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Identification of *De Novo* Germline Mutations in the *HRPT2* Gene in Two Apparently Sporadic Cases with Challenging Parathyroid Tumor Diagnoses

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Abstract The diagnosis of parathyroid carcinomas is often difficult. *HRPT2* mutations have been identified in familial [hyperparathyroidism-jaw tumor (HPT–JT) syndrome] and sporadic parathyroid carcinomas, supporting that *HRPT2* mutations may confer a malignant potential to parathyroid tumors. In this study, we report the clinical, histopathological, and genetic investigation of two unrelated cases, whom had apparently sporadic malignant parathyroid tumors, initially diagnosed as adenomas. In one case, the differential diagnosis was complicated by cervical seeding of parathyroid tumor cells. Genetic studies identified *de novo HRPT2* germline mutations in cases 1 (c.518_521delTGTC [p.Ser174LysfsX27]) and 2 (c.226 C>T [p.Arg76X]), unveiling the hereditary HPT-JT syndrome in both patients. Furthermore, the identification of somatic mutations in the patients parathyroid tumors provided evidence for complete inactivation of the *HRPT2* gene, which was consistent with the tumor malignant features. The sensitivity of parafibromin immunostaining to detect *HRPT2* mutations was limited. The present data suggests that patients with apparently sporadic parathyroid carcinomas, or parathyroid tumors with atypical histological features, should undergo molecular genetic testing, as it may detect germline *HRPT2* mutations. Establishing the diagnosis of hereditary HPT-JT syndrome is relevant for clinical counseling and management of the carriers and their relatives.

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Introduction

Primary hyperparathyroidism (HPT) is usually caused by a parathyroid adenoma, less commonly by parathyroid hyperplasia, and rarely by parathyroid carcinoma (0.5% to 5.2%) [1–4].

Parathyroid carcinomas are potentially life-threatening tumors. The management of these aggressive neoplasms is primarily surgical, with en bloc tumor resection and ipsilateral thyroid lobectomy and resection of adjacent soft tissues being the recommended as mode of therapy [3–6]. However, the diagnosis of parathyroid carcinoma is often challenging. Recent criteria indicate that the unequivocal diagnosis of parathyroid carcinoma requires the evidence of vascular invasion, perineural space invasion, capsular penetration with growth into adjacent tissues, and/or metastases [1]. Other criteria, such as trabecular growth pattern, fibrous bands, and mitotic figures, although common in parathyroid carcinomas, are not a definitive evidence of malignancy [1, 2, 4, 6]. It is, thus, common that the diagnosis of malignancy is made retrospectively following recurrence of the disease.

Sixty-seven to 100% (mean=77%) of parathyroid carcinomas harbor inactivating mutations of the *HRPT2* gene, as opposed to a small minority of adenomas [7–9]. Up to 33% of the mutations detected in apparently sporadic parathyroid carcinomas have been found to be of germline origin [10, 11], thus representing the hereditary hyperparathyroidism–jaw tumor (HPT-JT) syndrome (MIM ID #145001). This autosomal dominant disorder is characterized by the occurrence of parathyroid tumors, which are carcinomas in approximately 15% of patients, and ossifying fibromas of the mandible and/or the maxilla (~30% of the patients). Some HPT-JT patients may also develop uterine tumors and renal abnormalities such as Wilms' tumors, hamartomas, and polycystic disease [11–14]. However, in some of these families, parathyroid tumors may be the only clinical manifestation, and this variant of the HPT-JT syndrome is designated as familial isolated hyperparathyroidism (FIHP). FIHP is a genetically heterogeneous disease, which has also been related to mutations in the *MEN1* and *CASR* genes that cause the multiple endocrine neoplasia type 1 and the familial hypocalciuric hypercalcemia syndromes, respectively [15–17].

The *HRPT2* gene (also designated as *CDC73*) maps to chromosome 1q31.2 and consists of 17 exons, which encode a 531-amino acid ubiquitously expressed protein termed parafibromin [18]. Parafibromin is a component of the RNA polymerase II-associated factor 1 complex and has been suggested to induce cell cycle arrest, by integrating repressive histone H3 methylation during transcription, for cyclin D1 downregulation [19, 20].

