretation.
Each experiment was normalized and analysed separately. Scatter plots for each individual tumour and normal tissue were generated in Matlab (Figure 1) and anonymized.
To determine amplification and deletion in the analysis of the individual probes, a cut off value (amplification>1.3, deletion<0.7) was used. The evaluation of the regions was based on multiple (at least 2) probes and therefore we could use a less strict cut off; for amplification>1.2 and deletion<0.8.
To analyze the regions/chromosomal arms, we used 25 probes (region 1p: 4 probes; 1q:6 probes; 3q: 4 probes; 9p: 3 probes, 9q:2 probes, 11q: 6 probes; 13q: 5 probes). A region was considered "deleted" or "amplified" if more than 50% of the probes within

that particular region were "deleted" or "amplified" (i.e:2/2 (100%) of the probes, 2/3 (67%) of the probes, 3/4 of the probes (75%), 3/5 of the probes (60%), 4/6 of the probes (67%)) such that they had normalized peak heights of at least 0.2 below (deletion) or above (amplification) the median normalized peak height of the reference probes.

Sequence analysis
HRPT2 mutations and *MEN1* mutations were analysed in 27 and 23 sporadic parathyroid carcinoma samples/patients, respectively, as previously described.[6]

LOH analysis
From 20 parathyroid carcinoma samples, LOH status of chromosomes 1q and 11q was previously determined using microsatellite markers[16]

IHC parafibromin
From 27 patients, expression of parafibromin was previously determined with IHC as described [35].

Results

A MLPA probe set (Table 1) was constructed based on the following three criteria: a) the inclusion of genomic regions previously implicated in parathyroid tumorigenesis in the literature, such as chromosomes 1p, 1q, 3q, 9p, 9q, 11q, 13q and 19p ([2,14,21,26]); b) the inclusion of two crucial genes for parathyroid tumorigenesis; *HRPT2* on chromosome 1q and *MEN1* on chromosome 11q; and c) probes were included from several genes from a parathyroid carcinoma /*HRPT2* genecluster as identified by cDNA expression array analysis. [15]
We studied 49 parathyroid tumours, 16 adenomas and 33 carcinomas. In five parathyroid carcinomas and one adenoma, somatic and/or germline *HRPT2* mutations were identified. The average amount of deletions in adenomas was 3.3 (range 0-14), the average for amplification in adenomas was 5.9 (range 0-13). Parathyroid carcinomas showed an average amount of 6.7 deletions (range 0-12) and average amount of 5.8 amplifications (range 0-19). *HRPT*2 mutated samples had an average of 8.6 deletions (range 6-13) and 3 amplifications (range 1-8).

Deletion and amplification of chromosomal regions
In parathyroid carcinomas, deletion of chromosomes 1p (41%) and 13q (26%) are relatively frequently described [2,14,21,26], although for chromosome 13q the frequency is only slightly increased in comparison to adenomas (Table 1). We also observed losses of these chromosomes in parathyroid carcinomas using MLPA (1p, 27.3% 9/33; 13q, 21.2% 7/33 respectively), with the losses being most notable in the *HRPT2* mutated subset of carcinomas (3/5 of 1p; 5/5 of 13q). Chromosome 13q loss was also seen for one *HRPT2* mutated adenoma. On chromosome 13q, the probes for *BRCA2* (13q12), *ITM2B* (13q14), RB (13q14, less clear), *DACH* (13q21) and *ING1* (13q34) were deleted in *HRPT2* mutated samples (Figure 1). Also, chromosomes 3q and 9p were deleted in a relatively high percentage of *HRPT2* mutated carcinomas (3/5 and 2/5, respectively). The most frequently found chromosomal aberration in adenomas using CGH analysis is deletion of 11q. Using MLPA, the latter was not confirmed.
Using CGH, chromosomal gains were previously found in parathyroid carcinomas of the regions 1q (21%), 9q (12%) and 19p (13%). We found in both carcinomas and in *HRPT2* mutated samples amplification of chromosome 1q in 9.1% and 1/5, respectively. Adenomas showed no amplification. MLPA of chromosome 9q did not confirm the pattern observed by CGH (amplification in 12% of carcinomas and deletion in 8% of adenomas).

TABLE 1

	ref CGH	ref CGH	carcinomas(n=33)*		carc with HRPT2mut (n=5)		adenomas (n=16)		ad with HRPT2 mut (n=1)	
regions	adenoma	carcinoma	amplification	deletion	amplification	deletion	amplification	deletion	amplification	deletion
1p	-16%	-41%	0.0	27.3	0.0	60.0	0.0	6.3	0.0	0.0
1q	-3%	21%	9.1	0.0	20.0	0.0	0.0	6.3	0.0	0.0
3q	-4%	-5%	3.0	21.2	0.0	60.0	0.0	12.5	0.0	100.0
9p	-12%	-14%	6.1	12.1	0.0	40.0	12.5	12.5	0.0	0.0
9q	-8%	12%	3.0	6.1	0.0	20.0	0.0	6.3	0.0	100.0
11q	-32%	-9%	12.1	6.1	0.0	0.0	6.3	0.0	0.0	0.0
13q	-19%	-26%	0.0	21.2	0.0	100.0	0.0	12.5	0.0	100.0
19p	-5%	13%	45.5	0.0	20.0	0.0	43.8	0.0	0.0	0.0
probes	MA	in carc								
HRPT2	no data		6.1	12.1	20.0	0.0	12.5	6.3	0.0	0.0
CASR	Down	40%	6.1	33.3	0.0	60.0	12.5	18.8	0.0	100.0
MEN1	Down	0%	9.1	9.1	0.0	0.0	18.8	0.0	0.0	0.0
LMNA	Up	100%	9.1	0.0	20.0	0.0	6.3	6.3	0.0	0.0
FGFR1	Up	90%	12.1	0.0	0.0	0.0	6.3	12.5	0.0	0.0
FGFR4	Up	70%	36.4	0.0	0.0	0.0	37.5	6.3	0.0	0.0
DDEF1	Up	80%	21.2	3.0	20.0	20.0	25.0	0.0	100.0	0.0
CCND1	Up	80%	15.2	3.0	0.0	0.0	6.3	6.3	0.0	0.0
IGSF4	Down	nd	21.2	18.2	0.0	0.0	12.5	37.5	0.0	100.0
ITMB2	Down	90%	9.1	9.1	20.0	40.0	12.5	12.5	0.0	100.0
APP	Up	90%	15.2	3.0	0.0	20.0	18.8	12.5	0.0	100.0
CDH1	up	80%	30.3	18.2	60.0	0.0	18.8	25.0	0.0	0.0