Up to 52 heterozygous germline *HRPT2* mutations have already been reported in HPT-JT and FIHP families, and the vast majority are predicted to result in a truncated and hence inactivated form of parafibromin [10, 11]. Moreover, loss of heterozygosity or somatic inactivating mutations of *HRPT2* are also detected in HPT-JT-associated tumors [7–11, 21], suggesting that *HRPT2* is a tumor suppressor gene, in keeping with the "two-hit" model of hereditary cancer [22]. Several studies have evaluated the loss of nuclear immunoreactivity for parafibromin as a method of assessing *HRPT2* mutation status and/or parathyroid malignancy [23–28].

The present study reports the results of the clinical, histopathological, and genetic investigation of two unrelated cases—cases 1 and 2—which had apparently sporadic parathyroid tumors, initially diagnosed as adenomas, and that presented HPT recurrence 18 and 12 months, respectively, after initial surgery. The challenging diagnoses of carcinoma in these cases are discussed. Genetic studies identified *HRPT2* germline mutations, unveiling the hereditary HPT-JT syndrome in both patients, which supported a targeted genetic screening of their families.

Materials and Methods

Cases

Clinical features of patients 1 and 2 are summarized in Table 1.

Case 1

A 23-year-old female patient was referred to the hospital to evaluate a lower back pain in March 2006. Lumbar spine computed tomography (CT) scan revealed osteoporosis, and bone scintigraphy showed diffuse skeletal uptake. Biochemistry values were: serum calcium 12 mg/dl [reference interval (RI) 8.4–10.2 mg/dl], parathyroid hormone (PTH) 230 pg/ml (RI 12–65 pg/ml), serum phosphate 1.9 mg/dl (RI 2.3–4.7 mg/dl), urinary calcium 328 mg /24 h (RI 100–300 mg/24 h), urinary phosphate 835 mg/24 h (RI 400–1,300 mg/24 h), alkaline phosphatase 1,043 UI/l (RI 40–150 UI/l), and 25 OH vitamin D 9.5 ng/ml (RI 9–45 ng/ml). Neck ultrasound showed an extrathyroidal posterior right inferior nodule compatible with a parathyroid adenoma. She was diagnosed with primary hyperparathyroidism and submitted to right inferior parathyroidectomy. A parathyroid tumor, weighing

Table 1 Clinical and genetic findings in cases 1 and 2 analyzed for *HRPT2* gene mutations

Case	Sex	HPT age at diagnosis (years)	Calcemia (mg/dl) at diagnosis RI 8.4–10.2	PTH (pg/ml) at diagnosis RI 12–65	Parathyroid tumor initial diagnosis	Parathyroid tumor final diagnosis	Other HPT-JT related lesions	Family history	Disease diagnosis	*HRPT2* germline mutation			*HRPT2* somatic mutation				
										Exon	Mutation	Predicted effect		Exon	Mutation	Predicted effect	Parafibromin Immun.[a]
1	F	23	12	230	Adenoma	Carcinoma	No	No	HPT-JT	7	c.518_521delTGTC	p.Ser174LysfsX27	P1-PT1; P1-PT1onc; P1-PT2-nod1-3	2	c.162C>A	p.Tyr54X	Negative
													P1-NP	–	No	No	Positive
													P2-PT1	1	c.14T>C	p.Leu5Pro	Not done
2	F	34	13	705	Adenoma	Carcinoma	No	No	HPT-JT	2	c.226C>T	p.Arg76X	P2-PT3	1	c.14T>C	p.Leu5Pro	Positive

F female, *HPT* hyperparathyroidism, *HPT-JT* hyperparathyroidism–jaw tumor syndrome, *RI* reference interval, *P* patient, *PT1* first parathyroid tumor surgery, *PT2* second parathyroid tumor surgery, *PT3* third parathyroid tumor surgery, *onc* tumor oncocytic area, *nod* nodule, *NP* normal parathyroid, *Parafibromin Immun.* parafibromin immunostaining

[a] Anti-parafibromin antibody (2H1) targets amino acids 87–100 (encoded by exon 3 of the *HRPT2* gene)

3 g and measuring 3.0×2.5×2.0 cm, was excised with intraoperative capsule rupture. Microscopically, the mitotic count was low, and no extracapsular tissues invasion or cytological atypia was present, thus an adenoma was diagnosed.

Postoperatively, she developed a hungry bone syndrome. She was supplemented with calcium and calcitriol for 12 months. During this period, her PTH and calcium levels progressively raised to 143 pg/ml and 8.9 mg/dl, respectively. Eighteen months after surgery, PTH was 641 pg/ml and serum calcium was 11.9 mg/dl. She presented normal levels of prolactin, growth hormone, insulin-like growth factor-1, adrenocorticotropic hormone, and gastrin. There was no family history of hypercalcemia nor MEN1-associated neoplasms. HPT-JT was suspected, and she was found to carry a germline mutation in the *HRPT2* gene. She had no evidence of other HPT-JT tumors, such as jaw tumors (evaluated by CT scan), renal (CT scan), or uterine abnormalities (endovaginal ultrasound). Although imaging studies (magnetic resonance imaging, CT scan, ultrasound, and parathyroid scintigraphy) were repeatedly negative, she was submitted to an en bloc resection of thyroid and parathyroid glands, and soft subcutaneous tissues in the neck and in the superior mediastinum. Intraoperatively, PTH decreased from 1,043 (at baseline) to 0 pg/ml at 30 min after surgical neck tissue resection. Histology found uncountable nodules of parathyroid tissue, partially separated by fibrous septae, with low pleomorphism, and high proliferative activity (Ki-67 5–8%), raising the possibility of local seeding of the previously excised parathyroid tumor, since rupture of its capsule had occurred during the first surgery. The remaining three parathyroid glands showed normal histology. She was submitted to adjunct intensity-modulated radiation therapy (total dose 20 Gy) in neck and superior mediastinum. Twenty months after the second surgery and adjunct radiotherapy, she remains normocalcemic with measurable, although stable, levels of PTH (50–60 pg/ml). Thus, the diagnosis of carcinoma remains disputable in this case.

Case 2

A 34-year-old female patient was referred to the hospital to evaluate bone pain, in 2001. Together with osteopaenia, the patient also presented nephrolithiasis, polyuria (4.9 l/day; RI <3 l/day), constipation, asthenia, and depression. Physical examination revealed a palpable nodule on the lower right limit of the thyroid lobule. Biochemistry values were: serum calcium 13.0 mg/dl, PTH 705 pg/ml, serum phosphate 1.48 mg/dl, urinary calcium 242 mg/24 h, urinary phosphate 540 mg/24 h, alkaline phosphatase 221 UI/l, 25 OH vitamin D 12.4 ng/ml, osteocalcin N-Mid 123.4 ng/ml (RI <31.2 ng/ml), and B-crosslaps

1.68 ng/ml (RI <0.28 ng/ml). Further investigations were performed to exclude the presence of other tumors associated with the MEN1 syndrome. Bilateral kidney microlithiasis was present. She had no history of cervical irradiation and no family history of hyperparathyroidism.

Complete surgical excision of the right inferior parathyroid gland was performed. A parathyroid tumor, weighing 3 g and measuring 3.0×3.0×1.5 cm was excised. Histologically, the parathyroid neoplasm was composed predominantly by chief cells with atypia and anisocariosis. Mitotic count was low. Incomplete capsular penetration was present, no angioinvasion was found, and a parathyroid adenoma was diagnosed. After surgery, serum calcium was normalized (9.6 mg/dl), PTH was 34 pg/ml, and the patient initiated therapy with calcium and vitamin D. One year later, after discontinuation of oral calcium supplements, serum calcium was 11.2 mg/dl and PTH was 181 ng/ml. Neck ultrasound examinations revealed a tumor with 0.9×0.8×0.7 cm adjacent to the right thyroid lobe. In June 2004, she was submitted to right hemithyroidectomy and resection of adjacent fibroadipose tissue containing a recurrent parathyroid nodule measuring 0.9×0.6×0.2 cm. The parathyroid nodule was composed by cellular aggregates, involved by fibrous dense tissue. Mitotic index was low. Venous vascular invasion was observed. A diagnosis of parathyroid carcinoma was then established.