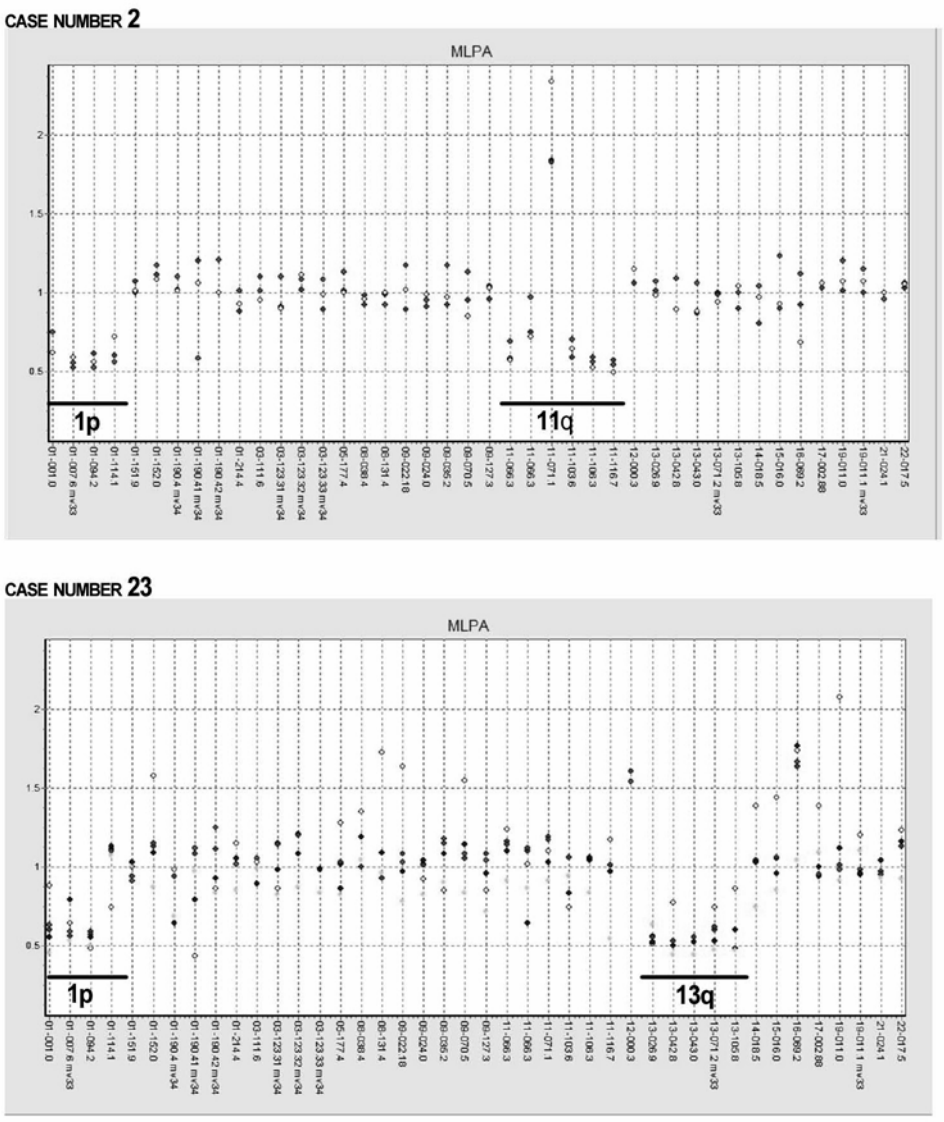
*The chromosomal locations of the probes are shown on the x-axis. The y-axis shows in log scale amplification (scoring in triplicate more than 1.3), retention (around 1) and deletion(scoring in triplicate less than 0.7). Abbreviations: ref CGH: average loss of regions found by comparative genomic hybridisation analysis as reported in previous papers; MA: microarray. * including three cases with somatic MEN1 mutations[6] and five cases with HRPT2 mutations. All data are percentages; the negative percentages indicate loss in the ref CGH columns, the positive percentages represent gain*

Using MLPA for chromosome 19p, the observation seen in CGH (amplification in 13% of carcinomas, deletion in 5% of adenomas) was not seen with a high amplification rate in both carcinomas and adenomas.
In conclusion, using MLPA in tumours with *HRPT2* mutations, there is a significant deletion of chromosomes 1p, 3q and 13q as compared to adenomas and carcinomas without a *HRPT2* mutation ($P<0.05$).

HRPT2 and MEN1 MLPA
Deletion of *HRPT2* was considered if more than 2 of the 3 *HRPT2* probes were deleted. This was the case in 3.6% (1/28) of overall carcinomas and in none of the *HRPT2* mutated samples and adenomas. Deletion of *MEN1* (in both probes) was not found in any of the adenomas and in only 9.1% of carcinomas, whereas frequently a low amplification was scored in both adenomas and carcinomas.

MLPA of differentially expressed genes
MLPA gene probes for 9 genes that were significantly up- (*LMNA*, *FGFR1*, *FGFR4*, *DDEF1*, *CCND1*, *APP* and *CDH1*) or downregulated (*CASR*, *IGSF4, ITMB2*) in *HRPT2* mutated samples using cDNA expression array analysis were analysed. Nonsignificant trends in the amplification/deletion of different probes were seen that mimicked the observed relative expression patterns. However, the *CASR* on chromosome 3q was scored as deleted in 33.3 % of carcinomas versus 18.8% of adenomas (nonsignificant) with frequent low amplification scores in both adenomas and carcinomas. Moreover, 4/6 *HRPT2* mutated tumours clearly showed loss of *CASR*. The trend towards amplification of CDH1 on chromosome 16q in carcinomas and particularly in *HRPT2* mutated carcinomas (3/5) correlated with a relatively high expression of this gene.

FIGURE 1 Scatter plot of 2 parathyroid carcinoma samples.



Case no.2 (carcinoma without *HRPT2*/*MEN1* mutation) showed loss of region 1p and 11q. Case no. 23 (carcinoma with HRPT2 mutation) showed loss of region 1p and 13q.

Discussion

MLPA was used to analyse copy number variation of chromosomal regions implicated in parathyroid tumorigenesis. In the *HRPT2* mutated carcinomas, chromosomes 3q and 9p and particularly chromosome 13q showed deletions. Loss of chromosome 13q is an event found in both sporadic adenomas (19%) and carcinomas (26%), although the prevalence is higher in carcinomas.[2,14,21,26]

MLPA analysis showed the same trend, with the deletion of 13q found in both adenomas and carcinomas but to a greater extent in carcinomas. Remarkable is that all carcinomas and the one adenoma with a *HRPT2* mutation showed deletion of 13q. The implication that 13q deletion plays a role in malignant parathyroid tumorigenesis was previously reported by Hunt et al.[19] The region of loss on chromosome 13q in parathyroid tumours has been shown to include two known tumour suppressor genes, the retinoblastoma gene (*RB, RB1*[10,12,27]) and *BRCA2.* [27] Cryns et al[10] were the first to suggest that inactivation of the *RB1* gene might help to distinguish benign from malignant parathyroid tumours and thus have potential prognostic and therapeutic implications. Other authors have cast doubts on the usefulness of *RB1* gene studies in the differential diagnosis between parathyroid carcinomas and adenomas, as abnormalities of the *RB* gene and/or protein are not a specific features of parathyroid malignancy.[22,27,34] The above illustrates that loss of 13q is more frequently found in carcinomas than in adenomas. Although this loss is not specific for parathyroid carcinomas, it now seems to be specific for *HRPT2* mutated tumours. Additional experiments are required to further support the role of 13q in *HRPT2* driven tumorigenesis.

Chromosome 1p is the only region of significant loss common to all tumour classes. This region is the area of most frequent loss in both malignant (41%) and benign tumours (secondary (72%) and tertiary HPT (73%), and adenomas (16%)).[26] Hunt et al[19] reported that although almost all adenomas and carcinomas showed loss of markers for 1p, the benign parathyroid diseases (adenomas and hyperplasia) had a low mean fractional allelic loss (11% and 15%, respectively). The parathyroid carcinomas, in contrast, showed high mean fractional allelic loss (63%). The current MLPA analysis confirms this; we also found loss in both tumour types, but the percentage of loss and the amount of probes lost was higher in parathyroid carcinomas.

Välimäki et al (2002)[36] reported, furthermore, that deletion mapping studies by LOH and CGH implicate that the 1p target regions in adenomas are more distally located at 1p34-pter and are thus different from parathyroid carcinomas where the deletions cluster at 1p21-p22.

The 1p21-22 region, found to be specifically deleted in carcinomas in the study of Välimäki et al, was also more frequently lost in carcinomas than in adenomas, but the loss of the distal part of chromosome 1p in parathyroid adenomas could not be confirmed. Downregulation of *CASR* mRNA has been described in adenomas[13] but has also recently been shown to be downregulated and to a higher extent in the *HRPT2*/parathyroid gene cluster.[15] On a protein level, a strong downregulation has also been reported in parathyroid carcinomas. Up till now, no mutations have been found in sporadic adenomas, although to our knowledge no mutation analysis has been carried out on malignant tumours.

In the current MLPA analysis, deletion of 3q and especially of the *CASR* region is a frequently seen event in parathyroid carcinomas. Once again this deletion is more specific for tumours with a *HRPT2* mutation than those without, suggesting that the *CASR* might also play a role in *HRPT2* driven tumorigenesis. We now also show that downregulation of *CASR* mRNA is partly based on the physical deletion of a region of chromosome 3q containing *CASR*. Mutation analysis should further prove the complete inactivation of this gene.