HPT-JT was suspected, and sequencing analysis identified a *HRPT2* gene germline mutation. A complete evaluation for the presence of other HPT-JT related tumors, including ossifying fibromas, was negative. Two years later, she presented again with increased levels of serum calcium and PTH. Ultrasonography revealed a 3.3×1.9×1.0 cm tumor at the same site of the previously removed right thyroid lobe. Meanwhile, she developed a hypercalcemia crisis (calcium 15.4 mg/dl) and was submitted to zoledronic acid perfusion (4 mg IV over 15 min) with clinical improvement. She completed the thyroidectomy, with removal of the parathyroid neoplasia jointly with tracheoesophageal, paratracheal, and eight upper mediastinal lymph nodes. Local recurrence of the parathyroid carcinoma was diagnosed, without evidence of lymph nodes metastasis. She underwent adjuvant neck radiotherapy (50 Gy in 25 fractions with linear accelerator 6 Mv) and was started on cinacalcet 90 mg tid. Currently, her serum calcium is 12.0 mg/dl and PTH is 495 pg/ml.

Biological Samples

Venous blood samples were obtained from the two patients and from ten of their first degree relatives, following written informed consent. This study was approved by the ethical committee of our institution.

Formalin-fixed paraffin-embedded samples, from the tumors of both patients, were used for genetic and immunohistochemical studies. Tissue from the parathyroid tumor excised in the first surgery, four recurrence nodules removed in the second surgery, and from a normal left parathyroid gland, were obtained in case 1. Parathyroid tissues from the tumor excised in the first surgery, and of its recurrence (third surgery), were obtained in case 2. The histopathological classification was performed by one pathologist and confirmed by another, following the criteria described in the World Health Organization classification of parathyroid tumors [1].

DNA Sequence Analysis of the *HRPT2* Gene

DNA from leukocytes and parathyroid specimens of cases 1 and 2 was extracted, using standard methods, and screened for mutations in the *HRPT2* gene. Seventeen pairs of primers were used for polymerase chain reaction (PCR) amplification of the 17 coding exons and adjoining splice junctions of the *HRPT2* gene, as previously reported [18]. Sequences of the PCR products were determined using the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an automated sequencer ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Primer sequences and assays conditions are available on request. Sequences were compared to consensus sequences obtained from the Ensembl Genome Browser (http://www.ensembl.org) and NCBI (http://www.ncbi.nlm.nih.gov) databases. DNA sequence abnormalities were confirmed by repeat PCR and sequencing and screened in the remaining family members.

Restriction Enzyme Analysis

Mutations, found by direct sequencing, were further confirmed by restriction endonuclease analysis. This approach was also used to confirm the segregation pattern of each mutation in the families of cases 1 and 2 and to assess its frequency in 50 unrelated Portuguese controls.

Immunohistochemistry

Parafibromin expression, on formalin-fixed paraffin-embedded parathyroid specimens, was evaluated by immunohistochemical staining, using a mouse monoclonal anti-parafibromin antibody (2H1), which targets amino acids 87–100 (encoded by exon 3 of the *HRPT2* gene; sc-33638, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Parafibromin immunostaining was optimized using a normal parathyroid tissue, which was also used as positive control. Antigen retrieval was done in 3 μm deparaffinized sections by pressure cooking (6 min) in a 0.01 M sodium citrate, pH 6.0 buffered

solution. Primary antibody (1:600) was incubated for 1 h, signal amplification was done with Envision™ FLEX kit (Dako, Glostrup, Denmark), and 3,3′-diaminobenzidine tetrahydrochloride was used as chromogen.

Results

DNA Sequencing and Restriction Enzyme Analysis of the *HRPT2* Gene

The results of DNA sequence analysis of exons 1–17 of the *HRPT2* gene, in leukocytes and parathyroid specimens from cases 1 and 2, are summarized in Table 1.

Case 1, carried a germline heterozygous frameshift mutation in exon 7 of the *HRPT2* gene (c.518_521delTGTC), which predicts a premature truncation of parafibromin at codon 201 (p.Ser174LysfsX27; Fig. 1). This deletion has been previously described in a sporadic parathyroid carcinoma and in a FIHP family [29, 30]. The c.518_521delTGTC genetic change was not detected in the patient's parents, indicating that it was a *de novo* mutation. DNA sequence analysis was also performed in an apparently normal left parathyroid (P1-NP) and in five different tumor samples from this patient: P1-PT1 (first surgery—parathyroid tumor), P1-PT1onc (first surgery—parathyroid tumor oncocytic region), P1-PT2-nod1-3 (second surgery—parathyroid tumor recurrence nodules 1, 2, and 3). A somatic c.162C>A heterozygous transversion in exon 2 (p.Tyr54X) was identified in all five tumor samples, but not in the normal parathyroid nor in the patient's genomic DNA (Fig. 2). This nonsense mutation, which is predicted to cause a premature termination of translation in codon 54, has been previously reported in three sporadic parathyroid tumors (two carcinomas and one adenoma) [7, 8, 31].

Case 2 carried a germline c.226C>T heterozygous transition in exon 2 (p.Arg76X). This nonsense mutation is expected to lead to the premature termination of the protein at codon 76. This mutation has been reported in a sporadic parathyroid carcinoma and in an HPT-JT family [8, 11]. Absence of this mutation in the patient's parents and five siblings showed that it was a mutation. The

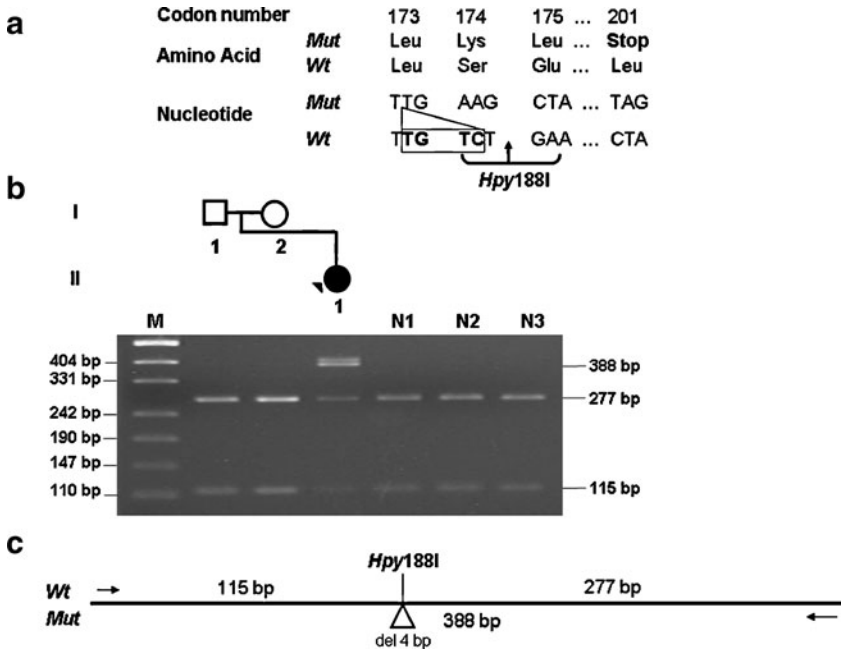

Fig. 1 Detection of a germline mutation in the *HRPT2* gene in case 1, who had an apparently sporadic parathyroid tumor. **a** DNA sequence analysis of the *HRPT2* gene in case 1 revealed a heterozygous TGTC deletion involving codons 173 and 174 (c.518_521delTGTC), which predicts a premature truncation of parafibromin at codon 201 (p.Ser174LysfsX27). **a**–**c** The mutation results in the loss of *Hpy*188I restriction endonuclease site (TCT/GA), thus providing a useful diagnostic test to confirm the presence of the mutation. **b**, **c** After amplification of a 392-bp PCR segment, cleavage of the wild-type sequence with *Hpy*188I resulted in two fragments of 115 and 277 bp, whereas the mutant sequence was not cleaved, thus corresponding to a single fragment of 388 bp (392 bp minus the 4 bp deletion). Case 1 (individual II .1) was heterozygous for the mutant sequence, whereas her parents, individuals I.1 and I.2, were homozygous for the wild-type sequence, thus confirming the results of sequencing analysis, and indicating that it was a *de novo* mutation. The absence of the TGTC deletion in 100 alleles of 50 unrelated normal controls (*N1*, *N2*, and *N3*, shown in **b**) indicates that it is not a common DNA sequence polymorphism. The positions of size marker pUC8 (*M*) are indicated. *Squares* represent males; *circles*, females; *open symbols*, unaffected individual; *full symbols*, affected with a parathyroid tumor; *arrow* indicates the proband