Recently we showed the use of 6K SNP-arrays on FFPE material in order to detect copy number variation and copy neutral LOH. The latter information cannot be obtained using the MLPA panel. For diagnostic purposes, however, MLPA is more easily applicable.

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Chapter 10

Concluding Remarks

In this thesis, parathyroid tumourigenesis was studied focusing on the underlying defects and the diagnosis.

HRPT2 gene

During the last 20 years, new insights in the pathogenesis, diagnosis and management of parathyroid tumours became apparent. An important milestone was the recent discovery of the *HRPT2* gene. The *HRPT2* gene is ubiquitously expressed, evolutionary conserved and consists of 17 exons encoding a protein of 531 amino acids, referred to as parafibromin.
Germ-line mutations in this gene are responsible for the *HPT-JT* syndrome. Furthermore, sporadic parathyroid carcinomas often show (somatic) mutations in the *HRPT2* gene (this thesis, chapter 4 and chapter 7). The percentage of identified *HRPT2* mutations in sporadic parathyroid carcinomas varies in different publications, partly due to different inclusion criteria. In 70% of carcinomas with local recurrence or metastasis *HRPT2* mutations have been observed.[3;6;9;11;16] In a Dutch cohort of parathyroid carcinomas selected primarily on histological grounds (i.e. with vasoinvasion and capsule invasion), the prevalence of *HRPT2* mutations was only 15%, although mutation analysis was performed in archival paraffin embedded tissue.
Somatic *HRPT2* mutations were also reported in HPT-JT associated tumours other than parathyroid. Somatic *HRPT2* mutations were found in two renal carcinomas, one clear cell carcinoma and one Wilms tumour.[32] Also, somatic mutations were identified in benign ossifying fibromas of the jaw.[26] Interestingly, these tumours showed retained expression of parafibromin. As IHC is not a quantitative analysis it could be possible that haploinsufficiency might play a role in tumour formation, which also might explain the benign behaviour in contrast to the aggressive behaviour of parathyroid tumours with total loss of expression of parafibromin due to double mutations in *HRPT2* or to the combination of one mutation and loss of the wildtype allele. Frequent allelic imbalance (LOH) of the *HRPT2* locus was detected in different subtypes of sporadic renal tumours and LOH analyzed by microsatellite markers and arrayCGH of the *HRPT2* locus is associated with an adverse clinical outcome. [18,24] A role of the *HRPT2* was also suggested in tumour types other than typically found in the HPT-JT spectrum, as illustrated in chapter 2 where tumours of the thyroid, testis and pancreas were found in a large HPT-JT family. Also uterine tumours are found to be associated with *HRPT2*.[3] Selvarajan et al showed altered immunohistochemical parafibromin staining in breast carcinomas.[28] In the future the development of knock-out mouse models for HPT-JT could help to gain more insight in the role of *HRPT2* in the development of all these tumours

HPT-JT syndrome

Patients with germ-line *HRPT2* mutations show a wide variation of clinical features. Such individuals can develop tumours in different organs or tissues, mostly in the parathyroids, kidneys, or jaws. Additionally, tumours in the thyroid, testes, pancreas (this thesis) and uterus [3] are described. HPT-JT has an autosomal dominant mode of inheritance, with incomplete penetrance as reported in the large Dutch family described in this thesis (chapter 2). The incomplete penetrance might also explain the relatively high percentage of germline mutations found in apparently sporadic parathyroid carcinomas (this thesis, chapters 7 and 4).[29] Some individuals with germ-line *HRPT2* mutations develop only parathyroid gland tumours. The latter is illustrated by the finding that about 5% of the patients suffering from familial isolated hyperparathyroidism (FIHP) carry *HRPT2* mutations.[6;22] Despite the reported rarity of

HRPT2 mutations in FIHP, FIHP patients with aggressive tumours are likely to carry *HRPT2* mutations and are therefore serious candidates for *HRPT2* germ-line testing. [14]

Parafibromin

Parafibromin is evolutionary conserved and binds to RNA polymerase II as part of a PAF1 transcriptional regulatory complex. PAF is comprised of five subunits that include PD2/hPaf1, parafibromin, hLeo1, hCtr9 and hSki8. The mechanism by which loss of parafibromin function can lead to neoplastic transformation is poorly understood. It has been suggested that parafibromin is involved in transcriptional regulation, histone modification, cell proliferation (including cell cycle progression[7;12], apoptosis[19] and wnt signalling.[23,2;27;30-32]
We suggested by both gene and protein expression that Histone 1 Family 2 (HIST1H1C), amyloid beta precursor protein (APP), and E-cadherin (CDH1) might play a role in HRPT2 driven tumourigenesis.
APP overexpression both at the mRNA and protein level[17] and abnormal cleavage is associated with the neuropathological abnormalities of Alzheimer's disease. It was recently shown that a soluble cleavage product of APP has a growth promoting effect in thyroid, skin, pancreas, colon and oral squamous cells by activating MAP kinase, epithelial growth factor[10;25], serine protease inhibitors[21], PKC and Ras pathways. [15]
Although a role for APP in EGF mediated growth of parathyroid cells similar to that of the mechanism in thyroid cells[25] can be expected, the direct interaction between parafibromin and APP has to be elucidated. Konishi et al[16] concluded that HIST1H1C has a role in transmitting apoptotic signals, while Lin et al[19] suggested that proapoptotic activity of endogenous parafibromin is also likely to be important in its role as a tumour suppressor.
E-cadherin is a cell adhesion molecule that interacts with the wnt signalling pathway. A role for parafibromin in Wnt signalling is also reported[23], in which parafibromin is thought to activate the Wnt/Wg target gene transcription by directly associating with beta catenin. Cyclin D1 (*CCND1*) was initially cloned and recognized as an oncogene in the development of the parathyroid tumours[1]. We demonstrated both on gene expression as well as on protein level overexpression of CCND1 in parathyroid carcinomas. Two recent publications showed evidence that parafibromin downregulation causes indeed an increase in CCND1 protein levels. [30;32]

Diagnosis of parathyroid carcinoma

Diagnosis based on histology alone is sometimes difficult because unequivocal diagnostic findings can be absent in individual cases and histological features of malignant and benign parathyroid tumours overlap. As a result of this histopathologic uncertainty, the best possible diagnosis can be unsatisfying referring to entities like "equivocal carcinoma" or "atypical adenoma". Recently in the WHO atlas[8] it is favoured to use the term atypical adenoma.
As the majority of parathyroid carcinomas with aggressive behaviour carry *HRPT2* mutations, somatic DNA sequence analysis of this gene in tumours is a valid approach for the diagnosis of both HPT-JT and sporadic parathyroid carcinoma. Despite the presence of mutation ''hot-spots'' in exons 1, 2, and 7 of HRPT2 where approximately 80% of all mutations occur[4;9;11], the time and resources for molecular analysis of *HRPT2* are beyond the means of most surgical pathology laboratories. We and others[5;9;13] showed the absence or reduced staining of parafibromin in sporadic and HPT-JT carcinomas. Conversely, two recent studies[5;13] have shown that negative parafibromin immunostaining is almost invariably associated with *HRPT2* mutations and confirm that loss of parafibromin staining strongly predicts parathyroid malignancy. A point to remember however is that HPT-JT adenomas might also show

reduced staining possibly indicating their potential to progress into carcinomas.[9;13] Also, additional information is needed regarding the reproducibility and the use of parafibromin in atypical adenomas/equivocal carcinomas in order to predict possible clinical behaviour.[20] Despite this, parafibromin testing seems to be a promising molecular marker for the diagnosis of parathyroid carcinoma. However, an exceptionally positive staining for parafibromin could still be compatible with *HRPT2* mutation in the case of missense mutations, for example. In addition, we have shown that molecules such as APP, E-cadherin, CASR might play a role in *HRPT2* driven tumourigenesis. Immunohistochemical analysis of APP, E-cadherin and CASR (i.e. strong cytoplasmic staining of APP, irregular membranous staining or deposits/ droplets in the cell of E-cadherin and absence of clear membranous staining of CASR) might give circumstantial evidence to support the diagnosis of malignancy. There is no role for *MEN1* mutation testing in parathyroid tumours suspected for malignancy since parathyroid adenomas often show somatic mutations of *MEN1* together with loss of the wild-type allele.