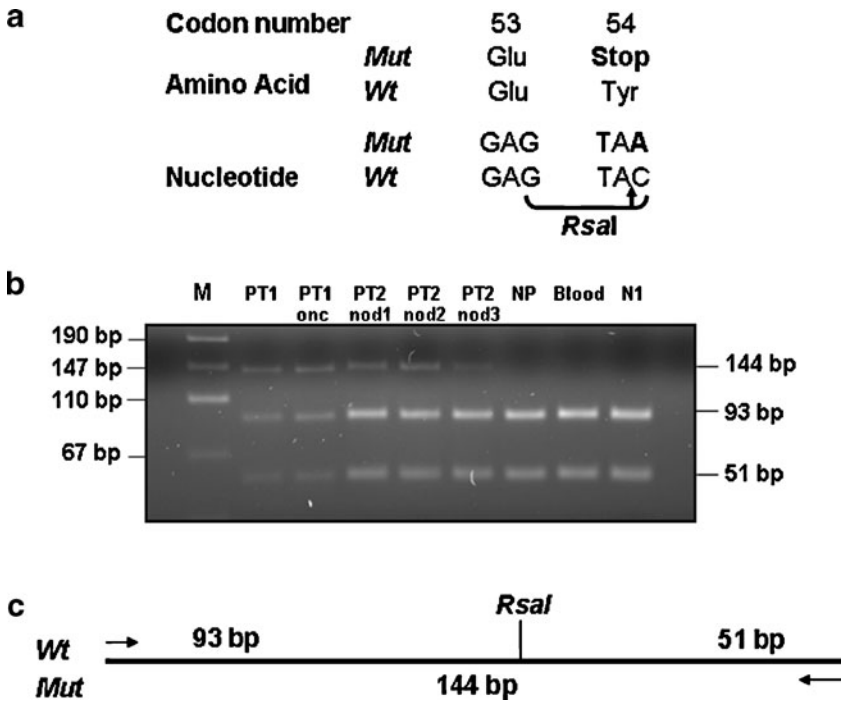

Fig. 2 Detection of a *HRPT2* gene somatic mutation in parathyroid tumor lesions from case 1. DNA sequence analysis of the *HRPT2* gene identified a somatic c.162C>A heterozygous transvertion in codon 54 (p.Tyr54X), which predicts a premature truncation of parafibromin, in five different tumor samples from patient 1 (P1): *PT1* (first surgery—parathyroid tumor), *PT1onc* (first surgery—parathyroid tumor oncocytic region), and *PT2-nod1-3* (second surgery—parathyroid tumor recurrence nodules 1, 2, and 3). **a**–**c** The mutation results in the loss of a *Rsa*I restriction endonuclease site (GTA/C), thus providing a useful test to confirm the presence of the mutation. **b**, **c** After amplification of a 144-bp PCR segment, cleavage of the wild-type sequence with *Rsa*I resulted in two fragments of 93 and 51 bp, whereas the mutant sequence was not cleaved . All five tumor samples were heterozygous for the mutant sequence, whereas a normal parathyroid (NP), the patient's genomic DNA (Blood), and a normal control (N1) were homozygous for the wild-type sequence, thus confirming the results of sequencing analysis. The positions of size marker pUC8 (*M*) are indicated

patient's 9-year-old daughter inherited the mutation, but no HPT-JT-related clinical or biochemical abnormalities were present. The *HRPT2* gene was also analyzed in DNA samples from the parathyroid tumor tissues, obtained in the patient's first (P2-PT1) and third surgery (recurrence; P2-PT3). In both samples, this analysis revealed an unreported c.14T>C transition in codon 5, exon 1, which is expected to result in the substitution of the hydrophobic leucine residue by a helix breaker proline residue (p.Leu5Pro). Therefore, this mutation, which affects an evolutionarily conserved amino acid residue [in *Canis familiaris* (dog), *Rattus norvegicus* (rat), *Mus musculus* (mouse), and *Xenopus tropicalis* (frog)], is likely to cause a significant alteration in the structure of parafibromin and impair its activity.

DNA sequence alterations were confirmed by restriction enzyme analysis (c.518_521delTGTC, c.162C>A, c.226C>T; Figs. 1 and 2) or repeated DNA sequence analyses (c.14T>C). In addition, restriction enzyme analysis of the DNA from 50 unrelated normal individuals confirmed the absence of the c.518_521delTGTC (Fig. 1) and c.226C>T germline mutations in 100 alleles.

Immunohistochemical Analysis of Parafibromin Expression

A normal parathyroid gland was used as positive control and showed diffuse nuclear staining in >99% of chief cells (data not shown). Parafibromin nuclear immunostaining was absent in almost all tumor cells (>99%) in the parathyroid tumor excised in the first surgery (Fig. 3a) and recurrent tumor nodules from case 1 (Fig. 3b). The immunohistochemical study in case 2, which was performed in parathyroid tumor tissue obtained in the third surgery (recurrence), showed an unexpected positive nuclear parafibromin immunostaining (>99% of parathyroid cells) with an intensity comparable to that observed in normal parathyroid (Fig. 3d).

Discussion

In this study, we describe two unrelated cases having clinical and histological features consistent with malignant parathyroid tumors. In both cases, a single parathyroid tumor was initially excised and diagnosed as sporadic

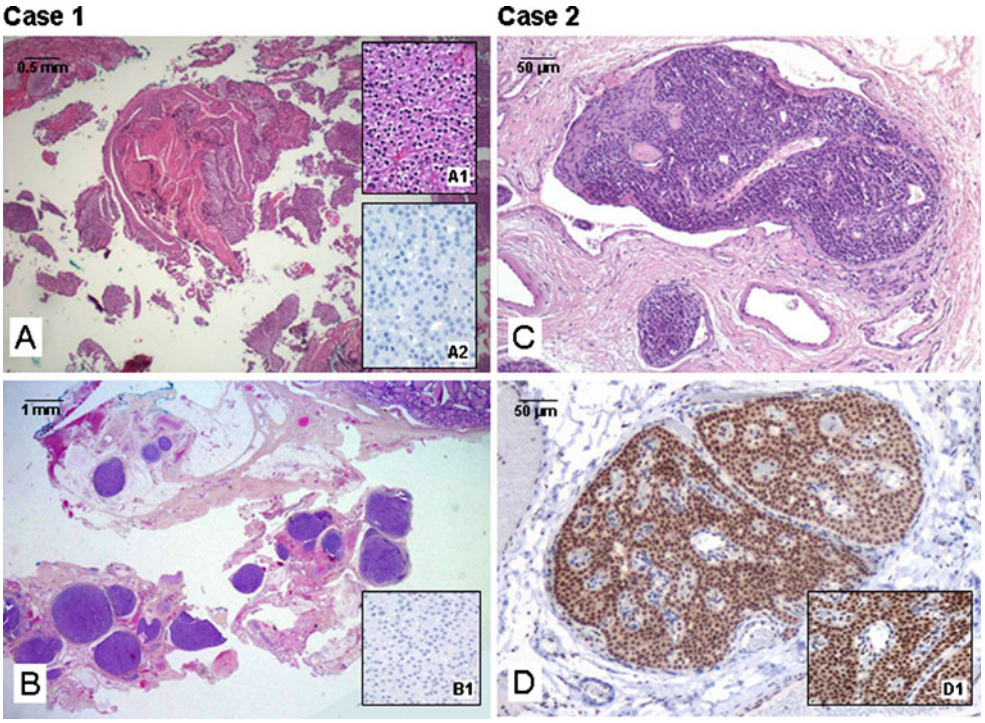

Fig. 3 Haematoxylin and eosin staining (H&E) and parafibromin immunohistochemistry (IHC) of parathyroid tumors from cases 1 and 2. **Case 1** *A* Histological evidence of tumor capsule rupture in the first surgery (H&E). *A1* Slight pleomorphic tumor cells (H&E). *A2* absence of parafibromin immunostaining in the neoplastic cells (IHC). *B* Multiple small recurrent parathyroid tumor nodules within the peri-thyroid soft tissue (H&E). *B1* Neoplastic cells negative for parafibromin immunostaining in one of the recurrent tumor nodules (IHC). **Case 2** *C* Angioinvasion by neoplastic cells (H&E). *D* Intense parafibromin immunostaining in the nuclei of the tumor cells (*D1* high power view) (IHC)

parathyroid adenoma. However, less than 2 years later, both patients presented with recurrent tumor disease.

In case 1, tumor capsule rupture occurred during the initial surgery, and at the second surgery, multiple parathyroid tumor nodules were removed from the cervical region. A literature review of 11 cases with recurrent/persistent hyperparathyroidism due to parathyroid hyperplasia/adenoma seeding reported that the mean time interval between seeding and recurrence was 9.9 years (range 1–23 years) [32]. Noteworthy, in the present case, the recurrence occurred in 2 years after initial surgery and was associated with a quick rise of PTH and calcium. Overall, these evidences, together with the high proliferative rate and fibrous septae in the recurrent tumor nodules, suggested that this case had an atypical parathyroid neoplasm with features consistent with parathyroid carcinoma.