Future perspective

There are still several aspects of parathyroid disease requiring further investigation: Can biomarkers be identified that can be used for molecular imaging of (abnormal) parathyroid glands? Such biomarkers might be highly expressed membrane bound molecules specific for parathyroid tissue. Although parathyroid carcinoma is a rare disease, in individual cases the disease can take a dramatic course. For such cases, the identification of specific parathyroid tumourigenesis pathways that can be targeted by designer molecules might be crucial. A third issue that should be addressed concerns the switch from secondary to tertiary hyperparathyroidism. What are the molecular switches that lead to such autonomous behaviour of an individual parathyroid gland? Only such insights might lead to the finding of novel therapies.

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Chapter 11

Summary

In this thesis tumourigenesis and tools for improved diagnosis of parathyroid tumours were studied with a special focus on parathyroid carcinomas.

In ***chapter 2*** we described a large, previously unreported Dutch kindred in which 13 affected members presented with either parathyroid adenoma or carcinoma; in 5 affected individuals, cystic kidney disease was found. Additionally, pancreatic adenocarcinoma, renal cortical adenoma, papillary renal cell carcinoma, testicular mixed germ cell tumour with major seminoma component, and Hürthle cell thyroid adenoma were also identified. We determined that the disease in this family was linked to the presumed *HRPT2* locus on chr 1q and we were able to localize the region of the gene to 14cM. We concluded that HPT-JT is a clinically heterogeneous syndrome and that the HRPT2 gene might play a role in development of several tumours.
The family described in chapter 2 was of great importance for the discovery of the *HRPT2* gene as described in ***chapter 3***. By combining data from 26 families suffering from the HPT-JT syndrome, the *HRPT2* region was narrowed down to 12 cM. Using a positional candidate cloning approach in 14/26 cases from fourteen families, heterozygous germline mutations in c1orf28 (*HRPT2*) were found. Inactivating somatic mutations in this gene were also found in 3/48 parathyroid (cystic) adenomas supporting the probable tumour suppressor effect of the gene. The gene is evolutionary conserved and consists of 17 exons encoding a protein of 531 amino acids and was named parafibromin. Parathyroid tumours show higher malignancy frequencies in HPT-JT syndrome than in other familial parathyroid tumour syndromes such as MEN1. Here we suggest that mutations in *HRPT2* may be responsible for this difference. To test this hypothesis we analyzed, as described in ***chapter 4***, 60 different (benign and malignant, mostly sporadic) parathyroid tumours. *HRPT2* somatic mutations were detected in all (4/4) sporadic parathyroid carcinoma cases and 5/5 HPT-JT tumours and 2/3 FIHP tumours. No mutations were detected in any of the other (benign) tumours. ‘‘Two-hits’’ (double mutations or one mutation and loss of heterozygosity at 1q24-32) affecting *HRPT2* were found in 2/4 sporadic carcinomas, 2/5 HPT-JT-related and 2/3 FIHP related tumours.
These data supported the role of *HRPT2* as a causative gene in the development of parathyroid malignancy both in familial and sporadic tumours and it provided evidence of a role for *HRPT2* as a tumour suppressor gene. We hypothesized that *HRPT2* mutation is an early event that may lead to parathyroid malignancy and therefore suggested that mutations of *HRPT2* are markers for malignant potential of parathyroid tumours, both familial and sporadic.
In ***chapter 5*** we hypothesized, based on this high prevalence of *HRPT2* gene mutations and biallelic inactivation in parathyroid carcinoma, that loss of parafibromin, the protein product of the *HRPT2* gene, could distinguish carcinoma from benign tissue. To study this, a novel antiparafibromin monoclonal antibody was generated and immunostaining on both benign and malignant parathyroid tumours was tested. We reported that the loss of parafibromin nuclear immunoreactivity had a high sensitivity and specificity in diagnosing carcinoma. Parafibromin thus seems a promising molecular marker in the diagnoses of parathyroid carcinoma.
In ***chapter 6*** the morphological, immunohistochemical and molecular characteristics of 26 primary parathyroid carcinomas and seven metastases were studied. Down-regulation of the calcium sensing receptor (CASR) was demonstrated in 31% of carcinomas, and this was significantly correlated with a high Ki-67 proliferation index. Chromosome 1q and chromosome 11q LOH were found in 12 of 22 (55%) and 11 of 22 (50%) carcinomas tested, respectively. Combined 1q and 11q LOH was seen in 8 of 22 (36%) carcinomas, in contrast to the low percentage of LOH reported in both regions in adenomas. We concluded that both loss of CASR protein expression in

combination with an increased proliferation rate and the combined 1q/11q loss could be used as supportive criteria in the diagnosis of parathyroid carcinoma.
Furthermore, the high percentage of LOH at 1q found in our set of sporadic parathyroid carcinomas seemed to confirm the tumour suppressor function of *HRPT2* also described in chapter 3 and the importance of the gene in the development of malignant parathyroid tumours. The high percentage of LOH of 11q also suggested involvement of the *MEN1* gene in the tumourigenesis of a part of parathyroid carcinomas. This hypothesis was tested in ***chapter 7*** where we evaluated the role of *MEN1* and *HRPT2* mutations in sporadic parathyroid tumours fulfilling histological criteria for malignancy. Formalin fixed, paraffin embedded (FFPE) parathyroid carcinoma tissue from 28 Dutch cases was studied. *HRPT2* (27/28 cases) and *MEN1* (23/28 cases) were analyzed by direct sequencing. Somatic MEN1 mutations were found in 3/23 (13%) sporadic parathyroid carcinoma cases, six *HRPT2* mutations were found in 4/27 cases (15%). These results again confirmed the role of *HRPT2* in sporadic parathyroid cancer formation, but also showed parathyroid carcinomas with *MEN1* mutations possibly suggesting that an adenoma with a *MEN1* mutation can progress into a carcinoma when untreated.
In ***chapter 8*** we undertook expression profiling of 53 hereditary and sporadic parathyroid tumours. A class discovery approach identified three distinct groups, mainly consisting of respectively (1) adenomas, (2) *HRPT2* mutated tumours, and (3) hyperplasia. The most robust cluster identified in this study consisted of sporadic parathyroid carcinomas, tumours from HPT-JT patients (both benign and malignant), and tumours from two FIHP patients. Eleven of 12 of these cases were shown to carry *HRPT2* mutations. We concluded that parathyroid tumours with *HRPT2* mutations follow pathways distinct from that of other tumours. Based on the expression data, we confirmed the differential expression of Histone H1, amyloid βA4 precursor protein, Cyclin D1 and E-cadherin using IHC.
The objective of the study described in ***chapter 9*** was to develop a Multiplex Ligation-dependent Probe Amplification (MLPA) based genomic assay for the rapid diagnosis of parathyroid carcinomas using a combination of known chromosomal amplifications and deletions. In this study we again confirmed that parathyroid tumours with *HRPT2* mutations follow pathways distinct from that of other benign and malignant tumours. We suggested that genes on chromosome 1p, 3q but especially chromosome 13 play a role in *HRPT2* driven tumourigenesis.

Chapter 11

Samenvatting

De mens heeft 4 bijschildklieren gelegen, zoals de naam al zegt, bij de schildklier in de hals. De bijschildklier heeft een belangrijke functie in de kalk (calcium) huishouding in ons lichaam. Calcium speelt een centrale rol in een groot aantal fysiologische processen zoals neuromusculaire signaaldoorgave, spiercontractie (waaronder van het hart), celdeling en celbeweging. Het door de bijschildklier geproduceerde hormoon (Parathyreoid Hormoon; PTH) houdt het bloed calcium niveau binnen de grenzen van normaal.
Overactiviteit van een of meerdere bijschildklieren leidt tot zogenaamde hyperparathyreoidie (HPT). Vaak gaat deze overactiviteit gepaard met vergrote bijschildklieren die teveel PTH maken waardoor het gehalte van dit hormoon in het bloed te hoog wordt.

HPT kan voorkomen als een primaire, een secundaire en een tertiaire afwijking. Met primair wordt bedoeld dat de oorzaak van de HPT gelegen is in de bijschildklier zelf, secundair als reactie op een biochemische verstoring in het bloed, meestal als gevolg van nierfalen. Secundaire reactieve hyperparathyreoidie kan medicamenteus worden behandeld. Op een bepaald moment reageert de patient(e) hier niet meer op en blijkt er sprake van een autonoom functionerende bijschildklier, waarbij gesproken wordt van een tertiaire HPT.
Primaire hyperparathyreoidie is een veel voorkomende afwijking met een prevalentie van 1-3 per 1000 individuen. Het merendeel van de tumoren ontstaat in een sporadische context, geen duidelijke oorzaak is aantoonbaar. In vijf procent van de primaire HPT ontstaat de ziekte echter in een familiaire (erfelijke) context (Multiple Endocrine Neoplasia syndrome (MEN) type 1 en 2A, Familiaire Geïsoleerde Hyperparathyreoidie (FIHP) en Hyperparathyreoidie-Kaak-Tumorsyndroom (HPT-JT)). De meeste gevallen van FIHP ontstaan ten gevolge van een erfelijk defect in de "calcium-sensing", het meten van de calcium waarden in het bloed door de bijschildklier. In alle overige familiaire gevallen toont een "tumor onderdrukkend" gen een DNA fout (de genen *MEN1*, *RET*, en *HRPT2* respectievelijk).

Het doel van dit proefschrift was om meer inzicht te krijgen in onbegrepen moleculaire mechanismen leidend tot tumorontwikkeling in de bijschildklier. Dit zou theoretisch tot betere diagnostiek en behandeling kunnen leiden van onbegrepen familiaire vormen van HPT. Met name ook de categorie van de kwaadaardige bijschildklier tumoren, de carcinomen, behoeft verbetering van diagnostiek en behandeling.

In ***hoofdstuk 2*** beschrijven we een grote Nederlandse familie waarvan 13 individuen zich presenteerden met bijschildklier tumoren waaronder de kwaadaardige vorm. Bij 5 van deze 13 individuen werden ook nog cysteuze nieren ontdekt. Er werd eveneens een breed scala van andere tumoren gediagnosticeerd in deze familie waaronder een kwaadaardig proces van de pancreas, een zaadbal tumor en een goedaardige schildklier tumor en kleine nier tumoren.
Eerst werd een MEN1 syndroom uitgesloten middels DNA onderzoek. Zogenaamd koppelings onderzoek toonde aan dat de ziekte in deze familie geassocieerd was met chromosoom 1q25-32, waar het reeds eerder veronderstelde, doch nog niet gevonden HRPT2 gen zou moeten liggen. Door onderzoek in onderhavige familie werd echter het chromosomale gebied waarin dit gen zou moeten liggen verkleind tot 14 cM (een genetische afstandsmaat). Wij postuleerden tevens dat het *HRPT2* gen mogelijk een rol speelt in de ontwikkeling van verschillende andere tumoren.
In de zoektocht naar het *HRPT2* gen werd de bovenbeschreven familie nogmaals geanalyseerd als onderdeel van een groot internationaal onderzoeksconsortium, beschreven in ***hoofdstuk 3***. Allereerst werd de potentiële regio verder verkleind naar 12 cM door gebruik te maken van de koppelingsdata van 26 HPT-JT families.

Daarna werd gericht naar DNA mutaties in kandidaat genen in het gebied gezocht. In 14 van deze 26 families werden enkelvoudige inactiverende kiembaan mutaties gevonden in het gen c1orf 28 (*HRPT2*). Ook werden er inactiverende DNA mutaties gevonden in *HRPT2* in 3/48 willekeurige doch cysteuze bijschildklier tumoren. Een tumor onderdrukkende functie van *HRPT2* leek aannemelijk. Het *HRPT2* gen wordt in min of identieke vorm bij diverse dieren of organismen gevonden (is evolutionair geconserveerd) en bestaat uit 17 coderende DNA blokken (exonen) die in een eiwit bestaande uit 531 aminozuren worden vertaald. Dit eiwit werd parafibromine genoemd. Bijschildkliertumoren gevonden in HPT-JT families zijn vaker kwaadaardig (15%) dan in andere familiaire bijschildklier tumorsyndromen zoals *MEN1*(<1%). Derhalve kon worden geconcludeerd dat een mutatie in het *HRPT2* gen hiervan de oorzaak zou kunnen zijn.
Deze hypothese werd getest als beschreven in hoofdstuk 4. Zestig willekeurige (sporadische) bijschildklier tumoren zowel goedaardig als kwaadaardig werden onderzocht op mutaties in het *HRPT2* gen. Zogenaamde somatische *HRPT2* mutaties werden gevonden in alle (4/4) sporadische kwaadaardige bijschildklier tumoren, in 5/5 HPT-JT tumoren en in 2/3 FIHP tumoren. In geen van alle andere (goedaardige) bijschildklier tumoren werden dergelijke mutaties gevonden.
Twee van de vier sporadische bijschildklier carcinomen, 2/5 HPT-JT en 2/3 FIHP tumoren toonden dubbele *HRPT2* mutaties of één mutatie gecombineerd met verlies van de normaal aanwezige kopie van chromosoom 1q24-32. Deze bevindingen bevestigden de rol van *HRPT2* als een oorzakelijk gen in het ontwikkelen van kwaadaardige bijschildklier tumoren in zowel een familiaire als in een sporadische context. *HRPT2* mutaties ontstaan in een vroege ontwikkelingsfase van de kwaadaardige bijschildklier tumoren en kunnen daarmee gebruikt worden als biomarkers in de diagnostiek hiervan.
In ***hoofdstuk 5*** wordt beschreven hoe een nieuw monoclonaal antilichaam tegen parafibromin werd gegenereerd. Immunohistochemisch verlies van de normale parafibromine kernkleuring werd gevonden in HPT-JT en sporadische kwaadaardige bijschildklier tumoren met een hoge sensitiviteit en specificiteit en lijkt daarmee eveneens een veelbelovende biomarker in de diagnose van bijschildklier tumoren.
De morfologische, immunohistochemische en moleculaire eigenschappen van 26 Nederlanse gevallen van een kwaadaardige bijschildklier tumor inclusief 7 uitzaaingen werden bestudeerd (***hoofdstuk 6***). Eén derde van de tumoren liet een afschakeling van de calcium sensing receptor (CASR) zien en dit was significant gerelateerd aan een hoge (>5%) "Ki67 proliferatie" index. Tevens werd er een "verlies van heterozygotie" analyse van de chromosomen 1q (*HRPT2* gen) en 11q (*MEN1* gen) uitgevoerd. Er werd verlies in ongeveer de helft van de carcinomen gevonden van respectievelijk DNA sequenties op chromosoom 1q (55%) en 11q (50%). Uniek voor carcinomen bleek het gecombineerde verlies van DNA sequenties op de chromosomen 1q én 11q, gevonden in 36% van de carcinomen in tegenstelling tot een zeer laag percentage hiervan bij de onderzochte goedaardige bijschildklier tumoren. Het onverwacht hoge percentage DNA verlies op chromosoom 11q kon een indicatie kunnen zijn dat ook het *MEN1* gen een rol in kwaadaardige bijschildklier tumoren zou kunnen spelen.
Om deze hypothese te testen werd in de *HRPT2* en *MEN1* genen naar DNA mutaties gezocht in het Nederlandse cohort kwaadaardige bijschildklier tumoren (**hoofdstuk 7**). Deze tumoren voldeden histologisch aan de criteria van kwaadaardigheid; aanwezigheid van bloed of lymfbaan invasie dan wel kapselinvasie en of uitzaaingen (in lymfeklieren of op afstand). DNA mutatieanalyse werd uitgevoerd in formaline gefixeerd paraffine ingebed tumor archief materiaal, soms wel 20 jaar oud.
Verrassenderwijs werden somatische *MEN1* mutaties werden gevonden in 23%(3/23) van de tumoren terwijl in er 6 tumoren *HRPT2* mutaties werden gevonden (4/27,