In case 2, the evidence of a tumor nodule with vascular invasion found in the second neck exploration, and the occurrence of a third local recurrence, established the diagnosis of parathyroid carcinoma.

Mutations in the *HRPT2* gene have been found in 67–100% of parathyroid carcinomas in three large series [7–9]. A review of all *HRPT2* mutations reported in the literature has shown that 33% of the mutations detected in apparently sporadic parathyroid carcinomas were present at the germline level [10, 11], indicating that these tumors may represent a manifestation of occult hereditary HPT-JT syndrome. Thus, despite the absence of a family history of the disease in the two present cases, we searched for *HRPT2* gene mutations in their leukocyte and tumor DNA.

Case 1 carried a *de novo* germline frameshift mutation in exon 7 (c.518_521delTGTC [p.Ser174LysfsX27]). DNA sequence analysis was also performed in an apparently normal parathyroid, and in five separate parathyroid tumor lesions from this patient collected in the two surgeries, including two regions of the primary parathyroid tumor and three recurrent tumor nodules. Besides the germline mutation, all the tumors shared the same inactivating somatic *HRPT2* nonsense mutation (c.162C>A [p.Tyr54X]), which was absent in the normal parathyroid. These findings indicated the inactivation of both copies of the *HRPT2* tumor suppressor gene, in the assessed tumors, as postulated by Knudson's two-hit model [22]. The finding of the same somatic mutation in all tumors indicates that the analyzed seeding nodules derived from the original tumor, and not from another parathyroid gland. The absence of parafibromin immunostaining in these parathyroid tumors is consistent with the coding of a less antigenic, or less stable, truncated parafibromin. The absence of nuclear staining for parafibromin has been suggested as diagnostic of parathyroid carcinomas, with a high specificity (88–100%) and a sensitivity ranging from 31–100% [23–28]. However, in a HPT-JT context, parathyroid adenomas may also show total *HRPT2* inactivation [10, 11], and several authors have raised concerns about the malignant potential of these apparently "benign" familial tumors [23, 25, 28, 33]. Thus, the biallelic inactivation of *HRPT2*, and absence of parafibromin nuclear staining, observed in the tumor specimens from case 1, was helpful to define a potentially aggressive tumor behavior.

The genetic analysis of case 2 identified a *de novo* germline nonsense mutation in exon 2 of the *HRPT2* gene (c.226C>T [p.Arg76X]). A novel *HRPT2* somatic missense mutation (c.14T>C [p.Leu5Pro]) was identified in the DNAs from parathyroid tumor tissues obtained in the patient's first and third surgery. However, although the two inactivating hits were present in tumor samples, a positive nuclear immunostaining for parafibromin was observed. This finding is not unexpected because the anti-parafibromin antibody used in the present study is directed against amino acid residues 87–100 and the p.Leu5Pro mutation only changes amino acid residue 5. Our result is in agreement with other studies, which reported *HRPT2* missense mutations, predicted to encode full-length parafibromin, which was recognized by the antibody, but was possibly less biologically active [24, 28, 34].

The identification of cases 1 and 2 as carriers of *de novo HRPT2* germline mutations will be relevant for the clinical counseling and management of their descendants, in particular, for the 9-year-old daughter of patient 2, who is still an asymptomatic carrier. The genetic testing also allowed the exclusion of the remaining first degree family members (parents and siblings) from further clinical follow-up on this issue.

The diagnosis of parathyroid carcinoma remains very difficult: 86% of the cases are not recognized intraoperatively [35] and 50% of recurrent cases are not diagnosed by the pathologists on the first parathyroid excision [36]. As shown in this study, and also reported by others [24, 25, 27, 28], the sensitivity of parafibromin immunostaining as a diagnostic marker alone is limited. Conversely, *HRPT2* genetic testing seems to be a useful tool to define a potential malignant behavior in parathyroid tumors. In addition, the present apparently sporadic cases underline the importance of *HRPT2* genetic testing to unveil the hereditary HPT-JT syndrome.

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