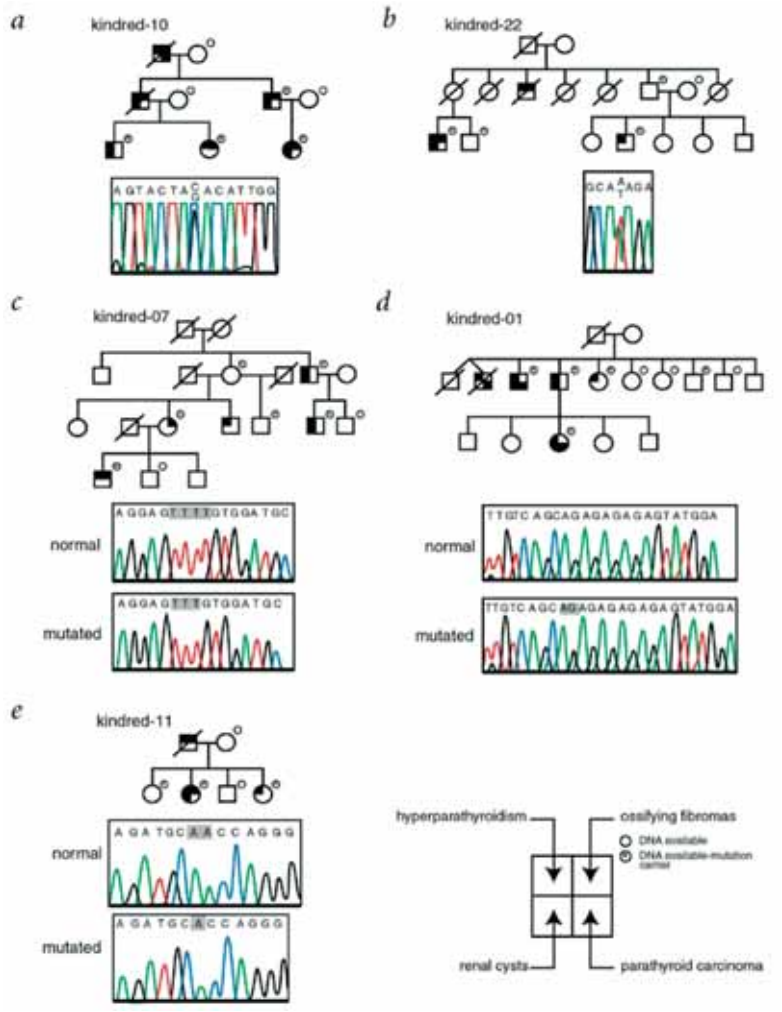
15%). Deze resultaten bevestigden weer de rol die *HRPT2* speelt bij sporadische bijschildkliercarcinomen, de resultaten lieten echter ook zien dat een aantal kwaadaardige tumoren *MEN1* mutaties bevatten. Dit zou kunnen betekenen dat in de loop der tijd een a priori goedaardige tumor met een *MEN1* mutatie toch kwaadaardig kan worden, het benadrukt tevens het belang van vroege opsporing en behandeling hiervan.

Hoofdstuk 8 beschrijft de expressie van praktisch alle genen die wij hebben (middels zogenaamde cDNA micro-array technieken) in 53 familiaire en sporadische tumoren. Met behulp van bioinformatica werden drie separate groepen geïdentificeerd. Deze bestonden hoofdzakelijk uit respectievelijk: (1) Adenomen (de benigne tumoren vaak gevonden bij primaire HPT), (2) Tumoren met een *HRPT2* mutatie (11 van de 12) en (3) Hyperplasieen (de benigne tumoren vaak gevonden bij secudaire hyperparathyreoidie). De meest robuuste groep in deze studie bleek groep 2. *HRPT2* gemuteerde tumoren lijken derhalve distincte karakteristieken te hebben aan de hand van een moleculair profiel. Immunohistochemische analyse met antilichamen gericht tegen enkele van deze moleculalen toonde inderdaad differentiële expressie (Histone H1, Amyloid βA4 precursor protein, Cycline D1 en Ecadherine).

In **hoofdstuk 9** wordt de zogenaamde "multiplex ligation dependent probe amplification" (MLPA) genomische analyse beschreven. Dit is een snelle diagnostische methode om de aan- of afwezigheid van bekende chromosomale amplificaties (chromosoom vermeerdering) en deleties (chromosoom verlies) in bijschildklier tumoren te analyseren. In deze studie bevestigden we wederom dat bijschildklier tumoren met een HRPT2 mutatie andere karakteristieke afwijkingen (op chr 1p, 3q maar in het bijzonder chr 13) tonen dan de overige tumoren.

Colour Figure Overview

FIGURE 3.2 Mutations in kindreds affected with HPT-JT.

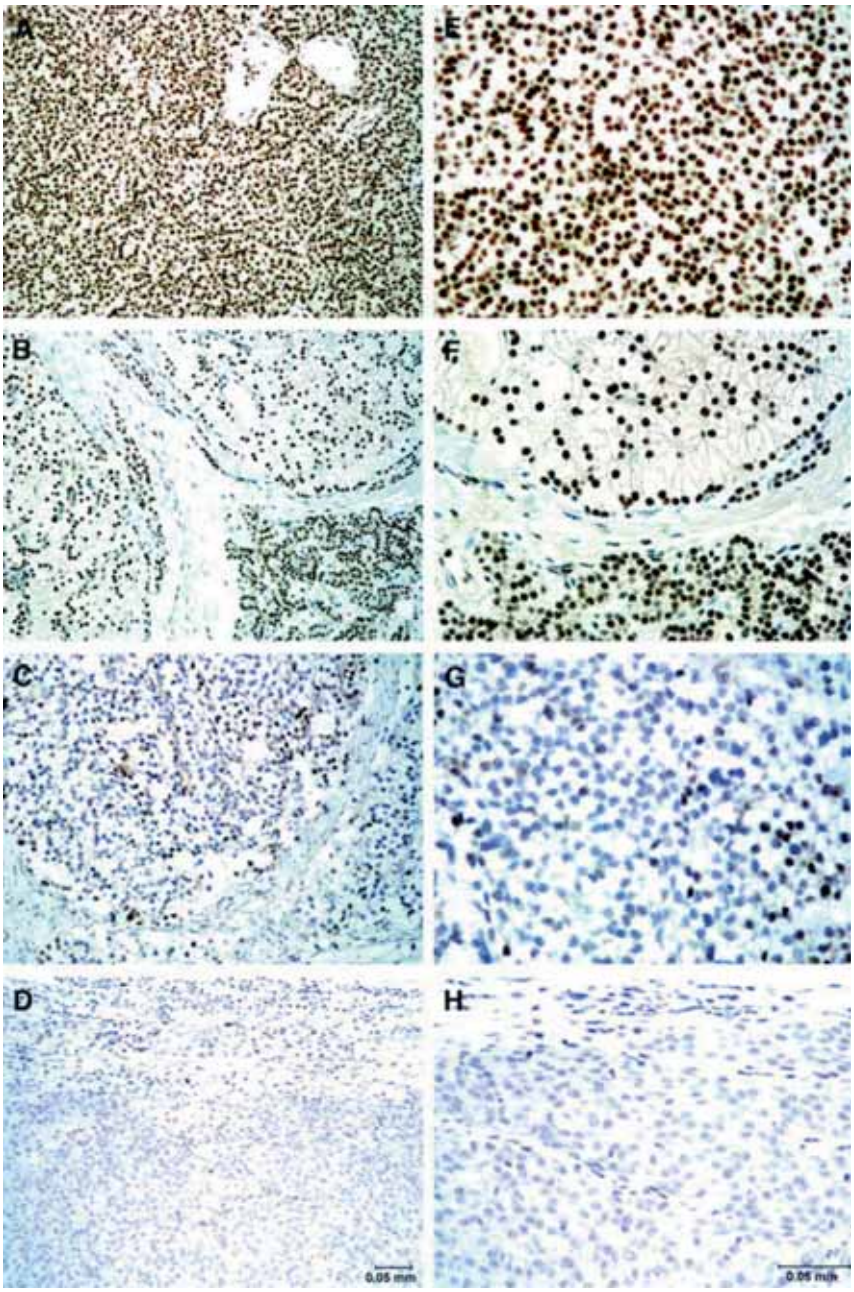


Shaded upper left quadrant represents hyperparathyroidism, upper right quadrant represents ossifying fibroma of the jaw, lower left quadrant represents renal cysts or other kidney tumors, and lower right quadrant represents parathyroid carcinoma. A line drawn through a symbol represents a deceased individual. Completely open symbols represent individuals who are currently unaffected. Small superscript circles to the upper right of family member symbols represent those individuals for whom DNA was available for mutational analysis. Small superscript circles with an asterisk (*) in the middle represent those individuals who are confirmed mutation carriers. a, Kindred-10 and chromatogram showing the heterozygous 165CG nonsense mutation in exon 2. b, Kindred-22 and chromatogram showing the heterozygous 406AT nonsense mutation in exon 5. c, Kindred-07 and chromatograms showing the normal allele and corresponding 636delT mutated allele in exon 7. d, Kindred-01 and chromatograms showing the normal allele and corresponding 679insAG mutated allele in exon 7. e, Kindred-11 and chromatograms showing the normal allele and corresponding 1238delA mutated allele in exon 14. For c-e, PCR products from single affected individuals carrying mutations were subcloned and subsequently sequenced to obtain sequences for both the mutated and normal alleles from the same individual.

FIGURE 5.1 Confocal images demonstrating co-localization of the GFP-parafibromin fusion protein with anti-parafibromin antibody within the nuclei of transfected HEK293 cells.

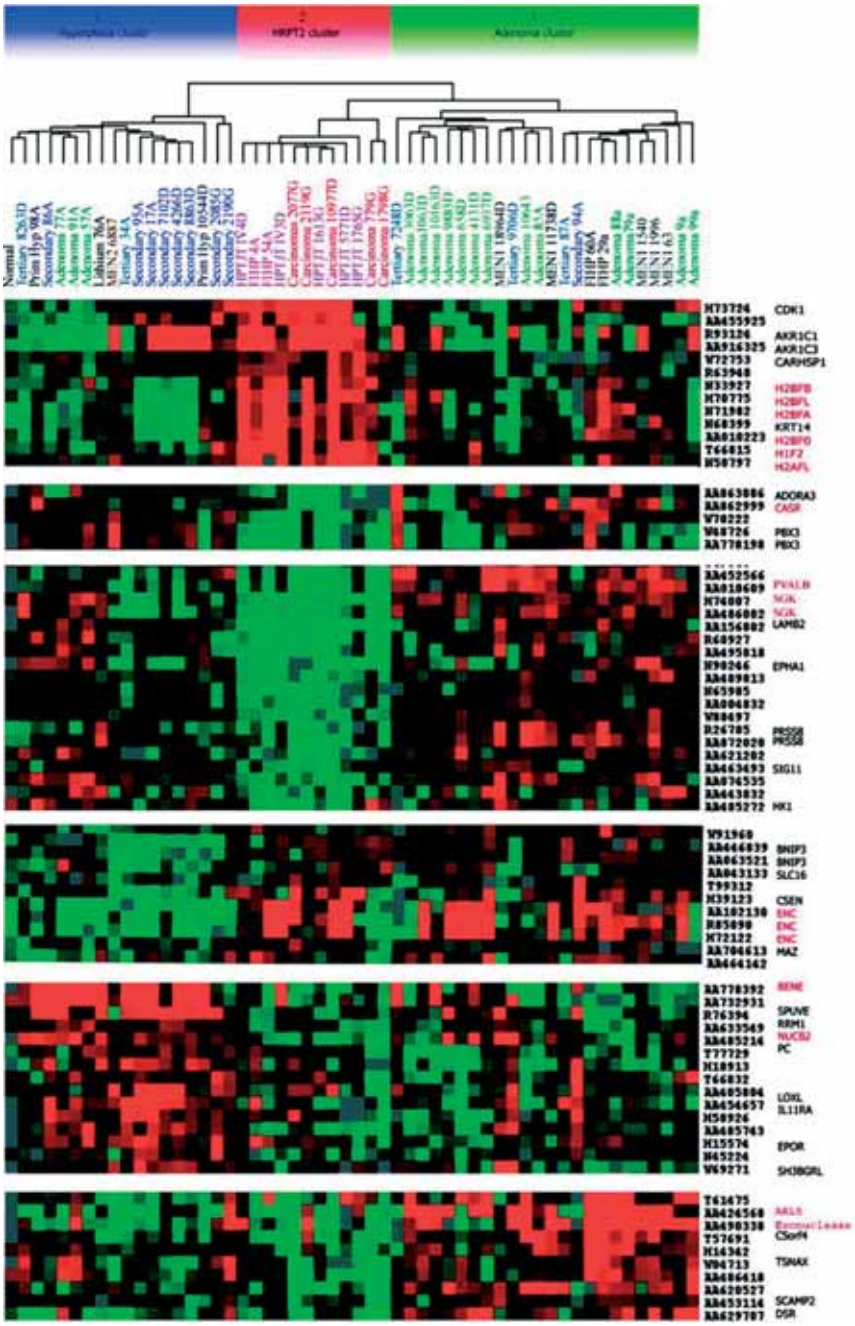
From left, GFP-parafibromin fusion protein expression (green); anti-parafibromin monoclonal antibody binding, as detected by secondary Rhodamine-Red goat antimouse antibody (red); Nomarski image of cells; 4',6-diamidino-2-phenylindole staining of nuclei (blue); superimposition of all images demonstrating co-localization within nuclei. All images captured with a Zeiss LSM510 META laser-scanning confocal microscope.

FIGURE 5.3 Immunohistochemical staining representing the various staining patterns manifested in the different pathologies through parafibromin immunostaining



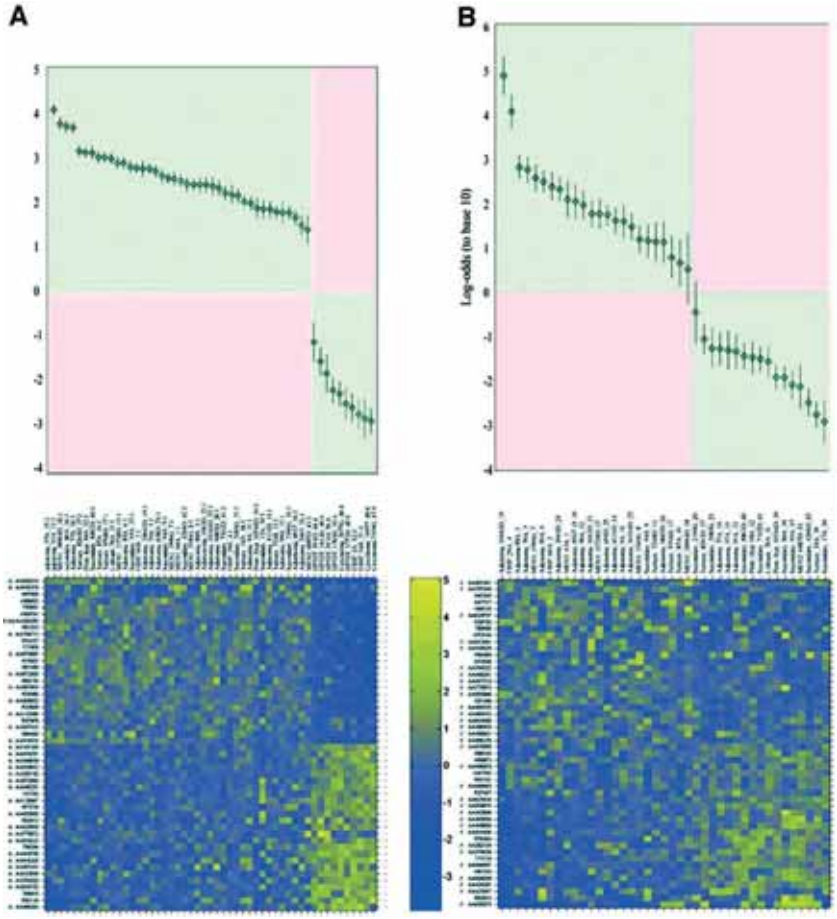
A-D, magnification, 200x. A, diffuse staining (primary parathyroid hyperplasia); B, diffuse staining (sporadic adenoma with a rim of normal tissue); C, focal loss (parathyroid carcinoma); and D, diffuse loss (parathyroid carcinoma). E-H, higher magnifications of the respective parathyroid pathologies at a magnification of 400x. All images were taken with a Spot Insight Camera on a Nikon Eclipse E600.

FIGURE 8.1 Detailed sample dendrogram of unsupervised hierarchical clustering.



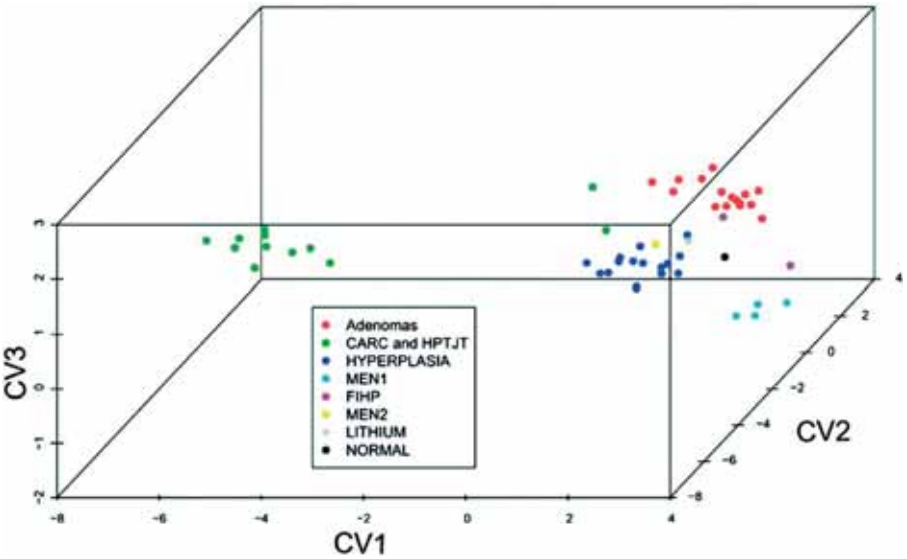
Red and green indicate transcript expression levels respectively above and below the median (black) for each gene across all samples. Grey squares indicate no results.

FIGURE 8.3 Results of classification with a penalized logistic regression model, based on two sets of 50 most significant genes (according to Significance Analysis of Microarray) present in all arrays.



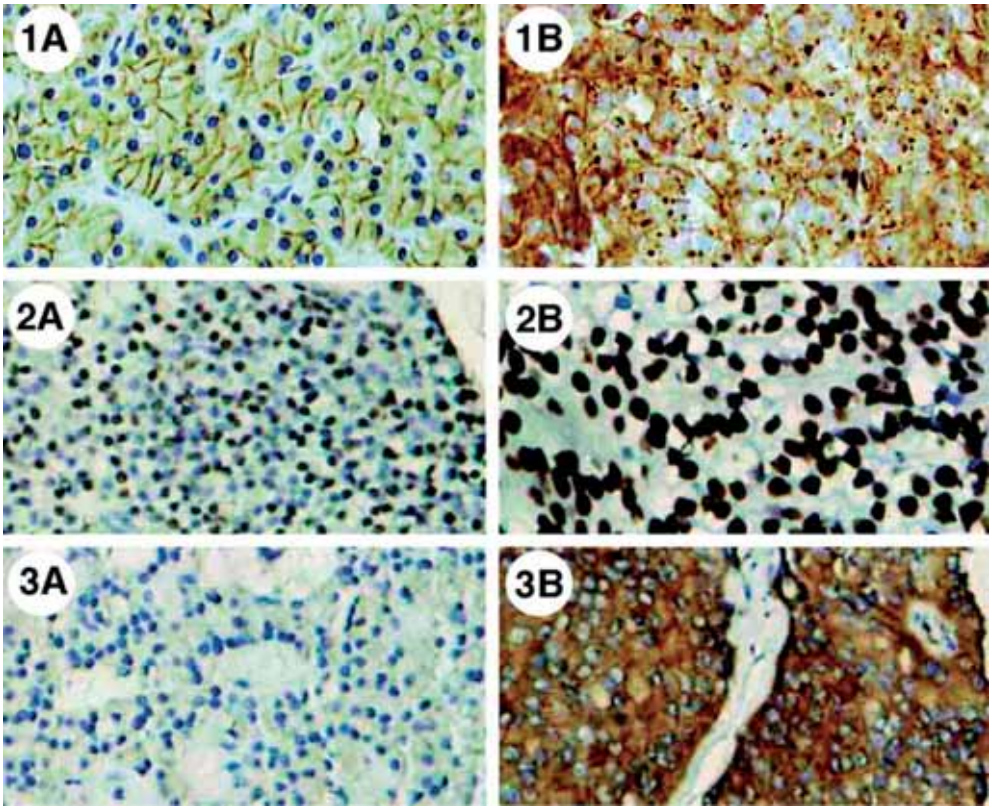
Top panel depicts the results of 100 bootstrap replications: means ([circle]) ±1 SD (lines) of log-odds per array. The vertical scale is log-odds to base 10, where "0" represents equal odds, so that in A, "2" indicates a probability of 100 to 1 that the specimen is not a cluster 2 tumor, and "−3" alternately indicates a probability of 1000 to 1 that the specimen is a cluster 2 tumor. Outcomes in the green (pink) regions are correctly (incorrectly) classified. A represents step-1 differentiation between non-Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma tumors (clusters 1 and 3) and Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma tumors (cluster 2) using 50 genes. B represents step-2 differentiation between adenomas (cluster 3) and the remainder (cluster 1) using a different set of 50 genes. Bottom panel provides graphical presentation of the log expressions with the arrays in the same order as in the top panel. The genes have been ordered along the values of their coefficients in the model with yellow squares indicating the highest expression.

FIGURE 8.4 Canonical variate plot of all arrays.



The plot shows the first three canonical variates (CV1–3). CV1 consisted of 125 genes, CV2, 57 genes, and CV3, 63 genes. Among genes of significance in CV1–3 were CDH1, APP, UCHL1, IGSF4, MOX2, and GAD1. A large separation between the carcinoma/Hyperparathyroidism-Jaw Tumor Syndrome group and the rest of the tumors is evident. The 2 familial isolated hyperparathyroidism specimens with HRPT2 mutations are included in the carcinoma and Hyperparathyroidism-Jaw Tumor Syndrome group that is depicted in green. The 2 (green) carcinomas distant from the main Hyperparathyroidism-Jaw Tumor Syndrome/carcinoma cluster are the outliers #779G and #1798G. Clear separation of the adenoma (red), hyperplasia (purple), and MEN 1 (light blue) groups is also evident. Two MEN 1 tumors are overlaid in this analysis and appear as one blue spot. The pooled normal (black) is among between the adenoma, hyperplasia, and MEN 1 groups. The 2 familial isolated hyperparathyroidism tumors with linkage at 11q13 (pink) are located between the adenomas and MEN 1 tumors. The MEN 2A (yellow) and lithium-associated tumor (gray) are situated closest to the hyperplasia group.

FIGURE 8.5 Immunohistochemical staining



Differential immunohistochemical staining at x200 magnification between (A) adenomas and (B) carcinomas (overexpression) for E-cadherin (1), histone H1 (2), and amyloid ßA4 precursor protein (3).

Curriculum Vitae

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1983-1989	VWO Rijks Scholen Gemeenschap, Heerenveen
1989-1993	Academie voor Fysiotherapie Rijks Hogeschool Groningen
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2000-2004	Promotie onderzoek afdeling Pathologie LUMC
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